

1 **The Antioxidant Defense System Keap1-Nrf2 Comprises a Multiple Sensing Mechanism for**
2 **Responding to a Wide Range of Chemical Compounds**

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23

1 ABSTRACT

2

3 Animals have evolved defense systems for surviving in a chemically diverse environment.**4 Such systems should bear plasticity, like adaptive immunity, enabling a response to even****5 unknown chemicals. The antioxidant transcription factor Nrf2 is activated in response to****6 various electrophiles and induces cytoprotective enzymes that detoxify them. We report here****7 the discovery of a multiple sensing mechanism for Nrf2 activation using zebrafish and eleven****8 Nrf2-activating compounds. Firstly, we showed that six of the compounds tested specifically****9 target Cys-151 in Keap1, the ubiquitin ligase for Nrf2, while two compounds target Cys-273.****10 Secondly, in addition to Nrf2 and Keap1, a third factor was deemed necessary for responding****11 to three of the compounds. Finally, we isolated a zebrafish mutant defective in its response to****12 seven compounds, but not to the remaining four. These results led us to categorize Nrf2****13 activators into six classes and hypothesize that multiple sensing allows enhanced plasticity in****14 the system.**

15

16 INTRODUCTION

17

18 Nrf2 is a transcription factor that transactivates cytoprotective genes through a common DNA

19 regulatory element, called the antioxidant response element or electrophile response element (18,

20 24). Nrf2-target genes are multifarious and encode phase 2 detoxifying enzymes, anti-oxidant

21 proteins, enzymes for glutathione biosynthesis, ABC transporters, scavenger receptors, transcription

22 factors, proteases, chaperone proteins and so forth (23). Under basal conditions, Nrf2 is rapidly

23 degraded by proteasomes and little induction of target genes is observed. This degradation is

24 controlled by Keap1, an Nrf2-specific adaptor protein for the Cul3 ubiquitin ligase complex (12, 20).

1 Nrf2-activating compounds block Keap1-dependent Nrf2 ubiquitination, leading to the stabilization
2 and nuclear translocation of Nrf2 and subsequent induction of Nrf2-target genes.

3 A number of Nrf2 activators have so far been found, but interestingly no common structures
4 were identified among them (23). Talalay and his colleagues classified Nrf2-activating compounds
5 into the following ten distinct classes based on their chemical structures (7): diphenols, Michael
6 reaction acceptors, isothiocyanates, thiocarbamates, trivalent arsenicals, 1,2-dithiole-3-thiones,
7 hydroperoxides, vicinal dimercaptans, heavy metals and polyenes. A current pursuit is unraveling
8 how cells detect these chemical compounds and transduce their signals into the activation of Nrf2.
9 Keap1 has many highly reactive cysteine residues that have the potential to sense electrophilic Nrf2
10 activators by forming covalent adducts with them. We and others have therefore proposed the model
11 that Nrf2-activating compounds directly modify the sulfhydryl groups of Keap1 cysteines by
12 oxidation, reduction or alkylation, which alters the conformation of Keap1 and ceases the
13 ubiquitination of Nrf2 (7, 24). In fact, mass spectrometry studies revealed that some Nrf2-activating
14 compounds can covalently react with cysteines in mouse or human Keap1. For example,
15 dexamethasone 21-mesylate with Cys-257, -273, -288, -297 and -613 (2),
16 iodoacetyl-*N*-biotinylnhexylenediamine/biotinylated iodoacetamide (IBA/BIA) with Cys-196, -226,
17 -241, -257, -288 and -319 (9) [in separate reports, Cys-151, -288 and -297 (4, 5) or Cys-13, -151,
18 -257, -288, -297, -613 and -622 (31)], sulforaphane with Cys-77, -226, -249, -257, -489, -513, -518
19 and -583 (8), xanthohumol with Cys-151, -319 and -613 (27), isoliquiritigenin with Cys-151 and
20 -226 (27) and 10-shogaol with Cys-151, -257 and -368 (27). Among these cysteine residues,
21 Cys-151, -273 and -288 were demonstrated to be essential for regulating Nrf2 degradation in cell
22 biological studies (21, 25, 33, 34, 43, 46), suggesting that these, and possibly other, cysteines are
23 sensor sites for Nrf2 activators. These findings support the model of Nrf2 activation by adduct
24 formation between Keap1 and activating compounds, but at the same time raise several fundamental

1 questions. For instance, which cysteines are the true *in vivo* sensor sites? Do different compounds
2 target different cysteines? Do modifying different cysteines regulate Keap1 functions in similar or
3 different manners? Are there any Nrf2-activating compounds that do not target Keap1 cysteines?

4 To address these questions, we systematically analyzed a variety of Nrf2 activators using
5 zebrafish as an experimental system to compare sensor requirements under physiological conditions.
6 We showed before that fish have a Keap1-based response system against electrophilic compounds,
7 as in mammals (22, 38, 39). In contrast to the mammalian system, zebrafish possess two Keap1
8 molecules, Keap1a and Keap1b, both of which regulate Nrf2 degradation (26), but have different
9 recognition specificities for Nrf2-activating compounds, as demonstrated in this paper. We now
10 report that the Keap1-Nrf2 system comprises discrete sensor sites, including the Keap1 cysteines
11 Cys-151 and Cys-273, for a variety of Nrf2-activating compounds.

12

13 MATERIALS AND METHODS

14

15 **Fish and chemical treatments.** Zebrafish embryos and larvae were obtained by natural mating.
16 For induction studies, fish were placed in culture dishes (embryos and larvae) or plastic chambers
17 (adult fish) containing 100 μ M diethylmaleate (DEM) (Wako), 2 μ M ebselen (Ebs) (gift from K.
18 Uchida), 40 μ M 1,2-dithiole-3-thione (D3T) (gift from T. Kensler), 30 μ M *tert*-butylhydroquinone
19 (tBHQ) (Sigma-Aldrich), 40 μ M sulforaphane (SF) (LKT laboratories), 3 μ M 1,2-naphthoquinone
20 (1,2-NQ) (Tokyo Kasei Industries), 50 μ M prostaglandin A₂ (PGA₂) (Wako), 2.5-10 μ M
21 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) (Cayman Chemical), 1 μ M auranofin (AF) (Alexis
22 Biochemicals), 1 mM hydrogen peroxide (H₂O₂) (Wako) or 40 μ M cadmium chloride (CdCl₂)
23 (Wako). In each case, chemical treatments were carried out for 6 hours. The gene expressions of
24 pi-class GST gene (*gstp1*) and γ -glutamylcysteine synthetase heavy subunit gene (*ygcsH*) were

1 analyzed by *in situ* hybridization or by RT-PCR, as described previously (22, 38). Real-time
2 RT-PCR was performed to quantitate *gstp1* expression using an ABI Prism 7700 (Applied
3 Biosystems) and probes labeled with a reporter fluorescent dye (TaqMan probe) as described
4 previously (26).

5
6 **Plasmid construction.** The plasmids pCS2FLkeap1a1bC1 and pCS2FLkeap1a1bC2 were
7 generated by inserting the cDNA encoding amino acids 2-54 or 2-148, respectively, of zebrafish
8 Keap1a into the *HindIII* and *PmaCI* or *HindIII* and *BglIII* sites, respectively, of pCS2FLkeap1b (26).
9 Plasmid pCS2FLkeap1a1bC3 was generated by inserting the cDNA encoding amino acids 299-593
10 of zebrafish Keap1b into the *BglIII* and *XbaI* sites of pCS2FLkeap1a (26). For constructing
11 pCS2FLmKeap1, cDNAs encoding the amino acid regions 2-7 and 8-624 were generated by
12 annealing synthetic oligonucleotides (Hokkaido System Science) or by PCR, respectively, and
13 inserted together into the *HindIII* and *XbaI* sites of pCS2FL (26). Point-mutations were introduced
14 by PCR into pCS2FLkeap1b to give plasmids pCS2FLkeap1bK124T, pCS2FLkeap1bC125S and
15 pCS2FLkeap1bC125W. To prepare pCS2FLmKeap1C151S, pCS2FLmKeap1C273S and
16 pCS2FLmKeap1C288S, Cys to Ser point-mutations were introduced by PCR into pCS2FLmKeap1.
17 All constructs were verified by DNA sequencing. Plasmids pCS2nrf2, pCS2keap1a, pCS2keap1b
18 and pCS2Nrf2NTnGFP were described previously (22, 26).

19
20 **Injection of mRNA and morpholino oligonucleotides.** Synthetic capped RNA was made with
21 an SP6 mMESSAGING mMACHINE *in vitro* transcription kit (Ambion) using linearized DNA of the
22 pCS2 derivatives described above. Nrf2-morpholino oligonucleotide (*nrf2*MO) was described
23 previously (22). mRNA or morpholino oligonucleotides were injected into yolk at the one-cell stage
24 using an IM300 microinjector (Narishige). GFP expression was examined under the GFP filter of a

1 BZ-8000 microscope (Keyence).

2

3 **Matrix-assisted laser desorption ionization time-of-flight mass spectrometry**

4 **(MALDI-TOF MS) analysis.** Expression in *E. coli* and purification of mouse Keap1 protein was
5 performed according to the method of Dinkova-Kostova *et al.* (2). Mouse Keap1 was incubated
6 with or without 100 μ M 15d-PGJ₂, 100 μ M PGA₂ or 500 μ M DEM for 60 min at 25°C in buffer
7 containing 20 mM Tris-HCl, pH 8.5. Trypsin-digested mouse Keap1 was mixed with dithiothreitol
8 and trifluoroacetic acid. To improve the ionization efficiency of mass spectrometry, samples were
9 purified with Zip-tip μ C18 (Millipore) before MS analysis. Peptides were mixed with
10 α -cyano-4-hydroxycinnamic acid (2.5 mg/ml) containing 50% acetonitrile and 0.1% trifluoroacetic
11 acid and dried on stainless steel targets at room temperature. Analyses were performed using an
12 AXIMA-TOF2 (Shimadzu) with a nitrogen laser, as described previously (14). All analyses took
13 place in the positive ion mode and the instrument was calibrated immediately prior to each series of
14 studies.

15

16 **Mutant screening.** Random mutations were generated by soaking male zebrafish of the AB
17 strain in 3 mM *N*-ethyl-*N*-nitrosourea (ENU) for 1 hour between 22 and 23°C and repeating this
18 process 4 times at weekly intervals. Resulting males were crossed with AB females and bred to
19 homozygosity over two generations, as previously described (3, 6).

20

21 **RESULTS**

22

23 **A variety of Nrf2-activating compounds induce *gstp1* expression in zebrafish.** To determine
24 if zebrafish can respond to a wide variety of Nrf2 activators, as do mammalian cells, we analyzed

1 the induction of *gstp1* by eleven Nrf2-activating compounds in 4-day old zebrafish larvae by
2 whole-mount *in situ* hybridization. The compounds tested were DEM, D3T, SF, tBHQ, 1,2-NQ,
3 Ebs, 15d-PGJ₂, PGA₂, H₂O₂, AF and CdCl₂ (Fig. 1A). All eleven compounds strongly induced
4 *gstp1* expression in gills, an organ of detoxification in fish (Fig. S1 in the supplemental material).
5 We also studied *gstp1* induction in adult gill by conventional and real-time RT-PCR analyses and
6 obtained the same results (Figs. 1B & S2 in the supplemental material). In the early larval stages,
7 Nrf2 expression can be knocked down using morpholino antisense oligonucleotides (22), allowing
8 us to elucidate the contribution of Nrf2 to each induction. One-cell stage embryos were injected
9 with or without *nrf2*MO and raised to 4-day old larvae before treatment with DEM, 15d-PGJ₂ or
10 H₂O₂ (Fig. 1C and D). The induction of *gstp1* by these compounds was decreased by the Nrf2
11 knockdown, indicating that Nrf2 mediates *gstp1* induction in all three cases. We concluded that
12 lineups of the Nrf2-activating compounds are equivalent in fish and mammals and thus it was
13 worthwhile to study the chemical sensing mechanisms using zebrafish as a model.

14
15 **Classification of Nrf2-activating compounds based on the requirement of Keap1a, Keap1b**
16 **and other factors.** In contrast to cultured cells, it is easy to monitor Nrf2-induced transactivation of
17 endogenous target genes in zebrafish embryos by mRNA injection assay (22), probably because of
18 the absence of artificial oxidative stress and the very low expression of endogenous Nrf2 mRNA
19 during embryonic development (Fig. S3 in the supplemental material). Using this experimental
20 system, we tried to decipher whether Keap1a and Keap1b have different specificities in the
21 recognition of Nrf2-activating compounds. The Nrf2-repression activities of both Keap1 molecules
22 were previously shown to be equivalent (26). No *gstp1* induction was observed for any of the
23 compounds tested in embryos without adding exogenous Nrf2 (Fig. 2B, no injection). Supplying
24 exogenous Nrf2 by mRNA injection induced *gstp1* expression in whole embryos (Fig. 2A and B,

1 control). This induction was abolished by co-overexpressing either Keap1a or Keap1b. Embryos
2 co-injected with Nrf2 and Keap1a, or Keap1b, mRNAs were treated with DEM, 15d-PGJ₂ or H₂O₂
3 and *gstp1* expression was analyzed. The expression of *gstp1* was induced by DEM treatment in
4 embryos overexpressing Keap1b, but not Keap1a (Fig. 2B, DEM). Surprisingly, 15d-PGJ₂
5 treatment cancelled suppression by both Keap1a and Keap1b (Fig. 2B, 15d-PGJ₂). The results
6 suggest that Keap1a has a sensor site for 15d-PGJ₂, but not for DEM, while Keap1b can sense both
7 compounds. On the other hand, H₂O₂ was unable to cancel the suppression elicited by either Keap1
8 protein (Fig. 2B, H₂O₂). Since H₂O₂ induced *gstp1* expression was Nrf2-dependent in larvae (see
9 Fig.1D), factors additional to Nrf2 and Keap1 are required for sensing H₂O₂ and these factors are
10 not expressed or functional at the embryonic stages. Both conventional and real-time RT-PCR using
11 total RNA from whole embryos confirmed these differences among three compounds (Figs. S4 &
12 S5 in the supplemental material). We then systematically analyzed the specificities of the remaining
13 eight Nrf2-activating compounds toward Keap1 molecules (Fig. S5 in the supplemental material)
14 and categorized them into three types: a Keap1b sensing-type (K1b), a Keap1a or Keap1b-sensing
15 type (K1a/b) and a posthatch factor-sensing type (posthatch) dependent on the putative factors
16 missing at the embryonic stages (see Fig. 2B). SF, D3T, Ebs, tBHQ and 1,2-NQ were grouped as
17 K1b-type, PGA₂ is K1a/b-type and CdCl₂ and AF are posthatch-type.

18 To ascertain whether or not induction was based on cancellation of the Keap1-promoting Nrf2
19 degradation, we overexpressed an Nrf2 GFP-fusion protein (Nrf2NTnGFP) in zebrafish embryos
20 and tested its stability by monitoring GFP fluorescence after DEM or 15d-PGJ₂ treatment (Fig. 2C
21 and D). Nrf2NTnGFP contains the Neh2 domain, a degron for Keap1-dependent degradation of
22 Nrf2, which has been shown to be functional not only in cultured cells, but also in mouse and
23 zebrafish (13, 26). GFP expression was almost undetectable in embryos overexpressing
24 Nrf2NTnGFP with either Keap1a or Keap1b (Nrf2NTnGFP, 25.0%, n=20; Nrf2NTnGFP+Keap1a,

1 2.4%, n=41; Nrf2NTnGFP+Keap1b, 0%, n=34). In Keap1b-overexpressing embryos, GFP
2 expression was induced after DEM treatment, while it remained low in Keap1a-overexpressing
3 embryos (Keap1a, 4.2%, n=47; Keap1b, 67.6%, n=34). In the case of 15d-PGJ₂, both Keap1a- and
4 Keap1b-overexpressing embryos showed strong GFP induction (Keap1a, 58.3%, n=48; Keap1b,
5 58.6%, n=29). The results were basically identical to those from experiments monitoring
6 endogenous *gstp1* induction, suggesting that the different DEM-responding activities of Keap1a and
7 Keap1b in inducing *gstp1* was due to their different activities in relieving Keap1-mediated Nrf2
8 degradation. In conclusion, we propose a model that the Keap1-Nrf2 system senses signals of a
9 variety of electrophiles by disparate mechanisms, yet all the signals lead to Nrf2 activation.

10

11 **Identification of a sensor cysteine for K1b-type Nrf2-activating compounds.** Previous *in*
12 *vitro* studies demonstrated that some cysteine residues in Keap1 bind directly to electrophilic Nrf2
13 activators and are presumed to function as electrophile sensors (2, 4, 5, 8, 9, 27, 31). The fact that
14 some of the activators were specific for Keap1b gave us the unique opportunity to identify the
15 putative sensor cysteine residues that rely on the molecular differences between Keap1a and
16 Keap1b. We predicted that a sensor site for K1b-type compounds is present in Keap1b, but not in
17 Keap1a. To identify these sites systematically, we constructed chimeras of Keap1a and Keap1b
18 proteins (Fig. 3A) and compared their DEM-responding activities by examining the induction of
19 *gstp1* expression by RT-PCR, as that can provide more quantitative data than *in situ* hybridization.
20 Only embryos overexpressing C1 protein, but not C2 or C3 proteins, were responsive to DEM (Fig.
21 3B). These findings indicate that the BTB domain of Keap1b is important for sensing DEM. In the
22 BTB domain, three cysteine residues are conserved among vertebrate Keap1 (Fig. 3C). We refer to
23 these cysteine residues as BTB cysteines (BCs) to ease comparison among various Keap1
24 molecules. Though all three BCs are conserved in both Keap1a and Keap1b, the amino acid

1 adjacent to BC2 is a threonine in Keap1a and a lysine in Keap1b and other vertebrate Keap1
2 proteins. It is known that adjacent basic residues stabilize the thiolate form of the cysteine residue,
3 thus enhancing its reactivity (2, 29). Based on the fact that DEM could cancel the Nrf2-repression
4 activity of Keap1b but not that of Keap1a, we predicted that BC2 is a sensor site for DEM and that
5 the adjacent lysine residue in Keap1b and other Keap1 proteins is critical for its reactivity. In order
6 to verify this possibility, we substituted the cysteine residue of Keap1b BC2 to serine (C125S) and
7 its adjacent lysine to threonine (K124T) and examined the effects of these substitutions on the
8 DEM-induced expression of *gstp1* (Fig. 3D). Both K124T and C125S substitutions reduced the
9 response of Keap1b to DEM, implying that BC2 is indeed a sensor site for DEM. Similar results
10 were obtained by real-time RT-PCR (Fig. S6 in the supplemental material).

11 We clarified that the function of BC2 in sensing DEM is conserved among vertebrate Keap1
12 proteins other than zebrafish. The corresponding BC2 residue of mouse Keap1 (Cys-151) was
13 mutated to serine and the DEM-induced expression of *gstp1* was assessed (Fig. 3E). As expected, a
14 C151S mutation significantly reduced the activity of mouse Keap1 to respond to DEM without
15 affecting its activity to repress Nrf2. This was also observed in the case of the DEM-induced
16 expression of *γgcsH*. Other K1b-type compounds (SF, D3T, tBHQ, 1,2-NQ and Ebs) gave identical
17 results to DEM (Figs. 3E & S7 in the supplemental material), indicating that BC2 is a sensor site for
18 K1b-type compounds. BC2 (Cys-151) has been shown to be required for sensing tBHQ and Ebs in
19 cultured cells (33, 46) and tBHQ in transgenic rescue mice (45). The important point here is that
20 BC1 and BC3 were not mutated, so we concluded that BC2 is the only *in vivo* sensing site for
21 certain types of Nrf2-activating compounds.

22 It is natural to assume that K1b-type compounds bind directly to Keap1 BC2. Indeed, we
23 previously demonstrated that Ebs bound to wild-type mouse Keap1, but not to Keap1C151S mutant
24 molecules (33). In addition, mass spectrometry studies revealed that some Nrf2-activating

1 compounds react covalently with the BC2 (Cys-151) of mouse and human Keap1 proteins (4, 5, 27),
2 albeit they also bind to many other cysteine residues in Keap1. It would be debatable to state that
3 modification of BC2 acts solely as a trigger to terminate Nrf2 degradation owing to the difficulty in
4 isolating Keap1 proteins with modification in only BC2. We overcame this obstacle by introducing
5 a cysteine to tryptophan mutation in Keap1b BC2 (C125W) and analyzed its effects (Fig. 3F).
6 Tryptophan (MW 186) is 83 kDa larger than cysteine (MW 103), so we expected that the
7 Cys-to-Trp substitution would mimic the binding of DEM (MW 172) or other K1b-type compounds
8 to BC2. As a result, Keap1bC125W failed to suppress Nrf2-mediated *gstp1* expression when the
9 protein was overexpressed to a similar amount as wild-type Keap1b and Keap1a (Fig. 3G). This
10 was not observed in the case of the C125S mutation, in which cysteine was substituted with an
11 amino acid of comparable size (serine, MW 87) (see Fig. 3D). These results suggest that
12 compounds binding to BC2 inhibit the Nrf2-repression activity of Keap1, probably due to the bulky
13 mass on BC2 masking important regions of Keap1. For instance, the interaction between Keap1 and
14 Cul3 would likely be perturbed, because the α -helix just after BC2 is believed to be critical for the
15 BTB-Cul3 interaction (44). In fact, Rachakonda *et al.* (31) reported recently that BC2 adduction
16 with IAB/BIA (MW 383) disrupts the Keap1-Cul3 interaction. We hypothesize that BC2 adduction
17 to Nrf2 activators may cease Nrf2 ubiquitination by disturbing the proper conformation of the
18 Keap1-Cul3 complex. This hypothesis is based on the “hinge and latch” model that depicts strict
19 regulation of Nrf2 ubiquitination (41).

20

21 **Identification of sensor cysteines for K1a/b-type Nrf2-activating compounds.** We found that
22 the BC2 mutation had no effect on sensing 15d-PGJ₂ and PGA₂ (Figs. 3E and S7 in the
23 supplemental material) and so the sensor site for these K1a/b-type compounds is not BC2 and is
24 different from the site for K1b-type compounds. Similar results were obtained by real-time RT-PCR

1 (Fig. S8 in the supplemental material). This is the first demonstration that different Nrf2-activating
2 compounds target different sensor sites in the Keap1-Nrf2 system. Although detailed mapping of
3 the 15d-PGJ₂-sensor site has not yet been carried out, we assume that it is located within the Keap1
4 protein, since direct binding between 15d-PGJ₂ and Keap1 has been demonstrated in cultured cells
5 by us and others (11, 21, 25). To determine the sites in Keap1 modified by 15d-PGJ₂, mouse Keap1
6 protein treated with or without 15d-PGJ₂ was digested with trypsin and analyzed by MALDI-TOF
7 MS. Peptide mass mapping by MALDI-TOF MS analysis of the trypsin fragments of native mouse
8 Keap1 provided identification of the peptides accounting for ~80% of the protein sequence (see
9 Table S1 in the supplemental material). Compared with the calculated masses of the unmodified
10 peptides, modified peptides P-1 and P-2 had an increased mass of +316 Da, corresponding to the
11 addition of a single molecule of 15d-PGJ₂ (Fig. 4A). The sequences and masses of the peptides
12 were: P-1 (CHALTPR, $m/z = 1113.7$) and P-2 (CEILQADAR, $m/z = 1334.8$), indicating that mouse
13 Keap1 is modified by 15d-PGJ₂ at Cys-273 and Cys-288. Binding sites for PGA₂ in the mouse
14 Keap1 protein were also examined by MALDI-TOF MS analysis and identified as Cys-273,
15 Cys-297 and Cys-489 (Fig. 4B; see Table S2 in the supplemental material). It was shown that
16 Cys-273 is the only common binding site for 15d-PGJ₂ and PGA₂, suggesting that Cys-273 acts as a
17 sensor cysteine for K1a/b-type compounds. We verified by MALDI-TOF MS analysis that the
18 K1b-type compound DEM has a binding specificity for mouse Keap1 Cys-151, but not Cys-273
19 (Fig. 4B; see Table S3 in the supplemental material). This observation not only indicates the
20 specificity of the analysis, but also supports our conclusion that Cys-151 is the sensor site for
21 K1b-type compounds.

22 Since MALDI-TOF MS pinpointed Cys-273 as the potential sensor site for K1a/b-type
23 compounds, we examined the effects of Cys-to-Ser mutations at Cys-273 (C273S) and also Cys-288
24 (C288S) in mouse Keap1 on Nrf2-repression and the response to compounds. Mutant

1 mKeap1C273S failed to suppress Nrf2-mediated *gstp1* expression when the amount of protein
2 overexpressed was similar to wild-type mouse Keap1 (Fig. 4C). This result is consistent with
3 previous reports demonstrating that a Cys-273 mutation reduces the Nrf2-repression activity of
4 mouse Keap1 in cultured cells (25, 43, 46) and in transgenic rescued mice (45). Basically identical
5 effects were seen with the C273S mutation and BC2 cysteine to tryptophan mutation (see Fig. 3G),
6 so we hypothesized that Cys-273 adducts with K1a/b-type compounds might disrupt the normal
7 structure of the Keap1-Cul3 complex, as in BC2 adducts with K1b-type compounds. The mouse
8 Keap1C288S mutation did not reduce Nrf2-repression (Fig. 4C), which was inconsistent with
9 previous reports (25, 43, 45, 46) perhaps due to differences in experimental conditions. Since
10 mKeap1C288S did not repress Nrf2 activity, we further analyzed its response to activators and
11 found that the C288S mutation did not affect responses to either DEM or 15d-PGJ₂ (Fig. 4D). This
12 suggested that mouse Keap1 Cys-288 might not be required for Nrf2-repression or response to K1b-
13 and K1a/b-type compounds, at least in this experimental condition. Though our present data do not
14 exactly deny the possibility that Cys-288 is a sensor site for K1a/b-type compounds, it is plausible
15 that Cys-273 is the major site for sensing such compounds.

16

17 **Isolation of a zebrafish mutant defective in responding to certain groups of**
18 **Nrf2-activating compounds.** Our present findings suggest that additional unknown factors regulate
19 the response to Nrf2-activating compounds. We therefore screened for mutant zebrafish with
20 defects in *gstp1* expression after treatment with or without the K1b-type compound DEM and
21 posthatch-type compound AF. Screening makes it possible to identify factors contributing to
22 regulation of the Keap1-Nrf2 system, for example factors related to the response to posthatch-type
23 compounds. In a large-scale genetic screen, we used induction of *gstp1* expression in 5-day old
24 larvae. We examined the F3 progeny of males mutagenized with ENU for changes in *gstp1*

1 expression. After screening about 800 F2 families, ten mutations were identified and confirmed in
2 the subsequent generation (Fig. 5A). Mutants were classified into 3 groups based on their
3 phenotypes (Fig. 5B): 1) DEM–AF– type mutants were defective in responding to both DEM and
4 AF, 2) DEM+AF– type mutants were defective in responding to AF, but not DEM and 3) Control+
5 type mutants showed basal *gstp1* expression in embryos without compound treatment. The only
6 mutant classed as DEM+AF– type was *it275*. Eleven Nrf2-activating compounds were tested for
7 induction of *gstp1* expression in *it275* larvae (Figs. 5C & S9 in the supplemental material). DEM,
8 H₂O₂, SF and D3T induced *gstp1* expression in *it275* larvae, while AF, 15d-PGJ₂, Ebs, CdCl₂,
9 tBHQ, 1,2-NQ and PGA₂ did not. This was unexpected since K1b-type compounds that are
10 supposed to bind directly to BC2 were divided into two classes. We speculated that the *it275* gene
11 product may play some roles in maintaining or enhancing electrophile activities. Although a
12 candidate gene for the *it275* mutation has not been identified, its gene locus was genetically mapped
13 to chromosome 24, where no known factors related to the Keap1-Nrf2 system exist (unpublished
14 results). Clarifying the roles and molecular nature of the *it275* gene product will provide new
15 insights into regulation of the Keap1-Nrf2 system.

16

17 **DISCUSSION**

18

19 In this paper, we propose a classification system for Nrf2-activating compounds based on the
20 combination of three mechanisms: 1) involvement of Keap1a and/or Keap1b, 2) requirement for
21 posthatch-specific factors and 3) *it275*-dependency. Our systematic approach classified
22 Nrf2-activating compounds into 6 classes (Fig. 6A) and indicated that Keap1-Nrf2 responds to
23 diverse chemical compounds via distinct molecular mechanisms constituting several molecular
24 cascades or networks. It was interesting to find that compounds with similar structures belong to the

1 same classes. For example, Class 2 compounds tBHQ and 1,2-NQ are quinone derivatives, Class 4
2 compounds 15d-PGJ₂ and PGA₂ are cyclopentenone prostaglandins and Class 6 compounds AF and
3 CdCl₂ contain heavy metals. Our classification therefore reflects some structural features of the
4 compounds. We assumed that compounds in the same class share target molecules and target
5 cysteine residues and thus show similar Nrf2-activating properties, such as dynamics of gene
6 induction, tissue and cell specificities and age-dependent regulation including larvae versus
7 embryos. Thus, it is important to identify the *it275* gene and posthatch factors to elucidate the
8 molecular bases of such classifications.

9 DEM and 15d-PGJ₂ are both Michael reaction acceptors, so the discovery that their sensor sites
10 in Keap1 are different was unexpected. We believe that the MS results are not *in vitro* artifacts for
11 two reasons. Firstly, the binding site for the K1b-type compounds DEM and 1,2-NQ was shown to
12 be Cys-151, while the K1a/b-type compounds 15d-PGJ₂ and PGA₂ bound to Cys-273 using the
13 same protein preparation (see Fig. 4B; unpublished results). Secondly, the Cys-to-Ser mutation in
14 Cys-151 disrupted the ability of mouse Keap1 to respond to DEM and other K1b-type compounds,
15 but not to 15d-PGJ₂ or PGA₂ (see Figs. 3E & S7 in the supplemental material). Instead, we
16 conjectured that the conformation formed by a cyclopentenone ring, double bonds, a carboxyl group
17 and other structures in 15d-PGJ₂ is suitable for interacting with Cys-273, while obstructing any
18 interaction with Cys-151. In other words, a strict intramolecular conformation of Nrf2-activating
19 compounds may be required for targeting Cys-273, but not Cys-151. Since 15d-PGJ₂ is an
20 endogenous Nrf2 activator, it is possible that other endogenous Nrf2-activating compounds, such as
21 8-nitro-cGMP (35) and 4-hydroxynonenal (10), target Cys-273 or other non-Cys-151 cysteines for
22 strict regulation. The target selection of a variety of Nrf2-activating compounds should be
23 investigated comprehensively in the future.

24 The biological effects of Nrf2-activating compounds are considerably diverse and range from

1 being therapeutic to toxic. It is hard to consider that Nrf2 activation solely contributes to all of these
2 effects. Instead, the interesting possibility exists that each compound reacts with specific target
3 proteins unrelated to the Keap1-Nrf2 system through specific cysteine residues. Indeed, the Class 2
4 compound 1,2-NQ was reported to target Cys-121 in protein tyrosine phosphatase 1B (PTP1B) (14).
5 Emitted as one of combustion products of fossil fuels and also formed in the atmosphere by
6 photochemical reactions of aromatic hydrocarbons, 1,2-NQ is considered to create a variety of
7 hazardous effects *in vivo*, including acute cytotoxicity, immunotoxicity and carcinogenesis.
8 Covalent attachment of 1,2-NQ to PTP1B reduced its activity and led to prolonged activation of
9 epidermal growth factor receptor, followed by abnormal cell proliferation. We speculated that
10 1,2-NQ forms adducts to Cys-151 in Keap1, to Cys-121 in PTP1B and to specific cysteines in other
11 proteins to instigate the cooperative induction of a variety of adverse health effects (Fig. 6B).
12 Deciphering the lineup of 1,2-NQ targets to understand its toxic effects on our health should prove
13 worthwhile. Similarly, the Class 4 compound 15d-PGJ₂ has been shown to react with some proteins
14 (19, 42), namely by covalent binding to specific cysteine residues identified as for example Cys-179
15 of IKK β (32), Cys-38 of NF κ B p65 (37), Cys-62 of NF κ B p50 (1), Cys269 of cJun (30) and
16 Cys-285 of PPAR γ (36). Protein functions are inhibited or activated by modification of these
17 cysteines by 15d-PGJ₂. It has been suggested that 15d-PGJ₂, a naturally occurring derivative of
18 prostaglandin D₂, exerts anti-inflammatory effects *in vivo* (19, 42). The carrageenan-induced
19 pleurisy model of acute lung injury was used to demonstrate the relationship between Nrf2
20 activation and anti-inflammation and that accumulation of 15d-PGJ₂ mediates this relation (11). In
21 the same manner, inhibition or activation of other target proteins are all related to anti-inflammation.
22 We therefore assumed that cooperative adducts of 15d-PGJ₂ to specific cysteines in proteins
23 including Keap1 are quite important for establishing anti-inflammatory states (Fig. 6B). As well as
24 in anti-inflammation, Nrf2-activating compounds have a variety of other clinical applications, such

1 as in cancer chemoprevention (SF) (17, 28, 40) and anti-rheumatism (AF) (15, 16). We now propose
2 a “cysteine code” hypothesis that converts a set of cysteine modifications into specific biological
3 effects. Breaking the cysteine code for each Nrf2-activating compound will serve to increase
4 understanding of its therapeutic and/or toxic effects. Please note that 1,2-NQ, 15d-PGJ₂, SF and AF
5 were grouped into different classes in our classification. We presume that compounds in the same
6 class share similar “cysteine codes” that lead to analogous biological effects.

7

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9

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18

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1 **FIGURE LEGENDS**

2

3 FIG 1. Induction of zebrafish *gstp1* by a variety of Nrf2 activators. (A) Structural representation
4 of Nrf2-activating compounds. (B) Induction of *gstp1* expression in adult gills was analyzed by
5 RT-PCR after 6 hours treatment with compounds. The expression of *eflα* was used to standardize
6 the amount of cDNA. (C) Experimental scheme for zebrafish Nrf2 gene knockdown. (D) Induction
7 of *gstp1* expression in 4-day old larvae. Strong *gstp1* induction was observed in larvae treated with
8 all three compounds, especially in the gills (red arrowheads). It was greatly reduced when *nrf2*MO
9 was injected at the embryonic stage (blue arrowheads).

10

11 FIG 2. Chemical-specific induction of *gstp1* in zebrafish embryos. (A) Experimental scheme for
12 *gstp1* induction in zebrafish embryos. (B) Induction of *gstp1* expression in 8-hour old embryos.
13 Nrf2 and Keap1 mRNAs were co-injected into embryos at the one-cell stage. After 2 hours,
14 embryos were treated with the compounds indicated for 6 hours. The expression of *gstp1* was
15 analyzed by whole-mount *in situ* hybridization. (C) Structure of Nrf2-GFP fusion protein. The
16 N-terminal half of Nrf2 protein and enhanced GFP (eGFP) protein were connected by two copies of
17 SV40 nuclear localizing signal (NLS). (D) Expression analysis of Nrf2-GFP fusion protein.
18 Nrf2NTnGFP mRNA was injected with or without mRNA encoding Keap1 proteins into one-cell
19 stage embryos. After 2 hours, embryos were treated with the compounds indicated for 6 hours and
20 GFP expression was analyzed.

21

22 FIG 3. Identification of a sensor cysteine for the K1b-type compounds. Induction of *gstp1* was
23 carried out as shown in Fig. 2A and its expression was analyzed by RT-PCR. FLAG-tagged Keap1
24 proteins were used instead of non-tagged proteins. The amount of mRNA was normalized to that of

1 Keap1 protein with a comparable activity in repressing Nrf2-induced *gstp1* expression in uninduced
2 conditions, except for in (G). The expression of *eflα* was used to standardize the amount of cDNA.
3 (A) Keap1a-Keap1b chimeric proteins used in the analysis. (B) DEM-induced expression of *gstp1*
4 in embryos overexpressing chimeric proteins. Note that *gstp1* induction was observed in embryos
5 overexpressing C1, but not C2 or C3 proteins. (C) Alignments of 3 cysteine residues and their
6 adjacent amino acid residues in the BTB domains of various Keap1 proteins. (D) DEM-induced
7 expression of *gstp1* in embryos overexpressing Keap1bK124T and Keap1bC125S. (E)
8 Electrophile-induced expression of *gstp1* and *γgcsH* in embryos overexpressing mouse Keap1 and
9 its C151S mutation. (F) Similarity between a Cys to Trp mutation and covalent binding of
10 Nrf2-activating compounds to Cys. (G) DEM-induced expression of *gstp1* in embryos
11 overexpressing Keap1bC125W. The amount of Keap1 mRNA was standardized by the expression
12 level of FLAG-tagged Keap1 protein analyzed by immunoblotting using anti-FLAG antibody.

13
14 FIG. 4. Identification of sensor cysteines for the K1a/b-type compounds. (A) MS analysis of
15 peptides from mouse Keap1 digested with trypsin following incubation with (bottom) or without
16 (top) 15d-PGJ₂. Keap1 protein (10 μg) was incubated at 25°C for 60 min in the absence (top) or
17 presence (bottom) of 100 μM 15d-PGJ₂ in a total volume of 10 μl containing 20 mM Tris-HCl,
18 pH8.5. (B) Modification sites in mouse Keap1 for Nrf2-activating compounds were determined by
19 MS analysis. (C) Nrf2-induced expression of *gstp1* in embryos overexpressing mKeap1C273S or
20 mKeap1C288S. The amount of Keap1 mRNA was standardized by the expression level of
21 FLAG-tagged Keap1 protein analyzed by immunoblotting using anti-FLAG antibody. (D)
22 Electrophile-induced expression of *gstp1* in embryos overexpressing mouse Keap1 and its C288S
23 mutation.

24

1 FIG. 5. Screen for zebrafish mutants with abnormal *gstp1* expression. (A) Summary of zebrafish
2 mutagenesis. (B) Phenotypic classes of mutations isolated. (C) Chemical-specific *gstp1* induction in
3 *it275* larvae 4 days after fertilization. The strong *gstp1* induction seen in the gills of wild-type fish
4 (red arrowheads) treated with certain Nrf2-activating compounds was greatly reduced in *it275*
5 larvae (blue arrowheads).

6
7 FIG. 6. Hypothetical models. (A) A cascade-based classification of Nrf2-activating compounds.
8 Chemical compounds belonging to Class 3 have not been identified. (B) A cysteine code hypothesis.
9 Electrophilic compounds that target different sets of cysteines may produce distinct biological
10 effects.