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MECHANISTIC STUDIES OF THE NRF2-KEAP1 SIGNALING PATHWAY

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Since eukaryotic cells constantly encounter various environmental insults, they have evolved defense mechanisms to cope with toxicant- and carcinogen-induced oxidative stress or electrophiles. One of the most important cellular defense mechanisms against oxidative stress or electrophiles is mediated by the transcription factor Nrf2. Under the basal condition, Nrf2-dependent transcription is repressed by a negative regulator Keap1. When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 escapes Keap1-mediated repression and activates antioxidant responsive element (ARE)-dependent gene expression to maintain cellular redox homeostasis. Beyond its antioxidant function, Nrf2 has recently been recognized as a key factor regulating an array of genes that defend cells against the deleterious effects of environmental insults. Since this Nrf2-dependent cellular defense response is able to protect multi-organs or multi-tissues, activation of Nrf2 has been implicated in conferring protection against many human diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, acute and chronic lung injury, autoimmune diseases, and inflammation. Therefore, understanding of Nrf2 regulation is crucial in the development of drugs for therapeutic intervention. This review will discuss recent progress in the field of the Nrf2-Keap1 signaling pathway, with emphasis on the mechanistic studies of Nrf2 regulation by Keap1, oxidative stress, or chemopreventive compounds.

Key Words: Nrf2; Keap1; Chemopreventive compounds; Oxidative stress; Ubiquitination; Degradation; Ubiquitin ligase.

INTRODUCTION

It is difficult to discuss the Nrf2 transcription factor without mentioning cancer prevention or chemoprevention, since the discovery of Nrf2 is attributed greatly to studies with anti-carcinogenic compounds. The very first concept of chemoprevention came from the observation that systemic administration of small quantities of xenobiotics, such as 3-methylcholanthrene, decreased the incidence of cancer in rats that were subsequently fed large doses of carcinogenic azo dyes (Richardson, 1951). Subsequent work over the last 50 years has firmly established that ingestion of small quantities of certain organic compounds,

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now commonly referred to as chemopreventive agents or anti-carcinogens, can lower the risk of cancer in mammals that are exposed to carcinogens (Kensler et al., 2000; Talalay and Fahey, 2001; Wolf, 2001). A number of chemopreventive compounds have been isolated from plants such as fruits and vegetables; other compounds are synthetic drugs. Interestingly, chemopreventive compounds are structurally diverse, including isothiocyanates (sulforaphane found in cruciferous vegetables), polyphenols [epigallocatechin-3-gallate (EGCG) in green tea, and caffeic acid phenethyl ester in honeybee propollis], and 1, 2, dithiole-3-thiones (oltipraz, a synthetic anti-cancer drug) (Zhang et al., 1994; Kensler et al., 2000; Orsolic et al., 2005; Shen et al., 2005). These compounds exert their chemopreventive activity by inducing expression of phase II enzymes and endogenous antioxidants that defend cells from oxidative stress or reactive carcinogenic intermediates (Kensler et al., 2000; Dinkova-Kostova et al., 2001; Talalay and Fahey, 2001; Wolf, 2001). The promoter regions of the phase II genes contain specific DNA sequences, termed the antioxidant response elements (AREs) or the electrophile response elements (EREs), that are required for induction by chemopreventive compounds, oxidative stress, or electrophiles (Jeyapaul and Jaiswal, 2000; Nioi et al., 2003). The search for the transcription factors that bind to ARE led to the identification of Nrf2 (Venugopal and Jaiswal, 1996; Wild et al., 1999; Nguyen et al., 2000). Subsequent studies with Nrf2 knockout mice have clearly established the pivotal role of Nrf2 in modulating the expression of phase II detoxification enzymes and endogenous antioxidants. In these mice, basal and inducible levels of phase II genes such as glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase (NQO1), and γ-glutamylcysteine synthetase (γGCS) are markedly reduced (Kwak et al., 2001; Chanas et al., 2002). Furthermore, Nrf2 knockout mice display increased sensitivity to chemical toxicants and carcinogens and are resistant to the protective actions of chemopreventive compounds (Aoki et al., 2001; Chan et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001; Cho et al., 2002; Cho et al., 2004; Iida et al., 2004; Rangasamy et al., 2004).

THE NRF2 TRANSCRIPTION FACTOR

Nrf2 was cloned by Kan and coworkers in 1996 as a factor that binds to the NF-E2 repeat of the β-globin gene promoter (Moi et al., 1994). It belongs to the cnc ("cap 'n' collar") subfamily of the basic region leucine zipper transcription factors. So far, six members in this family have been identified: NF-F2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2. In spite of the high homology in their DNA binding and leucine zipper domains, they have distinct biological roles. NF-E2 expression is erythroid-specific, and the NF-E2 knockout mouse suffers from mild anemia or excessive bleeding (Shivdasani et al., 1995). Nrf1 is expressed in virtually all tissues, and the absence of Nrf1 is lethal to embryonic development (Chan et al., 1998). Nrf2 is also ubiquitously expressed, but it is dispensable for normal development (Chan et al., 1996). However, the Nrf2 knockout mouse has decreased expression of both constitutive and inducible levels of phase II enzymes and endogenous antioxidants, as mentioned. Nrf3 is preferentially expressed in placenta, and the Nrf3 knockout mouse has no obvious phenotype (Derjuga et al., 2004).

Several homologue domains were identified when different species of the Nrf2 genes, such as human, mouse, and chicken, were aligned (Fig. 1). They are designated as Neh 1–6. The N-terminal Neh2 domain contains seven lysine residues for ubiquitin conjugation, so it confers negative regulation of the Nrf2 activity through proteasome-mediated degradation of Nrf2 (Zhang et al., 2004). It also binds to the Kelch domain of Keap1 (Itoh et al., 1999). The Neh4 and Neh5 are two independent transactivation domains that are

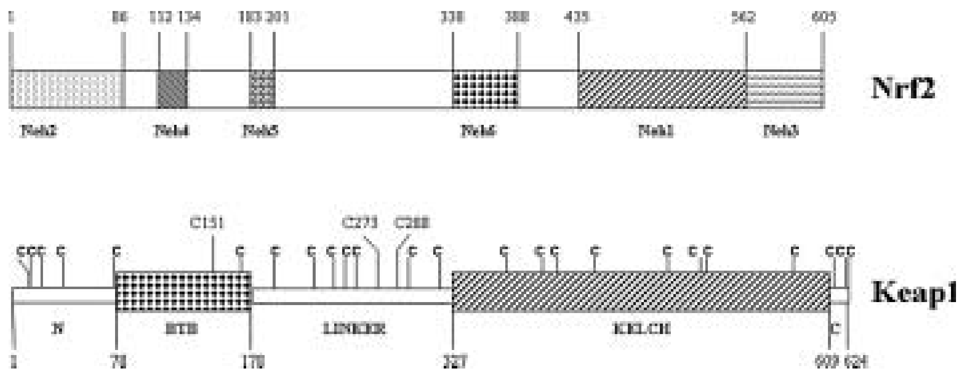

Figure 1 Schematic representation of the conserved regions in Nrf2 and Keap1. Nrf2 contains six discrete domains: a negative regulatory Neh2 domain, CPB-binding Neh4 and Neh5 domains, a serine-rich Neh6 domain, a CNC-type basic leucine zipper DNA-binding Neh1 domain, and a CHD6-binding Neh3 domain. Keap1 contains five discrete domains that are designated as N, BTB, Linker, Kelch, and C. The location of C151, C273, and C288 in Keap1 is indicated.

rich in acidic residues and interact with CREB-binding protein (CBP) (Katoh et al., 2001). The function of Neh6 remains largely unknown, although it is known to have a high content of serine residues. The Neh1 domain contains a CNC-type basic leucine zipper, which is necessary for DNA binding and dimerization with other transcription factors (Itoh et al., 1999; Nioi et al., 2005). In addition, there is a functional nuclear localization signal (NLS) within this domain (Jain et al., 2005). The C-terminal Neh3 is indispensable for transcriptional activity of Nrf2 by recruiting CHD6, a coactivator with both helicase domain and chromodomain (Nioi et al., 2005). However, the precise function of CHD6 is unclear.

ROLE OF NRF2-MEDIATED DEFENSE RESPONSE IN HUMAN DISEASES

Nrf2 was originally identified to have cancer preventive function through induction of phase II enzymes and endogenous antioxidants to inactive carcinogenic reactive intermediates. Recent micro-array technology has provided a powerful tool to monitor gene expression profiles controlled by Nrf2. Data derived from micro-array assays have provided evidence that Nrf2 regulates a battery of genes whose functions are beyond the classical phase II detoxification enzymes and antioxidants. Nrf2 downstream genes can be classified into categories according to their functions: cellular redox homeostasis, cell growth and apoptosis, inflammatory response, and the ubiquitin-mediated degradation pathway (Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Rangasamy et al., 2004; Cho et al., 2005). The orchestrated induction of these proteins, which was refered to as an Nrf2-mediated defense response, is crucial for cells to counteract the adverse effects from exogenous insults and to maintain cellular redox homeostasis. Since reactive oxygen species have been linked to the pathogenesis of many human diseases besides cancer, it is conceivable that Nrf2 may have functions beyond cancer prevention. Indeed, accumulating evidence has been provided recently indicating a protective role of Nrf2 against many human pathological conditions, such as Alzheimer's disease, Parkinson's disease, ischemia, aging and cardiovascular disease, pulmonary fibrosis and acute pulmonary

injury, inflammation, emphysema, asthma, lupus-like autoimmune nephritis, and macular degeneration (Aoki et al., 2001; Yoh et al., 2001; Braun et al., 2002; Cho et al., 2002; Cho et al., 2004; Gao and Talalay, 2004; Suh et al., 2004; Ishii et al., 2005; Rangasamy et al., 2005; Shih et al., 2005a; Shih et al., 2005b).

KEAP1, A NEGATIVE REGULATOR OF NRF2

As discussed, Nrf2 is a critical factor regulating the cellular defense response when cells are under oxidative stress or are stimulated with chemopreventive compounds. The activity of Nrf2 is tightly regulated by a negative regulator named Keap1, which was cloned using the Neh2 domain of Nrf2 as bait in a yeast two-hybrid system by Yamamoto and coworkers (Itoh et al., 1999). Keap1 contains three major domains: an N-terminal BTB (broad complex, tramtrack, and bric-a-brac) domain, a linker region, and a C-terminal Kelch domain (Fig. 1). The N-terminal BTB domain was implicated in homodimerization of the Keap1 protein (Zipper and Mulcahy, 2002). The linker region is a cysteine-rich domain that was proved to be indispensable for the activity of Keap1 (Zhang and Hannink, 2003). The C-terminal Kelch domain contains six conserved Kelch repeat sequences and binds to the Neh2 domain of Nrf2. Recently, the crystal structure of the Kelch domain has been solved and revealed a six β-propeller structure (Li et al., 2004; Padmanabhan et al., 2006).

Keap1 was initially described as a cytoplasmic factor that binds to actin cytoskeleton and Nrf2 to retain Nrf2 in the cytoplasm. Upon exposure of cells to oxidative stress or chemopreventive compounds, Nrf2 dissociates from Keap1, translocates to the nucleus, forms a heterodimer with its obligatory partner Maf, and ultimately activates ARE-dependent gene expression. Recently, findings from our laboratory and others indicate that Keap1 does not just passively sequester Nrf2 in the cytoplasm but plays an active role in targeting Nrf2 for ubiquitination and proteasomal degradation (Cullinan et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004; Furukawa and Xiong, 2005). In addition, both our *in vivo* data and the *in vitro* data from another group have challenged the Nrf2-Keap1 dissociation model (Zhang et al., 2004; Eggler et al., 2005). Both ubiquitin-mediated degradation of Nrf2 and failure of dissociation of the Nrf2-Keap1 complex in response to Nrf2-inducers will be discussed in the following sections.

SENSING MECHANISM OF KEAP1 TO OXIDATIVE STRESS OR CHEMOPREVENTIVE COMPOUNDS

Identification of Keap1 as the key repressor for Nrf2 leads to the prevailing model that one or more of the 27 cysteine residues in the human Keap1 protein are components of a molecular switch that is triggered by intracellular redox changes (Dinkova-Kostova et al., 2002). In support of this model, Nrf2-inducers with divergent structures share a common ability to react with protein thiols. Talalay and coworkers have demonstrated that four cysteine residues in mouse Keap1, C257, C273, C288, C297, in the linker region of Keap1 react with a thiol-specific reagent, preferentially under *in vitro* conditions (Dinkova-Kostova et al., 2002). Consistent with this finding, Liebler and coworkers found strong correlation between the ability of an electrophile to alkylate cysteine residues in the linker region of human Keap1 and the potency of the electrophile to activate the Nrf2-Keap1 pathway *in vitro* (Hong et al., 2005). Interestingly, another *in vitro* study using human Keap1 and iodoacetamide identified C151, C288, and C297 as the most reactive cysteine residues (Eggler et al., 2005).

By comparing the inhibitory activity of Keap1 between the wild-type and mutant Keap1 proteins in cultured cells, we identified two critical cysteine residues, C273 and C288, in the linker domain of human Keap1 that were required for Keap1-mediated repression of Nrf2 transcriptional activity under the basal condition (Fig. 2A) (Zhang and Hannink, 2003). The importance of C273 and C288 residues in controlling activation of Nrf2 was confirmed by two other independent groups (Levonen et al., 2004; Wakabayashi et al., 2004). In addition to C273 and C288, we also identified a third cysteine residue, C151, in the BTB domain of Keap1 that was required for activation of the ARE-dependent transcription in

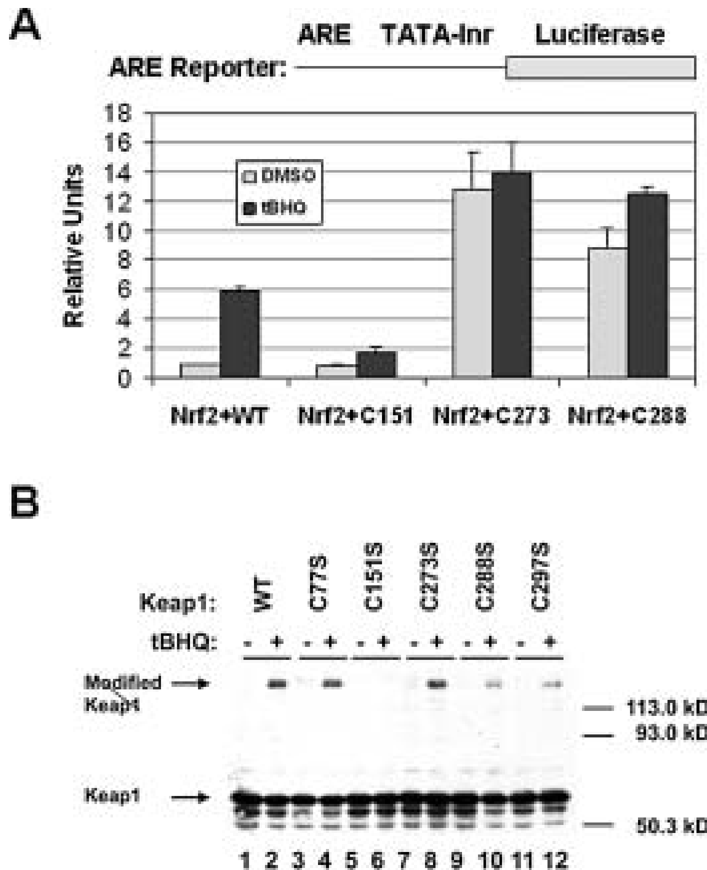

Figure 2 (A) Regulation of Nrf2-dependent transcriptional activity. NIH 3T3 cells were cotransfected with plasmids containing an ARE-dependent firefly luciferase reporter gene and expression plasmids for Nrf2 and either wild-type or mutant Keap1 proteins. A plasmid-encoding *Renilla* luciferase driven by the herpes simplex virus thymidine kinase promoter was included to normalize transfection efficiency. The transfected cells were exposed to 50 µM tBHQ for 16 h prior to analysis. All samples were run in duplicate for each experiment, and the data represent the means of the results from three independent experiments. (B) C151-dependent post-transcriptional modification of Keap1. COS1 cells transfected with expression vectors for either wild-type (WT) (lanes 1 and 2) or the indicated mutant Keap1 proteins (lanes 3 to 12) were treated with DMSO (–) or 25 µM tBHQ (+) for 16 h. Total cell lysates were subjected to immunoblot analysis with anti-Keap1 antibodies. The arrows on the left side of the figure indicate the location of Keap1 and of a tBHQ-induced form of Keap1 that migrates with retarded mobility. The positions of molecular mass markers are indicated on the right side of the figure. Fig. 2B is adapted from Zhang, D. D. and M. Hannink (2003). *Mol. Cell. Biol.* 23:8137–8151. © 2006 with permission from the American Society for Microbiology.

response to tBHQ (tert-butyhydroquinone) or sulforaphane treatment (Fig. 2A). Astonishingly, we also found that C151 in Keap1 is not only required for activation of Nrf2-dependent transcription, but also for a novel post-translational modification of Keap1 in cells exposed to tBHQ (Zhang and Hannink, 2003) (Fig. 2B). As shown in Fig. 2B, the Keap1 protein normally migrates on SDS-polyacrylamide gels with an apparent molecular size of approximately 60 kD, whereas the modified form of Keap1 has a size of approximately 130 kD. This modified form of Keap1 is stable to the reducing SDS-polyacrylamide gel electrophoresis conditions, such as 10-min heating in the presence of 100 mM DTT. It is unlikely that a disulfide bond is responsible for this modified form, although using a disulfide bond as a molecular switch is a very attractive model since disulfide linkage of Gpx3 and Yap1 has been used in yeast as a redox sensor (Delaunay et al., 2002). In spite of the fact that the Keap1 protein contains a high number of cysteine residues (27 cysteine residues in human Keap1), the modified form of Keap1 could not be an aggregation product because mutation of a single residue in Keap1, C151, completely abolished this modified form of Keap1 (Fig. 2B). The modified form of Keap1 could be a covalently adducted protein from two different proteins (an intermolecular adduct), an intramolecular adduct of Keap1, or two covalently linked Keap1 proteins (a dimeric form of Keap1). Formation of covalently adducted proteins in response to chemical treatment has been reported previously. For example, quinol-thioether-derived protein adducts that are resistant to reducing conditions have been detected in the kidneys of rats treated with hydroquinone or its derivatives (Kleiner et al., 1998). The chemical nature of this modified form of Keap1 and the important of the modification in activation of the Nrf2-Keap1 pathway are currently under investigation.

KEAP1: A SUBSTRATE ADAPTOR PROTEIN OF CUL3-CONTAINING E3 UBIQUTITIN LIGASE

Identification of the important roles of three cysteine mutants of Keap1 (C273, C288, and C151) in regulating the activity of Nrf2 has greatly speeded up our finding that Keap1 facilitates the degradation of Nrf2. As shown in Table 1, ectopically expressed Nrf2 had a half-life of 2.7 h that was significantly reduced to 0.6 h when the wild-type Keap1 was coexpressed. In contrast, the half-life of Nrf2 was 6.5 h in the presence of the mutant Keap1-C273S. Furthermore, sulforaphane treatment markedly increased the half-life of Nrf2 from 0.6 h to 2.5 h. Taken together, these data indicate that Keap1 speeds up the turnover rate of the Nrf2 protein and sulforaphane is able to reverse the effect of Keap1 on Nrf2

Table 1 The regulation of Nrf2 half-life by Keap1 and sulforaphane.

Nrf2	Keap1	SF	$T^1/_2$ (h)
+	–	–	2.7
+	–	+	3.2
+	WT	–	0.6
+	WT	+	2.5
+	C151S	–	0.6
+	C151S	+	0.6
+	C273S	–	6.5

HA-Nrf2 was expressed in MDA-MB-231 cells in the absence or presence of Keap1 proteins. Cells were either left untreated or treated with 4 μM sulforaphane (SF) for 16 h prior to and during the pulse-chase experiment. The half-life of HA-Nrf2 was determined by pulse-chase labeling.

turnover. Immunoblot analysis was also performed to verify that the steady-state levels of Nrf2 were reduced in the presence of the wild-type Keap1 but increased if the Keap1-C273S was cotransfected. Sulforaphane or tBHQ treatment markedly enhanced the levels of Nrf2. Collectively, these results led to our hypothesis that Keap1 is a novel E3 ubiquitin ligase that is specifically inhibited by oxidative stress or electrophiles.

The ubiquitin-mediated proteasomal degradation pathway has been extensively studied (Hochstrasser, 1996; Pickart, 2001). By controlling the degradation of many key proteins, this pathway has proved to be crucial in regulating many cellular processes, such as cell cycling, cell differentiation, and cell response to stress conditions. As shown in Fig. 3, ubiquitin conjugation reactions require the sequential actions of three major enzymes 1) ubiquitin-activating enzyme E1: forming a thioester bond with ubiquitin; 2) ubiquitin conjugation enzyme E2: accepting ubiquitin transferred from E1 enzyme; 3) ubiquitin ligase enzyme E3: transferring the activated ubiquitin from the E2 to a lysine residue of substrate proteins. The E2 enzymes typically function in the context of specific E3 ubiquitin ligase complexes (see the complex inside the circle), and it is E3 ubiquitin ligase that determines substrate specificity. The ubiquitin conjugation reaction can be repeated many times, and an additional ubiquitin molecule is added with each cycle to form a polyubiquitin chain. Polyubiquitin chains mainly serve as a signal for the destruction of the tagged protein by 26S proteasome (Fig. 3).

Cul-containing E3 ubiquitin ligase is one of the two classes of E3 ubiquitin ligases. So far, seven cullin members in the cullin family have been identified in human. There are

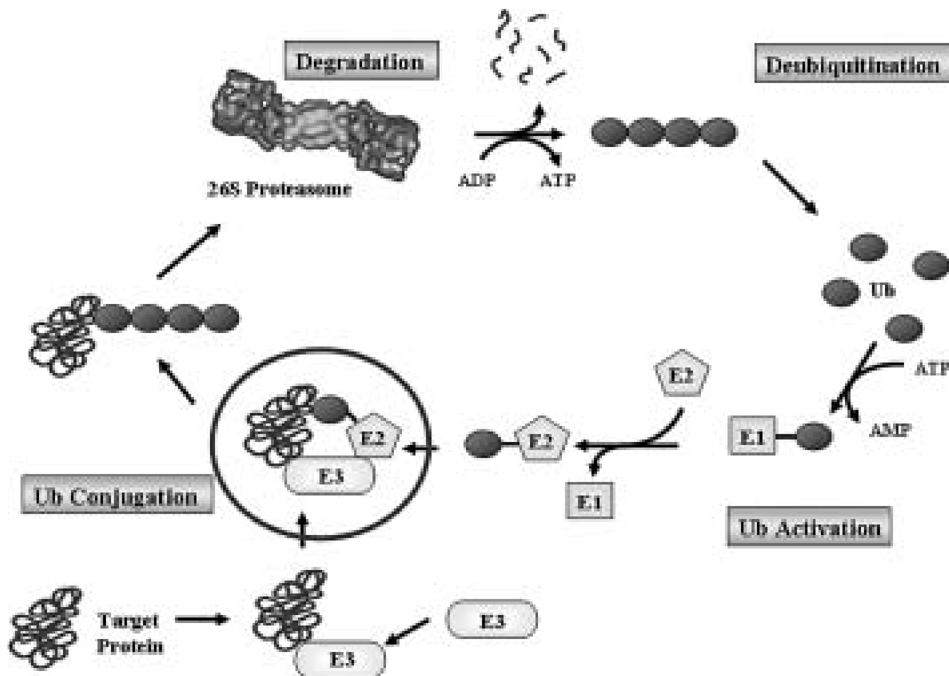

Figure 3 The ubiquitin-mediated degradation pathway. Ubiquitin conjugation reactions require the sequential actions of three major enzymes 1) ubiquitin-activating enzyme E1: forms a thioester bond with ubiquitin; 2) ubiquitin conjugation enzyme E2: accepts ubiquitin transferred from E1 enzyme; 3) ubiquitin ligase enzyme E3: transfers the activated ubiquitin from the E2 to a lysine residue of substrate proteins. Polyubiquitin chain mainly serves as a signal for the destruction of the tagged protein by 26S proteasome.

Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7. In the cullin-containing E3 ubiquitin ligase, each of the cullin proteins serves as a binding platform for other subunits. Our first approach was to identify which cullin protein coexisted with Keap1 in an E3 ubiquitin ligase. Using immunoprecipitation assay, we found that Keap1 associated strongly with Cul3 and slightly with Cul2. Next, we performed an *in vivo* ubiquitination assay to test the ability of Cul3 or Cul2 to facilitate ubiquitin addition onto the Neh2 domain of Nrf2, a domain with seven lysine residues that are the preferential targets for ubiquitin addition. Cul3, not Cul2, facilitated ubiquitination of Neh2 in a dose-dependent manner, indicating that Keap1 was in complex with Cul3, not Cul2 (Fig. 4A). We further verified the co-presence of the Rbx1, Cul3, and Nrf2 in the complex immunoprecipitated with an anti-Keap1 antibody. Furthermore, the functional importance of Rbx1, Cul3, and Keap1 in the facilitation of the ubiquitination of Nrf2 was confirmed in both *in vivo* and *in vitro* ubiquitination assays (Fig. 4B and 4C). These results provide clear evidence that Keap1 does not just passively sequester Nrf2 in the cytoplasm to block Nrf2 nuclear translocation, as originally proposed, but actively targets Nrf2 for ubiquitination and proteasomal degradation by functioning as a component of an E3 ubiquitin ligase (Zhang et al., 2004). The same conclusion was reached by three other groups. All identified Keap1 as a substrate adaptor protein for a Cul3-containing E3 ubiquitin ligase (Cullinan et al., 2004; Kobayashi et al., 2004; Furukawa and Xiong, 2005).

TBHQ OR SULFORAPHANE INHIBITS THE ACTIVITY OF KEAP1-CUL3 E3 UBIQUITIN LIGASE

In addition to the finding that Keap1 is one of more than 100 E3 ubiquitin ligases, we went further to investigate how the Keap1-containing E3 ubiquitin ligase is regulated. First, we compared the Keap1-Cul3 E3 ubiquitin ligase with the two other well-studied Cul1- and Cul2-containing E3 ubiquitin ligases (Fig. 5). SCF (Skp1-Cullin-F-box protein) is the best studied class of E3 ubiquitin ligases (Zheng et al., 2002). In this complex, the F-box protein and Skp1 together serve as a substrate adaptor that brings in a specific substrate for ubiquitin conjugation. Notably, the cullin-containing E3 ubiquitin ligases are dynamically undergoing assembly and disassembly under the tight control of other two families of proteins named CAND (also called Tip120A) and CSN (COP9 signalosome) (Wei and Deng, 2003). However, the common core complex, consisting of cullin and Rbx1, remains assembled and is shared by different substrate adaptor proteins to facilitate ubiquitination and degradation of different substrates. The advantage of using the same core complex for a variety of substrate adaptor proteins is to allow rapid degradation of diverse factors, according to the changes of intracellular environment, without *de novo* assemble of the entire E3 ubiquitin ligase complex. The F-box proteins in the SCF complex represent a large family, and each member can have several specific substrates. SCF regulates many important factors, including cyclin-dependent kinase inhibitors, cyclins, IkBα, and β-catenin. VCB (von Hippel-Lindau protein–elonginC-elonginB) is another cullin-containing E3 ubiquitin ligase that is responsible for degradation of HIFα (hypoxia-inducible transcription factor) under the normoxia (normal amount of oxygen) condition. In the VCB complex, the substrate adaptor function is accomplished by three proteins, pVHL, elonginC, and elonginB (Kamura et al., 1999) (Fig. 5). It is obvious that these three cullin-containing E3 ubiquitin complexes, SCF, VCB, and Keap1-Cul3, have similar overall architectures, in which the cullin protein constitutes a binding platform for other proteins, such as a substrate adaptor protein, a RING finger-containing Rbx1, and a ubiquitin-conjugated

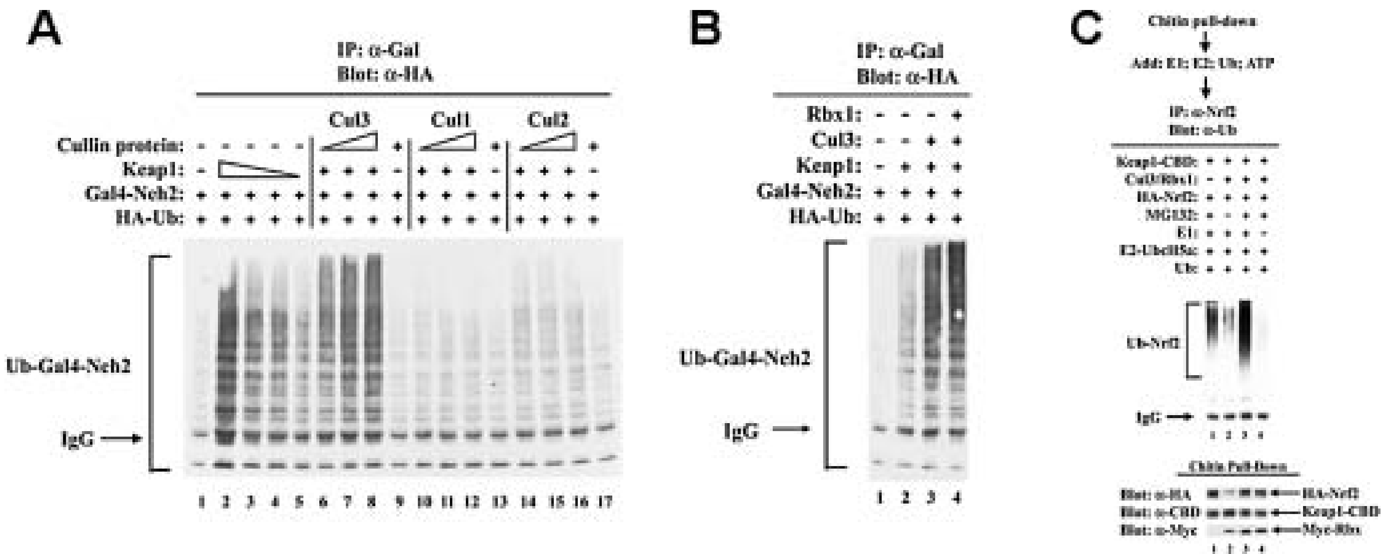

Figure 4 Keap1 is a substrate adaptor for a Cul3-containing E3 ubiquitin ligase. (A) MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub), Gal4-Neh2, Keap1, and each of the cullin proteins as indicated. Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. IgG, immunoglobulin G. (B) MDA-MB-231 cells were transfected with expression vectors for HA-Ub, Gal4-Neh2, Keap1 (lanes 2 to 4), Cul3 (lanes 3 to 4), and the Myc-Rbx1 expression plasmid (lane 4). Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. (C) COS1 cells were transfected with expression vectors for Nrf2 (lanes 1 to 4), Keap1-CBD (lanes 1 to 4), HA-Cul3 (lanes 2 to 4), and Myc-Rbx1 (lanes 2 to 4). Lysates from three 60-mm-diameter dishes were pooled for each sample and incubated with chitin beads. After washing, the chitin beads were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. Subsequently, the chitin beads were pelleted and washed, and proteins that were eluted from the beads after boiling were split into two sets of samples. One set was immunoprecipitated with anti-Nrf2 antibodies and then analyzed by immunoblotting with anti-ubiquitin antibodies (top panel). The other set was subjected to immunoblot analysis using anti-HA, anti-CBD, and anti-Myc antibodies (bottom three panels). Adapted from Zhang, D. D., et al. (2004). *Mol. Cell. Biol.* 24:10941–10953. © 2006 with permission from the American Society for Microbiology.

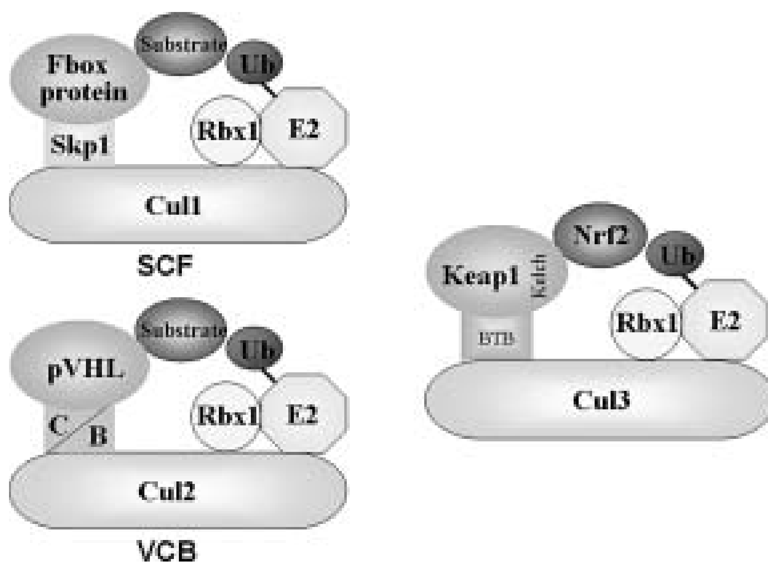

Figure 5 Comparison of the Keap1-Cul3 E3 ubiquitin complex with the two other E3 ubiquitin ligases, SCF and VCB. These three cullin-containing E3 ubiquitin complexes have similar overall architectures, in which each of the cullin proteins constitutes a binding platform for other proteins, such as a substrate adaptor protein, a RING finger-containing Rbx1, and a ubiquitin-conjugated E2 enzyme. In SCF, the substrate adaptor function is accomplished by two proteins, such as Skp1, and an F-box protein. In VCB, the substrate adaptor consists of pVHL, C, and B. In the Keap1-Cul3 E3 ubiquitin ligase complex, a single protein Keap1 functions as a substrate adaptor.

E2 enzyme. In the case of SCF and VCB complexes, regulation of the substrate stability is at the level of substrate binding. For example, the F-box protein β-TrCP in the SCF complex has very low affinity for IkBα under the basal condition. However, β-TrCP binds specifically and strongly with the phosphorylated IkBα, resulting in rapid degradation of phosphorylated IkBα upon treatment with TNFα or other NFκB inducers (Yaron et al., 1998). Similarly, pVHL binds with hydroxylated HIFα and degrades HIFα under the normoxia condition. It escapes destruction by VCB-mediated degradation machinery, whereas HIFα is unmodified by hydroxylation under hypoxia conditions (reduced oxygen). In analog with SCF and VCB complexes, we speculate that association of Nrf2 with Keap1 is also regulated by post-translational modification of Nrf2 in response to oxidative stress or electrophile treatments. In line with this notion, there are many reports describing Nrf2 as a downstream factor of many kinases. However, so far there is no modification reported to occur on Nrf2 that results in dissociation of the Keap1-Nrf2 complex *in vivo*. It is also possible that post-translational modification may occur on Keap1, leading to dissociation of the Nrf2-Keap1 complex. However, the fact that the three important Keap1 mutants (C151S, C273S, and C288S) bind to Nrf2 equally well as the wild-type Keap1 is inconsistent with this model.

We tested whether the association between Nrf2 and Keap1 is disrupted following tBHQ or sulforaphane treatment using immunoprecipitation assays in three different settings: 1) association of the endogenous Keap1 with the endogenous Nrf2, 2) association of the ectopically expressed Keap1 with the endogenous Nrf2, and 3) association of both ectopically expressed Keap1 and Nrf2. As expected, we observed increased steady-state levels of Nrf2 in cells exposed to tBHQ or sulforaphane. Nevertheless, there were proportional

increases in Nrf2 levels in Keap1 immunoprecipitates from tBHQ- or sulforaphane-exposed samples, indicating that neither tBHQ nor sulforaphane is able to disrupt the Nrf2-Keap1 complex (Fig. 6). The same conclusion was drawn from all three experimental settings. Our finding demonstrates that the original Nrf2-Keap1 dissociation model is likely incorrect. In an *in vitro* system with purified Keap1 and Neh2 proteins, Mesecar's group also demonstrated that Nrf2-inducers did not disrupt the Nrf2-Keap1 complex (Eggler et al., 2005). These results may imply that, unlike the SCF and VCB complexes, regulation of Nrf2 may not be rendered at the level of substrate binding with the Cul3-containing E3 ubiquitin ligase.

The important question then is how does tBHQ or sulforaphane block degradation of Nrf2? By performing *in vivo* ubiquitination assay in cells treated with tBHQ or sulforaphane, we found that ubiquitination was significantly blocked by tBHQ or sulforaphane treatment. Furthermore, co-transfection of Keap1-C273S or Keap1-C288S also repressed ubiquitination of Nrf2 (Fig. 7). Interestingly, tBHQ or sulforaphane was no longer effective in blockage of Nrf2 ubiquitination in the presence of Keap1-C151S (Fig. 7). Taken together, these results unambiguously demonstrate that tBHQ or sulforaphane activate ARE-dependent transcription primarily by inhibiting the activity of Keap1-Cul3 ubiquitin ligase, resulting in decreased Nrf2 ubiquitination, enhanced Nrf2 stability, elevated Nrf2 levels, and, ultimately, activation of ARE-dependent gene transcription. In support of our notion that Nrf2 inducers target the Keap1-containing E3 ubiquitin ligase rather than the Nrf2-Keap1 complex, Yamamoto and coworkers recently reported the same finding, i.e., tBHQ did not disrupt the Nrf2-Keap1 complex; rather, it blocked ubiquitination of Nrf2 (Kobayashi et al., 2006). However, the precise mechanism by which tBHQ or sulforaphane inhibits the Keap1-Cul3 E3 ubiquitin ligase is still unclear. Although speculative at this time, it is conceivable that C-151-dependent post-translational modification of Keap1 in response to tBHQ or sulforaphane treatment causes a conformational change of Keap1-containing E3 ubiquitin ligase, resulting in reduced activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase. The next challenge is to explain how Nrf2 translocates into the nucleus if Nrf2-inducers do not dissociate the Nrf2-Keap1 complex. Yamamoto and coworkers have proposed that it is the newly synthesized Nrf2 proteins that translocate into the nucleus by showing the reduced nuclear Nrf2 levels in cells treated with both tBHQ and the protein synthesis inhibitor cycloheximide (CHX), compared to the Nrf2 levels in cells treated with tBHQ alone (Kobayashi et al., 2006). However, the caveat of this experiment is that CHX treatment should certainly reduce the total amount of Nrf2 proteins, resulting in proportional reduction of Nrf2 in the nucleus. Furthermore, it is hard to explain how Keap1 can distinguish the "old" vs. the "new" Nrf2 molecules. We would like to use a saturation model: Under the basal conditions, there are significant amounts of Keap1 and very few molecules of Nrf2, due to proteasomal degradation of Nrf2. Upon treatment of tBHQ or sulforaphane, the levels of Nrf2 are markedly increased by blockage of proteasomal degradation of Nrf2. The amount of Nrf2 overwhelms the binding capacity of Keap1, resulting in existence of free Nrf2 molecules that activate the transcription of ARE-dependent genes. Our model of Nrf2 activation will be further discussed in the conclusion section.

MULTI-LEVELS OF NRF2 REGULATION

Although Keap1-mediated ubiquitination and degradation of Nrf2 are evidently major controls of Nrf2 activity, Nrf2 is also subjected to additional modes of regulation, including subcellular localization and post-translational modification. Studies investigating

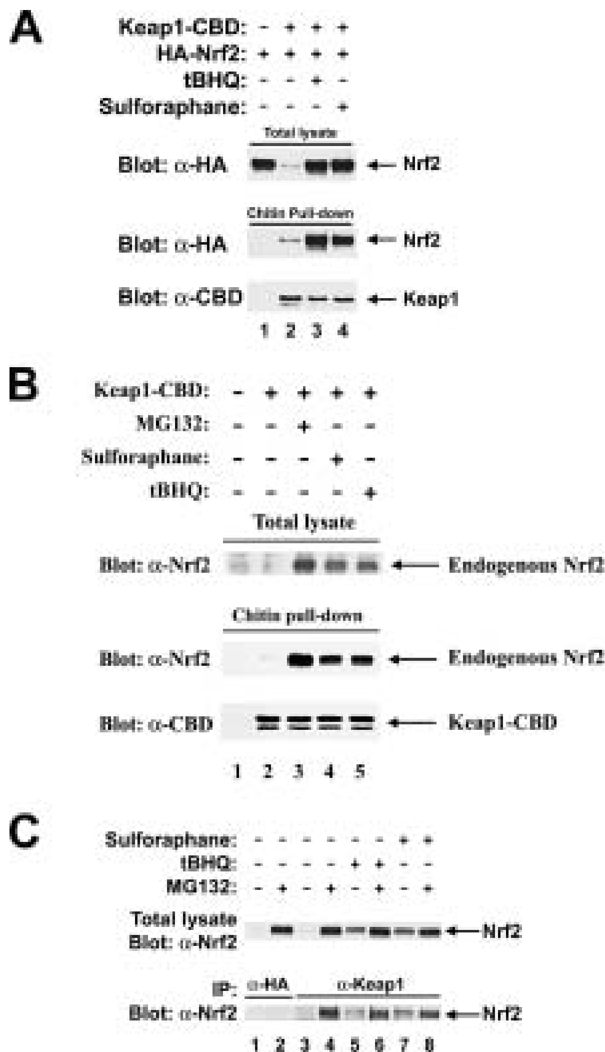

Figure 6 tBHQ or sulforaphane does not disrupt the Nrf2-Keap1 binding. (A) MDA-MB-231 cells were transfected with expression vectors for HA-Nrf2 and Keap1-CBD. The cells were either left untreated (lanes 1 and 2), treated with 50 μM tBHQ (lane3), or 25 μM sulforaphane (lane 4) for 5 h. Cell lysates were immunoblotted with anti-HA antibodies (top panel) or incubated with chitin beads. Proteins that remained bound to the chitin beads after extensive washing were analyzed by immunoblotting with either anti-HA (middle panel) or anti-CBD (bottom panel) antibodies. (B) MDA-MB-231 cells were transfected with an expression vector for Keap1-CBD and treated with 10 μM MG132 (lane 3), 20 μM sulforaphane (lane 4), or 50 μM tBHQ (lane 5) for 5 h. Total cell lysates were subjected to immunoblot analysis with anti-Nrf2 antibodies (top panels) or incubated with chitin beads. Proteins that remained bound to the chitin beads were analyzed by immunoblot analysis using either anti-Nrf2 antibodies (middle panel) or anti-CBD antibodies (bottom panel). (C) MDA-MB-231 cells were either untreated (lanes 1 and 3) or treated for 5 h with 10 μM MG132 (even-numbered lanes), 50 μM tBHQ (lanes 5 and 6), or 25 μM sulforaphane (lanes 7 and 8). Total cell lysates were collected, and 2% of the cell lysate was subjected to immunoblot analysis with anti-Nrf2 antibodies (top panel). The remainder of the lysate was subjected to immunoprecipitation with anti-HA antibodies (bottom panel, lanes 1 and 2) or anti-Keap1 antibodies (bottom panel, lanes 3 to 8). The immunoprecipitated proteins (IP) were analyzed by immunoblotting with anti-Nrf2 antibodies. Adapted from Zhang, D. D., et al. (2004). *Mol. Cell. Biol.* 24:10941–10953. © 2006 with permission from the American Society for Microbiology.

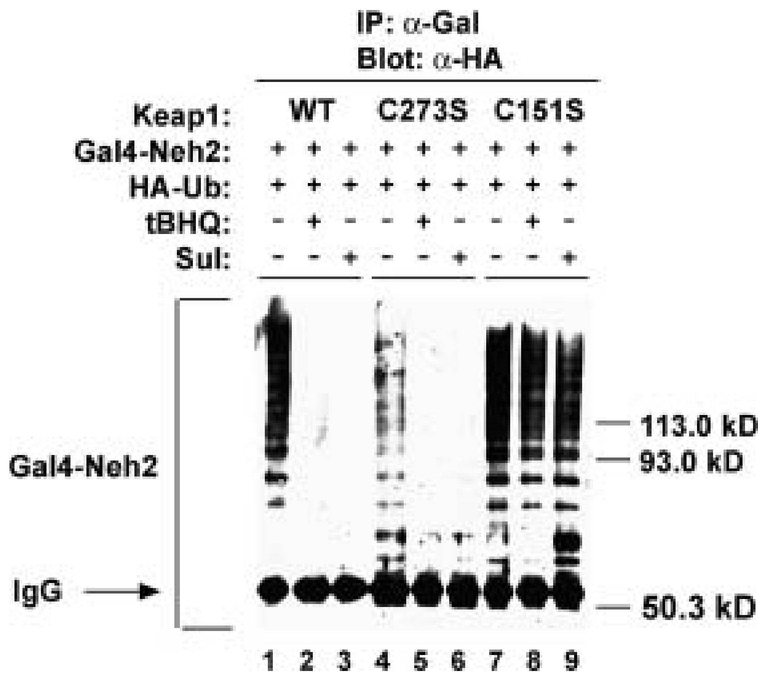

Figure 7 tBHQ or sulforaphane inhibits the Keap1-Cul3 E3 ubiquitin ligase. (A) MDA-MB-231 cells were cotransfected with an expression vector for HA-Ub, a Gal4-Neh2 fusion protein, or expression vectors for either wild-type (WT) Keap1, Keap1-C273S, or Keap1-C151S. The transfected cells were exposed to DMSO, tBHQ, or sulforaphane for 5 h before cell lysis. Anti-Gal4 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies.

subcellular localization of Nrf2 and Keap1 have given rise to controversial results. In contrast to the original notion that Keap1 is a cytoplasmic factor that binds to the actin cytoskeleton, Keap1 has been observed to accumulate in the nucleus in the presence of leptomycin B (LMB), a fungal-derived inhibitor of nuclear export (Karapetian et al., 2005; Nguyen et al., 2005; Velichkova and Hasson, 2005). More significantly, a LMB-sensitive nuclear export signal (NES), LVKIFEELTL, located in the linker region of Keap1 has been identified by three independent teams, indicating that Keap1 truly shuttles between the cytoplasm and the nucleus (Karapetian et al., 2005; Nguyen et al., 2005; Velichkova and Hasson, 2005). In addition, it is believed that Keap1 does not possess a nuclear localization signal (NLS). So it is conceivable that shuttling of Keap1 into the nucleus may be accomplished by the NLS from its partner Nrf2. Indeed, we identified that amino acid sequence 515–518 (RKRK) of human Nrf2 constitutes a classical basic-type NLS. We first observed a putative NLS sequence from 502–518 in the DNA binding domain of Nrf2. We generated two Nrf2-NLS mutations: 1) Nrf2-mNLS1, in which RRR (502–504) was replaced with AAA, and 2) Nrf2-mNLS2, in which RKRK (515–518) was replaced by AAAA. As shown in Fig. 8, although the ectopically expressed wild-type Nrf2 and Nrf2-mNLS1 localized in both the nucleus and the cytoplasm, the mutant Nrf2-mNLS2 was exclusively in the cytoplasm, indicating that the RKRK is the authentic NLS of Nrf2. Consistent with our data, Jaiswal's group showed that detection of RRRGKNKVAAQN-CRKRK (494–510) of mouse Nrf2 gave rise to a mutant Nrf2 with cytoplasmic localization

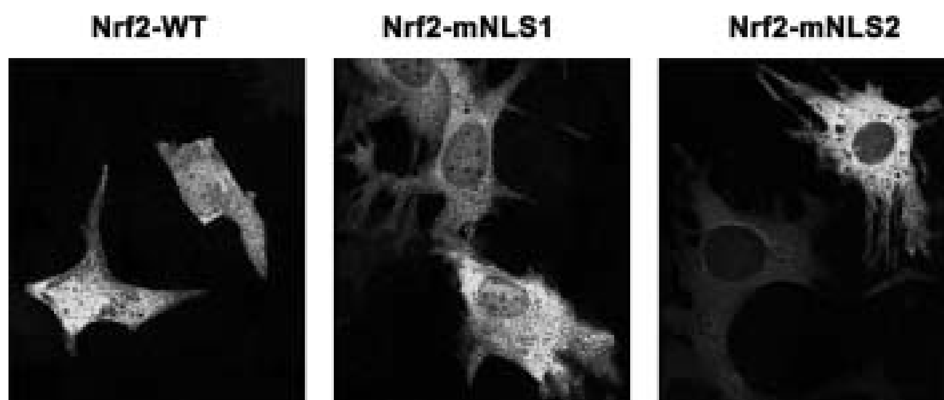

Figure 8 The nuclear localization signal in Nrf2. NIH 3T3 cells were transfected with expression vectors for HA-Nrf2, HA-Nrf2-mNLS1, or HA-Nrf2-mNLS2. The cellular localization of the Nrf2 proteins was determined by double-label indirect immunofluorescence with anti-HA antibodies. Image was obtained with a confocal microscope.

(Jain et al., 2005). Furthermore, they showed that RRRGKNKVAAQNCRKRK sequence is sufficient to support nuclear localization of the GFP-RRRGKNKVAAQNCRKRK fusion protein (Jain et al., 2005). Confusingly, in addition to the suggestion that the Keap1 NES regulates Nrf2 export, an NES located at the leucine zipper domain of the Nrf2 protein has been reported to regulate its nuclear export by two separate groups (Jain et al., 2005; Li et al., 2005). The reported NES sequence in human Nrf2 or in mouse Nrf2 is LKKQLSTLYL (553–562 in human) or LKRRLSTLYL (545–554 in mouse), respectively. So it seems that both Nrf2 and Keap1 are shuttling proteins and both have their own NES. Then an intriguing question is whether Nrf2 travels out of the nucleus alone, using its own NES, or in the complex with Keap1, using the NES signal from the Keap1 protein. More bewilderingly, Pickett's group recently presented very interesting data demonstrating that Nrf2 was localized in the nucleus and was constitutively degraded in the nucleus to maintain very low levels of Nrf2 under the basal condition. In addition, they showed that the E3 ubiquitin ligase function of Keap1 was performed inside the nucleus after Keap1 translocated into the nucleus (Nguyen et al., 2005). Therefore, many essential questions remain to be solved. For example, where is Nrf2 located in unstimulated cells? In which compartment does the Keap1-mediated ubiquitination and degradation of Nrf2 occur? How do cells maintain the constitutive levels of Nrf2-dependent genes? Is Nrf2 nuclear export controlled by the NES signal in Nrf2 or by the NES identified in Keap1? How do cells terminate the Nrf2-dependent defense response once cellular redox homeostasis is restored? Further investigations will warrant the clarification of these basic issues.

Phosphorylation of Nrf2 has been proposed to regulate the activity of Nrf2 by many investigators. The upstream kinases of Nrf2 reported in the literature include protein kinase C (PKC); PERK, an endoplasmic reticulum (ER)-transmembrane protein kinase that signals ER stress; the kinases in the MAP kinase pathway, such as extracellular-regulated kinase (ERK), P38, Jun N-terminal kinase (JNK); and phosphatidylinositol 3-kinase (PI3K). The details of these experiments will not be discussed in this review. However, it is clear that the selection of upstream kinases for Nrf2 phosphorylation depends on both inducers and cell types. Moreover, distinct kinase pathways can be activated by an extracellular stimulus, and these pathways may converge at certain point to integrate signals.

Although a large body of evidence demonstrates that Nrf2 is phosphorylated, the target residues for phosphorylation in Nrf2 that are absolutely required for the activity of Nrf2 are still unidentified. Nevertheless, it is clear that Nrf2 is phosphorylated in cells exposed to Nrf2-inducers. Multiple steps can be envisioned to explain Nrf2 activation by phosphorylation: 1) at the Nrf2-E3 ligase complex: in analog to the SCF complex, which only binds with phosphorylated substrates, the Keap1-Cul3 E3 complex may only bind with the unphosphorylated Nrf2. In response to Nrf2-inducers, Nrf2 is phosphorylated and it can no longer be recognized by Keap1-Cul3 E3, resulting in stabilization of Nrf2. However, the observation that the Nrf2-Keap1 complex still exists in cells treated with Nrf2-inducers is against this model. Nevertheless, this possibility is still worth exploring due to the limitation of the immunoprecipitation method used for detection of the Nrf2-Keap1 complex; 2) at the Nrf2-DNA complex: phosphorylation of Nrf2 may affect its binding affinity to ARE or enhance its interaction with the general transcriptional machinery; 3) at Nrf2-export machinery: Jaiswal and coworkers identified a tyrosine residue (Y568) in mouse Nrf2 that had to be phosphorylated for Nrf2 to travel out of the nucleus. Furthermore, they demonstrated that Fyn was the kinase that directly phosphorylated tyrosine-568 (Jain and Jaiswal, 2006). As reported by another group, glycogen synthase kinase-3 beta, a serine/threonine kinase, was required for phosphorylation of Nrf2 and for subsequent export of the phosphorylated Nrf2 out of the nucleus (Salazar et al., 2006). In both cases, phosphorylation of the Y568 or the unidentified serine/threonine plays a negative role in ARE-dependent gene activation by facilitating export of Nrf2. Furthermore, phosphorylation of Y568 was shown to be important for interaction of Nrf2 with Crm1/exportin 1 since substitution of tyrosine with alanine abolished Nrf2 binding with Crm1. Interestingly, this residue is not located within the NES sequence (545–554) of mouse Nrf2 that is known to interact with Crm1 to facilitate export of the NES-bearing proteins.

CONCLUSIONS

Great advances have been made in the understanding of activation of Nrf2 in response to oxidative stress, electrophiles, and chemopreventive compounds since the cloning of Keap1 in 1999. The negative regulator Keap1 has been identified to function as a molecular switch to turn on and off the Nrf2-mediated cellular defense response. To accomplish this task, Keap1 has to have dual properties: 1) the sensing function: Keap1 is able to "sense" the changes in intracellular redox environments, and 2) the switch function: Keap1 accomplishes this by functioning as a subunit of E3 ubiquitin ligase, which controls the levels of Nrf2 by ubiquitin-mediated degradation machinery. As shown in Fig. 9, under the basal condition, Keap1 constantly targets Nrf2 for ubiquitination and degradation by 26S proteasome, resulting in very few molecules of Nrf2. Hence, the majority of Nrf2 molecules associate with Keap1. Since the association of the Keap1-Nrf2 complex with the Cul3-Rbx E3 ligase core complex is a regulated dynamic process, the Nrf2-Keap1 complexes are either associating with the Cul3-Rbx1 E3 ubiquitin ligase transiently or are shuttling between the nucleus and the cytosol (Lo and Hannink, 2006). It is reasonable to assume that the nuclear Nrf2-Keap1 complexes are not active, possibly due to the Keap1-mediated interference of DNA binding, heterodimerization, or interactions with the general transcription factors. In response to changes of intracellular redox environment mediated by Nrf2-inducers, Keap1 undergoes a C151-dependent post-translational modification, resulting in a conformational change of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase to its suboptimal configuration. The decreased activity of the Keap1-containing E3 ubiquitin

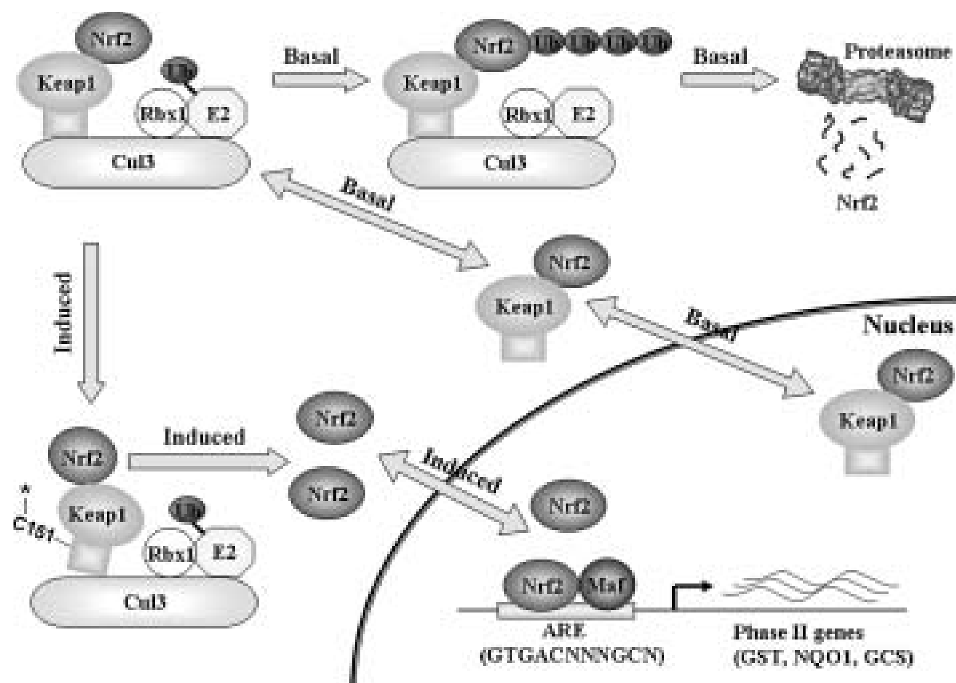

Figure 9 Schematic model of Nrf2 regulation by Keap1-mediated ubiquitination and degradation. Keap1, a substrate adaptor protein for Cul3-containing E3 ubiquitin ligase, constantly targets Nrf2 for ubiquitination and degradation by 26S proteasome under the basal condition. In response to changes of intracellular redox environment mediated by Nrf2-inducers, Keap1 undergoes a C151-dependent post-translational modification, resulting in a conformational change of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase. The decreased activity of the Keap1-containing E3 ubiquitin ligase leads to stabilization of Nrf2. Increased amounts of Nrf2 saturate the binding sites of Keap1, resulting in translocation of free Nrf2 into the nucleus, where it binds to ARE to activate the transcription of its downstream genes.

ligase leads to stabilization of Nrf2. Given that the levels of the Keap1 protein are relatively stable, increased amounts of Nrf2 should saturate the binding sites of Keap1, resulting in existence of free Nrf2. Free Nrf2 translocates into the nucleus, where it binds to ARE to activate the transcription of its downstream genes. Although the Keap1-mediated ubiquitination and degradation of Nrf2 is apparently the primary control of Nrf2, the activity of Nrf2 is also controlled at multiple stages, such as nucleocytoplasmic trafficking, post-translational modifications, and interaction of the Nrf2-ARE-containing complex with other gene regulatory factors. Understanding detailed regulatory mechanisms of Nrf2 will assure the success of medical prevention or intervention of many human diseases induced by oxidative stress.

ABBREVIATIONS

Nrf2	NF-E2-related factor 2
Keap1	Kelch-like ECH-associated protein 1
EGCG	epigallocatechin-3-gallate
ARE	antioxidant response element
ERE	electrophile response element

GST	glutathione S-transferase
NQO1	NAD(P)H quinone oxidoreductase
γGCS	γ-glutamylcysteine synthetase
NF-E2	nuclear factor (erythroid-derived 2)
cnc	“cap ‘n’ collar”
Neh	Nrf2-Ech homology
CBP	CREB-binding protein
BTB	broad complex, tramtrack and bric-a-brac
NLS	nuclear localization signal
NES	nuclear export signal
tBHQ	tert-butylhydroquinone
Cul	cullin
Rbx1	ring-box protein 1
SCF	Skp1-Cullin-F-box protein
pVHL	the von Hippel-Lindau tumor suppressor protein
VCB	pVHL, elongins C and B
CAND	cullin-associated and neddylation-dissociated
CSN	COP9 signalosome
PKC	protein kinase C
ERK	extracellular-regulated kinase
JNK	Jun N-terminal Kinase
PI3K	Phosphatidylinositol 3-kinasse

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REFERENCES

Aoki, Y., Sato, H., Nishimura, N., Takahashi, S., Itoh, K., Yamamoto, M. (2001). Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.* 173:154–160.

Braun, S., Hanselmann, C., Gassmann, M. G., auf dem Keller, U., Born-Berclaz, C., Chan, K., Kan, Y. W., Werner, S. (2002). 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.

Chan, J. Y., Kwong, M., Lu, R., Chang, J., Wang, B., Yen, T. S., Kan, Y. W. (1998). Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice. *Embo. J.* 17:1779–1787.

Chan, K., Han, X. D., Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc. Natl. Acad. Sci. USA* 98:4611–4616.

Chan, K., Lu, R., Chang, J. C., Kan, Y. W. (1996). 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.

Chanas, S. A., Jiang, Q., McMahon, M., McWalter, G. K., McLellan, L. I., Elcombe, C. R., Henderson, C. J., Wolf, C. R., Moffat, G. J., Itoh, K., Yamamoto, M., Hayes, J. D. (2002). 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.* 365:405–416.

Cho, H. Y., Jedlicka, A. E., Reddy, S. P., Kensler, T. W., Yamamoto, M., Zhang, L. Y., Kleeberger, S. R. (2002). Role of NRF2 in protection against hyperoxic lung injury in mice. *Am. J. Respir. Cell. Mol. Biol.* 26:175–182.

Cho, H. Y., Reddy, S. P., Debiase, A., Yamamoto, M., Kleeberger, S. R. (2005). Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radic. Biol. Med.* 38:325–343.

Cho, H. Y., Reddy, S. P., Yamamoto, M., Kleeberger, S. R. (2004). The transcription factor NRF2 protects against pulmonary fibrosis. *Faseb. J.* 18:1258–1260.

Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., Diehl, J. A. (2004). 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.* 24:8477–8486.

Delaunay, A., Pflieger, D., Barrault, M. B., Vinh, J., Toledano, M. B. (2002). A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. *Cell* 111:471–481.

Derjuga, A. Gourley, T. S., Holm, T. M., Heng, H. H., Shivdasani, R. A., Ahmed, R., Andrews, N. C., Blank, V. (2004). Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus. *Mol. Cell. Biol.* 24:3286–3294.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., Talalay, P. (2002). 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.

Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J., Talalay, P. (2001). 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.

Eggler, A. L., Liu, G., Pezzuto, J. M., van Breemen, R. B., Mesecar, A. D. (2005). Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. *Proc. Natl. Acad. Sci. USA* 102:10070–10075.

Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T., Yamamoto, M. (2001). 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.

Furukawa, M., Xiong, Y. (2005). BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol. Cell. Biol.* 25:162–171.

Gao, X., Talalay, P. (2004). Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. *Proc. Natl. Acad. Sci. USA* 101:10446–10451.

Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. *Annu. Rev. Genet.* 30:405–439.

Hong, F., Sekhar, K. R., Freeman, M. L., Liebler, D. C. (2005). Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. *J. Biol. Chem.* 280:31768–31775.

Iida, K., Itoh, K., Kumagai, Y., Oyasu, R., Hattori, K., Kawai, K., Shimazui, T., Akaza, H., Yamamoto, M. (2004). Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res.* 64:6424–6431.

Ishii, Y., Itoh, K., Morishima, Y., Kimura, T., Kiwamoto, T., Iizuka, T., Hegab, A. E., Hosoya, T., Nomura, A., Sakamoto, T., Yamamoto, M., Sekizawa, K. (2005). Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J. Immunol.* 175:6968–6975.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13:76–86.

Jain, A. K., Bloom, D. A., Jaiswal, A. K. (2005). Nuclear import and export signals in control of Nrf2. *J. Biol. Chem.* 280:29158–29168.

Jain, A. K., Jaiswal, A. K. (2006). Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *J. Biol. Chem.* 281:12132–12142.

Jeyapaul, J., Jaiswal, A. K. (2000). Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene. *Biochem. Pharmacol.* 59:1433–1439.

Kamura, T., Koepp, D. M., Conrad, M. N., Skowyra, D., Moreland, R. J., Iliopoulos, O., Lane, W. S., Kaelin, W. G., Jr., Elledge, S. J., Conaway, R. C., Harper, J. W., Conaway, J. W. (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* 284:657–661.

Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., Vartapetian, A. B. (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol.* 25:1089–1099.

Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., Yamamoto, M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 6:857–868.

Kensler, T. W., Curphey, T. J., Maxiutenko, Y., Roebuck, B. D. (2000). Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins. *Drug Metabol. Drug Interact.* 17:3–22.

Kleiner, H. E., Rivera, M. I., Pumford, N. R., Monks, T. J., Lau, S. S. (1998). Immunochemical detection of quinol--thioether-derived protein adducts. *Chem. Res. Toxicol.* 11:1283–1290.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., Yamamoto, M. (2004). 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.

Kobayashi, A., Kang, M. I., Watai, Y., Tong, K. I., Shibata, T., Uchida, K., Yamamoto, M. (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol. Cell. Biol.* 26:221–229.

Kwak, M. K., Itoh, K., Yamamoto, M., Sutter, T. R., Kensler, T. W. (2001). 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.

Kwak, M. K., Wakabayashi, N., Itoh, K., Motohashi, H., Yamamoto, M., Kensler, T. W. (2003). 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.

Lee, J. M., Calkins, M. J., Chan, K., Kan, Y. W., Johnson, J. A. (2003). 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.

Levonen, A. L., Landar, A., Ramachandran, A., Ceaser, E. K., Dickinson, D. A., Zanoni, G., Morrow, J. D., Darley-Usmar, V. M. (2004). Cellular mechanisms of redox cell signalling: role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem. J* .378:373–382.

Li, W., Jain, M. R., Chen, C., Yue, X., Hebbar, V., Zhou, R., Kong, A. N. (2005). Nrf2 Possesses a redox-insensitive nuclear export signal overlapping with the leucine zipper motif. *J. Biol. Chem.* 280:28430–28438.

Li, X., Zhang, D. D., Hannink, M., Beamer, L. J. (2004). Crystal structure of the Kelch domain of human Keap1. *J. Biol. Chem.* 279:54750–54758.

Lo, S. C., Hannink, M. (2006). CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1. *Mol. Cell. Biol.* 26:1235–1244.

Moi, P., Chan, K., Asunis, I., Cao, A., Kan, Y. W. (1994). 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.

Nguyen, T., Huang, H. C., Pickett, C. B. (2000). Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J. Biol. Chem.* 275: 15466–15473.

Nguyen, T., Sherratt, P. J., Nioi, P., Yang, C. S., Pickett, C. B. (2005). Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *J. Biol. Chem.* 280:32485–32492.

Nioi, P., McMahon, M., Itoh, K., Yamamoto, M., Hayes, J. D. (2003). Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem. J.* 374:337–348.

Nioi, P., Nguyen, T., Sherratt, P. J., Pickett, C. B. (2005). The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. *Mol. Cell. Biol.* 25:10895–10906.

Orsolic, N., Sver, L., Terzic, S., Basic, I. (2005). Peroral application of water-soluble derivative of propolis (WSDP) and its related polyphenolic compounds and their influence on immunological and antitumour activity. *Vet. Res. Commun.* 29:575–593.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M. I., Kobayashi, A., Yokoyama, S., Yamamoto, M. (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol. Cell* 21:689–700.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70:503–533.

Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., Kensler T. W. (2001). Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc. Natl. Acad. Sci. USA* 98:3410–3415.

Rangasamy, T., Cho, C. Y., Thimmulappa, R. K., Zhen, L., Srisuma, S. S., Kensler, T. W., Yamamoto, M., Petrache, I., Tuder, R. M., Biswal, S. (2004). Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J. Clin. Invest.* 114:1248–1259.

Rangasamy, T., Guo, J., Mitzner, W. A., Roman, J., Singh, A., Fryer, A. D., Yamamoto, M., Kensler, T. W., Tuder, R. M., Georas, S. N., Biswal, S. (2005). Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J. Exp. Med.* 202:47–59.

Richardson, H. L., Cunningham, L. (1951). The inhibitory action of methylcholanthrene on rats fed the azo dye 3'-methyl-4-dimethylaminobenzene. *Cancer Res.* 11:274.

Salazar, M., Rojo, A. I., Velasco, D., de Sagarra, R. M., Cuadrado, A. (2006). 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.* 281:14841–14851.

Shen, G., Xu, C., Hu, R., Jain, M. R., Nair, S., Lin, W., Yang, C. S., Chan, J. Y., Kong, A. N. (2005). Comparison of (-)-epigallocatechin-3-gallate elicited liver and small intestine gene expression profiles between C57BL/6J mice and C57BL/6J/Nrf2 (-/-) mice. *Pharm. Res.* 22:1805–1820.

Shih, A. Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P., Murphy, T.H. (2005a). Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J. Biol. Chem.* 280:22925–22936.

Shih, A. Y., Li, P., Murphy, T. H. (2005b). A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* 25:10321–10335.

Shivdasani, R. A., Rosenblatt, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Saris, C. J., Orkin, S. H. (1995). Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell* 81:695–704.

Suh, J. H., Shenvi, S. V., Dixon, B. M., Liu, H., Jaiswal, A. K., Liu, R. M., Hagen, T. M. (2004). Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc. Natl. Acad. Sci. USA* 101:3381–3386.

Talalay, P., Fahey, J. W. (2001.) Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J. Nutr.* 131:3027S–3033S.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res.* 62:5196–5203.

Velichkova, M., Hasson, T. (2005). Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Mol. Cell. Biol.* 25:4501–4513.

Venugopal, R., Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. USA* 93:14960–14965.

Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., Talalay, P. (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA* 101:2040–2045.

Wei, N., Deng, X. W. (2003). The COP9 signalosome. *Annu. Rev. Cell. Dev. Biol* .19:261–286.

Wild, A. C., Moinova, H. R., Mulcahy, R. T. (1999). Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J. Biol. Chem.* 274:33627–33636.

Wolf, C. R. (2001). Chemoprevention: increased potential to bear fruit. *Proc. Natl. Acad. Sci. USA* 98:2941–2943.

Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., Ben-Neriah, Y. (1998). Identification of the receptor component of the Ikappa-Balpha-ubiquitin ligase. *Nature* 396:590–594.

Yoh, K., Itoh, K., Enomoto, A., Hirayama, A., Yamaguchi, N., Kobayashi, M., Morito, N., Koyama, A., Yamamoto, M., Takahashi, S. (2001). Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney Int.* 60:1343–1353.

Zhang, D. D., Hannink, M. (2003). 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.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell. Biol.* 24:10941–10953.

Zhang, Y., Kensler, T. W., Cho, C. G., Posner, G. H., Talalay, P. (1994). Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc. Natl. Acad. Sci. USA* 91:3147–3150.

Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., Pavletich, N. P. (2002). Structure of the Cul1-Rbx1-Skp1-F-boxSkp2 SCF ubiquitin ligase complex. *Nature* 416:703–709.

Zipper, L. M., Mulcahy, R. T. (2002). The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J. Biol. Chem.* 277:36544–36552.