5

SURVIVAL STRATEGY AND DISEASE PATHOGENESIS ACCORDING TO THE Nrf2-SMALL Maf HETERODIMER

Masanobu Morita[1] and Hozumi Motohashi[2]

[1]*Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan*
[2]*Center for Radioisotope Sciences, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan*

5.1 INTRODUCTION

Organisms, including mammals, are constantly exposed to oxidative stress due to oxygen consumption during aerobic respiration and xenobiotic chemicals in their diet and/or the environment. Mammalian cells are equipped with sophisticated machinery for cell protection against oxidative and xenobiotic stress. NF-E2-related factor 2 (Nrf2) is a potent transcription activator that plays a central role in regulating the expression of genes encoding detoxifying enzymes and antioxidant proteins by binding to the antioxidant response element (ARE)/electrophile response element (EpRE). Nrf2 is a member of the Cap'n'Collar (CNC) transcription factor family that commonly contains a unique stretch of amino acids, designated the CNC domain, followed by a well-conserved basic region-leucine zipper (bZIP) motif. Under unstressed conditions, Nrf2 is ubiquitinated by kelch-like ECH-associated protein 1 (Keap1) and degraded by the proteasome in the cytoplasm. Upon exposure to oxidative or xenobiotic stress, Nrf2 is stabilized, translocates into the nucleus, heterodimerizes with small Maf, another bZIP protein, binds to the ARE/EpRE, and activates transcription. Recent studies have clarified the intricate molecular mechanisms of Nrf2 activation in response to stress and revealed the involvement of Nrf2 in many human diseases including neurodegeneration, airway disorders, cardiovascular disease, and cancer. This review covers the historical aspects of the discovery of Nrf2, recent advances in molecular studies of Nrf2 function, updated reports on the involvement of Nrf2 in various pathological conditions, and perspectives of Nrf2 utilization for human welfare.

5.1.1 Identification of Nrf2

The antioxidant response element (ARE), also known as an electrophile response element (EpRE) [TGA(G/C)NNNGC], was first identified in analyses of induction mechanisms of the detoxifying enzyme genes in response to electrophilic chemicals [1–3]. ARE/EpREs are often found in the promoter regions of cytoprotective genes and are considered to be critical for the inducible expression of these genes. Although the importance of this *cis*-regulatory element was clearly shown, the *trans*-acting factor responsible for inducible transcription activation remained unknown.

During the search for the *trans*-acting factor, an interesting coincidence occurred in research on transcriptional regulation. Nuclear factor erythroid 2 (NF-E2) is a transcriptional activator important for erythroid-specific gene expression through interaction with NF-E2 binding sites [ATGA(G/C)TCAGCA] [4, 5]. Nrf2 was originally identified as a homolog of NF-E2 and was found to associate with the NF-E2 binding site as expected [6, 7] (Fig. 5.1). Similarities between the ARE/EpRE sequence and the NF-E2 binding site led to the discovery of Nrf2 as the ARE/EpRE binding factor [8] (Fig. 5.2A).

Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling, First Edition.
Edited by Tahira Farooqui and Akhlaq A. Farooqui.

Nrf2 possesses a characteristic CNC domain followed by a well-conserved bZIP motif (Fig. 5.1). The CNC domain was defined based on the sequence homology of 43 amino acids to the *Drosophila* Cnc protein [9]. In addition to NF-E2 and Nrf2, four additional factors were isolated as members of the CNC family: Nrf1, Nrf3, Bach1, and Bach2 [10, 11, 12]. NF-E2, Nrf1, Nrf2, and Nrf3 are considered to be activators [4, 6, 7, 10, 11], while Bach1 and Bach2 possess characteristic Broad complex-Tramtrack-Bric-a-brac (BTB) domains at their N-terminal ends and are considered to be repressors [12–14] (Fig. 5.2B). Importantly, none of the members of the CNC family binds efficiently to DNA as monomer or homodimer, and each requires small Maf, another bZIP protein, as an obligate heterodimeric partner molecule [15] (Fig. 5.1 and Fig. 5.2B).

The CNC protein homolog in *Caenorhabditis elegans* is SKN-1, and it binds to DNA as a monomer because it does not possess the leucine zipper structure [16]. The SKN-1 recognition sequence is similar to half-sites of ARE/EpRE and NF-E2 binding site (Fig. 5.2A). One side of the site is recognized by CNC proteins, and small Maf proteins recognize the other side of the site, which contains a GC dinucleotide. Nrf2 shares a heterodimeric partner molecule with other CNC proteins, and all of the CNC-small Maf heterodimers recognize a nearly identical DNA binding sequence. This implicates cross talk between Nrf2 and other members of the CNC and small Maf transcription factor families.

5.1.2 Susceptibility of *Nrf2*-Null Mice to Oxidative and Xenobiotic Stress

Analysis of *Nrf2*-null mice clearly demonstrated the substantial contribution of Nrf2 to the inducible regulation of cytoprotective genes [8]. *Nrf2*-null mice display an increased formation of DNA adducts in the lung following exposure to diesel exhaust [17], a more severe liver toxicity after administration of acetaminophen [18], an increased susceptibility to cigarette smoke-induced emphysema [19], and an aggravated bleomycin-induced pulmonary fibrosis

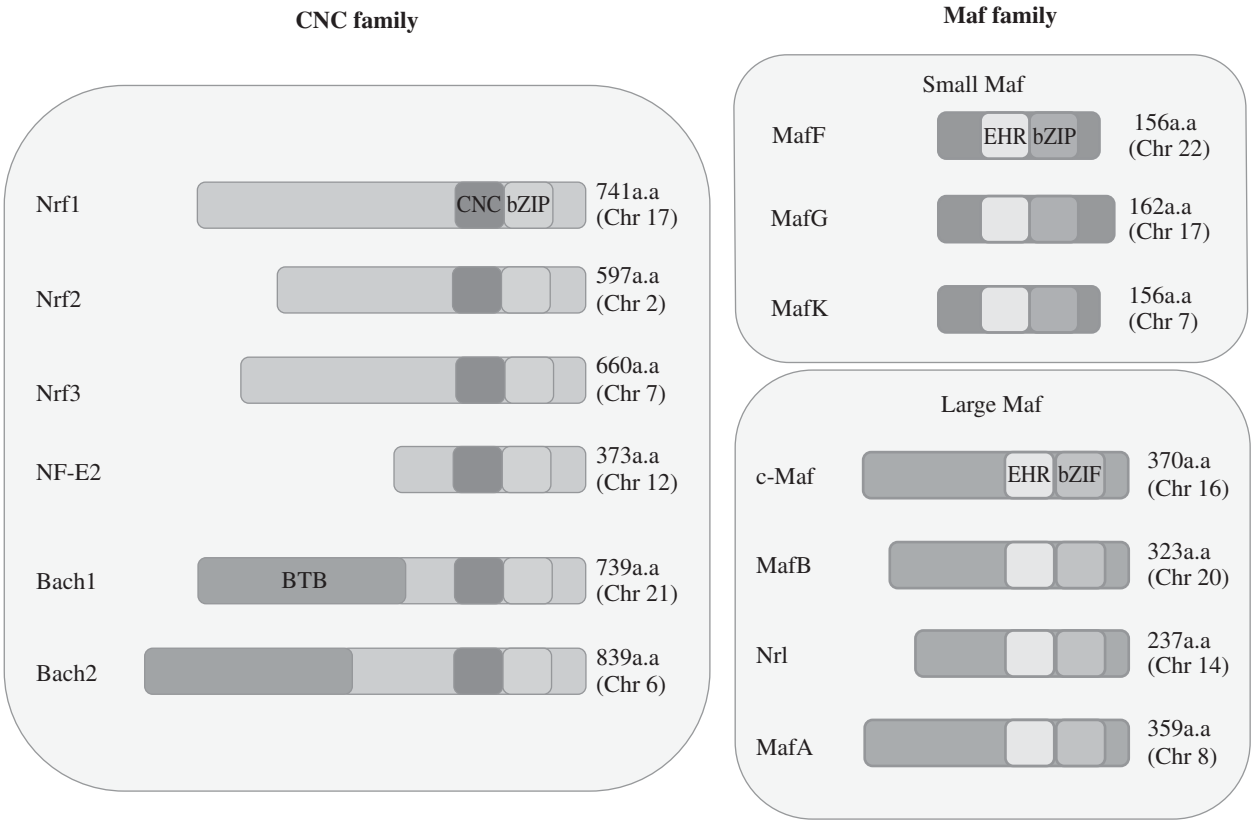

Fig. 5.1 The structures of CNC and Maf family proteins. The CNC family contains four transcriptional activators, Nrf1, Nrf2, Nrf3, and NF-E2, and two transcriptional repressors, Bach1 and Bach2. The Maf family consists of three small Maf transcription factors and four large Maf transcription factors. The bZIP motifs are a common structural feature of CNC and Maf family proteins. The CNC and EHR domains are unique to CNC and Maf family proteins, respectively. Bach1 and Bach2 possess BTB domains in their N-terminal regions. The amino acid number and chromosomal location of the human gene encoding each protein are shown.

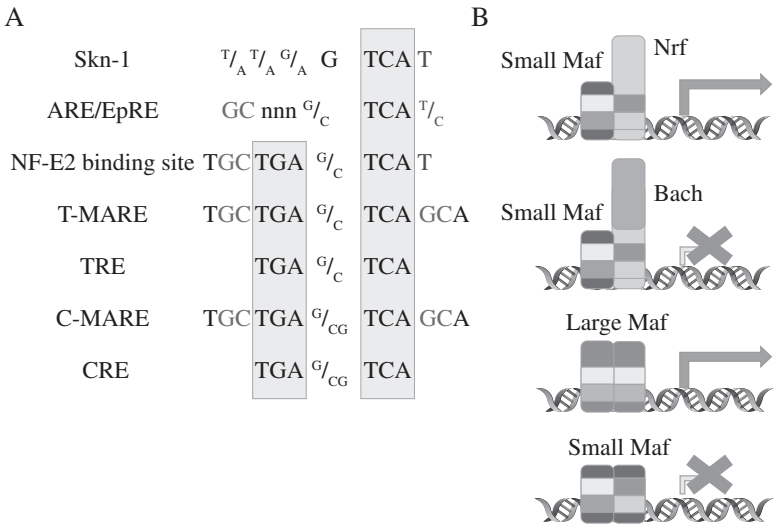

Fig. 5.2 DNA recognition sequences and the transcriptional activities of Maf-containing dimers. (A) Various MARE-related sequences are shown. The dinucleotide "GC" (marked in red) is essential for recognition by Maf proteins. The nucleotide "T" or "T/C" (marked in blue) enhances the binding of CNC proteins. Trinucleotides (boxed in gray) with the central G and GC sequence consist of the TRE and CRE, respectively, which make up the core region of the MARE. (B) Nrf1, Nrf2, Nrf3, and NF-E2 activate transcription by forming heterodimers with small Maf, while Bach1 and Bach2 repress transcription. A large Maf homodimer, possessing a *trans*-activation domain, activates transcription, while a small Maf homodimer, lacking a *trans*-activation domain, represses transcription. All of these Maf-containing dimers bind to T-MARE with high affinity. *(See color insert.)*

[20]. Nrf2 confers resistance to xenobiotic stress through activation of the genes encoding detoxifying enzymes, including glutathione *S*-transferases (GST), UDP glucuronate transferases (UGT), and NAD(P)H:quinone oxidoreductase 1 (NQO1) [8, 21–23]. The phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults are summarized in Table 5.1.

Nrf2 also plays an important role in response to oxidative stress by activating antioxidant proteins and enzymes for glutathione synthesis [21, 24]. Cellular capacities for elimination of reactive oxygen species (ROS) are limited in the absence of Nrf2 [25]. Nrf2 regulates intracellular ROS levels not only in pathological conditions but also during the physiological cellular differentiation process of megakaryocytic maturation [26]. With decreased capacities to eliminate ROS of endogenous origins, *Nrf2*-null mice tend to spontaneously develop various inflammatory disorders, including glomerulonephritis, immune-mediated hemolytic anemia, and multiorgan autoimmune inflammation [27–29], all of which appear to be due to a chronic increase in ROS. The phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults are summarized in Table 5.2.

The *Nrf2* gene is ubiquitously expressed in various cell lineages and at both the embryonic and adult stages. The expression level is relatively higher in the lung, kidney, and liver in adults and in the intestine, lung, and choroid plexus in embryos [30]. Chicken Nrf2 is highly expressed in the kidney and intestine [7]. The expression level of the *Nrf2* gene is regulated by a positive feedback mechanism dependent on an ARE/EpRE in the promoter region of the *Nrf2* gene [31]. The *Nrf2* gene is also transcriptionally induced by the aryl hydrocarbon receptor [32].

Analysis of a single nucleotide polymorphism (SNP) in the promoter region of the *Nrf2* gene demonstrated the significance of the transcriptional regulation of the *Nrf2* gene in the determination of Nrf2 activity. A SNP in the promoter region of the mouse *Nrf2* gene is linked to the reduced expression of the *Nrf2* gene and susceptibility to hyperoxic lung injury in the C57BL/6J mouse strain [33]. Indeed, exposure to hyperoxia causes more severe lung damage in *Nrf2*-null mice than in wild-type mice [34]. SNPs found in the promoter region of the human *NRF2* gene [35] are linked to a higher risk of acute lung injury [36]. These data demonstrate the importance of transcriptional regulation of the *Nrf2* gene in stress response.

5.2 THE KEAP1-NRF2 SYSTEM IN RESPONSE TO ELECTROPHILES

Electrophiles have substantial impacts on biomolecules including proteins and nucleic acids that are rich in electron-dense parts. An atom with stronger electronegativity, such as oxygen, attracts pi electrons, and consequently an electron-deficient part is generated, which

TABLE 5.1 Phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults

Organ	Reagent	Phenotype(s)	Inflammation-related genes affected in *Nrf2*-null mice	Reference
Skin	DMBA/TPA	Increased incidence of skin tumors and tumor numbers		119
	Diesel exhaust	Severe epithelial hyperplasia and increased levels of 8-OHdG in the lung		17
	Benzo[a]pyrene	Increased somatic mutations of the *Gpt* gene in lungs		121
Gastric	DSS	Susceptibility to DSS-induced colitis	IL-1β, IL-6, iNOS, COX-2, TNF-α	156
	AOM/DSS	Susceptibility to AOM/DSS-induced colitis	TNF-α, IL-1β, IL-12p40	157
	Benzo[a]pyrene	Increased tumor numbers in the stomach		65, 158, 159
	AOM/DSS	Increased incidence of colonic tumors	COX-2, 5-LOX	122
Liver	IQ	Increased incidence of liver tumors and tumor numbers		160
	D3T,CDDO-Im	Reduced expression of detoxifying genes and antioxidant genes		148, 161
	Acetaminophen	Acute hepatotoxicity		18
Bladder	BBN	Increased incidence of bladder tumors		23
Brain	LPS	Increased microglial infiltration	iNOS, IL-6, TNF-α	16
Lung	LPS, sepsis induced by cecal ligation and puncture	Exacerbated lung inflammation	TNF-α, enhanced activation of NF-κB	163
	Carrageenin	Exacerbated lung inflammation, increased neutrophil infiltration		133
	Ovalbumin sensitization and challenge	Exacerbated allergen-driven asthmatic inflammation, hyperresponsiveness to cholinergic challenge	IL-4, IL-13, enhanced activation of NF-κB	164
	Cigarette smoke	Susceptibility to cigarette smoke-induced emphysema	Activation of caspase-3, decreased SLPI	19, 134
	Elastase	Susceptibility to elastase-induced lung inflammation and emphysema	Decreased SLPI	135

DMBA/TPA, 7,12-Dimethylbenz(*a*)anthracene/12-O-tetradecanoylphorbol-13-acetate;

DSS, dextran sulfate sodium; AOM, azoxymethane; IQ, 2-amino-3-methylimidazo

[4,5-*f*]quinoline; D3T. 3*H*-1,2-dithiole-3-thione; CDDO-Im, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; BBN, *N*-nitrosobutyl(4-hydroxybutyl)amine; LPS, lipopolysaccharide; SLPI, secretory leukoprotease inhibitor.

TABLE 5.2 Phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults

Organ	Phenotype(s)	Reference
Skin	Prolonged inflammation	165
Brain	Astrogliosis and vacuolar leukoencephalopathy after 1 year of age	166
	Exacerbated leukocyte infiltration, increased infarct size, and severe behavioral deficits after the intracerebral hemorrhage	167, 168
Immune system	Autoimmune inflammation and lymphoproliferation in multiple organs	29
	Lupus-like autoimmune nephritis	27
Hematopoietic	Immune-mediated hemolytic anemia	28
Craniofacial	De-colorization of incisors	169

attacks electron-dense parts of nucleic acids and proteins. The protein adduct leads to acute toxicity due to cellular dysfunction, and the DNA adduct formation leads to carcinogenesis. The Keap1-Nrf2 system is a mechanism of cytoprotection from such electrophilic insults. The extremely sensitive nature of several thiol residues of Keap1 to electrophiles enables the system to respond to the stimuli in the first place.

5.2.1 Characteristic Features of Nrf2 Inducers

The most important feature of gene expression regulated by Nrf2 is inducibility upon exposure to xenobiotic and oxidative stress (Fig. 5.3). Nrf2 is a short-lived protein with a half-life of approximately 20 min [37, 38]. Proteasomal inhibitors dramatically increase the amount of Nrf2 protein, indicating that Nrf2 is constantly degraded by the proteasome. While Nrf2 is hardly detected during unstressed conditions, the half-life of Nrf2 becomes longer, allowing easy detection of Nrf2 in nuclei, during stressed conditions [37, 39]. Xenobiotic and oxidative stress inhibit proteasome-dependent degradation of Nrf2. Stabilization of Nrf2 upon exposure to stress is critical for the inducible expression of many cytoprotective genes.

A common feature of the diverse substances that act on Nrf2 is electrophilicity (Fig. 5.4). Typical Nrf2 inducers are diethyl maleate (DEM), *tert*-butylhydroquinone (tBHQ), and sulforaphane (SFN), all of which are electrophilic. Many other electrophiles, including ebselen (Ebs) and 8-nitroguanosine 3′, 5′-cyclic monophosphate (8-nitro-cGMP), have been shown to activate Nrf2 [40, 41]. Thus electrophiles can stabilize Nrf2, which allows Nrf2 to accumulate in nuclei and activate transcription.

5.2.2 Identification of Keap1

An important finding that led to the revelation of the molecular mechanism for how Nrf2 is stabilized by electrophiles originated from an analysis of the N-terminal region of Nrf2. Deletion of the N-terminal region of Nrf2 markedly enhances its transcriptional activity [42]. The N-terminal region corresponds to Nrf2-ECH homology 2 (Neh2), which is a highly homologous domain between human and chicken Nrf2. Nrf2 is divided into six domains, ranging from Neh1 to Neh6 [42] (Fig. 5.5A). Neh1 includes a bZIP motif and is involved in DNA binding, heterodimerization, and nuclear translocation. Neh4 and Neh5 are essential for strong transcriptional activity upon coactivator binding, such as CBP [43]. The Neh2 domain negatively regulates Nrf2 transcriptional activity.

The Kelch-like ECH associated protein 1 (Keap1) was identified as a novel cytoplasmic factor that interacts with the Neh2 domain of Nrf2 [42]. Keap1 is divided into three main parts (Fig. 5.5B). The BTB domain resides in the N-terminus, while the DC domain, which includes the double glycine repeat (DGR) and the carboxyl-terminal region (CTR), resides in the C-terminus. The region between the BTB and DC domains is called the intervening region (IVR). Molecular dissection of Nrf2 and Keap1 demonstrated that the Neh2 domain of Nrf2

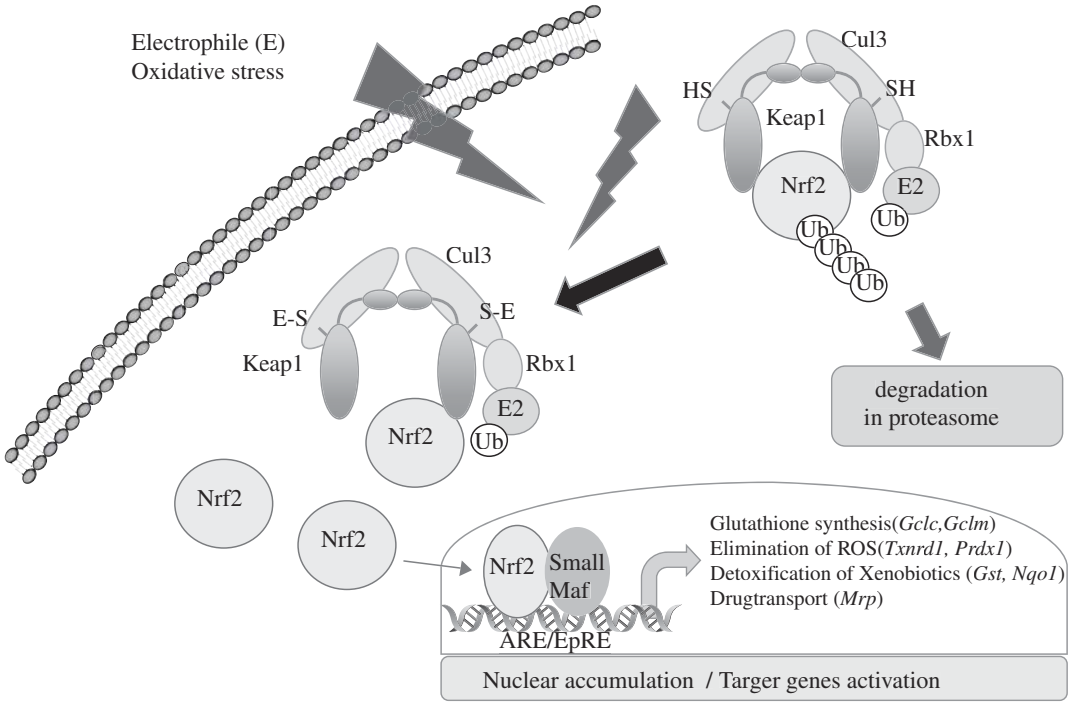

Fig. 5.3 The mechanism of Nrf2 activation in response to electrophiles and oxidative stress. In unstressed conditions, Nrf2 shows a rapid turnover due to ubiquitination and degradation via the proteasome. The Keap1-Cul3 complex serves as an E3 ubiquitin ligase for Nrf2. On exposure to electrophiles or oxidative stress, reactive thiols of Keap1 (-SH) are modified (-SE), resulting in a decline in enzymatic activity. Nrf2 is stabilized, and de novo synthesized Nrf2 translocates into nuclei, heterodimerizes with small Maf, and activates target genes encoding antioxidant proteins, detoxifying enzymes, and other cytoprotective proteins.

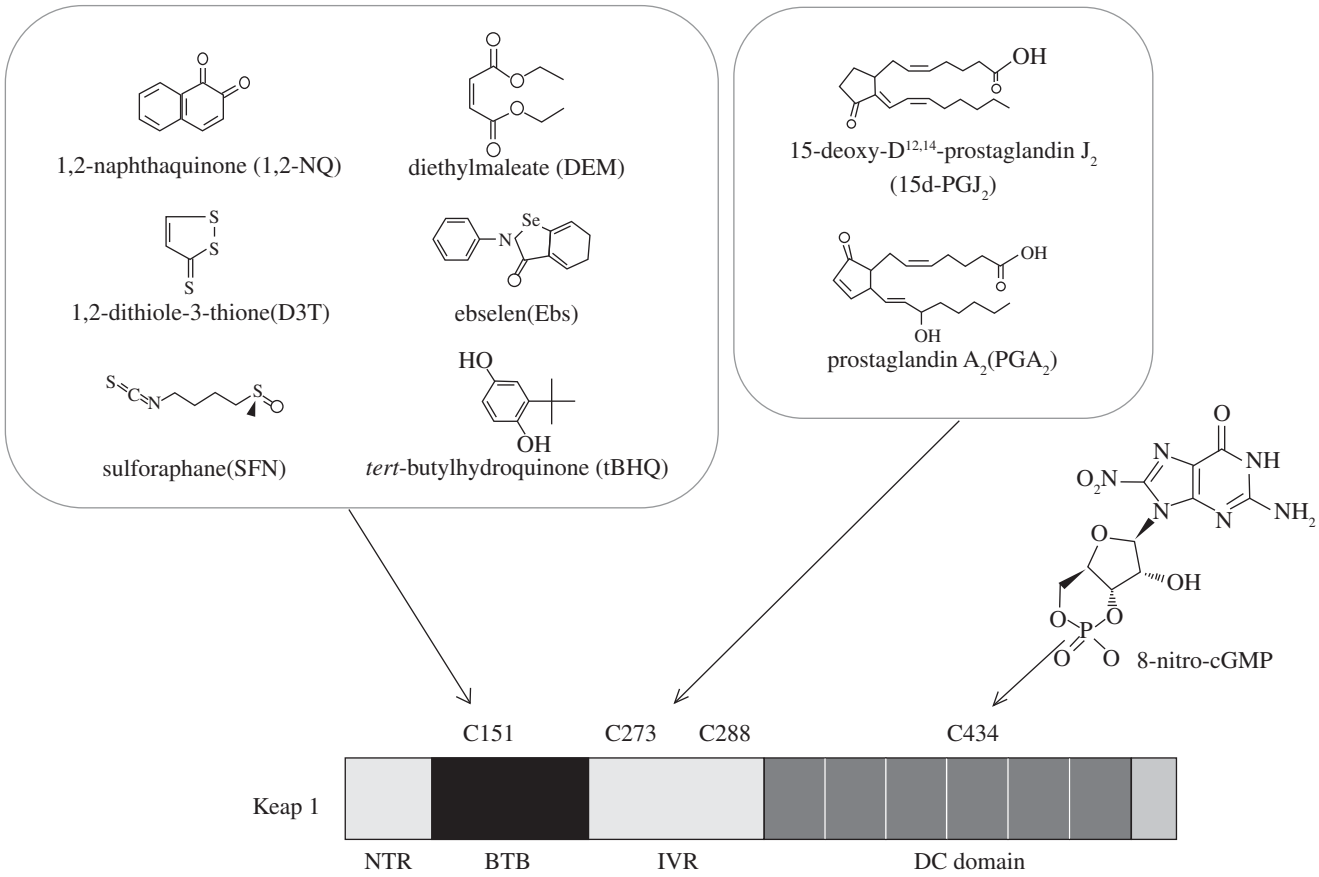

Fig. 5.4 Electrophile-induced Nrf2 activity and the Keap1 sensor system. Distinct cysteine residues are involved in sensing different types of electrophiles. Cys-151 is necessary for Keap1 to respond to a group of electrophiles. Bulky electrophiles, including 15d-PGJ_2 and PGA_2, are considered to target Cys-273 and Cys-288, while 8-nitro-cGMP modifies Cys-434.

interacts with the Keap1 DC domain [42]. An association between Keap1 and Nrf2 destabilizes Nrf2, inhibiting transcriptional activation of the stress response genes.

Keap1 is ubiquitously expressed in adult tissues, among which skin, the esophagus, and the forestomach display particularly abundant expression [44, 45]. An approximately 5.7-kbp region upstream of the mouse *Keap1* gene harbors the enhancer activity for recapitulating *Keap1* expression profiles in vivo [45]. A genomic fragment containing this region was utilized for expressing Keap1 or its mutant molecule in transgenic mice, which were then bred into the *Keap1*-null background to evaluate the function of the Keap1 mutant molecule in the *Keap1*-null background (see Section 5.2.5).

5.2.3 Nrf2 is the Most Substantial Target of Keap1

Disruption of the *Keap1* gene in mice revealed that Nrf2 is actually stabilized in the absence of Keap1. Subsequently, Nrf2 localizes to nuclei and activates target genes for cytoprotection. *Keap1*-null cells, which constitutively express high levels of cytoprotective genes, were expected to be resistant to xenobiotic and oxidative stress, and *Keap1*-null mice were expected to survive under severely stressed conditions. However, the phenotype of *Keap1*-null mice was unexpected. Instead of making mice more resistant to stress, *Keap1* deletion caused lethality 3 weeks after birth, and mice displayed severe hyperkeratosis of the esophagus and forestomach [44]. The lack of nutrition was considered to be the primary cause of the death, because keratinocyte-specific deletion of the *Keap1* gene results in the same preweaning lethality [46]. Mice can survive to adulthood if they escape this feeding problem [47].

An intriguing notion emerged from the analysis of mice harboring the hypomorphic allele of *Keap1* (*Keap1*^{flox}), in which the effect of the graded expression of Keap1 in vivo was examined [46]. Insertion of two loxP sequences into the *Keap1* locus for conditional disruption of the *Keap1* gene actually reduced expression of *Keap1*. *Keap1*$^{flox/-}$ mice displayed constitutive stabilization of Nrf2 in various tissues and were more resistant

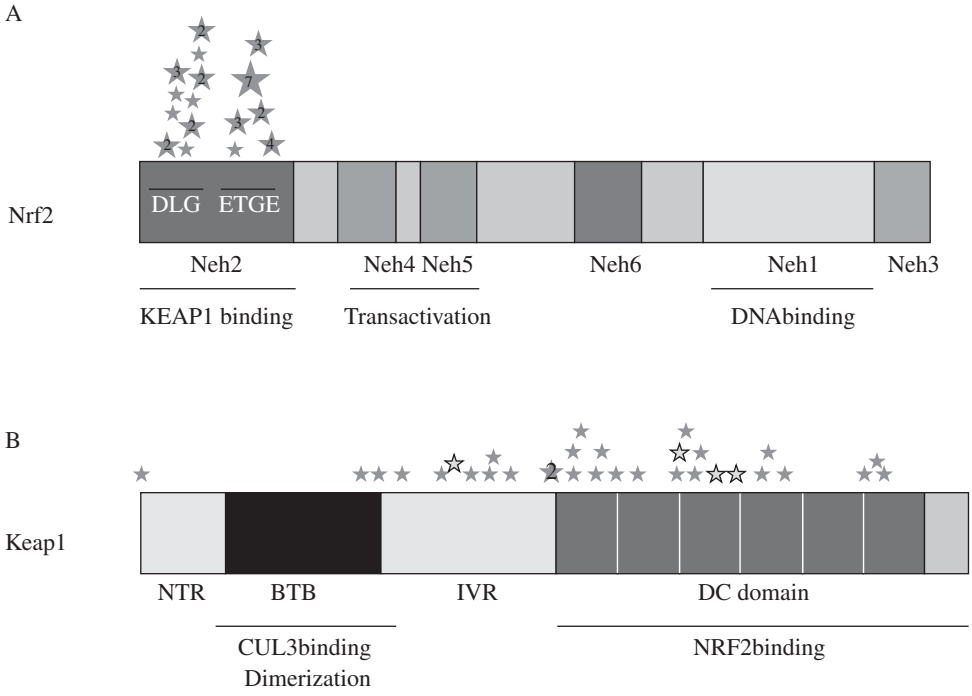

Fig. 5.5 The domain structures of Nrf2 and Keap1. (A) Nrf2 contains six well-conserved domains, Neh1 to Neh6. Neh2 is essential for binding to Keap1. Neh4 and Neh5 contribute to transactivation through interaction with CBP. Neh1 is involved in DNA binding and dimerization with small Mafs. Stars indicate the positions of amino acid substitution and deletion due to somatic mutations in the *NRF2* gene found in human cancers. When an amino acid is affected in more than two cases, the number of cases is shown in the star. All mutations are clustered within the DLG and ETGE motifs. The results of two reports are summarized [70, 71]. (B) Keap1 consists of a BTB domain, a DC (DGR and CTR) domain, and the IVR (between BTB and DC domain). The BTB domain contributes to homodimerization. BTB and IVR are involved in the interaction with Cul3. The DC domain is essential for the interaction with Nrf2. Stars without rims indicate the positions of amino acid substitution and deletion due to somatic mutations in the *KEAP1* gene found in human cancers. When an amino acid is affected in more than two cases, the number of cases is shown in the star. Stars with bold rims indicate the generation of stop codons due to somatic mutations, resulting in truncation of the KEAP1 protein. The results of six reports are summarized [68, 69, 72, 123, 124, 170].

to acetaminophen-induced liver toxicity than wild-type control mice. *Keap1*$^{flox/-}$:Albumin-Cre mice, in which the *Keap1* gene is completely disrupted in liver, are not as resistant as hypomorphic *Keap1*$^{flox/-}$ mice. Thus the inducible and transient activation of Nrf2 is beneficial for cytoprotection, while constant and intense activation of Nrf2 seems to be disadvantageous [48].

Importantly, simultaneous disruption of the *Nrf2* gene completely rescued lethality in *Keap1*-null pups [44], suggesting that constitutive activation of Nrf2 is the cause of the death and that Nrf2 is the most substantial target of Keap1.

5.2.4 The Keap1-Nrf2 Complex for Nrf2 Degradation

Keap1 is responsible for the degradation of Nrf2 in unstressed conditions. Further studies revealed that Keap1 is an adaptor molecule of the Cullin 3-based ubiquitin E3 ligase [38, 49–51] (Fig. 5.3). Ubiquitination of Nrf2 is inhibited in the absence of Keap1, and, consequently, Nrf2 is stabilized and accumulates in nuclei. In the absence of stimuli, low expression levels of Nrf2-dependent genes are maintained by the ubiquitin E3 ligase activity of the Keap1-Cul3 complex.

Structural and biochemical analyses revealed the molecular mechanisms of Nrf2 degradation. Keap1 homodimerizes through its BTB domain [52], and the overall structure of the homodimer resembles a "cherry-bob" [53] (Fig. 5.6A). The global structure of the cherry-bob is composed of a DC domain, the IVR and part of the BTB domain. A pair of Keap1 homodimers possesses two DC domains, in which one molecule of Nrf2 associates using two discrete motifs within the Neh2 domain, the ETGE and DLG motifs [54] (Fig. 5.6B). The two-site binding of Keap1 and Nrf2 seems to be beneficial for the ubiquitination and subsequent degradation of Nrf2 because seven lysine residues, which are targets of ubiquitination, are aligned at the same surface of the α-helix structure between the ETGE and DLG motifs.

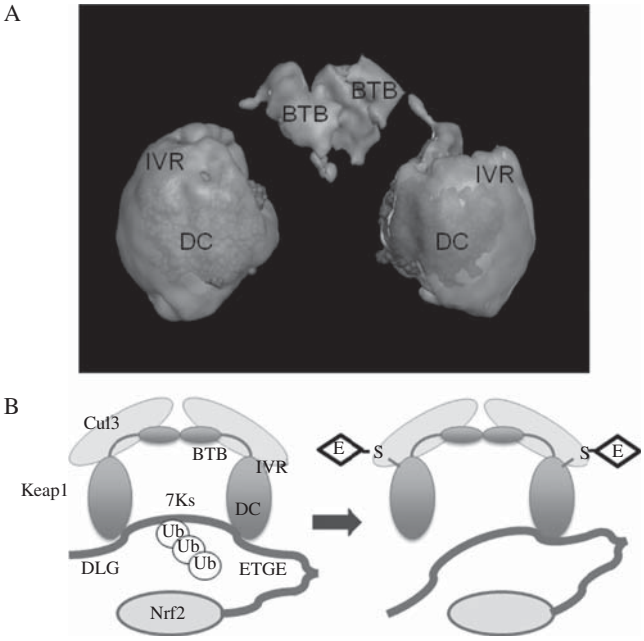

Fig. 5.6 The overall structure of the Keap1 homodimer and the regulation of Nrf2 activity by Keap1. (A) The three-dimensional structure of the Keap1 homodimer (cited from Ogura et al., *Proc Natl Acad Sci USA*. 2010:2842-7, 107). (B) Interaction between Nrf2 and the Keap1-Cul3 complex under unstressed conditions (left) and stressed conditions in which thiols are modified with electrophiles (E) (right). Each DC domain of the Keap1 homodimer binds to the DLG and ETGE motifs in the Neh2 domain of Nrf2. Keap1 is proposed to interact with Cul3 at the BTB domain and the IVR. Nrf2 is polyubiquitinated at 7 lysine residues (7Ks) between the DLG and ETGE motifs (left). Modification of Keap1 with electrophiles is thought to alter the overall conformation of the Keap1-Cul3 and Nrf2 complex, which inhibits Nrf2 ubiquitination (right). *(See color insert.)*

5.2.5 Keap1 as a Sensor Molecule for Electrophiles

Upon exposure to xenobiotic and oxidative stress, Keap1 stops the ubiquitination of Nrf2. Stabilized Nrf2 translocates into nuclei, heterodimerizes with small Maf, and activates the transcription of target genes (Fig. 5.3). An important question is how the stimuli repress the E3 ligase activity of the Keap1-Cul3 complex.

Keap1 is a thiol-rich protein and possesses many reactive cysteine residues neighboring basic amino acids. Covalent binding of electrophiles to cysteine residues has been observed in vitro and in vivo by mass spectrometry [55, 56]. The significance of the cysteine residues to Keap1 function was demonstrated *in transfecto* [57, 58] and in vivo [45]. The contributions of Cys-151 in the BTB domain and Cys-273 and Cys-288 in the IVR to Keap1 function were the focus of the in vivo studies. Transgenic mice expressing a mutant Keap1 under the regulation of the endogenous *Keap1* gene regulatory region were generated and crossed into the *Keap1*-null background. The in vivo contributions of each cysteine residue to Keap1 function was evaluated by this transgenic complementation rescue method. Alanine substitution of Cys-273 and Cys-288 abrogated the repressor function of Keap1, while serine substitution of Cys-151 did not affect the repressor function but impaired the responsiveness of Keap1 to a group of electrophiles [45] (Fig. 5.4). Thus Cys-273 and Cys-288 are essential for Keap1 to repress Nrf2 activity in unstressed conditions, while Cys-151 is essential for the response to electrophilic stimuli.

A more detailed analysis revealed that Keap1 senses various types of electrophiles utilizing distinct cysteine residues [59, 60] (Fig. 5.4). Many of the typical electrophiles, including DEM, tBHQ, SFN, Ebs, D3T, and 1,2-NQ, are sensed by Cys-151. Bulky electrophiles such as 15d-PGJ_2 and PGA_2 seem to attack Cys-273 and Cys-288. Cys-434 is involved in nitric oxide signaling [61]. Cys-226 and Cys-613, in addition to His-225, are critical for sensing metal ions such as Cd^{2+} and Zn^{2+} [60]. These results indicate that Keap1 is a multifaceted electrophile sensor. Diverse inputs to Keap1, which are sensed in multiple ways using discrete cysteine residues, are processed to a single output, namely, inhibition of Nrf2 ubiquitination and subsequent Nrf2 stabilization.

Keap1 possesses multiple thiols with a lower pKa, as 10 μM 8-nitro-cGMP, one of the endogenous electrophiles generated downstream of nitric oxide, modifies Keap1 even in the presence of 10 mM glutathione [41]. This explains why Keap1 detects low electrophile concentrations within cells and serves as a sensitive sensor. Such sensitivity enables the Keap1-Nrf2 system to play a central role in the electrophile counterattack response for cell protection.

Modification of Keap1 alters the overall conformation of the Keap1-Nrf2 complex and/or alters the interaction between Keap1 and Cul3 [54, 62]. Because the affinity between the Neh2 DLG motif and the Keap1 DC domain is lower than that of the Neh2 ETGE motif and the Keap1 DC domain, modification of Keap1 appears to alter the conformation of the complex, which leads to dissociation of the weaker interaction between DLG and DC. The stronger interaction between ETGE and DC seems to be maintained, which is consistent with the finding that alanine substitution of Keap1 C273 and C288 abrogates its ability to degrade Nrf2 but retains its binding affinity to Nrf2 [63]. In addition, de novo synthesized Nrf2, and not Nrf2 dissociated from Keap1, translocates into the nucleus [63]. The "hinge and latch model" is one of the molecular mechanisms for Nrf2 stabilization following Keap1 modification [64] (Fig. 5.6B). Alternatively, Cul3 dissociates from Keap1 upon exposure to stimuli [62]. The electrophiles targeting Cys-151 seem to disrupt the Keap1-Cul3 interaction. Loss of the Cul3-Keap1 interaction results in a decline in Keap1-Cul3 E3 ligase activity.

5.2.6 Electrophile-Independent Activation of Nrf2

An analysis of *Nrf2*-null mice showed that the activation of Nrf2 by electrophiles is effective for the inhibition of carcinogenesis following a challenge with carcinogens [65] (Table 5.2). A field study in China proved that Nrf2 activation is potentially effective for cancer chemoprevention [66, 67]. Nrf2 activation is beneficial for reducing the risk of carcinogenesis because many detoxifying enzymes induced by Nrf2 promote the elimination of strong electrophiles that could easily attack nucleic acids and proteins to form carcinogenic adducts.

Therefore, it is surprising that Nrf2 is constitutively stabilized in several cancer cells independent of electrophilic stimuli. Somatic mutations of the *KEAP1* gene have been found in lung adenocarcinomas [68, 69], while somatic mutations in the *NRF2* gene have been mainly identified in squamous carcinomas of the lung [70] (Fig. 5.5). Since the early reports about lung cancers, somatic mutations of *KEAP1* and *NRF2* have been documented in various solid cancers [71–73]. Mutant KEAP1 does not have the ability to ubiquitinate Nrf2, and, consequently, Nrf2 is constitutively stabilized. Intriguingly, *KEAP1* mutations often occur heterozygously, and heterozygous *KEAP1* mutations produce a dominant negative effect [68]. The cherry-bob structure and the two-site binding model offer a very rational explanation for this phenomenon [53, 74]. Keap1 exerts its function in the form of a dimer, and both Keap1 subunits need to be intact for the proper ubiquitination of Nrf2. A sophisticated study using a combination of *Keap1*-null mice and transgenic mice expressing wild-type and mutant Keap1 revealed that the heterodimer of wild-type Keap1 and mutant Keap1 is not functional as an adaptor for the E3 ubiquitin ligase [45]. Missense mutations in the *KEAP1* gene are one of the causes of the constitutive stabilization of Nrf2 (Fig. 5.5B).

Somatic mutations in the *NRF2* gene are another cause of the constitutive stabilization of Nrf2. Intriguingly, the DLG and ETGE motifs in the Neh2 domain, identified in the molecular dissection of Nrf2 protein, correspond to the hot spots of *NRF2* mutations in cancer cells [70]. Amino acid substitution in either the DLG or ETGE motifs results in inhibition of Nrf2 ubiquitination, which is consistent with the requirement for an intact two-site binding structure of the Keap1-Nrf2 complex for efficient ubiquitination of Nrf2 (Fig. 5.5A).

The third cause of the constitutive stabilization of Nrf2 is the repression of *KEAP1* gene expression by epigenetic mechanisms. DNA methylation of the *KEAP1* gene promoter has been observed frequently in several cancer cell lines [75, 76].

Although Keap1 and Nrf2 are functionally normal, several cases have been reported in which Nrf2 is constitutively stabilized. The accumulation of certain proteins that disrupt the association between Keap1 and Nrf2 was found to be responsible for stabilizing Nrf2. One example of these proteins is $p21^{Cip1/WAF1}$. The C-terminal region of p21 associates with the DLG motif of Nrf2 [77], preventing the formation of the two-site Keap1-Nrf2 binding complex. Thus p21 facilitates Nrf2 stabilization and promotes the expression of cytoprotective genes, which explains the cytoprotective function of p21 [78]. Another example is p62, a polyubiquitin-binding protein, which targets various substrates for autophagy [79]. The STGE motif of p62 interacts with the Keap1 DC domain in a similar manner to that of the DLG motif of Nrf2 [80]. When autophagy is impaired, p62 accumulates and disrupts the formation of the two-site Keap1-Nrf2 binding complex, which leads to Nrf2 stabilization. Intriguingly, abnormal accumulation of p62 is often observed in various human pathological conditions [81, 82]. Whether Nrf2 makes any contributions, favorable or unfavorable, to this pathogenesis remains to be elucidated.

5.2.7 Posttranslational Modification of Nrf2

Several reports have demonstrated the posttranslational modification of Nrf2. Phosphorylation of Nrf2 has been shown to promote or repress Nrf2 activity depending on the context. Activation of protein kinase C (PKC) enhances Nrf2 activity and is accompanied by the phosphorylation of Nrf2 at Ser-40 [83, 84]. In contrast, GSK-3β promotes nuclear exclusion of Nrf2 and the subsequent repression of the xenobiotic and antioxidant responses [85, 86]. Fyn kinase, activated by GSK-3β, phosphorylates Nrf2 at Tyr-568, which induces nuclear export and degradation of Nrf2 and attenuates the expression of cytoprotective genes [87].

Nrf2 is also modified by acetylation. CBP/p300 directly binds and acetylates Nrf2 in response to arsenite-induced oxidative stress [88]. Lysine residues of the acetylation target reside within the Neh1 domain of Nrf2, which contains the bZIP motif for DNA binding and dimerization. Consistently, this modification compromised the DNA binding activity of Nrf2 in a promoter-specific manner. The Neh3 domain of Nrf2 was also shown to be acetylated, which affects the subcellular localization of Nrf2 [89].

5.3 NRF2 IN THE CNC-SMALL Maf TRANSCRIPTION FACTOR NETWORK

Stabilized Nrf2 due to the exposure to electrophic stimuli translocates into the nucleus and forms a heterodimer with small Maf. Small Maf is an obligatory partner molecule of Nrf2, conferring DNA binding ability

and DNA recognition specificity. Functional deficiency of small Maf results in the summation of functional deficiency of each CNC protein. Because of the unique DNA recognition of small Maf, target genes regulated by CNC-small Maf heterodimer are distinct from those of other bZIP transcription factors including Jun and Fos families. Small Maf, being shared by all the CNC proteins, serves as a central node of the transcription factor network by CNC and small Maf proteins.

5.3.1 Small Maf Proteins as Heterodimerization Partners for Nrf2

Genetic evidence clarified the essential role of small Maf proteins as the heterodimeric partner molecules of CNC proteins, including Nrf2 [90–92]. Small Maf proteins comprise one of the subclasses of Maf family proteins. Maf proteins possess a unique bZIP motif accompanied by Maf-specific extended homology region (EHR) and bind to Maf recognition elements (MAREs; TGCTGA$^{G(C)}$/$_{C(G)}$TCAGCA) in the form of a homodimer [15] (Fig. 5.1 and Fig. 5.2). Large Maf proteins, for example, c-Maf, MafB, MafA, and NRL, additionally possess *trans*-activation domains in their N-terminal regions, whereas small Maf proteins, for example, MafG, MafK, and MafF, lack *trans*-activation domains. Small Maf homodimers thus repress transcription, while heterodimers composed of small Maf and CNC proteins with *trans*-activation domains activate transcription [93, 94]. CNC-small Maf heterodimers also bind to MAREs and related sequences including the ARE/EpRE and NF-E2 binding sites. A functional balance between the CNC and small Maf proteins may determine MARE-dependent transcription activity, ranging from repression to activation (Fig. 5.2B).

5.3.2 Small Maf Deficiency as the Summation of Each CNC Protein Deficiency

Because three small Maf proteins, MafF, MafG, and MafK, are highly homologous and functionally redundant, disruption of any single gene causes only a modest phenotype [90, 95, 96]. Double- or triple-mutant mice display more severe phenotypes, which are a composite of the phenotypes observed in mutant mice lacking each individual CNC protein. For example, small Maf-mutant mice display thrombocytopenia, neurodegeneration, and an impaired response to xenobiotic and oxidative stress [92, 97, 98]. The thrombocytopenia is characteristically observed in *NF-E2*-null mice [99]. The neurodegeneration is observed in neural tissue-specific *Nrf1*-null mice [100]. An impaired response to xenobiotic and oxidative stress is observed in *Nrf2*-null mice [8, 24]. Thus small Maf proteins are essential for CNC proteins to exert their activity as a transcription factor.

In addition to serving as a DNA-binding adaptor for the CNC proteins, small Maf plays additional roles in the regulation of the transcription activity of CNC proteins. Small Maf can be described as bidirectional in transcription, that is, it behaves as an activator or a repressor when it exists as a heterodimer with CNC or a homodimer, respectively (Fig. 5.2B). The latter function has been suggested to be dependent on the sumoylation of small Maf [101]. The former function requires proper subnuclear localization of the CNC-small Maf heterodimer, which is directed by the C-terminal region of small Maf [102]. Interestingly, the lysine residue for sumoylation and the C-terminal region for appropriate subnuclear targeting both reside outside of the bZIP motif, and their contributions are only detectable in vivo.

5.3.3 A Unique DNA Sequence Is Recognized by the Nrf2-Small Maf Heterodimer

As an obligatory heterodimeric partner molecule for CNC proteins, small Maf plays a critical role in the determination of the unique DNA binding specificity of the heterodimer [103]. A functional ARE/EpRE site characteristically requires the conserved GC sequence [GCNNN(G/C)TCA(T/C)] (Fig. 5.2A), which distinguishes the ARE/EpRE from the binding sequences of other bZIP transcription factors, including AP-1 [2].

The MARE is unique in terms of length. The complete MARE is a 13-bp [TGCTGA^{G}/$_{C}$TCAGCA] or 14-bp [TGCTGA^{GC}/$_{CG}$TCAGCA] palindromic sequence that is bound by Maf homodimers, whereas the dimers of other bZIP transcription factors, including Jun/Fos (AP-1), CREB, and C/EBP, recognize 7- or 8-bp palindromic sequences, for example, the TPA(12-*o*-tetradecanoylphorbol 13-acetate)-responsive element (TRE; TGA^{G}/$_{C}$TCA) or the cyclic AMP-responsive element (CRE; TGA^{GC}/$_{CG}$TCA) [104, 105] (Fig. 5.2A). The ARE/EpRE is regarded as a composite sequence with a half-site of the typical MARE and a half-site of the typical TRE. Small Maf and Nrf2 recognize the former and the latter portions, respectively [103]. Recognition of the GC bases flanking the core TRE or CRE is a unique feature of the small Maf-DNA interaction, which critically distinguishes the ARE/EpRE from a TRE or CRE.

The structural basis of the small Maf-DNA interaction was clarified through the crystallization of the bZIP motif of MafG and the MARE-containing DNA duplex [106]. Two structural components are specific to small Maf proteins. One is the presence of the EHR on the N-terminal side of the basic region, and the other is the tyrosine residue (Tyr-64) in the basic region, whose corresponding residue is alanine in other bZIP transcription factors (Fig. 5.7). The overall structure of MafG homodimers and DNA is almost identical to that of

AP-1 (c-Jun and c-Fos heterodimer), and the two Maf-specific components are not directly involved in the recognition of the GC bases outside of the TRE core. Maf-specific structural elements contribute to the unique orientation of the side chains of the invariant amino acids in the basic region, Arg-57 and Asn-61, which play direct roles in establishing unique DNA recognition specificity.

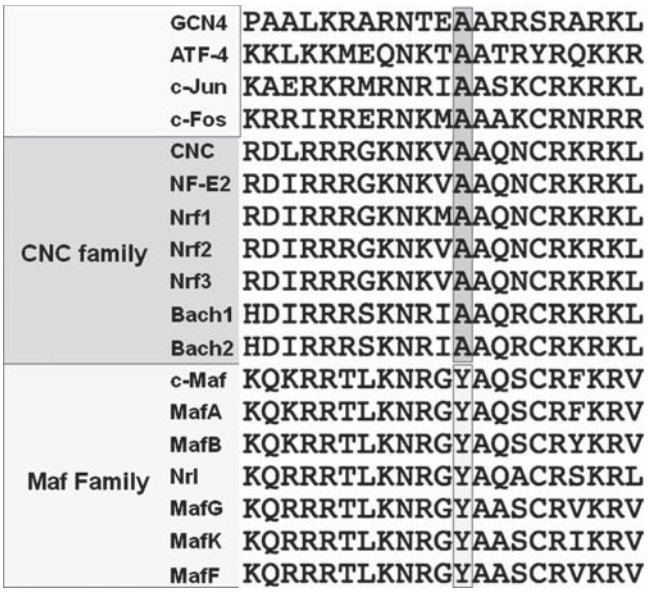

Fig. 5.7 Alignment of the basic domains of various bZIP transcription factors. The tyrosine residue is unique to Maf family proteins and corresponds to the alanine residue of the other bZIP proteins.

Comprehensive measurement of the binding affinities to MARE and related sequences was performed using the MafG homodimer and the Nrf2-MafG heterodimer [103, 107]. The comparison between the two results revealed that the MARE-related sequences can be categorized into three groups: homodimer-oriented MAREs, heterodimer-oriented MAREs, and ambivalent MAREs (Fig. 5.8). Homodimer MAREs are considered to be the exclusive targets of Maf proteins, while heterodimer MAREs are considered to be the exclusive targets of CNC-small Maf. The ambivalent MAREs should be bound competitively by both homodimers and heterodimers (Fig. 5.8). However, the in vivo significance of MARE-related sequence categorization into three classes remains to be evaluated.

Interestingly, swapping the unique tyrosine residue of MafG and the corresponding alanine residue of Nrf2 resulted in the simultaneous swapping of DNA recognition specificity [108]. The Nrf2 A502Y-MafG heterodimer preferentially recognizes homodimer-oriented MAREs as well as ambivalent MAREs (Fig. 5.8). Comparison of the target genes of the Nrf2 A502Y-MafG heterodimer with those of the wild-type Nrf2-MafG heterodimer demonstrates the significance of the distinct DNA recognition specificity of the Maf homodimer and the CNC-Maf heterodimer.

5.3.4 Regulation of Small Maf Expression

Genes encoding the small Maf proteins, MafF, MafG, and MafK, are expressed in a wide range of tissues [90, 96, 109, 110]. Dissection of the regulatory region of the *MafK* gene led to a new concept in which ubiquitous

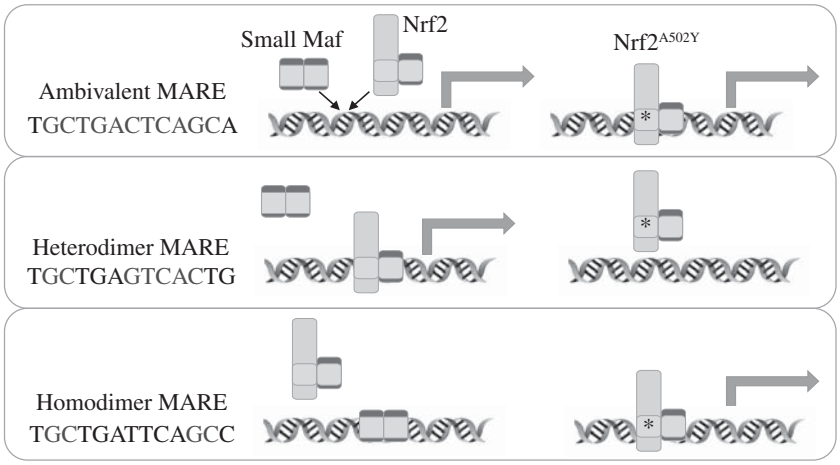

Fig. 5.8 Categorization of MARE-related sequences. Ambivalent MAREs are bound by both Nrf2-small Maf heterodimers and Maf homodimers. Heterodimer MAREs are preferentially bound by Nrf2-small Maf heterodimers, and homodimer MAREs are bound by Maf homodimers. Substitution of the alanine residue of the Nrf2 basic region with a tyrosine residue switches the DNA recognition specificity from the CNC type to the Maf type and results in the preferential binding of Nrf2 A502Y-small Maf heterodimers to homodimer MAREs and not to heterodimer MAREs. *(See color insert.)*

expression is achieved by the coordinate function of multiple tissue-specific enhancers rather than the activity of a single general enhancer [109, 111, 112]. Interestingly, many stimuli that induce Nrf2 activation enhance transcription of the *MafG* gene, including hydrogen peroxide, β-naphthoflavone, cadmium, zinc, and arsenite [113–115]. Consistently, the *MafG* gene possesses an ARE/EpRE in its promoter and is directly activated by Nrf2 [116], which constitutes a positive feedback loop. An increase in small Maf proteins must accompany Nrf2 stabilization for efficient use of the available Nrf2. The *MafG* gene is also induced by hypercapnic stimulation in the medulla oblongata by inhalation of 7% CO_2, where the central baroreceptive neurons are distributed [117]. Induction of the *MafG* gene is observed upon activation of the baroreceptors by the repressor agent phenylephrine [118]. The precise mechanism of *MafG* induction via these challenges in neural cells is still unknown.

5.4 DYSFUNCTION OF NRF2 IN PATHOLOGICAL CONDITIONS

Nrf2 dysfunction has been implicated in many pathological conditions. Functional impairment of Nrf2 is related to the susceptibility to exogenous chemical and physical insults and to the proinflammatory predisposition. Nrf2 inducers are beneficial for the treatment of these conditions. Naturally occurring electrophiles contained in vegetables and other synthetic drugs have been shown to be effective Nrf2 inducers. By contrast, aberrant hyperfunction of Nrf2 has been found in various human cancers. Nrf2 inhibitors, now under development, are expected to make promising anticancer drugs that increase the efficacy of chemotherapy and radiotherapy and inhibit the proliferation of cancer cells.

5.4.1 Dual Functions for Nrf2 in Cancer Pathology

Because Nrf2 is a key regulator of the inducible expression of detoxifying enzymes, chemical challenge produces more severe damage to *Nrf2*-null mice than to wild-type mice. One of the most obvious adverse effects in *Nrf2*-null mice is susceptibility to chemical carcinogenesis [23, 65, 119–122]. In the absence of Nrf2, elimination of highly reactive metabolites of chemicals is delayed, resulting in the increased formation of DNA adducts and the subsequent accumulation of DNA mutations. Nrf2 plays a protective role in carcinogenesis.

In contrast, Nrf2 expression in cancer cells contributes to malignant phenotypes during cancer progression and leads to a poor prognosis [70]. Somatic mutations in the *KEAP1* gene or the *NRF2* gene are found in a substantial portion of human cancers, including lung cancer, gallbladder cancer, head and neck cancer, and breast cancer [68–70, 73, 123–125] (Fig. 5.5). Reduced expression of *KEAP1* due to DNA methylation of the promoter region of the gene has been reported in lung cancer and prostate cancer [75, 76]. These genetic and epigenetic alterations cause constitutive stabilization of Nrf2 and elevated expression of cytoprotective genes in cancer cells. Constitutive stabilization of Nrf2 and sustained expression of its target genes confer resistance to anticancer drugs, such as cisplatin, carboplatin, etoposide, and 5-fluorouracil [68–70] and irradiation [126].

A recent study demonstrated the protective role of Nrf2 against cancer metastasis [127]. Nrf2 activity in cells of hematopoietic origin, which are thought to be myeloid-derived suppressor cells, is critical to the inhibition of lung metastasis of cancer cells. Thus Nrf2 is a double-edged sword in cancer pathology; Nrf2 activation in the host is beneficial, while Nrf2 activation in the cancer is detrimental.

5.4.2 The Contribution of Nrf2 to Inflammatory Processes

The Keap1-Nrf2 system responds not only to xenobiotic electrophiles but also to endogenous electrophiles, including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [128], 8-nitroguanosine 3′,5-cyclic monophosphate (8-nitro-cGMP) [41, 61], 4-hydroxynonenal (4-HNE) [129], and the oxidation products of omega-3 fatty acids [130–132]. 15d-PGJ_2 is generated during inflammatory responses in which cyclooxygenase-2 (COX-2) plays a critical role. 8-Nitro-cGMP is generated in the process of nitric oxide signaling, and 4-HNE is one of the products of lipid peroxidation caused by ROS. Derivatives of omega-3 fatty acids, which have received much attention because of their anti-inflammatory nature, are produced in the resolution phase of inflammation. Thus Nrf2 should be activated in processes in which these signaling pathways are operative.

Indeed, Nrf2 plays a critical role in the resolution phase of inflammation, when COX-2 activity is increased [128]. Carrageenan-induced pleurisy is aggravated and prolonged in *Nrf2*-null mice. Pleural macrophages express high levels of COX-2 in the resolution phase of acute inflammation, which promotes the synthesis of 15d-PGJ_2. Subsequent activation of Nrf2 facilitates the remission of cell infiltration [128, 133]. Nrf2 deficiency confers a predisposition toward inflammation, resulting in the exacerbation of emphysematous changes following insults from cigarette smoke or elastase [19, 133–135] and of fibrotic changes following bleomycin exposure [136]. *Nrf2*-null mice also display susceptibility to ConA-induced acute inflammatory liver injury [137]. In many cases, ablation of *Nrf2* in

mice results in upregulation of proinflammatory cytokines, implying an excessive inflammatory reaction in *Nrf2*-null mice (Table 5.1).

5.4.3 Nrf2 in Cell Proliferation and Tissue Regeneration

Microarray experiments have revealed that Nrf2 regulates not only detoxifying enzymes and antioxidant proteins but also several factors promoting cell proliferation [138–140]. Constitutive activation of Nrf2 promotes cell proliferation [68, 69], while disruption of the *Nrf2* gene compromises cell proliferation [139]. *Nrf2*-null cells display impaired cell cycle progression accompanied by a reduction in the phosphorylation of Akt and the protein abundance of ERK2 and Stat3 [141]. Importantly, supplementation of glutathione rescued the impaired proliferation, the compromised cell cycle progression, and the reduced kinase activities of *Nrf2*-null cells [139, 141]. Nrf2 sustains a high rate of cell proliferation by replenishing glutathione efficiently.

A new role for Nrf2 has been implicated in the process of liver regeneration [142, 143]. *Nrf2*-null mice showed delayed initiation of liver regeneration and impaired Akt phosphorylation following transient activation of insulin signaling after hepatectomy [142]. Notch1 was found to be a Nrf2 target gene responsible for liver regeneration [143]. Hepatocyte-specific expression of the intracellular domain of Notch1 rescued the impaired liver regeneration in *Nrf2*-null mice.

5.5 CONCLUSION AND FUTURE PERSPECTIVES

Because Nrf2 confers resistance against electrophilic and oxidative stress, Nrf2 activation by chemical compounds and natural products is a promising strategy for disease prevention and therapy. Synthesized compounds and natural products that activate Nrf2 have been tested for cancer chemoprevention. Oltipraz, dithiolethiones, triterpenoids, curcumin, and sulforaphane have been reported to be effective Nrf2 inducers [144–147]. The pharmacological effects of these chemicals are mediated by Nrf2, and they are ineffective in the absence of Nrf2 [65, 119, 146, 148]. The most potent Nrf2 inducer that has been identified to date is CDDO-Im (1-[2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oyl]imidazole), a synthetic triterpenoid. The effective dose of CDDO-Im for the prevention of carcinogen-induced hepatic and lung tumors is much lower than that of sulforaphane and oltipraz [148, 149]. In addition, administration of CDDO-Im attenuates cisplatin-induced renal damage [150], retinal injuries [151], and hyperoxia- and smoke-induced lung injuries [152, 153].

In contrast to Nrf2 inducers, few specific inhibitors of Nrf2, which are expected to be beneficial for anticancer therapy, have been reported. One of the most difficult but important issues in the development of Nrf2 inhibitors is how to achieve specificity. Recently, a compound purified from plant extracts, brusatol, was found to enhance the degradation of Nrf2 and inhibit Nrf2-mediated stress response and tumor growth [154]. The development of Nrf2 inhibitors based on the molecular mechanism of the Keap1-Nrf2 system is one of the most critical and challenging tasks in the development of effective therapies for cancer.

Another important issue that needs to be pursued is the cross talk between the redox response of Keap1 and other signaling pathways in the activation of Nrf2-mediated transcription. Activation of Nrf2 appears to be regulated by phosphorylation-signaling pathways, including mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), PKC, and casein kinase 2 (CK2) [155]. Ser40 and Tyr568 have been identified as phosphorylation sites of Nrf2 by PKC and the tyrosine kinase Fyn, respectively [83, 84, 87, 129]. p21 and p62 were identified as nonelectrophilic, endogenous inducers of Nrf2 [77, 80]. The former is a target gene of p53-mediated cell cycle arrest, and the latter is a polyubiquitin-binding protein targeting various substrates for autophagy. Elucidation of the physiological and pathological significance of the interaction between these pathways and the Keap1-Nrf2 system is an important future goal for the attainment of an overall understanding of mammalian stress response signaling.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aids for Scientific Research on Priority Areas (H.M.) and for Scientific Research (M.M. and H.M.) from the Ministry of Education, Science, Sports and Culture, and a Research Grant from the Princess Takamatsu Cancer Research Fund 09-24118 (H.M.).

REFERENCES

1. Rushmore TH and Pickett CB. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem* 1990; 265: 14648–14653.
2. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* 1991; 266: 11632–11639.

3. Friling RS, Bensimon A, Tichauer Y, Daniel V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proc Natl Acad Sci USA* 1990; 87: 6258–6262.
4. Mignotte V, Eleouet JF, Raich N, Romeo PH. *Cis*- and *trans*-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene. *Proc Natl Acad Sci USA* 1989; 89: 6548–6552.
5. Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH. Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* 1993; 362: 722–728.
6. Moi P, Chan K, Asunis I, Cao A, 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* 1994; 91: 9926–9930.
7. Itoh K, Igarashi K, Hayashi N, Nishizawa M, 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* 1995; 15: 84184–84193.
8. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997; 236: 313–322.
9. Mohler J, Vani K, Leung S, Epstein A. Segmentally restricted, cephalic expression of a leucine zipper gene during *Drosophila* embryogenesis. *Mech Dev* 1991; 34: 3–9.
10. Chan JY, Han XL, Kan YW. Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc Natl Acad Sci USA* 1993; 90: 11371–11375.
11. Kobayashi A, Ito E, Toki T, Kogame K, Takahashi S, Igarashi K, Hayashi N, Yamamoto M. Molecular cloning and functional characterization of a new Cap'n' collar family transcription factor Nrf3. *J Biol Chem* 1999; 274: 6443–6452.
12. Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M, Yamamoto M, Igarashi K. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. *Mol Cell Biol* 1996; 16: 6083–6095.
13. Muto A, Hoshino H, Madisen L, Yanai N, Obinata M, Karasuyama H, Hayashi N, Nakauchi H, Yamamoto M, Groudine M, Igarashi K. Identification of Bach2 as a B-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3′ enhancer. *EMBO J* 1998; 17: 5734–5743.
14. Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK. Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem* 2005; 280: 16891–16900.
15. Motohashi H, O'Connor T, Katsuoka F, Engel JD, Yamamoto M. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* 2002; 294: 1–12.
16. Blackwell TK, Bowerman B, Priess JR, Weintraub H. Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. *Science* 1994; 266: 621–628.
17. Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, Yamamoto M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol Appl Pharmacol* 2001; 173: 154–160.
18. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, 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* 2001; 59: 169–177.
19. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* 2004; 114: 1248–1259.
20. Cho HY, Reddy SP, Yamamoto M, Kleeberger SR. The transcription factor NRF2 protects against pulmonary fibrosis. *FASEB J* 2004; 18: 1258–1260.
21. McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, Hayes JD. The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res* 2001; 61: 3299–3307.
22. Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, Yamamoto M, and Hayes JD. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3, and Gstm4 genes in the livers of male and female mice. *Biochem J* 2002; 365: 405–416
23. Iida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, Kawai K, Shimazui T, Akaza H, Yamamoto M. Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res* 2004; 64: 6424–6431.
24. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa 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.
25. Hirayama A, Yoh K, Nagase S, Ueda A, Itoh K, Morito N, Hirayama K, Takahashi S, Yamamoto M, Koyama A. EPR imaging of reducing activity in Nrf2 transcriptional

factor-deficient mice. *Free Radic Biol Med.*, 2003; 34: 1236–1242.

26. Motohashi H, Kimura M, Fujita R, Inoue A, Pan X, Takayama M, Katsuoka F, Aburatani H, Bresnick EH, Yamamoto M. NF-E2 domination over Nrf2 promotes ROS accumulation and megakaryocytic maturation. *Blood* 2010; 115: 677–686.
27. Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, Kobayashi M, Morito N, Koyama A, Yamamoto M, Takahashi S. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int* 2001; 60: 1343–1353.
28. Lee JM, Chan K, Kan YW, Johnson JA. Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. *Proc Natl Acad Sci USA* 2004; 101: 9751–9756.
29. Ma Q, Battelli L, Hubbs AF. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. *Am J Pathol* 2006; 168: 1960–1974
30. Chan K, Lu R, Chang JC, 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* 1996; 93: 13943–13948.
31. Kwak MK, Itoh K, Yamamoto M, 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* 2002; 22: 2883–2892.
32. Miao W, Hu L, Scrivens PJ, Batist G. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *J Biol Chem* 2005; 280: 20340–20348.
33. Cho HY, Jedlicka AE, Reddy SP, Zhang LY, Kensler TW, Kleeberger SR. Linkage analysis of susceptibility to hyperoxia. Nrf2 is a candidate gene. *Am J Respir Cell Mol Biol* 2002; 26: 42–51.
34. Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, Kleeberger SR. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 2002; 26: 175–182.
35. Yamamoto T, Yoh K, Kobayashi A, Ishii Y, Kure S, Koyama A, Sakamoto T, Sekizawa K, Motohashi H, Yamamoto M. Identification of polymorphisms in the promoter region of the human NRF2 gene. *Biochem Biophys Res Commun* 2004; 321: 72–79.
36. Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lanken PN, Aplenc R, Yamamoto T, Yamamoto M, Cho HY, Kleeberger SR. Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *FASEB J* 2007; 21: 2237–2246.
37. Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 2003; 8: 379–391.
38. 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.
39. Katoh Y, Iida K, Kang MI, Kobayashi A, Mizukami M, Tong KI, McMahon M, Hayes JD, Itoh K, Yamamoto M. Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch Biochem Biophys* 2005; 433: 342–350.
40. 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.
41. Sawa T, Zaki MH, Okamoto T, Akuta T, Tokutomi Y, Kim-Mitsuyama S, Ihara H, Kobayashi A, Yamamoto M, Fujii S, Arimoto H, Akaike T. Protein S-guanylation by the biological signal 8-nitroguanosine 3′, 5′-cyclic monophosphate. *Nat Chem Biol* 2007; 3: 727–735.
42. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 1999; 13: 76–86.
43. Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 2001; 6: 857–868.
44. Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, Imakado S, Kotsuji T, Otsuka F, Roop DR, Harada T, Engel JD, Yamamoto M. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* 2003; 35: 238–245
45. 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.
46. Taguchi K, Maher JM, Suzuki T, Kawatani Y, Motohashi H, Yamamoto M. Genetic analysis of cytoprotective functions supported by graded expression of Keap1. *Mol Cell Biol* 2010; 30: 3016–3026.
47. Okawa H, Motohashi H, Kobayashi A, Aburatani H, Kensler TW, Yamamoto M. Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. *Biochem Biophys Res Commun* 2006; 339: 79–88.
48. Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? *Carcinogenesis* 2010; 31: 90–99.
49. Cullinan, SB, Gordan JD, Jin J, Harper JW, Diehl JA. The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol Cell Biol* 2004; 24: 8477–8486.

50. 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.
51. Furukawa M, Xiong Y. BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol Cell Biol* 2005; 25: 162–171
52. Zipper LM, Mulcahy RT. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J Biol Chem* 2002; 277: 36544–36552.
53. 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.
54. Tong KI, Padmanabhan B, Kobayashi A, Shang C, Hirotsu Y, Yokoyama S, Yamamoto M. Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol* 2007; 27: 7511–7521.
55. 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.
56. 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.
57. 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.
58. Kang MI, Kobayashi A, Wakabayashi N, Kim SG, 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* 2004; 101: 2046–2051.
59. 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.
60. 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.
61. Fujii S, Sawa T, Ihara H, Tong KI, Ida T, Okamoto T, Ahtesham AK, Ishima Y, Motohashi 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.
62. Rachakonda G, Xiong Y, Sekhar KR, Stamer SL, Liebler DC, Freeman ML. Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3. *Chem Res Toxicol* 2008; 221: 705–710.
63. 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.
64. 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.
65. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, 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 USA* 2001; 98: 3410–3415.
66. Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, Qian GS, Kuang SY, Zarba A, Egner PA, Jacobson LP, Muñoz A, Helzlsouer KJ, Groopman JD, Kensler TW. Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. *J Natl Cancer Inst* 1999; 91: 347–354.
67. Kensler TW, Chen JG, Egner PA, Fahey JW, Jacobson LP, Stephenson KK, Ye L, Coady JL, Wang JB, Wu Y, Sun Y, Zhang QN, Zhang BC, Zhu YR, Qian GS, Carmella SG, Hecht SS, Benning L, Gange SJ, Groopman JD, Talalay P. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol Biomarkers Prev* 2005; 14: 2605–2613.
68. Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, Brock MV, Biswal S. Dysfunctional KEAP1-NRF2 interaction in non-small-cell lungcancer. *PLoS Med* 2006; 3: e420.
69. Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M, Hirohashi S. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* 2008; 68: 1303–1309.
70. Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M, Hirohashi S. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc Natl Acad Sci USA* 2008; 105: 13568–13573.
71. Kim YR, Oh JE, Kim MS, Kang MR, Park SW, Han JY, Eom HS, Yoo NJ, Lee SH. Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J Pathol* 2010; 220: 446–451.
72. Takahashi T, Sonobe M, Menju T, Nakayama E, Mino N, Iwakiri S, NagaiS, Sato K, Miyahara R, Okubo K, Hirata T, Date H, Wada H. Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. *J Surg Oncol* 2010; 101: 500–506.

73. Li QK, Singh A, Biswal S, Askin F, Gabrielson E. KEAP1 gene mutations and NRF2 activation are common in pulmonary papillary adenocarcinoma. *J Hum Genet* 2011; 56: 230–234. DOI:10.1038/jhg.2010.172.
74. Suzuki T, Maher J, Yamamoto M. Select heterozygous Keap1 mutations have a dominant-negative effect on wild-type Keap1 in vivo. *Cancer Res* 2011; 71: 1700–1709.
75. Wang R, An J, Ji F, Jiao H, Sun H, Zhou D. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem Biophys Res Commun* 2008; 373: 151–154.
76. Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, Wu H, Bova SG, Biswal S. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol Cancer Ther* 2010; 9: 336–346.
77. Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, Zhang DD. Direct interaction between Nrf2 and p21 (Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol Cell* 2009; 34: 663–673.
78. O'Reilly MA. Redox activation of $p21^{Cip1/WAF1/Sdi1}$: a multifunctional regulator of cell survival and death. *Antioxid Redox Signal* 2005; 7: 108–118.
79. Komatsu M, Ichimura Y. Selective autophagy regulates various cellular functions. *Genes Cells* 2010; 15: 923–933.
80. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou YS, Ueno I, Sakamoto A, Tong KI, Kim M, Nishito Y, Iemura S, Natsume T, Ueno T, Kominami E, Motohashi H, Tanaka K, Yamamoto M. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol* 2010; 12: 213–223.
81. Lu M, Nakamura RM, Dent ED, Zhang JY, Nielsen FC, Christiansen J, Chan EK, Tan EM. Aberrant expression of fetal RNA-binding protein p62 in liver cancer and liver cirrhosis. *Am J Pathol* 2001; 159: 945–953.
82. Strnad P, Stumptner C, Zatloukal K, Denk H. Intermediate filament cytoskeleton of the liver in health and disease. *Histochem Cell Biol* 2008; 129: 735–749.
83. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* 2002; 277: 42769–42774.
84. Bloom DA, Jaiswal AK. Phosphorylation of Nrf2 at Ser40 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* 2003; 278: 44675–44682.
85. Jain AK, Jaiswal AK. GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *J Biol Chem* 2007; 282: 16502–16510.
86. Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *J Biol Chem* 2006; 281: 14841–14851.
87. Jain AK, Jaiswal AK. Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *J Biol Chem* 2006; 281: 12132–12142.
88. Sun Z, Chin YE, Zhang DD. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol Cell Biol* 2009; 29: 2658–2672.
89. Kawai Y, Garduno L, Theodore M, Yang J, Arinze IJ. Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleo-cytoplasmic localization. *J Biol Chem* 2011; 286: 7629–7640
90. Shavit JA, Motohashi H, Onodera K, Akasaka J, Yamamoto M, Engel JD. Impaired megakaryopoiesis and behavioral defects in mafG-null mutant mice. *Genes Dev* 1998; 12: 2164–2174.
91. Motohashi H, Katsuoka F, Engel JD, Yamamoto M. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc Natl Acad Sci USA* 2004; 101: 6379–6384.
92. Katsuoka F, Motohashi H, Ishii T, Aburatani H, Engel JD, Yamamoto M. Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. *Mol Cell Biol* 2005; 25: 8044–8851.
93. Nagai T, Igarashi K, Akasaka J, Furuyama K, Fujita H, Hayashi N, Yamamoto M, Sassa S. Regulation of NF-E2 activity in erythroleukemia cell differentiation. *J Biol Chem* 1998; 273: 5358–5365.
94. Motohashi H, Katsuoka F, Shavit JA, Engel JD, Yamamoto M. Positive or negative MARE-dependent transcriptional regulation is determined by the abundance of small Maf proteins. *Cell* 2000; 103: 865–875.
95. Kotkow KJ, Orkin SH. Complexity of the erythroid transcription factor NF-E2 as revealed by gene targeting of the mouse p18 NF-E2 locus. *Proc Natl Acad Sci USA* 1996; 93: 3514–3518.
96. Onodera K, Shavit JA, Motohashi H, Katsuoka F, Akasaka JE, Engel JD, Yamamoto M. Characterization of the murine mafF gene. *J Biol Chem* 1999; 274: 21162–21169.
97. Onodera K, Shavit JA, Motohashi H, Yamamoto M, Engel JD. Perinatal synthetic lethality and hematopoietic defects in compound mafG::mafK mutant mice. *EMBO J* 2000; 19: 1335–1345.
98. Katsuoka F, Motohashi H, Tamagawa Y, Kure S, Igarashi K, Engel JD, Yamamoto M. Small Maf compound mutants display central nervous system neuronal degeneration, aberrant transcription, and Bach protein mislocalization coincident with myoclonus and abnormal startle response. *Mol Cell Biol* 2003; 23: 1163–1174.

99. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, Jackson CW, Hunt P, Saris CJ, Orkin SH. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell* 1995; 81: 695–704.

100. Kobayashi A, Tsukide T, Miyasaka T, Morita T, Mizoroki T, Saito Y, Ihara Y, Takashima A, Noguchi N, Fukamizu A, Hirotsu Y, Ohtsuji M, Katsuoka F, Yamamoto M. Central nervous system-specific deletion of transcription factor Nrf1 causes progressive motor neuronal dysfunction. *Genes Cells* 2011; 16: 692–703.

101. Motohashi H, Katsuoka F, Miyoshi C, Uchimura Y, Saitoh H, Francastel C, Engel JD, Yamamoto M. MafG sumoylation is required for active transcriptional repression. *Mol Cell Biol* 2006; 26: 4652–4663.

102. Motohashi H, Fujita R, Takayama M, Inoue A, Katsuoka F, Bresnick EH, Yamamoto M. Molecular determinants for small Maf protein control of platelet production. *Mol Cell Biol* 2011; 31: 151–162.

103. Yamamoto T, Kyo M, Kamiya T, Tanaka T, Engel JD, Motohashi H, Yamamoto M. Predictive base substitution rules that determine the binding and transcriptional specificity of Maf recognition elements. *Genes Cells* 2006; 11: 575–591.

104. Kataoka K, Noda M, Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol Cell Biol* 1994; 14: 700–712.

105. Kerppola, TK, Curran T. Maf and Nrl can bind to AP-1 sites and form heterodimers with Fos and Jun. *Oncogene* 1994; 9: 3149–3158.

106. Kurokawa H, Motohashi H, Sueno S, Kimura M, Takagawa H, Kanno Y, Yamamoto M, Tanaka T. Structural basis of alternative DNA recognition by Maf transcription factors. *Mol Cell Biol* 2009; 29: 6232–6244.

107. Kyo M, Yamamoto T, Motohashi H, Kamiya T, Kuroita T, Tanaka T, Engel JD, Kawakami B, Yamamoto M. Evaluation of MafG interaction with Maf recognition element arrays by surface plasmon resonance imaging technique. *Genes Cells* 2004; 9: 153–164.

108. Kimura M, Yamamoto T, Zhang J, Itoh K, Kyo M, Kamiya T, Aburatani H, Katsuoka F, Kurokawa H, Tanaka T, Motohashi H, Yamamoto M. Molecular basis distinguishing the DNA binding profile of Nrf2-Maf heterodimer from that of Maf homodimer. *J Biol Chem* 2007; 282: 33681–33690.

109. Motohashi H, Igarashi K, Onodera K, Takahashi S, Ohtani H, Nakafuku M, Nishizawa M, Engel JD, Yamamoto M. Mesodermal- vs. neuronal-specific expression of MafK is elicited by different promoters. *Genes Cells* 1996; 11: 223–238.

110. Murphy P, Kolsto A. Expression of the bZIP transcription factor TCF11 and its potential dimerization partners during development. *Mech Dev* 2000; 97: 141–148.

111. Motohashi H, Ohta J, Engel JD, Yamamoto M. A core region of the mafK gene IN promoter directs neurone-specific transcription in vivo. *Genes Cells* 1998; 3: 671–684.

112. Katsuoka F, Motohashi H, Onodera K, Suwabe N, Engel JD, Yamamoto M. One enhancer mediates mafK transcriptional activation in both hematopoietic and cardiac muscle cells. *EMBO J* 2000; 19: 2980–2991.

113. Crawford DR, Leahy KP, Wang Y, Schools GP, Kochheiser JC, Davies KJ. Oxidative stress induces the levels of a MafG homolog in hamster HA-1 cells. *Free Radic BiolMed.*, 1996; 21: 521–525.

114. Wild AC, Moinova HR, Mulcahy RT. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J Biol Chem* 1999; 274: 33627–33636.

115. Suzuki T, Blank V, Sesay JS, Crawford DR. Maf genes are involved in multiple stress response in human. *Biochem Biophys Res Commun* 2001; 280: 4–8.

116. Katsuoka F, Motohashi H, Engel JD, Yamamoto M. Nrf2 transcriptionally activates the mafG gene through an antioxidant response element. *J Biol Chem* 2005; 280: 4483–4490.

117. Shimokawa N, Okada J, Miura M Cloning of MafG homologue from the rat brain by differential display and its expression after hypercapnic stimulation. *Mol Cell Biochem* 2000; 203: 135–141.

118. Kumaki I, Yang D, Koibuchi N, Takayama K. Neuronal expression of nuclear transcription factor MafG in the rat medulla oblongata after baroreceptor stimulation. *Life Sci* 2006; 78: 1760–1766.

119. Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH, Kong AN. Inhibition of 7,12-dimethylbenz [*a*]anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer Res* 2006; 66: 8293–8296.

120. auf dem Keller U, Huber M, Beyer TA, Kümin A, Siemes C, Braun S, Bugnon P, Mitropoulos V, Johnson DA, Johnson JA, Hohl D, Werner S. Nrf transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing. *Mol Cell Biol* 2006; 26: 3773–3784.

121. Aoki Y, Hashimoto AH, Amanuma K, Matsumoto M, Hiyoshi K, Takano H, Masumura K, Itoh K, Nohmi T, Yamamoto M. Enhanced spontaneous and benzo[a]pyrene-induced mutations in the lung of Nrf2-deficient gpt delta mice. *Cancer Res* 2007; 67: 5643–5648.

122. Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, Cheung WK, Chan JY, Reddy BS, Yang CS, Kong AN. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. *Cancer Prev Res* 2008; 1: 187–191.

123. Padmanabhan B, Tong KI, Ohta T, Nakamura Y, Scharlock M, Ohtsuji M, Kang MI, Kobayashi A, Yokoyama S, Yamamoto M. Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell* 2006; 2: 689–700.

124. Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, Hirohashi S. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* 2008; 135: 1358–1368.

125. Lee DF, Kuo HP, Liu M, Chou CK, Xia W, Du Y, Shen J, Chen CT, Huo L, Hsu MC, Li CW, Ding Q, Liao TL, Lai CC, Lin AC, Chang YH, Tsai SF, Li LY, Hung MC. KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta. *Mol Cell* 2009; 36: 131–140.

126. McDonald JT, Kim K, Norris AJ, Vlashi E, Phillips TM, Lagadec C, Della Donna L, Ratikan J, Szelag H, Hlatky L, McBride WH. Ionizing radiation activates the Nrf2 antioxidant response. *Cancer Res* 2010; 70: 8886–8895.

127. Satoh H, Moriguchi T, Taguchi K, Takai J, Maher JM, Suzuki T, Winnard PT Jr, Raman V, Ebina M, Nukiwa T, Yamamoto M. Nrf2-deficiency creates a responsive microenvironment for metastasis to the lung. *Carcinogenesis* 2010; 31: 1833–1843.

128. Itoh K, Mochizuki M, Ishii Y, Ishii T, Shibata T, Kawamoto Y, Kelly V, Sekizawa K, Uchida K, Yamamoto M. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin j_2. *Mol Cell Biol* 2004; 24: 36–45.

129. Numazawa S, Ishikawa M, Yoshida A, Tanaka S, Yoshida T. Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am J Physiol Cell Physiol* 2003; 285: C334–C342.

130. Gao L, Wang J, Sekhar KR, Yin H, Yared NF, Schneider SN, Sasi S, Dalton TP, Anderson ME, Chan JY, Morrow JD, Freeman ML. Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. *J Biol Chem* 2007; 282: 2529–2537.

131. Groeger AL, Cipollina C, Cole MP, Woodcock SR, Bonacci G, Rudolph TK, Rudolph V, Freeman BA, Schopfer FJ. Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids. *Nat Chem Biol* 2010; 6: 433–441.

132. Majkova Z, Layne J, Sunkara M, Morris AJ, Toborek M, Hennig B. Omega-3 fatty acid oxidation products prevent vascular endothelial cell activation by coplanar polychlorinated biphenyls. *Toxicol Appl Pharmacol* 2011; 251: 41–49.

133. Mochizuki M, Ishii Y, Itoh K, Iizuka T, Morishima Y, Kimura T, Kiwamoto T, Matsuno Y, Hegab AE, Nomura A, Sakamoto T, Uchida K, Yamamoto M, Sekizawa K. Role of 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 and Nrf2 pathways in protection against acute lung injury. *Am J Respir Crit CareMed*., 2005; 171: 1260–1266.

134. 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.

135. 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; 175: 6968–6975.

136. Kikuchi N, Ishii Y, Morishima Y, Yageta Y, Haraguchi N, Itoh K, Yamamoto M, Hizawa N. Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. *Respir Res* 2010; 11: 31.

137. Osburn WO, Yates MS, Dolan PD, Chen S, Liby KT, Sporn MB, Taguchi K, Yamamoto M, Kensler TW. Genetic or pharmacologic amplification of Nrf2 signaling inhibits acute inflammatory liver injury in mice. *Toxicol. Sci* 2008; 104: 218–227

138. Cho HY, Reddy SP, Debiase A, Yamamoto M, Kleeberger SR. Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radic Biol Med* 2005; 38: 325–343

139. Reddy NM, Kleeberger SR, Yamamoto M, Kensler TW, Scollick C, Biswal S, Reddy SP. Genetic dissection of the Nrf2-dependent redox signaling-regulated transcriptional programs of cell proliferation and cytoprotection. *Physiol Genomics* 2007; 32: 74–81.

140. Malhotra D, Portales-Casamar E, Singh A, Srivastava S, Arenillas D, Happel C, Shyr C, Wakabayashi N, Kensler TW, Wasserman WW, Biswal S. Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res* 2010; 38: 5718–5734.

141. Reddy NM, Kleeberger SR, Bream JH, Fallon PG, Kensler TW, Yamamoto M, Reddy SP. Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. *Oncogene* 2008; 27: 5821–5832.

142. Beyer TA, Xu W, Teupser D, auf dem Keller U, Bugnon P, Hildt E, Thiery J, Kan YW, Werner S. Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated insulin/IGF-1 resistance. *EMBO J* 2008; 27: 212–223.

143. Wakabayashi N, Shin S, Slocum SL, Agoston ES, Wakabayashi J, Kwak MK, Misra V, Biswal S, Yamamoto M, Kensler TW. Regulation of notch1 signaling by nrf2: implications for tissue regeneration. *Sci Signal* 2010; 3: ra52.

144. Zhang Y, Munday R. Dithiolethiones for cancer chemoprevention: where do we stand? *Mol Cancer Ther* 2008; 7: 3470–3479.

145. Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T, Gribble GW, Johnson DA, Johnson JA, Burton NC, Guilarte TR, Yamamoto M, Sporn MB, Kensler TW. Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol Cancer Ther* 2007; 6: 154–162.

146. Shen G, Xu C, Hu R, Jain MR, Gopalkrishnan A, Nair S, Huang MT, Chan JY, Kong AN. Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin. *Mol Cancer Ther* 2006; 5: 39–51.

147. Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell Mol Life Sci* 2007; 64: 1105–1127.

148. Yates MS, Kwak MK, Egner PA, Groopman JD, Bodreddigari S, Sutter TR, Baumgartner KJ, Roebuck BD,

Liby KT, Yore MM, Honda T, Gribble GW, Sporn MB, Kensler TW. Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3-,12-dioxooleana-1,9 (11)-dien-28-oyl]imidazole. *Cancer Res* 2006; 66: 2488–2494.

149. Liby K, Royce DB, Williams CR, Risingsong R, Yore MM, Honda T, Gribble GW, Dmitrovsky E, Sporn TA, Sporn MB. The synthetic triterpenoids CDDO-methyl ester and CDDO-ethyl amide prevent lung cancer induced by vinyl carbamate in A/J mice. *Cancer Res* 2007; 67: 2414–2419.
150. Aleksunes LM, Goedken MJ, Rockwell CE, Thomale J, Manautou JE, Klaassen CD. Transcriptional regulation of renal cytoprotective genes by Nrf2 and its potential use as a therapeutic target to mitigate cisplatin-induced nephrotoxicity. *J Pharmacol Exp Ther* 2010; 335: 2–12.
151. Pitha-Rowe I, Liby K, Royce D, Sporn M. Synthetic triterpenoids attenuate cytotoxic retinal injury: cross-talk between Nrf2 and PI3K/AKT signaling through inhibition of the lipid phosphatase PTEN. *Invest Ophthalmol Vis Sci* 2009; 50: 5339–5347.
152. Reddy NM, Kleeberger SR, Kensler TW, Yamamoto M, Hassoun PM, Reddy SP. Disruption of Nrf2 impairs the resolution of hyperoxia-induced acute lung injury and inflammation in mice. *J Immunol* 2009; 182: 7264–7271.
153. Sussan TE, Rangasamy T, Blake DJ, Malhotra D, El-Haddad H, Bedja D, Yates MS, Kombairaju P, Yamamoto M, Liby KT, Sporn MB, Gabrielson KL, Champion HC, Tuder RM, Kensler TW, Biswal S. Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proc Natl Acad Sci USA* 2009; 106: 250–255.
154. Ren D, Villeneuve NF, Jiang T, Wu T, Lau A, Toppin HA, Zhang DD. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc Natl Acad Sci USA* 2011; 108: 1433–1438.
155. Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med*., 2008; 74: 1526–1539.
156. Khor TO, Huang MT, Kwon KH, Chan JY, Reddy BS, Kong AN. Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. *Cancer Res* 66: 2006; 11580–11584.
157. Osburn WO, Karim B, Dolan PM, Liu G, Yamamoto M, Huso DL, Kensler TW. Increased colonic inflammatory injury and formation of aberrant crypt foci in Nrf2-deficient mice upon dextran sulfate treatment. *Int J Cancer* 2007; 121: 1883–1891.
158. Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A. Sulforaphane inhibits extracellular, intracellular, and antiobiotic-resistant strains of *Helicobacter pylori* and prevents benzo [*a*]pyrene-induced stomach tumors. *Proc Natl Acad Sci USA* 2002; 99: 7610–7615.
159. Ramos-Gomez M, Dolan PM, Itoh K, Yamamoto M, Kensler TW. Interactive effects of *nrf2* genotype and oltipraz on benzo[*a*]pyrene-DNA adducts and tumor yield in mice. *Carcinogenesis* 2003; 24: 461–467.
160. Kitamura Y, Umemura T, Kanki K, Kodama Y, Kitamoto S, Saito K, Itoh K, Yamamoto M, Masegi T, Nishikawa A, Hirose M. Increased susceptibility to hepatocarcinogenicity of Nrf2-deficient mice exposed to 2-amino-3-methylimidazo[4,5-f]quinoline. *Cancer Sci* 2007; 98: 19–24.
161. Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, Yamamoto M, Kensler TW. Role of phase 2 enzyme induction in chemoprotection bydithiolethiones. *Mutat Res* 2001; 480–481: 305–315.
162. Innamorato NG, Rojo AI, García-Yagüe AJ, Yamamoto M, de Ceballos ML, Cuadrado A. The transcription factor Nrf2 is a therapeutic target against brain inflammation. *J Immunol* 2008; 181: 680–689.
163. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, Biswal S. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006; 116: 984–995.
164. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med*., 2005; 202: 47–59.
165. Braun S, C. Hanselmann, M.G. Gassmann, U. auf dem Keller, C. Born-Berclaz, K. Chan, Y.W. Kan and S. Werner., 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* 2002; 22: 5492–5505.
166. Hubbs AF, Benkovic SA, Miller DB, O'Callaghan JP, Battelli L, Schwegler-Berry D, Ma Q. Vacuolar leukoencephalopathy with widespread astrogliosis in mice lacking transcription factor Nrf2. *Am J Pathol* 2007; 170: 2068–2076.
167. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, Grotta JC, Aronowski J. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. *Stroke* 2007; 38: 3280–3286.
168. Wang J, Fields J, Zhao C, Langer J, Thimmulappa RK, Kensler TW, Yamamoto M, Biswal S, Doré S. Role of Nrf2 in protection against intracerebral hemorrhage injury in mice. *Free Radic Biol Med*., 2007; 43: 408–414.
169. Yanagawa T, Itoh K, Uwayama J, Shibata Y, Yamaguchi A, Sano T, Ishii T, Yoshida H, Yamamoto M. Nrf2 deficiency causes tooth decolourization due to iron transport disorder in enamel organ. *Genes Cells* 2004; 9: 641–651.
170. Nioi P and Nguyen T. A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochem Biophys Res Commun* 2007; 362: 816–821.