ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 3 & 4, 2005
© Mary Ann Liebert, Inc.

# Forum Review

# Molecular Mechanisms Activating the Nrf2-Keap1 Pathway of Antioxidant Gene Regulation

MAKOTO KOBAYASHI and MASAYUKI YAMAMOTO

## ABSTRACT

**Several years have passed since NF-E2-related factor 2 (Nrf2) was demonstrated to regulate the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes. Following a number of studies, it was realized that Nrf2 is a key factor for cytoprotection in various aspects, such as anticarcinogenicity, neuroprotection, antiinflammatory response, and so forth. These widespread functions of Nrf2 spring from the coordinated actions of various categories of target genes. The activation mechanism of Nrf2 has been studied extensively. Under normal conditions, Nrf2 localizes in the cytoplasm where it interacts with the actin binding protein, Kelch-like ECH associating protein 1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway. Signals from reactive oxygen species or electrophilic insults target the Nrf2-Keap1 complex, dissociating Nrf2 from Keap1. Stabilized Nrf2 then translocates to the nuclei and transactivates its target genes. Interestingly, Keap1 is now assumed to be a substrate-specific adaptor of Cul3-based E3 ubiquitin ligase. Direct participation of Keap1 in the ubiquitination and degradation of Nrf2 is plausible. The Nrf2-Keap1 system is present not only in mammals, but in fish, suggesting that its roles in cellular defense are conserved throughout evolution among vertebrates. This review article recounts recent knowledge of the Nrf2-Keap1 system, focusing especially on the molecular mechanism of Nrf2 regulation.** *Antioxid. Redox Signal.* 7, 385–394.

## INTRODUCTION

The accumulation of reactive oxygen species (ROS) or electrophilic insults contributes to a wide variety of diseases, including cancer, diabetes, and neurodegenerative diseases. Cytoprotection is provided by the expression of antioxidant proteins and phase 2 detoxifying enzymes that are strongly induced upon exposure to low levels of electrophiles or oxidative stress. For convenience, in this review we have referred to induction as phase 2 induction. Activation of the defense system by phase 2 induction renders cells more resistant to the potential challenges of a subsequent, even greater stress. This coordinated response is regulated through a *cis*-acting element called the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) within the regulatory region of each gene. A number of studies were performed to identify ARE/EpRE binding factors, and NF-E2-related factor 2 (Nrf2) finally got into the limelight as the major contributor to phase 2 induction.

Nrf2 was first isolated as a closely related protein of p45 NF-E2 by an expression cloning procedure using an oligonucleotide containing the NF-E2 site as a probe (37, 65). p45 NF-E2 is the larger subunit of a heterodimer with binding activity at the NF-E2 site (5′-TGCTGAGTCAC-3′), a key *cis*-acting regulator of globin gene expression (5). The smaller subunit was shown to be one of the small Maf proteins, MafK, MafG, or MafF (34). Four members of the p45 NF-E2-related proteins, p45 NF-E2, Nrf1, Nrf2, and Nrf3, have been isolated in mammals and referred to as Cap'n'collar (CNC)-type basic leucine zipper (bZIP) proteins (68). This term was derived from their sequence similarity to *Drosophila* CNC protein. CNC-type bZIP proteins require a member of the small Maf proteins as a heterodimeric partner molecule for DNA binding. Although Nrf2 was assumed to be an important

ERATO-JST, Center for TARA and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba 305-8577, Japan.

regulator of hematopoiesis like p45 NF-E2, Nrf2-deficient mice did not display any abnormality in blood formation (13, 38, 50, 62). Instead, they showed a drastic reduction in the electrophilic-induced gene expression of phase 2 detoxifying enzymes (38). Many subsequent studies demonstrated that most known ARE/EpRE-driven cytoprotective genes, including those encoding antioxidant proteins, are transcriptionally regulated by Nrf2. This shifted the interest of researchers to the regulatory mechanism of Nrf2 activity. As a result, Kelch-like ECH associating protein 1 (Keap1) was isolated and demonstrated to regulate the intracellular localization of Nrf2 by sequestering Nrf2 in the cytoplasm (39). Phase 2 inducers cause the dissociation of Nrf2 from Keap1, allowing for nuclear accumulation of Nrf2 and enhanced expression of its target cytoprotective genes. In this review, we have selected four topics related to the Nrf2-Keap1 system: target genes, roles in the defense mechanism, regulatory mechanism, and evolutional conservation.

## TARGET GENES OF Nrf2

When Nrf2 was clarified to be a transcriptional regulator of phase 2 detoxifying enzymes, it was thought to control a relatively small set of genes. However, following various extensive studies, a substantial number of genes are considered to be under Nrf2 regulation. In this section, we have listed Nrf2 target genes, mainly identified through Nrf2-deficient mouse analysis, and classified them into several categories (Fig. 1).

Data from *in vivo* studies using Nrf2-deficient mice clearly implicated Nrf2 as a protein critical in regulating the expression of glutathione *S*-transferases (GSTs) and NAD(P)H quinone oxidoreductase (38). Nrf2 was shown to control genes encoding other phase 2 detoxifying enzymes, such as UDP-glucuronyl transferase 1A6, aflatoxin B1 aldehyde reductase, and microsomal epoxide hydrolase (12, 53). In addition to phase 2 detoxifying enzymes, we demonstrated that induction of antioxidant proteins during oxidative stress depends on Nrf2 activation (35). In this category of genes, heme oxygenase-1, ubiquitin/PKC-ζ-interacting protein A170, peroxiredoxin 1, the heavy and light chain of ferritin, catalase, glutathione peroxidase, superoxide dismutase, and thioredoxin were shown to be regulated by Nrf2 (12, 17, 35, 46, 52, 74).

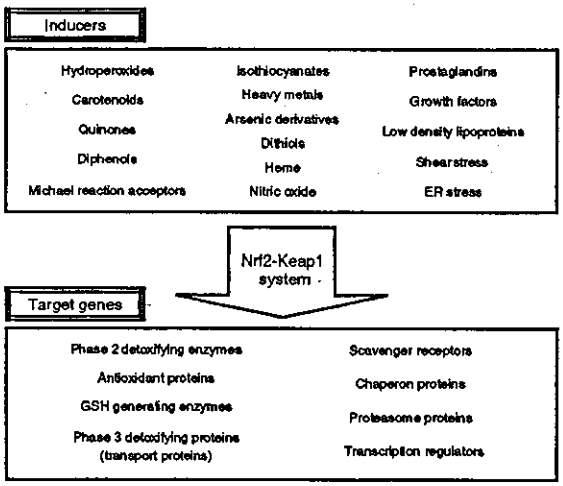


**FIG. 1. Inducers and target genes of the Nrf2-Keap1 system.**

Glutathione (GSH) is an effective scavenger of electrophiles and ROS that are generated during chemical metabolism within cells. Thus, it is important that the gene expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH biosynthesis, is well regulated in order to maintain intracellular levels of GSH. Nrf2 controls both the basal and inducible expression of genes encoding the heavy and light chains of γ-GCS (11, 12, 92). In some cells, cystine/glutamate exchange transport by system $X_c^-$ is crucial for the maintenance of GSH levels. Nrf2 has also been demonstrated to control expression of the gene encoding xCT, one of two protein components of system $X_c^-$ (78).

Chemicals conjugated to GST or similar are actively removed from cells, and factors involved in this elimination process are now designated as phase 3 detoxifying proteins. Multidrug resistance-associated protein 1/ATP-binding cassette transporter C plays an important role in the cellular extrusion of conjugated metabolites and is induced by electrophiles in an Nrf2-dependent manner (29). We recently found that CD36, a gene encoding the scavenger receptor that mediates the uptake of oxidized low-density lipoproteins, is also a target of Nrf2 in vascular smooth muscle cells (36). This result implicates Nrf2 as an important signaling pathway component in atherosclerosis.

Some transcription factors, including regulatory proteins of phase 2 genes, are also regulated by Nrf2. The level of Nrf2 transcription itself is basically unchanged before and after treating cells with phase 2 inducers. However, in keratinocytes, Nrf2 appears to autoregulate its own expression through an ARE/EpRE-like sequence (54). Some oxidative stress was shown to induce the expression levels of small Maf proteins and Keap1 (19, 61, 66, 83, 84). It is suggested that induction of these genes results in a negative feedback regulation of phase 2 induction. Nrf3, another member of CNC-type bZIP proteins, was up-regulated in Nrf2-deficient skin (9).

Finally, several groups have recently tried to identify Nrf2-target genes systematically by use of a microarray-based survey (56, 58, 59, 85). Their results suggested that the Nrf2-Keap1 pathway might modulate in excess of 200 genes. We identified Nrf2-dependent induction of most subunits of the 26S proteasome by antioxidants (56). The promoter of the PSMB5 subunit of the 26S proteasome was analyzed by reporter gene and chromatin immunoprecipitation assays, and its tandem ARE/EpRE sequences were shown to be direct targets for Nrf2 (55). Induction of the 26S proteasome may provide an efficient means for cells to survive conditions of various stresses that collectively enhance the likelihood of chronic disease. Heat shock proteins are also inducible by the Nrf2-dependent pathway (56). Accumulation of unfolded polypeptides following oxidative stress can disturb normal cellular functions and trigger apoptosis. These chaperone proteins, together with the proteasome system, play an essential role in

response to stress by repairing and removing damaged proteins.

## ROLES OF Nrf2 IN THE DEFENSE MECHANISM

As Nrf2 regulates various cytoprotective genes, it seems to serve as a key factor in the protection against toxic xenobiotics. Without Nrf2, induction of cytoprotective enzymes is insufficient and the susceptibility of cells to toxic xenobiotics, including acetaminophen, butyrated hydroxytoluene, and diesel exhaust, is increased (6, 12, 25). Moreover, Nrf2 has been implicated in the protection against oxidative damage induced by acute pulmonary injury and hyperoxia (14, 17, 18). Elimination of Nrf2 also enhances the sensitivity of neurons and astrocytes to oxidative stress by reducing both constitutive and inducible gene expression of cytoprotective genes (58, 59). These studies demonstrate that Nrf2 is fundamental to defense against ROS and imply that Nrf2 is involved in the pathogenesis of lung, neural, and other chronic diseases. The redox status of wild-type and Nrf2-deficient mice was measured using a combination of real-time electron paramagnetic resonance imaging and spin probe kinetic analysis (31) and clearly showed that Nrf2 functions in the reduction of ROS *in vivo* (31). Nrf2-deficient mice also form higher levels of DNA adducts following exposure to carcinogens such as aflatoxin B1, diesel particulate matter, and benzo[*a*]pyrene (6, 52, 77). In addition, the effects of cancer chemopreventive reagents such as oltipraz and sulforaphane are abolished in mice deficient in Nrf2 (26, 52, 53, 76, 77). Functions of Nrf2 in cell survival are also clear (20, 58, 59, 67) and thought to be mediated at least partially by inhibition of the FAS pathway (49, 67).

Recently, Nrf2 target genes were suspected to play antiinflammatory roles, and the influence of Nrf2 during acute inflammation was explored. The persistence of inflammatory cells in Nrf2-deficient mice was observed during carrageenan-induced pleurisy (41). In endothelial cells, overexpression of Nrf2 inhibited the tumor necrosis factor-α-mediated induction of vascular cell adhesion molecule-1 gene expression, which is important for monocyte recruitment during the inflammatory response (16). Laminar shear stress, which acts as an anti-inflammatory signal, activated phase 2 genes in an Nrf2-dependent manner. The induced expression of proinflammatory cytokines in wounded skin was delayed in Nrf2-deficient mice (9). Aged Nrf2-deficient female mice developed lupus-like autoimmune nephritis (94). All these results suggest that Nrf2 plays important roles in antiinflammation.

## REGULATION OF Nrf2

The activities of Nrf2 in the defense system allowed us to imagine that constitutive expression of Nrf2 causes animals to become more resistant to stress, but this is not the case. Keap1-deficient mice in which Nrf2 is constitutively active die within 3 weeks after birth (90). Therefore, controlled Nrf2 activity is quite important for our health.

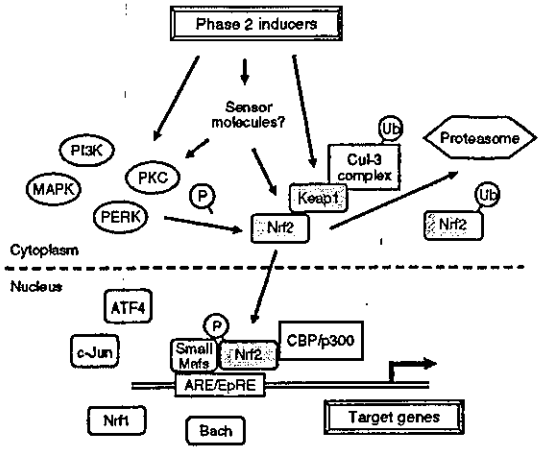


**FIG. 2. Model of Nrf2-Keap1 system regulation.**

Nrf2 activation is regulated in several steps. Some key features emerged from an extensive study of the molecular mechanism of Nrf2 activation in phase 2 induction. In this section, we discuss current models for Nrf2 regulation (Fig. 2).

### *DNA binding*

The regions homologous between mouse Nrf2 and chicken Nrf2 (ECH) are called Neh (Nrf2-ECH homology) domains. Six Neh domains, Neh1 to Neh6, have been identified (39) (Fig. 3). The Neh1 domain contains a bZIP structure that is required for DNA binding and dimer formation. Nrf2 cannot bind to the ARE/EpRE as a monomer or a homodimer and must heterodimerize with one of the small Maf proteins for DNA binding and transactivation (37, 44, 84). The requirement for a "GC" motif in the ARE/EpRE consensus sequence strongly supports the contention that small Maf proteins serve as the heterodimeric partner molecules for Nrf2 (51). Indeed, we recently demonstrated genetically that small Maf proteins are required for Nrf2 activities *in vivo* using compound mutant mice (69). c-Jun and activating transcription factor 4 (ATF4) were also reported to form heterodimers with Nrf2 *in vitro* and to enhance the activity of ARE/EpRE-driven reporter genes. It is possible that these proteins also act as partner molecules for Nrf2 in some conditions (30, 88).

DNA binding was also controlled through competition with other ARE/EpRE-binding proteins. Among these factors, the roles of the transcriptional repressors Bach1 and Bach2 are the most intriguing, particularly because it has been established that Bach1 antagonizes the function of Nrf2, especially in heme oxygenase-1 gene expression (82), and that oxidative stress induces the nuclear accumulation of Bach2 while reducing ARE/EpRE-dependent reporter gene expression (70). Nrf1 is also fascinating. Chimeric mouse analysis using Nrf1-deficient embryonic stem cells indicated that loss of Nrf1 results in impaired expression of antioxidant genes and increased oxidative stress in the liver (15). Mouse embryonic fibroblasts (MEF) from Nrf1-deficient embryos displayed enhanced sensitivity to oxidative stress and an increased accumulation of free radicals (57). MEF deficient in

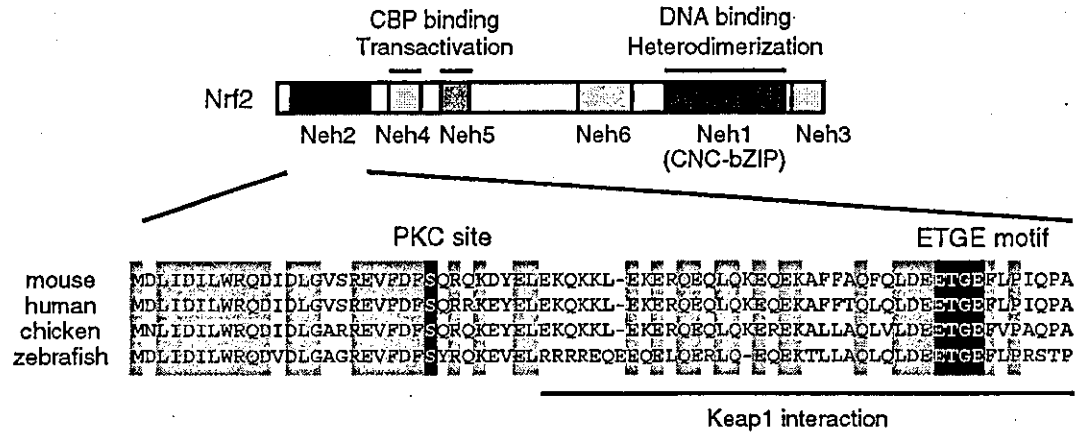


FIG. 3. Neh domains in Nrf2.

both Nrf1 and Nrf2 contained a higher level of intracellular ROS and were more sensitive to oxidative stress than Nrf2-single deficient MEF (60). These results indicate that the functions between Nrf2 and Nrf1 are redundant, especially in liver cells.

## *Transactivation*

Neh4 and Neh5 domains have both been shown to be important for the transactivation activity of Nrf2 (39, 48) (Fig. 3). Neh5 is highly similar to the domain in p45 NF-E2 that is responsible for associating with coactivator CREB binding protein (CBP). The Neh4 domain contains a TRAM binding motif to which CBP and its inhibitor adenovirus E1A protein were shown to interact. Indeed, CBP or p300 was shown to mediate Nrf2 transactivation activity (45, 97). Among CNC-type bZIP family proteins, Nrf2 was found to be the most potent transcriptional activator and typically activates reporter gene transcription by nearly 100-fold (47, 86). The synergistic activity of Neh4-CBP and Neh5-CBP can explain the strong activation potential of Nrf2 (45).

## *Intracellular localization*

Deletion of the N-terminal Neh2 domain enhanced the transcriptional activity of Nrf2 (39) (Fig. 3). This observation suggested that the Neh2 domain recruits a negative regulator of Nrf2. This repressor, Keap1, was identified in a yeast two-hybrid screen using the Neh2 domain as bait (39). Keap1 is a member of the Kelch family of proteins that possess two characteristic domains, the broad complex/tramtrack/bric-a-brac (BTB) domain and the double glycine repeat (DGR) domain (1) (Fig. 4). In common with other Kelch family proteins, Keap1 directly interacts with actin through the DGR domain, thus colocalizing with the actin cytoskeleton in the cytoplasm (43). In the absence of phase 2 inducers, Nrf2 associates with Keap1 in the cytoplasm, but upon the addition of electrophiles, Nrf2 translocates into nuclei and concludes in activation of target gene transcription (22, 39).

As the association and dissociation of the Nrf2-Keap1 complex was considered to be the most significant step for regulating Nrf2 activity, residues essential for the interaction of each protein were analyzed. From this analysis, the ETGE motif in the Neh2 domain was identified as a Keap1-interacting site by a yeast reverse two-hybrid screen (48) (Fig. 3). In the case of Keap1, a point mutation at Ser104 in the BTB domain of Keap1 decreased the association of Keap1 with Nrf2 (99). Keap1 was demonstrated to self-associate, and the mutation at Ser104 disrupts this Keap1 dimerization. In contrast, deletion of the BTB domain did not impair Keap1 activity in our transfection analysis (43). Therefore, the importance of Keap1 dimerization should be elucidated.

The interaction between Nrf2 and Keap1 was also demonstrated at the genetic level (90). Keap1-deficient mice died within 3 weeks after birth due to hyperkeratosis in the esophagus and forestomach. In the liver of these mice, a high steady-state nuclear accumulation of Nrf2 and constitutive expression of phase 2 genes were observed. Importantly, these phenotypes were all rescued in compound Keap1-Nrf2-deficient mice. Our results strongly suggest that Keap1 acts as an indispensable regulator of Nrf2.

## *Protein stability*

Recently, we and other groups demonstrated the rapid degradation of Nrf2 by the ubiquitin-proteasome pathway and the stabilization of Nrf2 by phase 2 inducers (3, 40, 63, 72, 80, 81). By analyzing LacZ or green fluorescent protein fusion proteins, the Neh2 domain was shown to be responsible

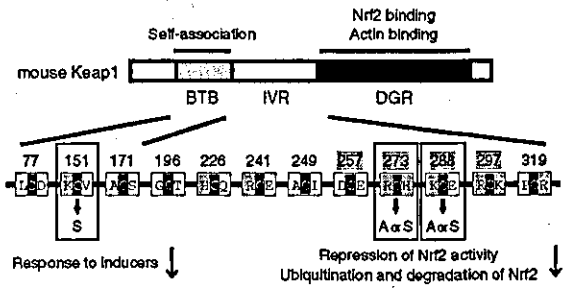


FIG. 4. Critical cysteine residues in mouse Keap1.

for mediating the rapid degradation of Nrf2, in turn suggesting that Keap1 participates in the regulation of Nrf2 degradation (40). Indeed, the addition of Keap1, but not an ETGE motif-deleted mutant, destabilizes Nrf2 (63), and Cys273 and Cys288 in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 (96) (Fig. 4). Interestingly, BTB proteins, including Kelch family proteins, were recently reported to be substrate-specific adaptors of Cul3-based E3 ubiquitin ligase complexes (27, 28, 75, 93). One plausible model is that Keap1 binds to Cul3 and facilitates Nrf2 degradation as an Nrf2-specific adaptor of E3 ubiquitin ligase.*

### *Sensing inducers*

Identifying molecules that sense phase 2 inducers and transduce their signals to Nrf2 have become hot topics. Inducers of phase 2 genes vary as in nine structurally diverse chemical groups (23). Although these inducers share only a few properties, they can all modify sulfhydryl groups by alkylation, oxidation, or reduction. Recognition of these properties suggested that cells possess primary sensors equipped with highly reactive cysteine residues. Interestingly, Keap1 contains 27 cysteine residues, and several of them are reactive, implying that Keap1 might be a direct target of phase 2 inducers. Recently, we showed in a cell-free system that selective cysteine amino acids in Keap1 could react directly with dexamethasone mesylate, a sulfhydryl reactive inducer, and trigger the release of Nrf2 from Keap1 (24). The direct interaction of Keap1 and the phase 2 inducer 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) was also demonstrated (41). The most reactive residues in Keap1 were Cys257, Cys273, Cys288, and Cys297 present in the intervening region (IVR) (24) (Fig. 4). Among them, mutation of Cys273 or Cys288 resulted in the inability of Keap1 to repress Nrf2 activity (91, 96). These cysteine residues were further demonstrated to be required for Keap1-dependent ubiquitination of Nrf2 (96). It is possible that phase 2 inducers directly target these residues, with the resulting modification decreasing ubiquitination activity. The BTB domain may be an alternative target for phase 2 inducers, because Zhang and Hannink (96) further elucidated that a Cys151 mutation in the BTB domain makes Keap1 a constitutive repressor of Nrf2 (Fig. 4).

In addition to Keap1, protein kinases might be candidates as sensor molecules of electrophiles or oxidative stress, because activation of protein kinase C (PKC) (8, 32, 73), extracellular signal-regulated kinases (ERK) (10, 98, 100), p38 mitogen-activated protein kinase (MAPK) (2, 7, 10, 98, 100), MAPK/ERK kinase-1 (79), MEK kinase 1 (95), phosphatidylinositol 3-kinase (PI3K) (42, 71), and PKR-like endoplasmic reticulum kinase (PERK) (20) was observed after treatment with phase 2 inducers. Furthermore, phase 2 gene and ARE/EpRE-driven reporter gene induction was blocked by specific kinase inhibitors. Among the kinases, PKC and PERK are remarkable because both can phosphorylate Nrf2 directly *in vitro* and *in vivo* (20, 32, 33). A coimmunoprecipitation assay revealed that phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1 and that a Ser to Ala mutation at amino acid 40 in Nrf2, which is the target site for PKC, decreased this PKC-dependent dissociation (33) (Fig. 3). On the other hand, PERK-dependent phosphorylation of Nrf2 also triggers dissociation of the Nrf2-Keap1 complex (20). It is possible that PKC and/or PERK or their upstream signaling molecules may be sensors for oxidative stress.

*Specific association of Keap1 with Cul3 has been confirmed during editorial process of this review (101).

## EVOLUTIONARY CONSERVATION OF THE Nrf2-Keap1 SYSTEM

The importance of the bZIP protein in cellular defense has been shown in yeast cells (87). The bZIP protein Yap1 in budding yeast and Pap1 in fission yeast regulate the gene expression of various cytoprotective proteins, such as γ-GCS, thioredoxin, the hsp70 family member, NAD(P)H oxidoreductase, glutathione transferase, catalase, and ATP binding cassette-type transporters. Both Yap1 and Pap1 are cytoplasmic in unstressed cells and translocate into nuclei in response to treatment with oxidants, electrophiles, or heavy metals. These characteristics are quite similar to those of Nrf2. The clear difference between the Yap1/Pap1 and Nrf2 systems is the regulatory mechanism of cytoplasmic retention and nuclear translocation. In budding yeast, redox signals promote the formation of disulfide bonds between the intermolecular cysteines of Yap1 that mask the C-terminal nuclear export signal domain, resulting in inhibition of Yap1 nuclear export (21). Cytoplasmic retention molecules such as Keap1 are not required for Yap1, and Nrf2 probably does not contain a nuclear export signal domain as in Yap1.

In nematode, SKN-1 was demonstrated to regulate phase 2 detoxifying genes through constitutive and stress-inducible mechanisms (4). Its binding sites exist in the upstream regions of γ-GCS heavy chain, glutathione synthetase, NADH quinone oxidoreductase, GST, catalase, and superoxide dismutase. SKN-1 mutants are sensitive to oxidative stress and have shortened life spans. Analysis of green fluorescent protein fusion proteins revealed that heat or paraquat treatment induced the nuclear accumulation of SKN-1. Again, the functions of SKN-1 seem to be similar to those of Nrf2. Although SKN-1 shares homology with Nrf2 in both the N-terminal halves of the Neh2 and Neh1 domains, it lacks an ETGE motif or leucine zipper domain. Indeed, homologues for Keap1 or small Maf proteins have not been found in *C. elegans*, implying that the regulatory mechanisms of SKN-1 activation may be different from those for Nrf2.

In fruit fly, CNC protein has homology with Nrf2. CNC was originally identified as a regulatory protein for labral and mandibular development (64). So far, no study has been reported about CNC functions in the defense system. Interestingly, CNCC protein, one of three isoforms of CNC, possesses a Neh2-related region containing an ETGE motif (48). In addition, a Keap1-related gene and a small Maf protein were identified in *Drosophila* (48, 89). In common with Nrf2 in vertebrates, it is possible that CNCC plays important roles in the defense mechanism in fruit flies.

Nrf2 was identified in mouse, human, chicken, and zebrafish and supposedly exists in all other vertebrates (48). Gene knock-down analysis of zebrafish Nrf2 using morpholinophosphorodiamidate-modified antisense oligonucleotide

revealed that Nrf2 is required for phase 2 induction in fish, as it is in mammal. Keap1 also exists in zebrafish and was shown to interact with and repress the activity of zebrafish Nrf2. The molecular mechanism regulating the Nrf2-Keap1 system may be conserved among vertebrates.

## CONCLUSION

Recently, Nrf2 has been found to be activated by endogenous products of oxidative stress or other stress generated inside the body, such as 4-hydroxynonenal (36, 73), oxidized low-density lipoproteins (36), heme (2, 46, 71), and nitric oxide (10, 42). In addition, prostaglandin 15d-$PGJ_2$ (41) and keratinocyte growth factor (9) can induce Nrf2-dependent gene expression. As these agents function as signaling molecules in many systems, the Nrf2-Keap1 system may be considered as a central component of cellular defense networks. Identification of molecules sensing phase 2 inducers and transducing their signals to Nrf2 will greatly contribute to a better understanding of these networks. A number of significant findings were reported in the last couple of years, and the molecular mechanism activating the Nrf2-Keap1 pathway is gradually being unveiled. The complete picture of the Nrf2-Keap1 system should come into view in the near future.

## ACKNOWLEDGMENTS

We thank Dr. Tania O'Connor for critical reading of the manuscript, and Drs. Ken Itoh, Hozumi Motohashi, Akira Kobayashi, and Tetsuro Ishii for discussion. This work was supported by grants from ERATO-JST, the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare.

## ABBREVIATIONS

ARE, antioxidant responsive element; ATF4, activating transcription factor 4; BTB, broad complex, tramtrack, and bric-a-brac; bZIP, basic leucine zipper; CBP, CREB binding protein; CNC, Cap'n'collar; DGR, double glycine repeat; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$; EpRE, electrophile responsive element; ERK, extracellular signal-regulated kinase; γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione *S*-transferase; IVR, intervening region; Keap1, Kelch-like ECH associating protein 1; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; Neh, Nrf2-ECH homology; Nrf2, NF-E2-related factor 2; PERK, PKR-like endoplasmic reticulum kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species.

## REFERENCES

1. Adams J, Kelso R, and Cooley L. The kelch repeat superfamily of proteins: propellers of cell function. *Trends Cell Biol* 10: 17–24, 2000.
2. Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, Choi AM, Burow ME, and Tou J. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *J Biol Chem* 275: 27694–27702, 2000.
3. Alam J, Killeen E, Gong P, Naquin R, Hu B, Stewart D, Ingelfinger JR, and Nath KA. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. *Am J Physiol Renal Physiol* 284: F743–F752, 2003.
4. An JH and Blackwell TK. SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes Dev* 17: 1882–1893, 2003.
5. Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, and Orkin SH. Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* 362: 722–728, 1993.
6. Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, and Yamamoto M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol Appl Pharmacol* 173: 154–160, 2001.
7. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J, and Motterlini R. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 371: 887–895, 2003.
8. Bloom DA and Jaiswal AK. Phosphorylation of Nrf2 at $Ser^{40}$ by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J Biol Chem* 278: 44675–44682, 2003.
9. Braun S, Hanselmann C, Gassmann MG, auf dem Keller U, Born-Berclaz C, Chan K, Kan YW, and Werner S. Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. *Mol Cell Biol* 22: 5492–5505, 2002.
10. Buckley BJ, Marshall ZM, and Whorton AR. Nitric oxide stimulates Nrf2 nuclear translocation in vascular endothelium. *Biochem Biophys Res Commun* 307: 973–979, 2003.
11. Chan JY and Kwong M. Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. *Biochim Biophys Acta* 1517: 19–26, 2000.
12. Chan K and Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci USA* 96: 12731–12736, 1999.
13. Chan K, Lu R, Chang JC, and Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc Natl Acad Sci USA* 93: 13943–13948, 1996.
14. Chan K, Han XD, and Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc Natl Acad Sci U S A* 98: 4611–4616, 2001.
15. Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L, and Chan JY. Nrf1 is critical for redox balance and sur-

vival of liver cells during development. *Mol Cell Biol* 23: 4673–4686, 2003.
16. Chen XL, Varner SE, Rao AS, Grey JY, Thomas S, Cook CK, Wasserman MA, Medford RM, Jaiswal AK, and Kunsch C. Laminar flow induction of antioxidant response element-mediated genes in endothelial cells. A novel anti-inflammatory mechanism. *J Biol Chem* 278: 703–711, 2003.
17. Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, and Kleeberger SR. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 26: 175–182, 2002.
18. Cho HY, Jedlicka AE, Reddy SP, Zhang LY, Kensler TW, and Kleeberger SR. Linkage analysis of susceptibility to hyperoxia. *Nrf2* is a candidate gene. *Am J Respir Cell Mol Biol* 26: 42–51, 2002.
19. Crawford DR, Leahy KP, Wang Y, Schools GP, Kochheiser JC, and Davies KJA. Oxidative stress induces the levels of a *MafG* homolog in hamster HA-1 cells. *Free Radic Biol Med* 21: 521–525, 1996.
20. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, and Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* 23: 7198–7209, 2003.
21. Delaunay A, Pflieger D, Barrault MB, Vinh J, and Toledano MB. A thiol peroxidase is an $H_2O_2$ receptor and redox-transducer in gene activation. *Cell* 111: 471–481, 2002.
22. Dhakshinamoorthy S and Jaiswal AK. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* 20: 3906–3917, 2001.
23. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, and Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc Natl Acad Sci USA* 98: 3404–3409, 2001.
24. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, and 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* 99: 11908–11913, 2002.
25. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, and Yamamoto M. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol Sci* 59: 169–177, 2001.
26. Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, and Lozniewski A. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[*a*]pyrene-induced stomach tumors. *Proc Natl Acad Sci USA* 99: 7610–7615, 2002.
27. Furukawa M, He YJ, Borchers C, and Xiong Y. Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. *Nat Cell Biol* 5: 1001–1007, 2003.
28. Geyer R, Wee S, Anderson S, Yates J, Wolf DA, Zhou C, Seibert V, Rhee E, Lyapina S, Cope G, and Deshaies RJ. BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Mol Cell* 12: 783–790, 2003.
29. Hayashi A, Suzuki H, Itoh K, Yamamoto M, and Sugiyama Y. Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochem Biophys Res Commun* 310: 824–829, 2003.
30. He CH, Gong P, Hu B, Stewart D, Choi ME, Choi AMK, and Alam J. Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J Biol Chem* 276: 20858–20865, 2001.
31. Hirayama A, Yoh K, Nagase S, Ueda A, Itoh K, Morito N, Hirayama K, Takahashi S, Yamamoto M, and Koyama A. EPR imaging of reducing activity in Nrf2 transcriptional factor-deficient mice. *Free Radic Biol Med* 34: 1236–1242, 2003.
32. Huang HC, Nguyen T, and Pickett CB. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci USA* 97: 12475–12480, 2000.
33. Huang HC, Nguyen T, and Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* 277: 42769–42774, 2002.
34. Igarashi K, Kataoka K, Itoh K, Hayashi N, Nishizawa M, and Yamamoto M. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* 367: 568–572, 1994.
35. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, and Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 275: 16023–16029, 2000.
36. Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M, and 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* 94: 609–616, 2004.
37. Itoh K, Igarashi K, Hayashi N, Nishizawa M, and Yamamoto M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol Cell Biol* 15: 4184–4193, 1995.
38. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, and Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236: 313–322, 1997.
39. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, and Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 13: 76–86, 1999.
40. Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, and Yamamoto M. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 8: 379–391, 2003.
41. Itoh K, Mochizuki M, Ishii Y, Ishii T, Shibata T, Kawamoto Y, Kelly V, Sekizawa K, Uchida K, and Ya-

mamoto M. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$. *Mol Cell Biol* 24: 36–45, 2004.
42. Kang KW, Choi SH, and Kim SG. Peroxynitrite activates NF-E2-related factor 2/antioxidant response element through the pathway of phosphatidylinositol 3-kinase: the role of nitric oxide synthase in rat glutathione *S*-transferase A2 induction. *Nitric Oxide* 7: 244–253, 2002.
43. Kang M-I, Kobayashi A, Wakabayashi N, Kim S-G, and Yamamoto M. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proc Natl Acad Sci USA* 101: 2046–2051, 2004.
44. Kataoka K, Igarashi K, Itoh K, Fujiwara KT, Noda M, Yamamoto M, and Nishizawa M. Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. *Mol Cell Biol* 15: 2180–2190, 1995.
45. Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, and Yamamoto M. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 6: 857–868, 2001.
46. Kim YC, Masutani H, Yamaguchi Y, Itoh K, Yamamoto M, and Yodoi J. Hemin-induced activation of the thioredoxin gene by Nrf2. A differential regulation of the antioxidant responsive element by a switch of its binding factors. *J Biol Chem* 276: 18399–18406, 2001.
47. Kobayashi A, Ito E, Toki T, Kogame K, Takahashi S, Igarashi K, Hayashi N, and Yamamoto M. Molecular cloning and functional characterization of a new Cap'n'-collar family transcription factor Nrf3. *J Biol Chem* 274: 6443–6452, 1999.
48. Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, Takagi Y, and Yamamoto M. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* 7: 807–820, 2002.
49. Kotlo KU, Yehiely F, Efimova E, Harasty H, Hesabi B, Shchors K, Einat P, Rozen A, Berent E, and Deiss LP. Nrf2 is an inhibitor of the Fas pathway as identified by Achilles' Heel Method, a new function-based approach to gene identification in human cells. *Oncogene* 22: 797–806, 2003.
50. Kuroha T, Takahashi S, Komeno T, Itoh K, Nagasawa T, and Yamamoto M. Ablation of Nrf2 function does not increase the erythroid or megakaryocytic cell lineage dysfunction caused by p45 NF-E2 gene disruption. *J Biochem (Tokyo)* 123: 376–379, 1998.
51. Kusunoki H, Motohashi H, Katsuoka F, Morohashi A, Yamamoto M, and Tanaka T. Solution structure of the DNA-binding domain of MafG. *Nat Struct Biol* 9: 252–256, 2002.
52. Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, Yamamoto M, and Kensler TW. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat Res* 480–481: 305–315, 2001.
53. Kwak MK, Itoh K, Yamamoto M, Sutter TR, and Kensler TW. Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1,2-dimethiole-3-thione. *Mol Med* 7: 135–145, 2001.
54. Kwak MK, Itoh K, Yamamoto M, and Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the *nrf2* promoter. *Mol Cell Biol* 22: 2883–2892, 2002.
55. Kwak MK, Wakabayashi N, Greenlaw JL, Yamamoto M, and Kensler TW. Antioxidants enhance mammalian proteasome expression through the Keap1-Nrf2 signaling pathway. *Mol Cell Biol* 23: 8786–8794, 2003.
56. Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, and Kensler TW. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. *J Biol Chem* 278: 8135–8145, 2003.
57. Kwong M, Kan YW, and Chan JY. The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. Role for Nrf1 in $\gamma$-*gcs*$_L$ and *gss* expression in mouse fibroblasts. *J Biol Chem* 274: 37491–37498, 1999.
58. Lee JM, Calkins MJ, Chan K, Kan YW, and Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J Biol Chem* 278: 12029–12038, 2003.
59. Lee JM, Shih AY, Murphy TH, and Johnson JA. NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. *J Biol Chem* 278: 37948–37956, 2003.
60. Leung L, Kwong M, Hou S, Lee C, and Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. *J Biol Chem* 278: 48021–48029, 2003.
61. Li J, Lee JM, and Johnson JA. Microarray analysis reveals an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells. *J Biol Chem* 277: 388–394, 2002.
62. Martin F, van Deursen JM, Shivdasani RA, Jackson CW, Troutman AG, and Ney PA. Erythroid maturation and globin gene expression in mice with combined deficiency of NF-E2 and nrf-2. *Blood* 91: 3459–3466, 1998.
63. McMahon M, Itoh K, Yamamoto M, and Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 278: 21592–21600, 2003.
64. Mohler J, Mahaffey JW, Deutsch E, and Vani K. Control of *Drosophila* head segment identity by the bZIP homeotic gene *cnc*. *Development* 121: 237–247, 1995.
65. Moi P, Chan K, Asunis I, Cao A, and Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the $\beta$-globin locus control region. *Proc Natl Acad Sci USA* 91: 9926–9930, 1994.
66. Moran JA, Dahl EL, and Mulcahy RT. Differential induction of *mafF*, *mafG* and *mafK* expression by electrophile-response-element activators. *Biochem J* 361: 371–377, 2002.

67. Morito N, Yoh K, Itoh K, Hirayama A, Koyama A, Yamamoto M, and Takahashi S. Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. *Oncogene* 22: 9275–9281, 2003.
68. Motohashi H, O'Connor T, Katsuoka F, Engel JD, and Yamamoto M. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* 294: 1–12, 2002.
69. Motohashi H, Katsuoka F, Engel JD, and Yamamoto M. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc Natl Acad Sci U S A* 101: 6379–6384, 2004.
70. Muto A, Tashiro S, Tsuchiya H, Kume A, Kanno M, Ito E, Yamamoto M, and Igarashi K. Activation of Maf/AP-1 repressor Bach2 by oxidative stress promotes apoptosis and its interaction with promyelocytic leukemia nuclear bodies. *J Biol Chem* 277: 20724–20733, 2002.
71. Nakaso K, Yano H, Fukuhara Y, Takeshima T, Wada-Isoe K, and Nakashima K. PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. *FEBS Lett* 546: 181–184, 2003.
72. Nguyen T, Sherratt PJ, Huang HC, Yang CS, and Pickett CB. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 278: 4536–4541, 2003.
73. Numazawa S, Ishikawa M, Yoshida A, Tanaka S, and Yoshida T. Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am J Physiol Cell Physiol* 285: C334–C342, 2003.
74. Pietsch EC, Chan JY, Torti FM, and Torti SV. Nrf2 mediates the induction of ferritin H in response to xenobiotics and cancer chemopreventive dithioletliones. *J Biol Chem* 278: 2361–2369, 2003.
75. Pintard L, Willis JH, Willems A, Johnson JLF, Srayko M, Kurz T, Glaser S, Mains PE, Tyers M, Bowerman B, and Peter M. The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature* 425: 311–316, 2003.
76. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, and Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in *nrf2* transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 98: 3410–3415, 2001.
77. Ramos-Gomez M, Dolan PM, Itoh K, Yamamoto M, and Kensler TW. Interactive effects of *nrf2* genotype and oltipraz on benzo[*a*]pyrene-DNA adducts and tumor yield in mice. *Carcinogenesis* 24: 461–467, 2003.
78. Sasaki H, Sato H, Kuriyama-Matsumura K, Sato K, Maebara K, Wang H, Tamba M, Itoh K, Yamamoto M, and Bannai S. Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem* 277: 44765–44771, 2002.
79. Sekhar KR, Spitz DR, Harris S, Nguyen TT, Meredith MJ, Holt JT, Gius D, Marnett LJ, Sunmar ML, Freeman ML, and Guis D. Redox-sensitive interaction between KIAA0132 and Nrf2 mediates indomethacin-induced expression of γ-glutamylcysteine synthetase. *Free Radic Biol Med* 32: 650–662, 2002.
80. Sekhar KR, Yan XX, and Freeman ML. Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. *Oncogene* 21: 6829–6834, 2002.
81. Stewart D, Killeen E, Naquin R, Alam S, and Alam J. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J Biol Chem* 278: 2396–2402, 2003.
82. Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, and Igarashi K. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J* 21: 5216–5224, 2002.
83. Suzuki T, Blank V, Sesay JS, and Crawford DR. Maf genes are involved in multiple stress response in human. *Biochem Biophys Res Commun* 280: 4–8, 2001.
84. Takagi Y, Kobayashi M, Li L, Suzuki T, Nishikawa K, and Yamamoto M. MafT, a new member of the small Maf protein family in zebrafish. *Biochem Biophys Res Commun* 320: 62–69, 2004.
85. Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, and Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res* 62: 5196–5203, 2002.
86. Toki T, Itoh J, Kitazawa J, Arai K, Hatakeyama K, Akasaka J, Igarashi K, Nomura N, Yokoyama M, Yamamoto M, and Ito E. Human small Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif. *Oncogene* 14: 1901–1910, 1997.
87. Toone WM and Jones N. AP-1 transcription factors in yeast. *Curr Opin Genet Dev* 9: 55–61, 1999.
88. Venugopal R and Jaiswal AK. Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* 17: 3145–3156, 1998.
89. Veraksa A, McGinnis N, Li X, Mohler J, and McGinnis W. Cap 'n' collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress deformed homeotic function in the *Drosophila* head. *Development* 127: 4023–4037, 2000.
90. Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, Harada T, Engel JD, and Yamamoto M. *Keap1*-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* 35: 238–245, 2003.
91. Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, Kensler TW, and Talalay P. Protection against electrophiles and oxidant stress by induction of the phase 2 response: fate of the cysteine residues of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A* 101: 2040–2045, 2004.
92. Wild AC, Moinova HR, and Mulcahy RT. Regulation of γ-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J Biol Chem* 274: 33627–33636, 1999.

93. Xu L, Wei Y, Reboul J, Vaglio P, Shin TH, Vidal M, Elledge SJ, and Harper JW. BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature* 425: 316–321, 2003.
94. Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, Kobayashi M, Morito N, Koyama A, Yamamoto M, and Takahashi S. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int* 60: 1343–1353, 2001.
95. Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tan TH, and Kong ANT. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. *J Biol Chem* 275: 39907–39913, 2000.
96. Zhang DD and 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* 23: 8137–8151, 2003.
97. Zhu M and Fahl WE. Functional characterization of transcription regulators that interact with the electrophile response element. *Biochem Biophys Res Commun* 289: 212–219, 2001.
98. Zipper LM and Mulcahy RT. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochem Biophys Res Commun* 278: 484–492, 2000.
99. Zipper LM and Mulcahy RT. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem* 277: 36544–36552, 2002.
100. Zipper LM and Mulcahy RT. Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. *Toxicol Sci* 73: 124–134, 2003.
101. Kobayashi A, Kang M-I, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, and Yamamoto M. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* 24: 7130–7139, 2004.

Address reprint requests to:
*Makoto Kobayashi, Ph.D.*
*Institute of Basic Medical Sciences*
*University of Tsukuba*
*Tsukuba 305-8575, Japan*

*E-mail:* kobayash@tara.tsukuba.ac.jp

Received for publication April 30, 2004; accepted October 9, 2004.