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Validation of the Multiple Sensor Mechanism of the Keap1-Nrf2 System

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Abstract

The Keap1-Nrf2 system plays a critical role in cellular defense against electrophiles and reactive oxygen species. Keap1 possesses a number of cysteine residues, some of which are highly reactive and serves as sensors for these insults. Indeed, point mutation of Cys151 abrogates the response to certain electrophiles. However, this mutation does not affect the other set of electrophiles, suggesting that multiple sensor systems reside within the cysteine residues of Keap1. Precise contribution of each reactive cysteine to the sensor function of Keap1 remains to be clarified. To elucidate the contribution of Cys151 *in vivo*, in this study we adopted transgenic complementation rescue assays. Embryonic fibroblasts and primary peritoneal macrophages were prepared from mice expressing the Keap1-C151S mutant. These cells were challenged with various Nrf2 inducers. We found that some of the inducers triggered only marginal responses in Keap1-C151S-expressing cells, while others evoked responses in a comparable magnitude to those observed in the wild-type cells. We found that *tert*-butyl hydroquinone, diethylmaleate, sulforaphane and dimethylfumarate were Cys151-preferable, whereas 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PG-J₂), 2-cyano-3,12 dioxooleana-1,9 diene-28-imidazolide (CDDO-Im), ebselen, nitro-oleic acid and cadmium chloride were Cys151-independent. Experiments with embryonic fibroblasts and primary macrophages yielded consistent results. Experiments testing protective effects against the cytotoxicity of 1-chloro-2,4-dinitrobenzene of sulforaphane and 15d-PG-J₂ in Keap1-C151S-expressing macrophages revealed that the former inducer was effective, while the latter was not. These results thus indicate that there exists distinct utilization of Keap1 cysteine residues by different chemicals that trigger the response of the Keap1-Nrf2 system, and further substantiate the notion that there are multiple sensing mechanisms within Keap1 cysteine residues.

Keywords

Keap1; Nrf2; reactive cysteine; stress response

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Introduction

The transcription factor Nrf2 (NF-E2-related factor 2) plays an important role in cellular defense against electrophilic and oxidative insults [1,2]. Under unstressed conditions, the cellular level of Nrf2 is maintained at a low level, as Keap1 (Kelch-like ECH-associated protein 1), an adaptor component of Cul3 (Cullin 3)-based ubiquitin E3 ligase complex, promotes ubiquitination and proteasomal degradation of Nrf2. Upon exposure to electrophiles or reactive oxygen species, the ubiquitination of Nrf2 ceases, leading to the stabilization and nuclear translocation/ accumulation of Nrf2 and the subsequent induction of Nrf2 target genes [1]. A variety of Nrf2 inducers have been reported, most of which are electrophilic and readily react with cysteine thiols [3]. Keap1 is a cysteine-rich protein possessing 27 and 25 cysteine residues in the human and mouse proteins, respectively. A number of studies detected covalent modifications of Keap1 cysteine residues following exposure to electrophiles using *in vitro* labeling and mass spectrometry [4–13]. The functional significance of these cysteine residues has been examined in several experimental systems exploiting site-directed mutagenesis of Keap1. The function of mutant Keap1 molecules has been tested by transfection into culture cell lines (*in transfecto*) or by ectopic overexpression in zebrafish embryos [8,9,11,14–17]. We also established the significance of three cysteine residues, *i.e.*, Cys151, 273 and 288, for the regulation of Nrf2 activity using a transgenic approach [18].

Of the Nrf2-inducing chemicals, *tert*-butylhydroquinone (tBHQ) is readily auto-oxidized to the electrophilic metabolite *tert*-butylbenzoquinone and modifies the cysteine residues of Keap1 [9,13]. Because cells and mice exclusively expressing the Keap1-C151S mutant showed decreased accumulation of Nrf2 in response to tBHQ [8,14,18], Cys151 has been assumed critical for the Keap1 sensor activity against tBHQ. It has also been suggested that diethylmaleate (DEM), ebselen, dimethylfumarate (DMF), sulforaphane (SFN) and 2-cyano-3,12 dioxooleana-1,9 diene-28-imidazolide (CDDO-Im) require Cys151 to trigger the Keap1-Nrf2 response *in vitro* or *in transfecto* [8,14,17,19,20], although several conflicting results have also been reported [5,12].

On the other hand, the presence of Cys151-independent Nrf2 inducers has also been recognized. For instance, the cells or zebrafish embryos expressing the Keap1-C151S mutant responded to 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) at comparable levels to that of wild-type cells or zebrafish embryos. Furthermore, exposure of Keap1 to 15d-PGJ₂ resulted in covalent modification of Cys273 but not Cys151 [8]. Similarly, heavy metals, such as cadmium chloride (CdCl₂) and arsenic compounds activated Nrf2 signaling in a Cys151-independent manner [9,16]. These observations suggest that the modification of distinct cysteine residues results in a common downstream consequence, namely Keap1 halts to ubiquitinate Nrf2. Thus, we surmise that there are multiple sensor mechanisms within the cysteine residues of Keap1. However, critical target cysteine residues have not been identified for each compound *in vivo*, so that precise contribution of each reactive cysteine residue to the sensor function of Keap1 remains to be clarified.

One of the critical parameters affecting the responsiveness of the Keap1-Nrf2 system is the expression ratio of Keap1 and Nrf2 proteins. Different ratios produced in overexpression experiments *in transfecto* gives rise to different outcomes. Indeed, as for the necessity of the BTB (broad complex-tramtrack-bric a brac) domain of Keap1, it has been difficult to draw a conclusion based on overexpression experiments. In this regard we recently developed a transgenic complementation rescue assay in which the expression levels of Keap1 and Nrf2 were regulated to within a range comparable to that in wild-type mice, and demonstrated the necessity of the BTB domain *in vivo* [18].

In order to rigorously verify validity of the multiple cysteine sensor system for the Keap1-Nrf2 system, in this study we systematically and comprehensively examined the responsiveness of the C151S mutant of Keap1 against a variety of Nrf2 inducers exploiting the transgenic complementation rescue approach. The results demonstrated the presence of Cys151-preferable and Cys151-independent inducers of Nrf2 *in vivo*, indicating that various chemical inducers utilize reactive Keap1 cysteine residues distinctly. This study has established a basis for the analysis of *in vivo* function of Keap1 and Nrf2 inducers.

Materials and methods

Chemical reagents

DEM, CdCl₂ and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Wako chemicals, and tBHQ and ebselen were purchased from Sigma Aldrich. MG132, SFN, 15d-PGJ₂ and DMF were purchased from Peptide Institute Inc., LKT Laboratories, Cayman Chemicals and Tokyo Chemical Industry, respectively. CDDO-Im and 9- and 10-nitro-octadec-9-enoic acids (OA-NO₂) were kind gifts from REATA Co. and Bruce Freeman, respectively.

Transfection and luciferase assay

Transfection experiments were performed using X-tremeGENE 9 (Roche). The luciferase assay was performed using the Dual-Luciferase reporter system (Promega). After culturing in 24-well plates, HEK293 cells were transfected with 100 ng pNqo1-ARE-Luc plasmid, 10 ng pRL-TK transfection control plasmid, 40 ng p3xFlag-Nrf2 plasmid [21] and 40 or 200 ng wild-type or C151S Keap1 expression plasmid [18]. The total amount of DNA was maintained at 400 ng with empty expression plasmid. Luciferase activity was measured 36 hours after transfection. The transfected cells were exposed to 50 μM tBHQ for 12 hours prior to the analysis of luciferase activity.

Mouse embryonic fibroblasts (MEFs)

Transgenic mice expressing wild-type Keap1 or the Keap1-C151S mutant were generated as described previously [18]. Transgenic mice were crossed into the *Keap1*-null background to obtain compound (*Keap1*^{-/-::Tg}) mice. MEFs were prepared from individual embryos at embryonic day 13.5 (E13.5). The head and internal organs were removed, and the torso was minced and dispersed in 0.25% trypsin-EDTA. MEFs were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical) containing 10% fetal bovine serum and penicillin-streptomycin (10 units/0.1 mg/ml), at 37°C and 5% CO₂. MEFs at less than 10 passages were used for the analyses.

Immunoblot analyses

The cells were lysed in buffer A (20 mM HEPES-KOH (pH 8.0), 10-mM potassium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, 100 μM PMSF, 10% glycerol, protease inhibitor cocktail (Roche) and 0.025% NP-40). After cell centrifugation, the supernatants were saved as the cytoplasmic fractions, and the nucleus pellets were lysed in Laemmli sample buffer. The samples were subjected to immunoblot analysis using anti-Nrf2 [22], anti-Keap1 [23], anti-Lamin B (a nuclear fraction marker; Invitrogen) and anti-Tubulin (a cytoplasmic fraction marker; Sigma) antibodies. Quantitative measurement of immunoreactive bands was performed by densitometry analyses and Nrf2 protein level was normalized against Lamin B. All experiments were performed three times and data are expressed as means ± SE. The unpaired t-test was used and differences between groups were considered statistically significant if *p* < 0.05.

RNA extraction and quantitative real-time PCR

Total RNA was prepared from MEFs or macrophages using an Isogen RNA extraction kit (Nippon Gene). The cDNAs were synthesized from the isolated RNAs by Superscript III (Invitrogen). Real-time quantitative PCR was performed using ABI 7300 (Applied Biosystems). The primer and probe sequences used for detecting *Nqo1* and glutamate-cysteine ligase catalytic subunit (*Gclc*) were described previously [24].

Isolation of thioglycollate-elicited peritoneal macrophages

Macrophages were isolated by lavage from mice that had received a 2-ml intraperitoneal injection of 4% thioglycollate broth 4 days before. The cells were transferred onto a 6 cm dish at a density of 5×10^5 cells/ml for immunoblot analyses or RNA extraction, or onto a 96-well dish at a density of 1×10^5 cells/ml for cell viability assays. The macrophages were maintained in RPMI 1640 medium containing 10% FBS and penicillin-streptomycin (10 units/0.1 mg/ml).

Determination of glutathione (GSH) levels

Intracellular glutathione (GSH) level was determined using a GSH-Glo glutathione assay (Promega).

Results

Cys151 is critical sensor for responding to tBHQ

Importance of the Cys151 residue of Keap1 as a sensor has been recognized based on transfection overexpression experiments introducing the C151S mutant of Keap1 into culture cells. However, the derepression effects induced by tBHQ were clearly different for wild-type Keap1 and the Keap1-C151S mutant only when a suitable dose ratio between Nrf2 and Keap1 was adopted. The robust induction of the reporter gene activity by tBHQ was observed when 40 ng of Keap1 expression vector was applied, but not when 200 ng of plasmid was applied (Fig. 1A). Moreover, tBHQ-mediated induction of reporter activity was observed in the absence of exogenous Keap1 (Fig. 1A), suggesting that endogenous Keap1 also affected the reporter activity. Thus, overexpression experiments *in transfecto* can be misleading due to the arbitrary expression levels of Keap1 mutant molecules and interference from endogenous Keap1. A refined evaluation system was required to clarify the Keap1 function *in vivo*.

To develop a more stable system for examining the response of the Keap1-Nrf2 system, we decided to employ the transgenic complementation rescue assays [18]. Transgenic mice expressing wild-type Keap1 or the Keap1-C151S mutant under the regulation of the *Keap1* gene regulatory domain (KRD) were crossed into a *Keap1*-null background. In this study we obtained the following compound mutant mice: *Keap1*^{-/-}::*Tg-Keap1*^{WT} and *Keap1*^{-/-}::*Tg-Keap1*^{C151S}. Mouse embryonic fibroblasts (MEFs) derived from *Keap1*^{-/-}::*Tg-Keap1*^{WT} and *Keap1*^{-/-}::*Tg-Keap1*^{C151S} mice were prepared and referred to as Keap1-MEF and C151S-MEF, respectively (Fig. 1B). The protein expression levels of transgene-derived wild-type Keap1 (Line 34) and Keap1-C151S (Line 18) were comparable to those of endogenous Keap1 (Fig. 1C). Incubation with the proteasome inhibitor MG132 for three hours caused an accumulation of Nrf2 protein at a similar level between wild-type, Keap1-MEF and C151S-MEF cells, suggesting that the protein level of Nrf2 degraded via the proteasome was also comparable between the three genotypes of mice (Fig. 1C). Keap1 was hardly degraded by the proteasome (Fig. 1C and our unpublished observation).

We previously found that following treatment with 50-μM tBHQ, C151S-MEFs showed impaired Nrf2 activation compared with Keap1-MEFs [18]. In this study, we newly

prepared Keap1-MEFs and C151S-MEFs from distinct lines of transgenic rescue mouse (i.e., *Keap1*^{-/-}::*Tg-Keap1*^{WT} and *Keap1*^{-/-}::*Tg-Keap1*^{C151S}). We found that consistent with the previous finding nuclear accumulation of Nrf2 following 30- or 100-μM tBHQ treatment was markedly decreased in C151S-MEFs compared with Keap1-MEFs (Fig. 1D). When 100-μM tBHQ was added to the medium, a 22-fold increase over baseline of nuclear Nrf2 protein was observed in Keap1-MEFs (closed circle), whereas only a 6-fold increase was observed in C151S-MEFs (open circle) (Fig. 1E). Upon treatment with 100-μM tBHQ, *Nqo1*, a prototype Nrf2 target gene, was induced at a level 35-fold higher than that under the basal conditions in Keap1-MEFs, whereas only a 10-fold increase was observed in C151S-MEFs (Fig. 1F). Another Nrf2 target gene, *Gclc*, was induced at a level 8-fold higher than that under the basal conditions in Keap1-MEFs by 100-μM tBHQ, whereas only a three-fold increase was observed in C151S-MEFs (Fig. 1G). MEFs from *Nrf2*^{-/-} mouse did not show any response to tBHQ (closed triangle in Fig. 1F and G).

We normalized the results by using *18S ribosomal subunit* (Fig. 1F and G) and also with *Hprt* (Supplementary Fig. 1A and B). Both results showed good agreement, indicating the reproducibility of the analyses. These results demonstrate that the C151S-MEF system is suitable for the evaluation of Cys151 function and Cys151 is indeed a sensor residue of Keap1 in the response to tBHQ. However, it should also be noted that there is a modest accumulation of Nrf2 and detectable activation of *Nqo1* and *Gclc* genes in the C151S-MEFs in response to tBHQ and this is in stark contrast to the response of *Nrf2*^{-/-} MEFs. The latter results suggest that other functional sensor cysteine(s) may reside within Keap1.

DEM and SFN are Cys151-preferable Nrf2 inducers

We next examined other Nrf2 inducers utilizing the C151S-MEF system. First, we tested DEM. DEM was previously found not to activate Nrf2 in zebrafish embryos ectopically expressing Keap1-C151S [8]. As was the case for tBHQ, upon addition of 100-μM DEM to the culture, nuclear Nrf2 was increased more than 18-fold over baseline in Keap1-MEFs, whereas only a 6-fold increase of nuclear Nrf2 was observed in C151S-MEFs (Fig. 2A and B). Expression of *Nqo1* and *Gclc* were increased 31- and 27-fold in Keap1-MEFs, but increased only 6- and 8-fold in C151S-MEFs, respectively (Fig. 2C and D). These results thus indicate that Cys151 of Keap1 is essential for DEM-mediated activation of Nrf2 signaling.

Although Cys151 appears to be a likely target of SFN [8,9,14,24], there still remains controversy as to which cysteine residues of Keap1 are modified by SFN [5,12]. We therefore adopted the C151S-MEF system to elucidate this issue. When exposed to 10-μM SFN, Keap1-MEFs showed a 35-fold increase in Nrf2 protein levels compared with basal levels, but C151S-MEFs showed only a 7-fold increase (Fig. 2E and F). The *Nqo1* and *Gclc* gene expression was 27-fold and 20-fold higher in Keap1-MEFs, respectively, but these genes were increased only 8- and 6-fold in C151S-MEFs, respectively (Fig. 2G and H). These results support the contention that Keap1 Cys151 is essential for the SFN-mediated activation of Nrf2 signaling.

15d-PGJ₂ and ebselen are Cys151-independent inducers

We then examined how 15d-PGJ₂ induces Nrf2 and its target genes in the C151S-MEF system. Although 15d-PGJ₂ is reported to modify Cys273 [8], another report shows that

Supplementary Fig. 1

(A and B) Keap1-MEF (closed circle), C151S-MEF (open circle) and *Nrf2*^{-/-}-MEF (triangle) were incubated with 0-, 30- and 100-μM tBHQ for 12 hours and the expression levels of *Nqo1* (A) and *Gclc* (B) were examined by RT-qPCR with the *Hprt* as internal control. The representative results were shown from multiple independent experiments.

Nrf2 induction by 15d-PGJ₂ is reduced in the presence of Keap1-C151S [25]. When exposed to 15d-PGJ₂, Keap1-MEFs and C151S-MEFs showed comparable levels of nuclear Nrf2 accumulation (Fig. 3A and B). Consistent with the Nrf2 level, *Nqo1* and *Gclc* gene expression was similar in Keap1-MEFs and C151S-MEFs following the 15d-PGJ₂-treatment (Fig. 3C and D). It should be noted that the induction of *Nqo1* and *Gclc* gene expression by 15d-PGJ₂ was Nrf2-preferable, as 15d-PGJ₂ was not affect the expression of these genes in *Nrf2*^{-/-} MEFs. These results clearly demonstrate that Cys151 is dispensable for the full activation of Nrf2 signaling in response to 15d-PGJ₂.

Ebselen is an Nrf2 inducer that has been speculated to be Cys151-preferable. One previous study showed that ebselen failed to activate the *Gstp1* and *Gsta1* genes in rat hepatoma cells overexpressing Keap1-C151S [19]. Another study reported that Nrf2 activation by ebselen did not occur in zebrafish embryos that ectopically expressed Keap1-C151S [8]. However, when Keap1-MEFs and C151S-MEFs were exposed to ebselen, nuclear accumulation of Nrf2 was comparable in both cells (Fig. 3E and F). Consistent with the Nrf2 level, ebselen induced expression of *Nqo1* and *Gclc* genes in C151S-MEFs equally to that in Keap1-MEFs (Fig. 3G and H). Thus, contrary to our original expectation, Cys151 was found to be dispensable for activation of Nrf2 signaling in response to ebselen.

Application of the C151S-MEF system for the assessment of Nrf2 inducers

We then applied the C151S-MEF system to other Nrf2 inducers of biological interest. We first analyzed DMF, which has attracted attention due to its use in patients with relapsing-remitting multiple sclerosis. DMF has been shown to be both safe and highly efficacious in the population [29]. Nrf2 appears to mediate the neuroprotective effect of DMF [20]. Based on the *in vitro* Keap1-DMF binding assay [20], it has been assumed that DMF activates the Nrf2 signaling in a Cys151-preferable manner. We found that upon DMF treatment, nuclear Nrf2 levels were robustly increased in Keap1-MEFs, whereas they were increased only marginally in C151S-MEFs (Fig. 4A and B). This result indicates that DMF is a Cys151-preferable inducer of Nrf2.

CDDO-Im and its derivatives are another examples of clinically potent Nrf2 inducers. CDDO-Im is considered to be a promising therapeutic agent for the alleviation of various pathological conditions [26–29]. Activation of Nrf2 signaling by CDDO-Im [25] and dihydro-CDDO-trifluoroethyl amide [31] was attenuated in cells overexpressing Keap1-C151S, suggesting that CDDO-Im is Cys151-preferable. However, C151S-MEFs responded to CDDO-Im, and Nrf2 protein levels in C151S-MEFs increased to levels similar to Keap1-MEFs (Fig. 4C and D). CDDO-Im induced expressions of *Nqo1* and *Gclc* genes in C151S-MEFs equally to those in Keap1-MEFs (Supplementary Fig. 2A and B). These results indicate that CDDO-Im is a Cys151-independent Nrf2 inducer.

Heavy metals, including cadmium, are also known to activate Nrf2 response, but the precise mechanism remains to be elucidated. CdCl₂ has been shown to activate Nrf2 signaling by modulating the zinc binding capacity of Keap1 [32]. Overexpression experiments in cell lines and in zebrafish embryos showed that Cys151 is not required for Keap1 to respond to CdCl₂ [8,9]. Indeed, C151S-MEFs were able to respond to CdCl₂. Nrf2 protein levels in C151S-MEFs were rather significantly increased compared to those in Keap1-MEFs (Fig. 4E and F), indicating that Cys151 is not required, but the lack of Cys151 rather stimulates the Nrf2 activation by CdCl₂.

Supplementary Fig. 2

(A and B) Keap1-MEF (closed circle), C151S-MEF (open circle) and *Nrf2*^{-/-}-MEF (triangle) were incubated with 0, 30 and 100 nM CDDO-Im for 12 hours and the expression levels of *Nqo1* (A) and *Gclc* (B) were examined by RT-qPCR with the 18S ribosomal subunit as internal control. The representative results were shown from multiple independent experiments.

Finally, nitro-fatty acids are electrophilic signaling mediators formed via nitric oxide and nitrite-preferable reactions [33]. Previous reports have indicated that 9- and 10-nitro-octadec-9-enoic acid (OA-NO₂) modifies Keap1 cysteine residues other than Cys151 [10,11]. We found that C151S-MEFs responded to OA-NO₂. As was the case for the cadmium chloride, Nrf2 protein levels were also increased in C151S-MEFs compared with those in Keap1-MEFs, albeit the increase was not statistically significant (Fig. 4G and H). These results thus indicate that Cys151 is dispensable for the Nrf2 activation by nitro-fatty acids OA-NO₂.

Utilization of Keap1 Cys151 in macrophages

To further verify the physiological importance of the Cys151-mediated sensing mechanism, we adopted thioglycollate-elicited peritoneal macrophages prepared from the Keap1 compound mutant mice as an experimental system, as many studies have revealed critical roles for the Keap1-Nrf2 system in the inflammation process, including emphysema [34,35], sepsis [36,37] and atherosclerosis [38–40]. Thioglycollate-elicited peritoneal macrophages were collected from *Keap1*^{-/-}::*Tg-Keap1*_{WT} (Line 34) and *Keap1*^{-/-}::*Tg-Keap1*_{C151S} (Line 18) mice, which were referred to as Keap1-Mφ and C151S-Mφ, respectively (Fig. 5A). Nrf2 accumulation upon treatment with electrophiles was analyzed in Keap1-Mφ and C151S-Mφ. Keap1 protein levels in Keap1-Mφ and C151S-Mφ were comparable to that of endogenous Keap1 (Fig. 5B). Nrf2 protein levels following treatment with MG132 were also comparable to those observed in peritoneal macrophages prepared from wild-type mice (Fig. 5B).

In agreement with the results obtained from MEFs, C151S-Mφ showed reduced accumulation of Nrf2 in response to DEM or SFN (Fig. 5C and D, Supplementary Fig. 3A), but Nrf2 levels did not change upon treatment with 15d-PGJ₂ or ebselen (Fig. 5E and F, Supplementary Fig. 3B). A time-course study revealed that treatment with 100-μM DEM immediately increased the level of Nrf2 protein in Keap1-Mφ. Nrf2 protein remained elevated for three hours and then gradually decreased to basal levels. However, the same dose of DEM did not increase the level of Nrf2 protein in C151S-Mφ (Fig. 5G). Thus, the reduced accumulation of Nrf2 protein following DEM treatment in the dose-response study was not due to delayed Nrf2 accumulation. The substitution of Cys151 to serine affected neither the Nrf2 accumulation triggered by 15d-PGJ₂ in the dose study nor in the time-course study (Fig. 5E and H).

SFN enhances resistance to oxidative stress via a Cys151-preferable mechanism

Finally, we examined whether the loss of Cys151 in Keap1 would shut off specific electrophilic input to the Keap1-Nrf2 system and whether the cytoprotective activity primed by the signal would be impaired. To examine the effect of Cys151-deficiency on cellular antioxidant activity, Keap1-Mφ and C151S-Mφ were pretreated 24 hours after the addition of DMF and CDDO-Im to the medium, which are Cys151-preferable and -independent Nrf2 inducers, respectively. We found that 1- and 3-μM DMF treatments significantly enhanced intracellular GSH level in Keap1-Mφ but not in C151S-Mφ (Fig. 6A), suggesting that the activation of Nrf2 by DMF via Cys151 indeed increased the intracellular GSH level.

Both types of cells were then incubated for an additional 30 minutes with 1-μM CDNB [39], a strong oxidant and GSH depletor. The CDNB treatment markedly decreased the intracellular GSH level. However, consistent with the Cys151-preference, pretreatment with DMF relatively sustained the intracellular GSH level in Keap1-Mφ but this did not occur in

Supplementary Fig. 3

(A and B) Graphical representations of the results by Western blot shown in Fig. 5C and F. n=3; asterisks indicate statistically significant differences (p < 0.05).

C151S-M ϕ In contrast, CDDO-Im treatment increased the intracellular GSH levels both in Keap1-M ϕ and C151S-M ϕ , and the level was in C151S-M ϕ higher than that in Keap1-M ϕ . Similarly, in presence of CDNB pretreatment of CDDO-Im relatively sustained the GSH level in both type of cells (Fig. 6D). These results thus support the contention that there are multiple discrete sensor mechanisms for Nrf2 induction on Keap1 and DMF and CDDO-Im indeed utilizes distinct sensor pathways.

Discussion

In this study, we examined contributions of the Keap1 Cys151 residue to cytoprotection *in vivo*. The contributions were monitored via Nrf2 accumulation and expression of its target genes. The results allowed us to unequivocally categorize Nrf2 inducers into at least two classes: Cys151-preferable inducers and Cys151-independent inducers. We found that tBHQ, DEM and DMF are in the Cys151-preferable class, whereas 15d-PGJ₂, CdCl₂ and OA-NO₂ are in the Cys151-independent class. These results are in close agreement with previous experiments utilizing *in vitro* binding analyses or transfection overexpression analyses.

One salient aspect of the present study is that our *in vivo* evaluation system has settled a discrepancy pertaining to the Cys151-dependency of ebselen, CDDO-Im and SFN. Ebselen and CDDO-Im have been reported to be Cys151-preferable [17,19]. However, in the present study, we found that both ebselen and CDDO-Im act to induce Nrf2 in a Cys151-independent manner. We do not have solid explanation for this discrepancy in the Cys151-preference of CDDO-Im and ebselen, and this issue remains to be clarified. One plausible explanation for the discrepancy may be that we have used mouse Keap1 MEFs and macrophages, while the preceding studies have used human Keap1 *in transfecto*. We surmise that the difference in the experimental system or difference in human and mouse Keap1 might be responsible to this discrepancy. In contrast, the Cys151-dependency of SFN has not been supported by mass spectrometry, perhaps due to the highly unstable state of cysteine-sulforaphane adducts [5,12]. However, the present study provides convincing evidence that SFN-mediated activation of Nrf2 signaling is Cys151-preferable.

Importantly, the classifications deduced from experiments utilizing MEFs were reproducible in experiments utilizing peritoneal macrophages. Our results further revealed that the cytoprotective effect of SFN was indeed Cys151-preferable, while that of 15d-PGJ₂ was Cys151-independent. These results establish the fact that multiple distinct sensor mechanisms in the Keap1-Nrf2 system are in operation under physiological conditions.

One interesting observation is that CdCl₂ activates Nrf2 in C151S-MEFs to a significantly higher level than in Keap1-MEFs, and OA-NO₂ also shows similar tendency. This phenomenon has not been reported before. The hyper-response of C151S MEFs to CdCl₂ and OA-NO₂ is observed exclusively in the stable evaluation system employed in this study, but has never been observed in the study using transfection overexpression assay. Although the reason why the hyper-response of C151S MEFs in terms of Nrf2 activation occurs remains unknown, one plausible explanation is that loss of Cys151 residue may cause compensatory enhancement of other sensing activity of Keap1, suggesting that Cys151 may act as a sensor, but also as a modulator of the Nrf2 activation in response to certain type of Nrf2 inducers.

It is noteworthy that the input of signals from the Cys151-preferable class of Nrf2 inducers is not restricted exclusively to the Cys151 pathway, but that these Nrf2 inducers did stabilize Nrf2 and activate target genes in C151S-MEFs and C151S-M ϕ , although the response is modest and limited. The contribution of endogenous Keap1 can be ignored in the transgenic

complementation rescue approach, as there is no wild-type Keap1 in C151S-MEFs and C151S-M ϕ , we surmise that the Cys151-preferable inducers attack other cysteine residues to transduce their signal.

We could not find any common structural features in the Cys151-preferable inducers. In this regard, this study has revealed two interesting features. DEM and DMF, which both belong to the Cys151-preferable class of inducers, share a similar structural motif. Additionally, the molecular structures of 15d-PGJ₂ and OA-NO₂, which both belong to the Cys151-independent class of inducers, also resemble each other. These observations suggest that the molecular structure of each Nrf2 inducer may be one of the determinants for the preference of target cysteine residues in the Keap1 molecule.

An important question that remains is how Nrf2 degradation is stopped upon exposure to the Cys151-preferable class of Nrf2 inducers. As a response mechanism dependent on Cys151, disruption of the Keap1-Cul3 interaction has been proposed, based on the observation that Cul3 associates with the N-terminal region of Keap1, including the BTB domain [45]. This model is compatible with the fact that Cys151 is located in the BTB domain of Keap1. Substitution of Cys151 with the bulky amino acid tryptophan, which mimics the cysteine residue conjugated with electrophiles, abrogates the ability to suppress Nrf2 activity [25]. A similar result was obtained in an experiment using zebrafish [8]. However, substitution of Cys151 with the small hydrophobic amino acid alanine never disrupts the Keap1-Cul3 interaction but does inhibit the degradation of Nrf2 [9]. These observations imply that the Cys151-mediated stress response mechanism cannot be explained solely by the disruption of the Keap1-Cul3 interaction.

Cys273 and Cys288 have been suggested as alternative sensor residues of Keap1 responsible for the 15d-PGJ₂- and OA-NO₂-mediated signals, based on analysis of modification sites using mass spectrometry [8,9,11]. Single particle electron microscopy demonstrated that the overall structure of the Keap1 dimer resembles a “cherry-bob,” in that two globular structures are connected with a stem-like structure. The globular domains are mostly composed of an intervening region (IVR), which is where Cys273 and Cys288 are located, and a double glycine repeat or Kelch plus C-terminal (DC) domain. The proximity between the IVR and the DC domain suggest that modification of Cys273 and Cys288 in the IVR is conveyed as a conformational distortion to the DC domain where Nrf2 binds [42,46]. These observations imply that a conformational change in Keap1 would be expected to disrupt or distort the Keap1-Nrf2 interaction resulting in Nrf2 stabilization. This mechanism has been proposed as the Hinge and Latch mechanism [42].

His225, Cys226 and Cys613 residues are reported to be zinc sensors [9] and these residues are also expected to lie proximal to Cys273 and Cys288. This zinc sensing mechanism may be utilized similarly to the Cys273 and Cys288-based sensor. This model is consistent with the results of an immunoprecipitation assay that found that mutations of Cys273 and Cys288 do not affect the interaction between Keap1 and Nrf2 [47] or between Keap1 and Cul3 [48].

While the mechanisms responsible for detecting and transducing environmental stressors within the body need further analysis, the results of the present study strongly argue that Keap1 comprises multiple sensor residues that detect various stress stimuli. This study provides an important method for the categorization of electrophilic stresses into at least two classes: Cys151-preferable inducers and Cys151-independent inducers. More precise data on the mechanisms of the Keap1 sensor function should provide important clues for the development of Nrf2 inducers as drugs for chronic disorders.

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Abbreviations

tBHQ	tert-butylhydroquinone
BTB	broad complex-tramtrack-bric a brad
CDDO-Im	2-cyano-3, 12 dioxooleana-1,9 diene-28-imidazolide
CDNB	1-chloro-2,4-dinitrobenzene
DEM	diethyl maleate
DMF	dimethylfumarate
Gclc	glutamate-cysteine ligase catalytic subunit
Hprt	hypoxanthine guanine phosphoribosyl transferase
Keap1	Kelch-like ECH-associated protein 1
MEF	Mouse embryonic fibroblast; Mφmacrophage
Nqo1	NAD(P)H:quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
OA-NO₂	nitro-oleic acid
SFN	sulforaphane
15d-PGJ₂	15-deoxy-Δ ^{12,14} -prostaglandin J ₂

REFERENCES

1. Kobayashi M, Yamamoto M. Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.* 2006; 46:113–140. [PubMed: 16887173]
2. Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? *Carcinogenesis.* 2010; 31:90–99. [PubMed: 19793802]
3. Kobayashi M, Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal.* 2005; 7:385–394. [PubMed: 15706085]
4. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. USA.* 2002; 99:11908–11913. [PubMed: 12193649]
5. Hong F, Freeman ML, Liebler DC. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chem. Res. Toxicol.* 2005; 18:1917–1926. [PubMed: 16359182]
6. Egger AL, Liu G, Pezzuto JM, van Breemen RB, Mesecar AD. Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. *Proc. Natl. Acad. Sci. USA.* 2005; 102:10070–10075. [PubMed: 16006525]
7. Egger AL, Luo Y, van Breemen RB, Mesecar S. Identification of the highly reactive cysteine 151 in the chemopreventive agent-sensor Keap1 protein is method-dependent. *Chem. Res. Toxicol.* 2007; 20:1878–1884. [PubMed: 17935299]

8. Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y, Eguchi M, Wada Y, Kumagai Y, Yamamoto M. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* 2009; 29:493–502. [PubMed: 19001094]
9. McMahon M, Lamont DJ, Beattie KA, Hayes JD. Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proc. Natl. Acad. Sci. USA.* 2010; 107:18838–18843. [PubMed: 20956331]
10. Tsujita T, Li L, Nakajima H, Iwamoto N, Nakajima-Takagi Y, Ohashi K, Kawakami K, Kumagai Y, Freeman BA, Yamamoto M, Kobayashi M. Nitro-fatty acids and cyclopentenone prostaglandins share strategies to activate the Keap1-Nrf2 system: a study using green fluorescent protein transgenic zebrafish. *Genes Cells.* 2011; 16:46–57. [PubMed: 21143560]
11. Kansanen E, Bonacci G, Schopfer FJ, Linna S, Tong KI, Leinone H, Woodcock SR, Yamamoto M, Carlberg C, Ylä-Herttuala S, Freeman AL, Levonen AL. Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism. *J. Biol. Chem.* 2011; 286:14019–14027. [PubMed: 21357422]
12. Hu C, Eggler AL, Mesecar AD, van Breeman RB. Modification of keap1 cysteine residues by sulforaphane. *Chem. Res. Toxicol.* 2011; 24:515–521. [PubMed: 21391649]
13. Abiko Y, Miura T, Phuc BH, Shinka Y, Kumagai Y. Participation of covalent modification of Keap1 in the activation of Nrf2 by tert-butylbenzoquinone, an electrophilic metabolite of butylated hydroxyanisole. *Toxicol. Appl. Pharmacol.* 2011; 255:32–39. [PubMed: 21651925]
14. Zhang DD, Hannink M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol.* 2003; 23:8137–8151. [PubMed: 14585973]
15. Wakabayashi N, Dinkova-Kostiva AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, Kensler TW, Talalay P. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA.* 2004; 101:2040–2045. [PubMed: 14764894]
16. Wang XJ, Sun Z, Chen W, Li Y, Villeneuve NF, Zhang DD. Activation of Nrf2 by arsenite and monomethylarsonous acid is independent of Keap1-C151: enhanced Keap1-Cul3 interaction. *Toxicol. Appl. Pharmacol.* 2008; 230:383–389. [PubMed: 18417180]
17. Eggler AL, Small E, Hannink M, Mesecar AD. Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1. *Biochem. J.* 2009; 422:171–180. [PubMed: 19489739]
18. Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H, Yamamoto M. Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. *Mol. Cell. Biol.* 2008; 28:2758–2770. [PubMed: 18268004]
19. Sakurai T, Kanayama M, Shibata T, Itoh K, Kobayashi A, Yamamoto M, Uchida K. Ebselen, a seleno-organic antioxidant, as an electrophile. *Chem. Res. Toxicol.* 2006; 19:1196–1204. [PubMed: 16978024]
20. Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, Zeng W, Hronowsky X, Buko A, Chollate S, Ellrichmann G, Bruck W, Dawson K, Goelz S, Wiese S, Scannevin RH, Lukashev M, Gold R. Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain.* 2011; 134:678–692. [PubMed: 21354971]
21. Zhang J, Hosoya T, Maruyama A, Nishikawa K, Maher J, Ohta T, Motohashi H, Fukamizu A, Shibahara S, Itoh K, Yamamoto M. Nrf2 Neh5 domain is differentially utilized in the transactivation of cytoprotective genes. *Biochem. J.* 2007; 404:459–466. [PubMed: 17313370]
22. Maruyama A, Tsukamoto S, Nishikawa K, Yoshida A, Harada N, Motojima K, Ishii T, Nakane A, Yamamoto M, Itoh K. Nrf2 regulates the alternative first exons of CD36 in macrophages through specific antioxidant response elements. *Arch. Biochem. Biophys.* 2008; 477:139–145. [PubMed: 18585365]
23. Watai Y, Kobayashi A, Nagase H, Mizukami M, McEvoy J, Singer JD, Itoh K, Yamamoto M. Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes Cells.* 2007; 12:1163–1178. [PubMed: 17903176]

24. Suzuki T, Kelly VP, Motohashi H, Nakajima O, Takahashi S, Nishimura S, Yamamoto M. Deletion of the selenocysteine tRNA gene in macrophages and liver results in compensatory gene induction of cytoprotective enzymes by Nrf2. *J. Biol. Chem.* 2008; 283:2021–2030. [PubMed: 18039655]
25. Egglar AL, Small E, Hannink M, Mesecar AD. Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1. *Biochem. J.* 2009; 422:171–180. [PubMed: 19489739]
26. Thimmulappa RK, Fuchs RJ, Malhotra D, Scollick C, Traore K, Bream JH, Trush MA, Liby KT, Sporn MB, Kensler TW, Biswal S. Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. *Antioxid. Redox. Signal.* 2007; 9:1963–1970. [PubMed: 17822364]
27. Reisman SA, Buckley DB, Tanaka Y, Klaassen CD. CDDO-Im protects from acetaminophen hepatotoxicity through induction of Nrf2-dependent genes. *Toxicol. Appl. Pharmacol.* 2009; 236:109–114. [PubMed: 19371629]
28. Yates MS, Tran QT, Dolan PM, Osburn WO, Shin S, McCulloch CC, Silkworth JB, Taguchi K, Yamamoto M, Williams CR, Liby KT, Sporn MB, Sutter TR, Kensler TW. Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid mice. *Carcinogenesis.* 2009; 30:1024–1031. [PubMed: 19386581]
29. Pergola PE, Raskin P, Toto RD, Meyer CJ, Huff JW, Grossman EB, Krauth M, Ruiz S, Audhya P, Christ-Schmidt H, Wittes J, Warnock DG. BEAM Study Investigators. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N. Engl. J. Med.* 2011; 365:327–336. [PubMed: 21699484]
30. Kappos L, Gold R, Miller DH, Macmanus DG, Havrdova E, Limmroth V, Polman CH, Schmierer K, Yousry TA, Yang M, Eraksoy M, Meluzinova E, Rektor I, Dawson KT, Sandrock AW, O'Neill GN. Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomized, double-blind, placebo-controlled phase IIb study. *Lancet.* 2008; 372:1463–1472. [PubMed: 18970976]
31. Ichikawa T, Li J, Meyer CJ, Janicki JS, Hannink M, Cui T. Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. *PLoS One.* 2009; 4:e8391. [PubMed: 20027226]
32. Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N. Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. *Biochemistry.* 2005; 44:6889–6899. [PubMed: 15865434]
33. Freeman BA, Baker PR, Schopfer FJ, Woodcock SR, Napolitano A, d'Ischia M. Nitro-fatty acid formation and signaling. *J. Biol. Chem.* 2008; 283:15515–15519. [PubMed: 18285326]
34. Iizuka T, Ishii Y, Itoh K, Kiwamoto T, Kimura T, Matsuno Y, Morishima Y, Hegab AE, Homma S, Nomura A, Sakamoto T, Shimura M, Yoshida A, Yamamoto M, Sekizawa K. Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes Cells.* 2005; 10:1113–1125. [PubMed: 16324149]
35. Ishii Y, Itoh K, Morishima Y, Kimura T, Kiwamoto T, Iizuka T, Hegab AE, Hosoya T, Nomura A, Sakamoto T, Yamamoto M, Sekizawa K. Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J. Immunol.* 2005; 15:6968–6975. [PubMed: 16272357]
36. Ashino T, Yamanaka R, Yamamoto M, Shimokawa H, Sekikawa K, Iwakura Y, Shioda S, Numazawa S, Yoshida T. Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages. *Mol. Immunol.* 2008; 45:2106–2115. [PubMed: 18022235]
37. Reddy NM, Suryanarayana V, Kalvakolanu DV, Yamamoto M, Kensler TW, Hassoun PM, Kleeberger SR, Reddy SP. Innate immunity against bacterial infection following hyperoxia exposure is impaired in NRF2-deficient mice. *J. Immunol.* 2009; 183:4601–4608. [PubMed: 19734219]

38. Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M, Mann GE. Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ. Res.* 2004; 94:609–616. [PubMed: 14752028]
39. Sussan TE, Jun J, Thimmulappa R, Bedja D, Antero M, Gabrielson KL, Polotsky VY, Biswal S. Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PLoS One.* 2008; 3:e3791. [PubMed: 19023427]
40. Barajas B, Che N, Yin F, Rowshanrad A, Orozco LD, Gong KD, Wang X, Castellani LW, Reue K, Lusis AJ, Araujo JA. NF-E2-related factor 2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* 2011; 31:58–66. [PubMed: 20947826]
41. Ishii T, Itoh K, Takahashi S, Sato H, Tanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem.* 2000; 275:16023–16029. [PubMed: 10821856]
42. Tong KI, Kobayashi A, Katsuoka F, Yamamoto M. Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol. Chem.* 2006; 387:1311–1320. [PubMed: 17081101]
43. Fujii S, Sawa T, Ihara H, Tong KI, Ida T, Okamoto T, Ahtesham AH, Ishima Y, Moyohashi H, Yamamoto M, Akaike T. The critical role of nitric oxide signaling, via protein S-guanylation and nitrated cyclic GMP in the antioxidant adaptive response. *J. Biol. Chem.* 2010; 285:23970–23984. [PubMed: 20498371]
44. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell. Biol.* 2004; 24:10941–10953. [PubMed: 15572695]
45. Rachakonda G, Xiong Y, Sehkar KR, Stamer SL, Liebler DC, Freeman ML. Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3. *Genes Cells.* 2008; 7:807–820.
46. Ogura T, Tong KI, Mio K, Maruyama Y, Kurokawa H, Sato C, Yamamoto M. Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening, double glycine repeat, and C-terminal domains. *Proc. Natl. Acad. Sci. USA.* 2010; 107:2842–2847. [PubMed: 20133743]
47. Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, Yamamoto M. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol. Cell. Biol.* 2006; 26:221–229. [PubMed: 16354693]
48. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol.* 2004; 24:7130–7139. [PubMed: 15282312]

Highlights

- We establish a reliable assay for testing necessity of sensor cysteines of Keap1.
- We identify a subset of Nrf2 inducers that require Keap1 Cys151.
- Keap1 utilizes distinct sets of cysteines for sensing different chemicals.
- This study substantiates the multiple sensing mechanism of Keap1.

Fig. 1.

Effect of tBHQ treatment on Nrf2 activity in overexpression experiments by transfection or MEFs derived from transgenic rescue mice. (A) HEK293 cells were transfected with p*Nqo1*-ARE-luciferase plasmid, Nrf2 expression vector, and 40 or 200 ng of wild-type or C151S Keap1 expression vector and incubated for 24 hours. The transfected cells were exposed to 50 μ M tBHQ for 12 hours prior to analysis of luciferase activity. (B) The experimental scheme for generating MEFs derived from Keap1^{-/-}::Tg-Keap1^{WT} or Keap1^{-/-}::Tg-Keap1^{C151S} mice (Keap1-MEFs and C151S-MEFs, respectively) is shown. (C) Cytoplasmic (Cyt) or nuclear (Nuc) fractions of wild-type, Keap1-MEFs and C151S-MEFs in presence or absence of 5 μ M MG132 were examined by Western blot. Asterisk indicates non-specific

band. (D) Nuclear Nrf2 proteins of Keap1-MEF (closed circle) or C151S-MEF (open circle) following incubation with 0, 30 or 100 μ M tBHQ for three hours were examined by Western blot. (E) A graphical representation of the results in D is shown. N=3; asterisks indicate statistically significant differences ($p < 0.05$). (F and G) Keap1-MEFs (closed circle), C151S-MEFs (open circle) and *Nrf2*^{-/-}-MEFs (triangle) were incubated with 0, 30 or 100 μ M tBHQ for 12 hours, and the expression levels of *Nqo1* (F) and *Gclc* (G) were examined by RT-qPCR with the 18S ribosomal subunit as an internal control. Representative results are shown from at least two-independent experiments.

Fig. 2. Effect of DEM or SFN treatment on Keap1-MEFs and C151S-MEFs (A and E) Nuclear Nrf2 proteins of Keap1-MEF or C151S-MEF following incubation with 0, 30 and 100 μ M DEM (A) or 0, 3 and 10 μ M SFN (E) for three hours were examined by Western blot. (B and F) Graphical representation of the results is shown in A or E. N=3; asterisks indicate statistically significant differences ($p < 0.05$). (C, D, G and H) Keap1-MEF (closed circle), C151S-MEF (open circle) and *Nrf2*^{-/-}-MEF (triangle) were incubated with 0, 30 and 100 μ M DEM (C and D) or 0, 3 and 10 μ M SFN (G and H) for 12 hours and the expression levels of *Nqo1* (C and G) and *Gclc* (D and H) were examined by RT-qPCR with the 18S

ribosomal subunit as internal control. The representative results were shown from at least two-independent experiments.

Fig. 3. Effect of 15d-PGJ₂ or ebselen treatment on Keap1-MEFs and C151S-MEFs. (A and E) Nuclear Nrf2 proteins of Keap1-MEFs or C151S-MEFs following incubation with 0, 3 or 10 μM 15d-PGJ₂ (A) or 0, 3 or 10 μM ebselen (E) for three hours were examined by Western blot. (B and F) Graphical representation of the results is shown in A or E. N=3; asterisks indicate statistically significant differences ($p < 0.05$). (C, D, G and H) Keap1-MEFs (closed circle), C151S-MEFs (open circle) and Nrf2^{-/-}-MEFs (triangle) were incubated with 0, 3 or 10 μM 15d-PGJ₂ (C and D) or 0, 3 or 10 μM ebselen (G and H) for 12 hours, and the expression levels of *Nqo1* (C and G) and *Gclc* (D and H) were examined by RT-qPCR with

the 18S ribosomal subunit as internal control. The representative results were shown from at least two-independent experiments.

Fig. 4. Effect of DMF, CDDO-Im, CdCl₂ or OA-NO₂ treatment on Keap1-MEFs or C151S-MEFs. (A, C, E and G) Nuclear Nrf2 proteins in Keap1-MEFs or C151S-MEFs following incubation for 3 hours with 0-, 10- or 30- μ M DMF (A), 0-, 30- or 100-nM CDDO-Im (C), 0-, 30- or 100- μ M CdCl₂ (E), or 0-, 3- or 10- μ M OA-NO₂ (G) were examined by Western blot. (B, D, F and H) Graphical representations of the results are shown in A, C, E and G. n=3; asterisks indicate statistically significant differences ($p < 0.05$).

Fig. 5. Effect of DEM, SFN, 15d-PGJ₂ or ebselen treatment on peritoneal macrophages derived from *Keap1*^{-/-}::*Tg-Keap1*^{WT} or *Keap1*^{-/-}::*Tg-Keap1*^{C151S} mice. (A) The experimental scheme for isolation of thioglycollate-elicited peritoneal macrophages derived from *Keap1*^{-/-}::*Tg-Keap1*^{WT} (Line 34) or *Keap1*^{-/-}::*Tg-Keap1*^{C151S} (Line 18) mice (Keap1-Mφ and C151S-Mφ). (B) Cytoplasmic (Cyt) or nuclear (Nuc) fractions of wild-type, Keap1-Mφ and C151S-Mφ in presence or absence of 10 μM MG132 were examined by Western blot. (C-F) Nuclear Nrf2 proteins of Keap1-Mφ or C151S-Mφ following incubation for three hours with 0, 30, 100 or 300 μM DEM (C), 0, 1, 3 or 10 μM SFN (D), 0, 1, 3 or 10 μM 15d-PGJ₂ (E), or 0, 1, 3 or 10 μM ebselen (F) were examined by Western blot. G and H, Nuclear

Nrf2 proteins of Keap1-M ϕ or C151S-M ϕ following incubation for 0, 3, 6 or 12 hours with 100 μ M DEM (G), or 10 μ M 15d-PGJ₂ (H) were examined by Western blot.

Fig. 6. Effect of DMF and CDDO-Im on intracellular GSH level in Keap1-M ϕ and C151S-M ϕ . Keap1-M ϕ (closed circle) and C151S-M ϕ (open circle) were treated for 24 hours with 1- or 3- μ M DMF (A and B), or 30- or 100-nM CDDO-Im, and then incubated for additional 30 minutes with vehicle (A and C) or 1- μ M CDNB (B and D). The GSH level of untreated macrophage was set at 1. N=3; asterisks indicate statistically significant differences compared to unstimulated controls ($p < 0.05$).

Fig. 7.
The model for the multiple stress-sensing mechanism of Keap1. A classification of Nrf2 inducers into Cys151-preferable and Cys151-independent inducers is shown.