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THE KEAP1-NRF2 SYSTEM: A THIOL-BASED SENSOR-EFFECTOR APPARATUS FOR MAINTAINING REDOX HOMEOSTASIS

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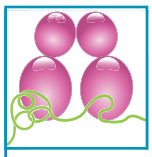

Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 System: a Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol Rev* 98: 1169–1203, 2018. Published May 2, 2018; doi:10.1152/physrev.00023.2017.—The Kelch-like ECH-associated protein 1-NF-E2-related factor 2 (KEAP1-NRF2) system forms the major node of cellular and organismal defense against oxidative and electrophilic stresses of both exogenous and endogenous origins. KEAP1 acts as a cysteine thiol-rich sensor of redox insults, whereas NRF2 is a transcription factor that robustly transduces chemical signals to regulate a battery of cytoprotective genes. KEAP1 represses NRF2 activity under quiescent conditions, whereas NRF2 is liberated from KEAP1-mediated repression on exposure to stresses. The rapid inducibility of a response based on a derepression mechanism is an important feature of the KEAP1-NRF2 system. Recent studies have unveiled the complexities of the functional contributions of the KEAP1-NRF2 system and defined its broader involvement in biological processes, including cell proliferation and differentiation, as well as cytoprotection. In this review, we describe historical milestones in the initial characterization of the KEAP1-NRF2 system and provide a comprehensive overview of the molecular mechanisms governing the functions of KEAP1 and NRF2, as well as their roles in physiology and pathology. We also refer to the clinical significance of the KEAP1-NRF2 system as an important prophylactic and therapeutic target for various diseases, particularly aging-related disorders. We believe that controlled harnessing of the KEAP1-NRF2 system is a key to healthy aging and well-being in humans.

I. INTRODUCTION

A. Pre-NRF2 Era

Recognition of the concept that our environment has an impact on our health, especially in the case of carcinogenesis, was first described by Percival Pott in 1775 in his publication *Chirurgical Observations: Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures, and the Mortification of the Toes and Feet* (16). Pott noticed an unusually high incidence of scrotal cancers among chimney sweeps in London and suggested that coal soot is an environmental factor triggering carcinogenesis. In 1915, the causal link between environmental chemicals and carcinogenesis was clearly demonstrated by Yamagiwa and Ichikawa (257). They showed that chronic exposure of rabbit ears to coal tar induced skin cancers. Nowadays, it is well appreciated that avoiding and eliminating exposure to adverse environmental factors is critical in the prevention of chemical carcinogenesis in humans.

In the 1960s and 70s, it was demonstrated that coal tar carcinogens and carcinogens from other environmental and occupational settings typically required biotransformation to electrophilic metabolites that formed covalent adducts between "ultimate" carcinogens and proteins, RNA and DNA (34). This metabolic activation is typically catalyzed by the microsomal cytochrome *P*-450 system and other oxygenases, sometimes termed phase I enzymes (139). While increasing attention was being paid to chemicals as a cause of human cancers, others searched for effective inhibitors of chemical carcinogenesis, small molecules that inhibit the metabolic activation step(s) and/or enhance the detoxication of procarcinogens or their reactive intermedi-

ates. Initial interest in these "cancer chemopreventive" compounds focused on food antioxidants and natural products, such as flavonoids and isothiocyanates, which were presumed to be safe for humans. Pretreatment of rodents with the food antioxidant 2 (3)-tert-butyl-4-hydroxyanisole (BHA) effectively inhibited carcinogenesis, resulting from a wide variety of carcinogens that included benzo[*a*]pyrene, dimethyl-benz[*a*]anthracene, diethylnitrosamine, 4-nitroquinoline-*N*-oxide, urethane, and aflatoxin B1 (250).

Benson and colleagues (10) reported that BHA elevated the enzymatic activities of glutathione *S*-transferases, epoxide hydratase, NAD(P)H:quinone oxidoreductase, and other detoxication enzymes in multiple tissues of the mouse. Many classes of compounds (phenolic antioxidants, azo dyes, polycyclic aromatics, flavonoids, coumarins, cinnamates, indoles, isothiocyanates, 1,2-dithiole-3-thiones, and thiocarbamates) were identified as inducers of these enzymes. Several of these inducers contain a distinctive chemical feature, or acquired this feature after metabolism, that regulates the synthesis of these protective enzymes. Such inducers are Michael reaction acceptors, which are characterized by olefinic bonds that are rendered electrophilic (positively charged) by conjugation with electron-withdrawing substrates. The potency of inducers paralleled their efficiency in Michael reactions and shared the common property of modifying sulfhydryl groups by oxidation, reduction, or alkylation (229). Exposure of cells to low doses of "soft" (non-DNA reactive) sulfhydryl reactive electrophiles evoked a coordinated increase in these enzyme activities (historically termed phase II enzymes), thereby protecting cells against the toxicity of high doses of "hard" (DNA-reactive) electrophiles. This cellular adaptation is called the "electrophile counterattack response" (173). Stemming from these observations, the hypothesis emerged that the cellular "sensor" molecule that transduces the chemical signal of these inducers does so by virtue of unique and highly reactive sulfhydryl functions that recognize and react with them (41).

B. Discovery of NRF2: a Key Effector of the Electrophile Counterattack Response

The elevation of detoxication enzyme activities by electrophiles is now attributable primarily to the increased transcription of their respective genes. *Cis*-regulatory elements critical for the inducible expression of genes encoding phase II enzymes were identified and designated the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) (55, 189, 190). However, *trans* regulatory elements encoding transcription factors for the ARE/EpRE were unknown until Itoh et al. established *Nrf2*-null mice and analyzed their expression of phase II enzymes (78). NRF2 (NF-E2-related factor 2) was originally isolated as a homolog of the hematopoietic transcription factor NF-E2 p45, but its function was unknown (77, 143). They found that the electrophile counterattack response was completely absent in *Nrf2*-null mice, as the induction of phase II enzymes by BHA was abrogated. Identification of NRF2 as a key regulator of the electrophile counterattack response opened a door in toxicology to a new stage where underlying regulatory mechanisms of various defense reactions and adaptations continue to be deciphered and understood at the molecular level.

C. Discovery of KEAP1: a Sensor for Electrophiles

It has been shown that reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion, and electrophiles of extrinsic and intrinsic origin exert beneficial and toxic effects on cellular functions. However, it has been a long-standing question as to how our body or cells sense these redox-disruptive stimuli and respond effectively to them. Although NRF2 was identified as a key regulator of the electrophile counterattack response, the mode of regulation of NRF2 activity in response to chemical insults was still a mystery.

However, the missing factor, the sensor of environmental insults and regulator of NRF2 activity, was soon discovered. In 1999, Itoh et al. (79) isolated, by yeast two-hybrid screening, a new thiol-rich protein and negative regulator of NRF2 and named it KEAP1 (Kelch-like ECH-associated protein 1). KEAP1 promotes NRF2 degradation in unstressed conditions, whereas redox-disrupting stimuli directly modify KEAP1 thiols, leading to inactivation of KEAP1 function, stabilization of NRF2, and induction of cytoprotective genes (32, 58, 108, 266). Thus KEAP1 is a biosensor for electrophiles and ROS.

D. Concise Overview of the KEAP1-NRF2 System

With these discoveries, the KEAP1-NRF2 system has become recognized as the body's dominant defense mechanism against environmental insults. The KEAP1-NRF2 system is a typical two-component system: KEAP1 as a sensor for electrophiles, and NRF2 as an effector for the coordinated activation of cytoprotective genes **(FIGURE 1)**. In the cytoplasm, KEAP1 forms a ubiquitin E3 ligase complex with CULLIN3 (CUL3) and polyubiquitinates NRF2, which marks NRF2 for rapid degradation through the proteasome system. Thus in unstressed conditions, NRF2 is synthesized, but constantly degraded. However, on exposure to electrophiles or ROS, the reactive cysteine residues of KEAP1 are directly modified, which reduces the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex and results in NRF2 stabilization. Nascent NRF2 can then directly translocate into the nucleus, heterodimerize with one of the small musculo-aponeurotic fibrosarcoma (sMAF)

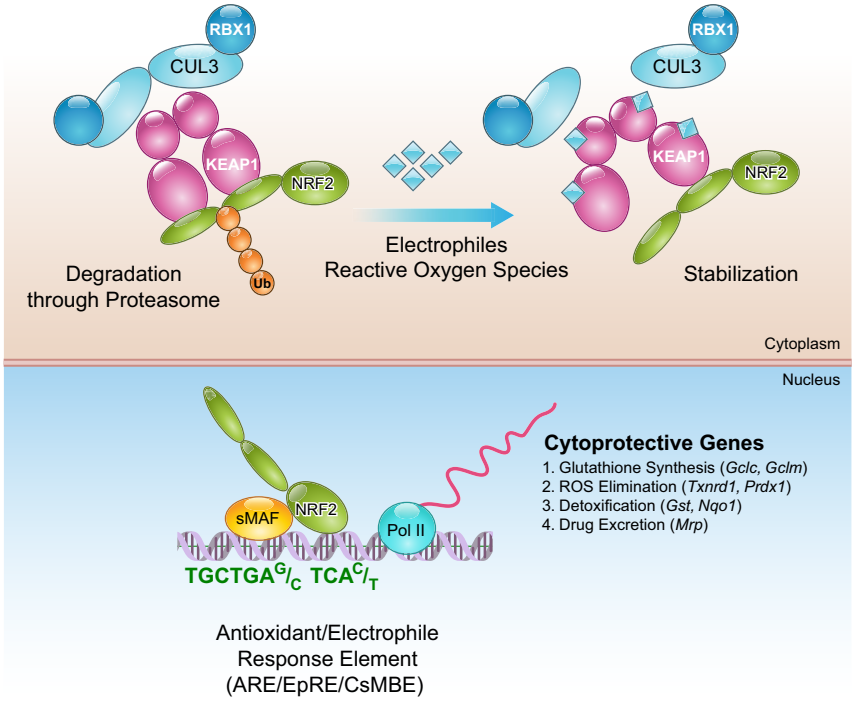

FIGURE 1. KEAP1-NRF2 is a two-component system. In the cytoplasm, NRF2 is ubiquitinated by the KEAP1-CUL3 ubiquitin E3 ligase complex to mark it for degradation by the proteasome. When cells are exposed to electrophiles or reactive oxygen species, KEAP1 is modified and the KEAP1-CUL3 ubiquitin E3 ligase activity declines, which results in the stabilization of NRF2. Stabilized and accumulated NRF2 translocates to the nucleus and activates a battery of cytoprotective genes.

proteins, bind to the ARE/EpRE, and robustly activate a battery of phase II genes, thereby acting as a master regulatory transcription factor.

Vigorous worldwide research on the KEAP1-NRF2 system led to substantial accumulation of evidence demonstrating the critical significance of NRF2 activity and its regulatory mechanisms to the maintenance of our health. Importantly, dysregulation of the KEAP1-NRF2 system underlies the pathogenesis of various human diseases. It is now widely recognized that the KEAP1-NRF2 system provides an attractive target for drug development. In this review, we provide a comprehensive overview of the KEAP1-NRF2 system. We discuss the molecular mechanisms by which the KEAP1-NRF2 system senses and responds to intrinsic and extrinsic redox disturbances and the roles the system plays in organismal defense mechanisms. We consider the generally mitigating, but sometimes exacerbating, contributions of the KEAP1-NRF2 system to various human pathological conditions, such as diabetes, inflammation, and cancer.

II. IDENTIFICATION OF CNC-SMAF TRANSCRIPTION FACTORS

A. Starting From *cis*-Regulatory Elements

A *cis*-regulatory element, termed the ARE/EpRE ($TGA^G/_C$-NNNGC), was first defined from studies in 1990 in the context of toxicology through studies on the mechanism of induction of a glutathione *S*-transferase gene in response to electrophiles (55, 189, 190). ARE/EpREs were also found in promoter regions of other cytoprotective genes within the phase II battery, confirming the inducible and concerted nature of this gene expression response. Although the importance of the ARE/EpRE was clearly shown, the transcription factors that bind to the element and elicit transcriptional activation remained unidentified.

Meanwhile, researchers in the molecular biology field identified a critical *cis*-regulatory element for erythroid-specific transcriptional activation and named it the NF-E2 binding site ($ATGA^G/_CTCAGCA$) (138). The NF-E2 binding activity was successfully purified from a large amount (700 g) of cultured mouse erythroleukemia cells (4). NF-E2 p45 was identified and isolated and became a founding member of the mammalian cap 'n' collar (CNC) transcription factor family. Following the discovery of p45, other members of the CNC family were identified as NRF1, NRF2, NRF3, BACH1, and BACH2 (21, 77, 107, 143, 169) **(FIGURE 2)**.

A seminal discovery related to the vertebrate CNC family of proteins is that they require sMAF factors as obligatory heterodimeric partner molecules for efficient binding to the ARE/EpRE/NF-E2 motif (68, 146). Both CNC proteins and sMAF factors possess a well-conserved basic region-leucine zipper (bZIP) motif. The CNC proteins also retain a characteristic CNC domain that was defined based on sequence homology of 43 amino acids with the *Drosophila* Cnc protein (142). The sMAF factors also retain a characteristic extended homology region **(FIGURE 2)**. As the sMAF factors do not appear to possess any substantial extra-functional do-

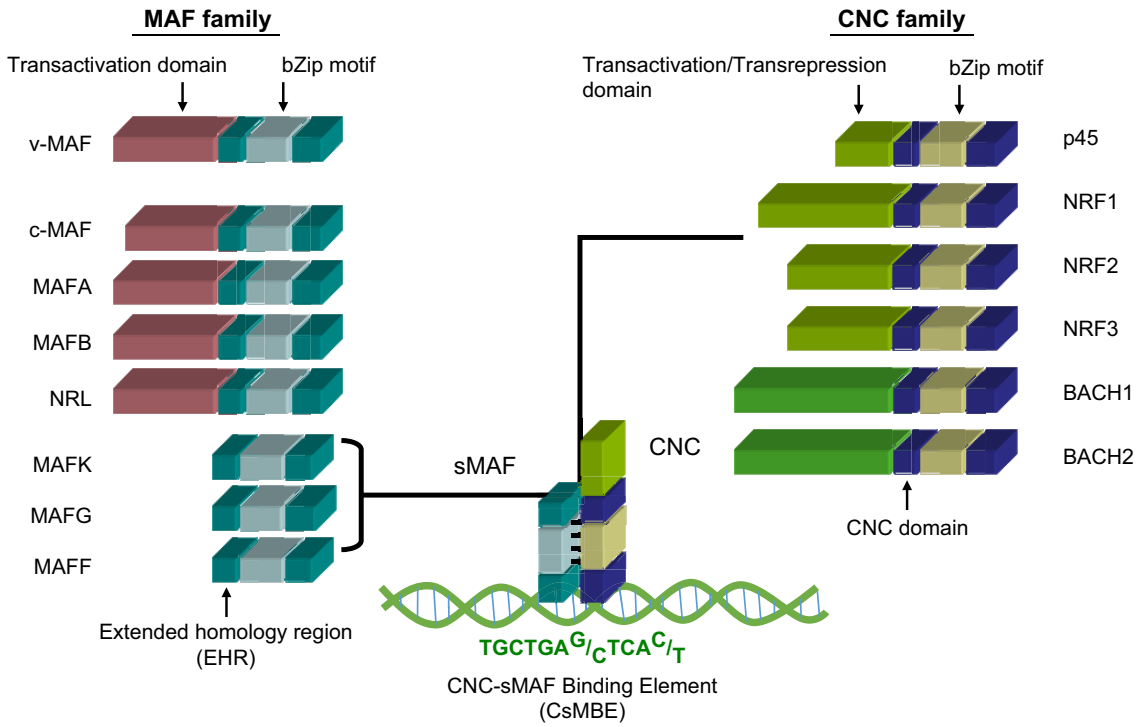

FIGURE 2. Members of Maf and CNC families of transcription factors. Both family members possess well-conserved basic region-leucine zipper motifs. The basic regions mediate DNA recognition, and the leucine zippers mediate dimerization. Small Maf proteins lacking canonical transactivation domains are obligatory heterodimeric partner molecules of CNC family proteins.

mains, including transactivation domains, the activity of the CNC-sMAF heterodimers seems to be defined mainly by that of the CNC factors. Of the CNC factors, NF-E2 p45, NRF1, NRF2, and NRF3 possess transactivation domains and are considered to be transcriptional activators (21, 77, 107, 143). In contrast, BACH1 and BACH2 possess characteristic broad complex-tramtrack-bric-a-brac (BTB) domains and lack any canonical transactivation domains, thus representing transcriptional repressors (39, 94, 153, 169). Consequently, based on the binding sequence similarity and related information, CNC-sMAF heterodimers emerged as strong candidates for the transcriptional activation of phase II enzyme genes.

B. sMAF as an Obligatory Partner of CNC Proteins

MAF family factors are DNA-binding transcription factors that possess a well-conserved bZIP motif and an extended homology region on the NH_2-terminal side of the bZIP motif **(FIGURE 2)**. MAF transcription factors can form homodimers and bind to the MAF recognition element, referred to as the MARE (TGCTGA$^{G}/_{C}$TCAGCA), that contains an activating protein-1 binding site known as the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) (TGA$^{G}/_{C}$TCA) (92, 102). MAF factors are divided into two groups: large MAF factors that possess transactivation domains, and sMAF factors that lack such domains (15, 89, 144, 146) **(FIGURE 2)**. A founding member of the MAF family is v-MAF, which was isolated from avian musculo-aponeurotic fibrosarcoma virus (159). Other large MAF factors include the cellular counterpart of v-MAF called c-MAF and also MAFA, MAFB, and NRL. These large MAFs act as homodimers that activate transcription (9, 159). In contrast, the three mammalian sMAFs, named MAFF, MAFG, and MAFK, form homodimers that repress transcription (145, 148, 150, 154). Homodimers of sMAF are assumed to behave as competitive inhibitors of bZIP transcription factors acting on MAREs.

Another important role of sMAFs is that of obligatory heterodimeric partner molecules of CNC proteins, allowing them to bind to DNA and exert their function as transcription regulators (5, 68). Genetic evidence that sMAF deficiency recapitulates CNC deficiency strongly validates the critical contribution of sMAFs to the function of CNC proteins (261). For example, mice deficient in NF-E2 p45, NRF2, or NRF1 display severe thrombocytopenia, an impaired response to oxidative stress, and neuronal dysfunction, respectively (78, 110, 205). In good agreement to this observation, disruptions to the three sMAF factors in various combinations result in all of these phenotypes with various severities, depending on the extent of the sMAF reduction (96, 97, 147, 163, 199, 261). Thus CNC-sMAF heterodimers form a new functional unit of transcription factors that are distinct from other bZIP family transcription factors and regulate several biological processes, par-

ticularly those related to cell differentiation, maturation, and maintenance.

C. Unique Features of CNC-sMAF Binding Elements

The NF-E2 p45-sMAF heterodimer is a critical regulator of megakaryopoiesis through its binding to NF-E2 binding sites (24, 38, 56, 121, 228). Importantly, NRF2 also associates with the NF-E2 binding site (77, 143), which led us to recognize the sequence resemblance between the ARE/EpRE and the NF-E2 binding site and to then discover NRF2 as an ARE/EpRE binding transcription factor **(FIGURE 3)**. This notion has been supported unequivocally by genetic analyses demonstrating that ARE/EpRE-regulated gene expression is severely impaired in *Nrf2*-null mice (78), as well as in sMAF-deficient mice (97). To date, genetic studies and other approaches have clearly established the NRF2-sMAF heterodimer as the *trans*-acting factor that interacts with the ARE/EpRE and activates the expression of cytoprotective genes.

The NF-E2 binding site and the ARE/EpRE are collectively recognized as binding sites of CNC-sMAF heterodimers. Based on recent analyses of the genomewide distribution of CNC proteins, including NRF2, NF-E2 p45, NRF1, and BACH2 (6, 29, 56, 62, 65, 131), the sequence GCTGA$^{G}/_{C}$TCA$^{C}/_{T}$ has arisen as a common consensus binding site and has been designated as the CNC-sMAF binding element (CsMBE) (168). Asymmetry in the CsMBE reflects the distinct binding preferences of CNC and sMAF proteins. Alignment of the DNA binding domains of various bZIP transcription factors (including those belonging to the CNC, ATF, JUN, FOS, and MAF families) revealed that a single amino acid in the basic region is a unique feature of MAF family proteins **(FIGURE 4A)**. MAF proteins commonly possess a tyrosine (Tyr: Y) residue in their basic region, which corresponds to alanine (Ala: A) in the other bZIP transcription factors that include NRF2. The tyrosine residue in the basic region of MAF family proteins is a key to understanding the unique DNA binding preference of MAF family proteins.

A comprehensive analysis revealed the DNA binding preferences of the MAFG homodimer and the NRF2-MAFG heterodimer **(FIGURE 4B)** (259). The "GC" dinucleotides on each side of the MARE binding site are sufficient for DNA binding of the MAFG homodimer **(FIGURE 3)**. Instead, the "GC" dinucleotide on one side and one-half of the TRE on the other side of the binding site are required for DNA binding of the NRF2-MAFG heterodimer **(FIGURE 3)**. As deduced from these observations, MAF proteins preferentially recognize the flanking "GC" dinucleotide, whereas CNC proteins recognize the core TRE. It should be noted that a single base outside the core TRE substantially influences the DNA binding affinity of the NRF2-MAFG heterodimer, suggesting that CNC proteins recognize a region that is one base wider than that recognized by JUN and FOS family proteins (104). Thus the "GC" in one-half of the CsMBE (underlined in $\underline{\text{GCTGA}}^{G}/_{C}\text{TCA}^{C}/_{T}$) is recognized by sMAF, whereas the other one-half (underlined in $\text{GCTGA}^{G}/_{C}\underline{\text{TCA}^{C}/_{T}}$) is recognized by CNC.

To understand the structural basis of the unique DNA recognition mode of MAF proteins, a DNA binding domain of MAFG was cocrystallized with the MARE-containing DNA duplex (117). Crystallization of the MAFG homodimer with the MARE revealed a critical role of the Tyr/Ala difference in the basic region that discriminates MAF proteins from other bZIP proteins, such as JUN, FOS, and ATF family proteins. The MAF-specific tyrosine residue is responsible for the unique DNA recognition mode. Swapping the unique tyrosine residue Y64 of MAFG and the corresponding alanine residue A502 of NRF2 resulted in the simultaneous swapping of DNA recognition specificity. MAFG Y64A did not require the "GC" dinucleotide for DNA binding. The NRF2 A502Y mutant partially mimicked the DNA binding preference of the MAFG ho-

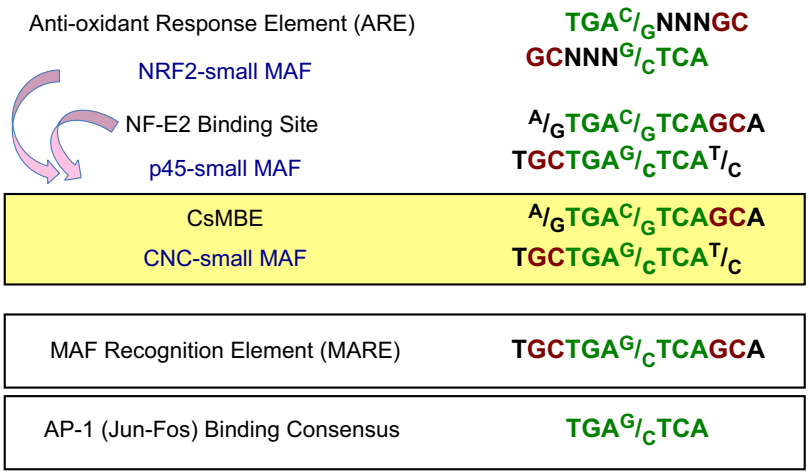

FIGURE 3. DNA binding consensus sequences of CNC and MAF family proteins. Core sequences common with the AP1 binding consensus sequence are indicated in green. Flanking "GC" dinucleotides unique to the MAF binding consensus are indicated in red.

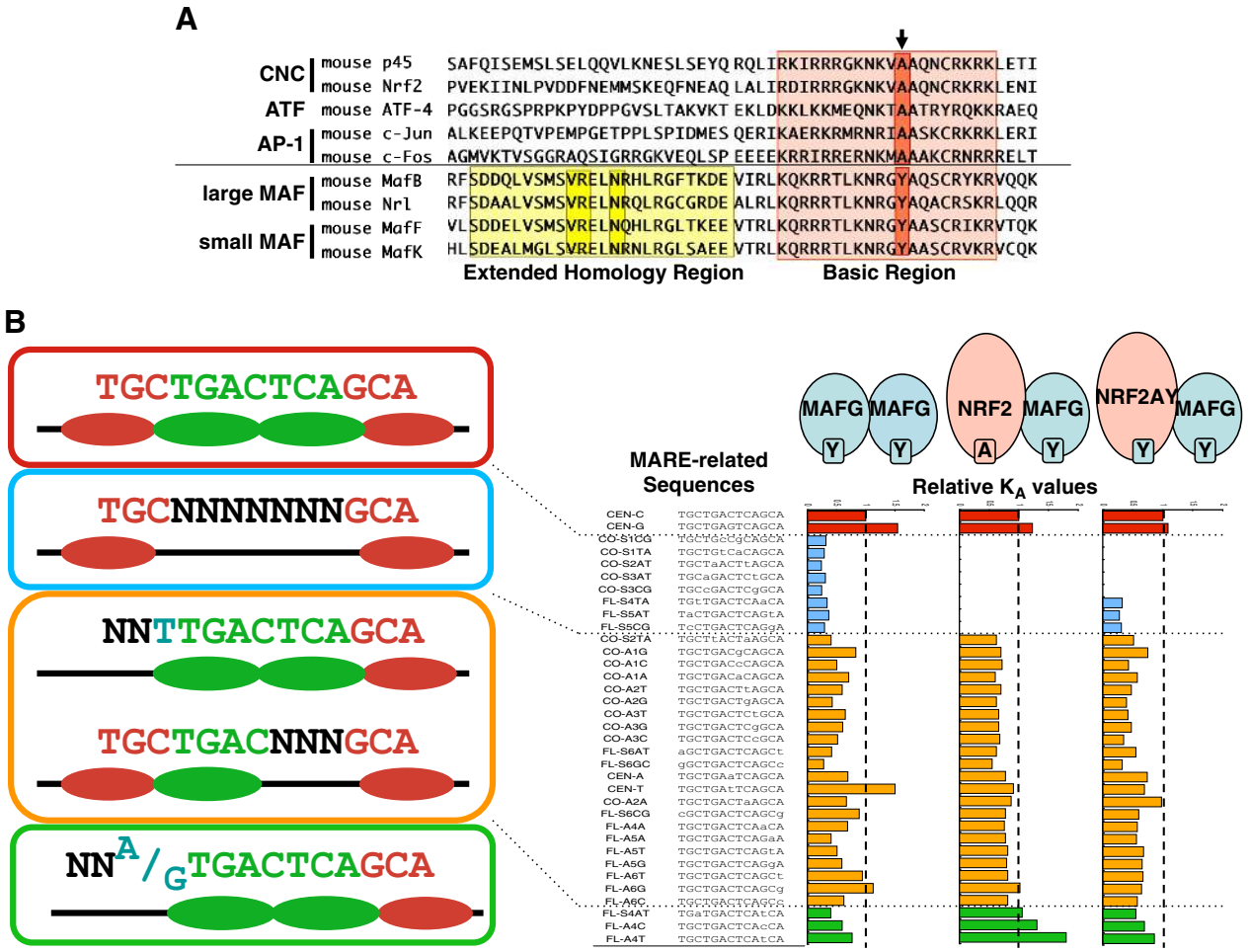

FIGURE 4. Unique DNA recognition preference of MAF family proteins. *A*: alignment of the DNA binding domains of various bZIP transcription factors. Arrow indicates a conserved tyrosine residue for the MAF family and a conserved alanine residue for the others. *B*, *right*: relative K_A values of MAFG homodimer, NRF2-MAFG heterodimer, and NRF2A502Y-MAFG heterodimer to various MARE-related sequences. *Left*: categorization of the MARE-related sequences.

modimer. Thus the heterodimer of NRF2 A502Y and MafG acquired the ability to bind to MAF homodimer-specific binding sites (MARE) and lost their ability to bind to the CsMBE with high affinity (104). The significance of high-affinity binding to the CsMBE was highlighted in a study of mice expressing NRF2 A502Y instead of wild-type NRF2 (168). Mice homozygously expressing NRF2 A502Y recapitulated the major phenotypes of *Nrf2*-null mice, indicating a strict requirement of the CsMBE for the NRF2-mediated stress response and cytoprotection.

III. DEREPRESSION REGULATION OF NRF2 BY KEAP1

A. KEAP1-Based Ubiquitin E3 Ligase Complex and Proteasomal Degradation of NRF2

Phylogenetic conservation of the structure of NRF2 among species revealed the presence of six functional domains: Neh1 (NRF2-ECH homology domain-1) to Neh6 (79) **(FIGURE 5*A*)**. Neh1 contains the CNC and bZIP domains that mediate DNA binding and dimer formation, whereas Neh3, Neh4, and Neh5 are transactivation domains. Of the transactivation domains, Neh4 and Neh5 make a major contribution to transcriptional activation by recruiting histone acetyl-transferase cAMP responsive element binding protein (CBP) (93) and Mediator complex (197).

Truncated NRF2 lacking the Neh2 domain exhibited markedly increased transcriptional activity, suggesting the existence of a repressor molecule that interacts with Neh2. To this end, KEAP1 was isolated as this Neh2-interacting molecule by yeast two-hybrid screening (79) **(FIGURE 5*B*)**. Soon after its identification, KEAP1 was found to be a component of ubiquitin E3 ligase (108). Thus Neh2 was found to be a KEAP1-specific degron of NRF2, and this led to the discovery of a unique derepression mechanism. Neh6 is a KEAP1-independent degron of NRF2 as it harbors a cluster of ser-

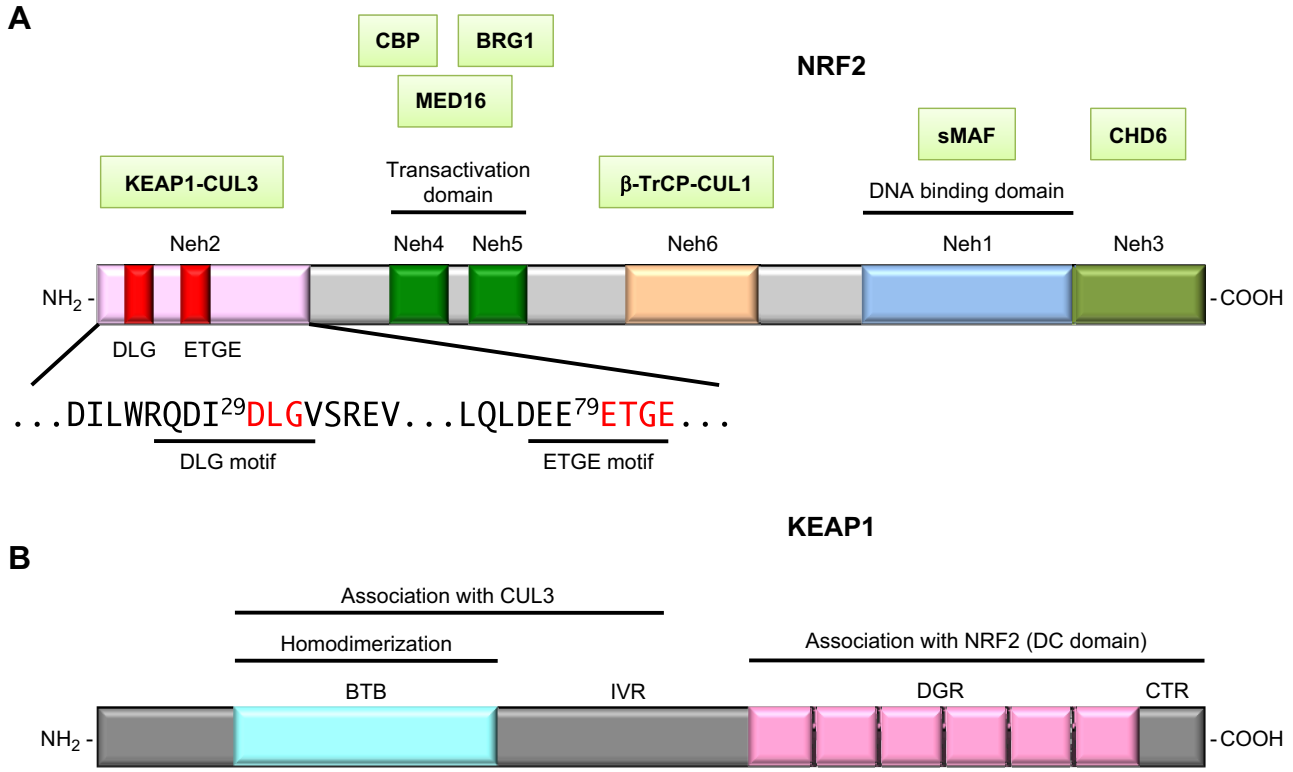

FIGURE 5. Domain structures of NRF2 (*A*) and KEAP1 (*B*). NRF2-interacting molecules are shown in green boxes and placed above their interacting domains.

ine residues that are phosphorylated by glycogen synthase kinase 3 (GSK-3), resulting in the facilitation of NRF2 degradation.

The most important feature of NRF2 is its inducibility. KEAP1 creates the inducible nature of NRF2 function by serving as a substrate recognition component of the E3 ubiquitin ligase complex in cooperation with CUL3 and RBX (32, 58, 108, 266). Under normal conditions, ubiquitinated NRF2 undergoes proteasomal degradation, and it is this constitutive degradation of NRF2 that maintains the quantity and activity of NRF2 at a low level. The E3 ubiquitin ligase activity of the KEAP1-CUL3 complex is disrupted on exposure to electrophiles and ROS that modify the cysteine residues of KEAP1, allowing newly synthesized NRF2 to accumulate in the nucleus and activate transcription (109). Thus the possession of highly reactive cysteine residues renders KEAP1 an efficient and sensitive biosensor of redox disturbance through switching on or off the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex.

Quantitative immunoblot analyses indicated that, in the basal state, the cellular level of NRF2 was kept lower than the levels of KEAP1 and CUL3 proteins. Challenge with an electrophilic agent dramatically increased NRF2 to a level greater than that of KEAP1 and CUL3, resulting in the accumulation of NRF2 in the nucleus (76). By contrast, KEAP1 and CUL3 did not display any changes in their abundance, subcellular localization, or interaction in response to electrophilic stimuli. Higher resolution immunostaining examination revealed that KEAP1 localizes in the perinuclear cytoplasm (248) with loose attachment to the actin cytoskeleton (88). This localization leads to a model in which KEAP1 acts as a floodgate (76) **(FIGURE 6)**. NRF2 moves into nucleus only when the floodgate (KEAP1) is modified and opened. In summary, the regulation of levels of NRF2 protein during a stress response is mediated by the activity, but not the composition, of the KEAP1-CUL3 ubiquitin ligase complex.

B. Structure of CUL3-KEAP1-NRF2 Complex Is Permissive for NRF2 Ubiquitination

To elucidate how NRF2 is ubiquitinated under normal conditions, multiple biochemical and biophysical analyses were conducted. Three domains have been identified in KEAP1: a BTB, an intervening region (IVR), and a double glycine repeat and COOH-terminal region (DC) domain **(FIGURE 5*B*)**. Structure-function and molecular-dissection studies have shown that the NRF2 Neh2 domain directly interacts with the KEAP1 DC domain (79). Two discrete motifs in the NRF2 Neh2 are critical for KEAP1-dependent NRF2 degradation (136, 233), implying that these two motifs mediate the interaction between the NRF2 Neh2 domain and the KEAP1 DC domains.

The NRF2 Neh2 and KEAP1 DC domains show a unique mode of interaction, referred to as the two-site binding

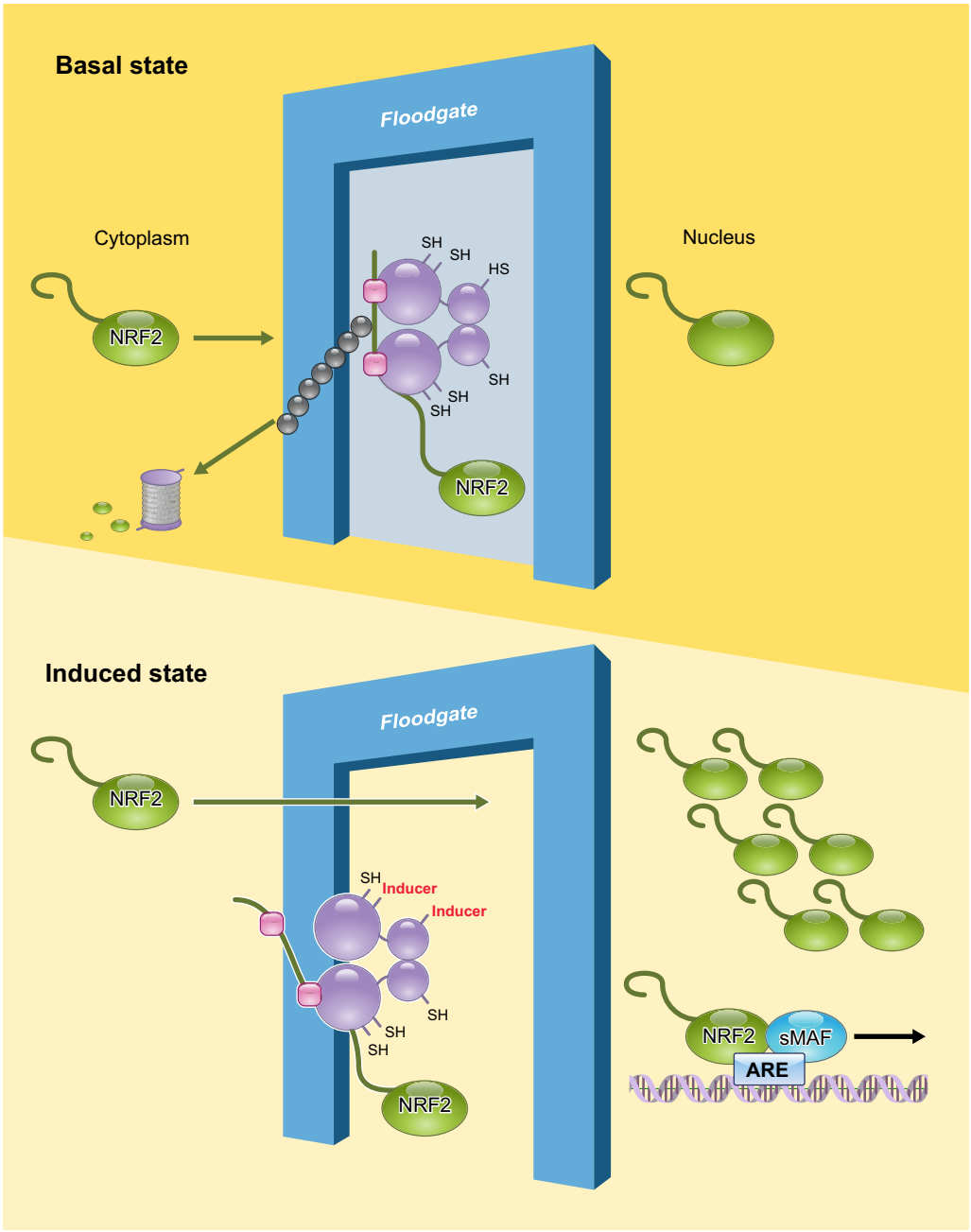

FIGURE 6. Molecular dynamics of the NRF2-KEAP1-CUL3 complex in cells. KEAP1 acts as a floodgate for NRF2 and usually blocks NRF2 entry to the nucleus by facilitating its degradation. The KEAP1 floodgate opens when cells are assaulted by stresses, and NRF2 translocates into nucleus to activate cytoprotective gene expression.

model. This binding model was first suggested from studies of isothermal calorimetry and subsequently verified by NMR structural analyses and molecular dissection analyses (233, 234). The two distinct motifs DLG and ETGE in the Neh2 domain are critical for its interaction with the KEAP1 DC domain. Together, the DLG and ETGE motifs bind to a KEAP1 homodimer, with each motif interacting individually with its own molecule of KEAP1 (57, 76). Thermodynamic examination revealed a significant difference between the motifs when they interact with the KEAP1 DC domain: the ETGE motif exclusively utilizes hydrogen bonding, whereas the DLG motif relies on both hydrophobic interactions and hydrogen bonding (57).

Kinetic analysis using surface plasmon resonance further consolidated the qualitative difference suggested by the thermodynamic examination (57). Surface plasmon resonance signals indicated that the DLG and ETGE motifs form low- and high-affinity binding sites, respectively, for the KEAP1 DC domain (234). Detailed analyses of association and dissociation rate constants indicated that the DLG exhibits a single-state binding with fast association and fast dissociation. In contrast, the ETGE shows two-state binding with slow association and slow dissociation steps, suggesting that a quick transient binding conformation is slowly transferred to a fully stable binding conformation (57). The qualitative difference in the two binding

sites provides an important clue for understanding how NRF2 ubiquitination starts and stops in response to electrophilic signals.

Structural analyses of the KEAP1-NRF2 system were carried out using NMR, X-ray, and electron microscopy. The solution structure of NRF2 Neh2 was examined using NMR (233) and showed that NRF2 Neh2 adopts a rodlike conformation with a central single α-helix flanked by the DLG and ETGE motifs. Seven lysine residues that are ubiquitinated by the KEAP1-CUL3 E3 ubiquitin ligase complex are clustered in the α-helix. Six of these lysines are aligned on a single half-side of the helix surface, resulting in their optimal presentation for high-efficiency ubiquitination.

The crystal structure of the KEAP1 DC domain obtained by X-ray disclosed a β-barrel structure with six blades (57, 125, 170). Cocrystallization of the KEAP1 DC domain with a peptide containing either the DLG motif or the EGTE motif showed that the peptides interact at the bottom of the β-barrel structure. The ETGE motif forms a β-hairpin structure by hydrogen bonding and binds to the KEAP1 DC domain in a key (ETGE) and keyhole (pocket in KEAP1 DC domain) manner, whereas the extended DLG motif forms a triple-helix structure using both hydrogen bonding and hydrophobic interaction and loosely attaches onto the bottom surface of the KEAP1 DC domain (57). The interface between the DLG motif and the KEAP1 DC domain is wider than the binding surface between the ETGE and the KEAP1 DC domain, which is in harmony with the kinetic and thermodynamic differences between the two interactions.

Single-particle analysis using electron microscopy revealed a breathtaking structure of the KEAP1 homodimer and rendered all of the existing biological, biochemical, and biophysical results to be consistent with each other (161). Two large spheres are joined at the end of their stems, just like a cherry-bob **(FIGURE 7)**. The stemlike structure and the globular portion of KEAP1 correspond to the BTB and DC domains, respectively. Superimposition of the DC crystal structure to the globular portion of the cherry-bob indicated that the NRF2 binding sites are located on the bottom of the two globular portions. The distance between the two binding pockets in the two globular domains is ~80 Å (161), which is very close to the expected distance between the DLG and ETGE motifs, as determined by the NMR solution structure analysis of Neh2 (233).

The next step was to find out how CUL3 is integrated into the KEAP1-NRF2 complex. The interaction between KEAP1 and CUL3 is another important issue for understanding the molecular mechanism underlying NRF2 ubiquitination. Interaction between the NH_2-terminal region of CUL3 and the IVR domain of KEAP1 was detected in a molecular dissection analysis (108). Analysis of the crystal structure of KLHL11, a BTB-Kelch family protein that is closely related to KEAP1, was consistent with this observation (20). A hydrophobic groove called the "3-box" motif located between the BTB and IVR domains of KLHL11 associates with the NH_2-terminal domain of CUL3. Further analysis is necessary to clarify the composite structure of the CUL3-KEAP1-NRF2 ternary complex.

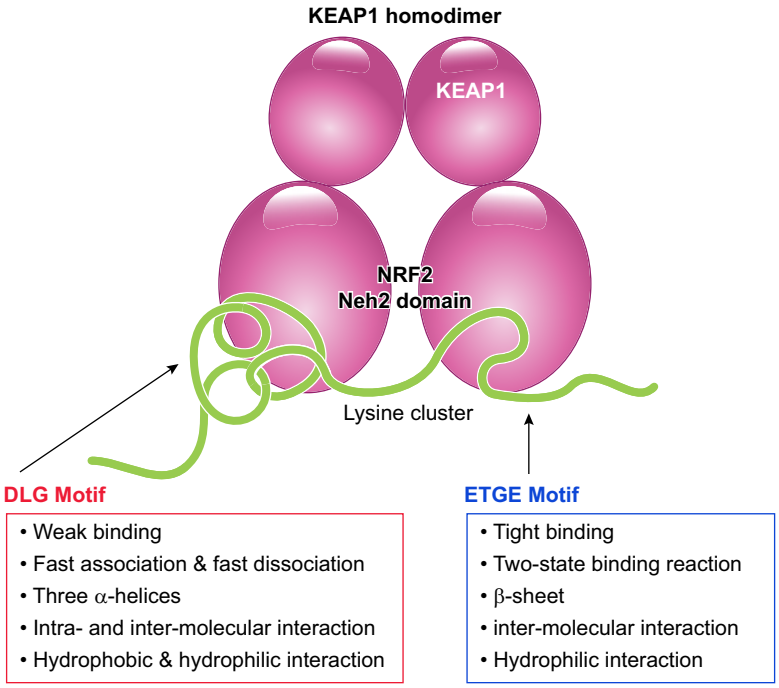

FIGURE 7. Schematic illustration of the formation of the complex between the KEAP1 homodimer and the Neh2 domain of NRF2. Biophysical characteristics and structural features are summarized.

C. Functional Analyses of KEAP1 Domain Structures

Transgenic complementation rescue is a powerful and sophisticated approach for establishing the in vivo functions of proteins (145, 148, 150). Lethality in *Keap1*-null mice occurs before weaning due to marked stenosis in the esophagus, thus providing a phenotype for in vivo evaluation of structure-function (148, 240). The esophageal lesions and subsequent lethality observed in *Keap1*-null mice were completely rescued by the simultaneous disruption of *Nrf2* (240). Phenotypes resulting from *Keap1* deficiency can also be rescued by transgene-derived KEAP1 expressed under the influence of the *Keap1* gene regulatory region (260). This approach of transgenic complementation rescue enabled us to evaluate the function of KEAP1 as a repressor of NRF2 by monitoring the phenotypes caused by *Keap1* deficiency. Replacing wild-type KEAP1 with mutant KEAP1 in this experimental setting provided reliable answers concerning the in vivo functions of various domains in KEAP1. For instance, KEAP1 C273S mutant (Cys273 substituted with serine) did not rescue the lethality of *Keap1*-null mice, indicating that Cys273 is essential for KEAP1 to repress NRF2 activity (260).

The essential functional contribution of the KEAP1 BTB domain, which mediates homodimer formation, was also demonstrated by transgenic complementation rescue experiments. Cross-breeding with transgenic mice expressing wild-type KEAP1 rescued the lethality of *Keap1*-null mice, whereas cross-breeding with transgenic mice expressing a BTB-deficient KEAP1 did not (260). Five amino acids in the BTB domain that are critical for dimerization were mutated, and the resulting KEAP1 BTB mutant failed to restore the function of KEAP1 (218). These results, in combination with recognition that the stoichiometry of the interaction between NRF2 Neh2 and KEAP1 is 1:2 (233), consolidate the critical significance of homodimer formation of KEAP1 through the BTB domain for the ubiquitination and degradation of NRF2. Collectively, these results have been integrated into the "two-site binding model" of NRF2 and KEAP1 that defines the structural basis for the efficient ubiquitination of NRF2 **(FIGURE 7)**.

D. Phylogenic Distribution of the KEAP1-NRF2 System

The KEAP1-NRF2 system is very well conserved among vertebrates, from fish to mammals. An NRF2 ortholog in zebrafish heterodimerizes with zebrafish sMAF, and the heterodimer binds to DNA and activates genes involved in the adaptive stress response. Zebrafish KEAP1 regulates the activity of the zebrafish NRF2-sMAF heterodimer in a manner similar to that in mammals (112). The system is also well conserved in invertebrates. For example, the NRF2 ortholog in *Drosophila* is called cap 'n' collar (CNC). The *cnc* gene in *Drosophila* produces multiple alternative splice variants, with the products CncA, CncB, and CncC being the major isoforms. These isoforms possess different lengths of NH_2-terminal regions and a common COOH-terminal region containing a CNC domain and a bZIP motif. CncC is the longest isoform and best resembles mammalian NRF2. Similar to vertebrate NRF2, *Drosophila* CncC requires sMAF for carrying out its NRF2-like functions. A KEAP1 ortholog in *Drosophila* interacts with CncC and acts as a negative regulator, just like KEAP1 regulates NRF2 in mammals (172).

In contrast, *C. elegans* possesses an NRF2 ortholog called SKN-1 and operates through a different system of defense against oxidative stress (13). Whereas SKN-1 possesses a CNC domain and a basic region that highly resembles those of *Drosophila* and vertebrate CNC proteins, it lacks a leucine zipper for dimerization with sMAF. Thus SKN-1 binds to DNA as a monomer that recognizes the sequence GTCAT (12), which coincides with the CNC-side half-site of CsMBE (GCTGA$^{G}/_{C}$-$\underline{\text{TCA}}^{C}/_{T}$). Therefore, it is not surprising that there is no ortholog of sMAF in *C. elegans*. In addition, there is no KEAP1 ortholog in *C. elegans*. Instead of electrophilic signaling, which is directly sensed by KEAP1 in *Drosophila* and vertebrates, SKN-1 activity is regulated by phosphorylation signals mediated by p38 and AKT/mammalian target of rapamycin (mTOR). SKN-1 translocates to the nucleus when it is phosphorylated by p38 MAPK in response to oxidative stress (73), whereas nuclear localization of SKN-1 is inhibited when it is phosphorylated by GSK-3 (3).

It should be noted that, due to their short life span, *Drosophila* and *C. elegans* are often utilized for aging studies. Activation of CncC by KEAP1 mutation extends the life span of *Drosophila* (221). Disruption of SKN-1 in *C. elegans* reduces resistance to stress and shortens longevity (236). The contribution of SKN-1 to longevity is more prominent in the presence of oxidative stress than in unstressed conditions, suggesting that reinforcement of an oxidative stress response is an effective antiaging mechanism. A recent study using naked mole-rats, rodents with naturally long life spans, revealed that a higher NRF2 activity is closely associated with their longevity (123). Naked mole-rats have lower levels of KEAP1 protein and higher levels of NRF2 target gene than mice with shorter life spans. Maximum lifetime correlated positively with the ARE/CsMBE-binding activity of NRF2. These reports strongly support the notion that a higher NRF2 activity is advantageous for longevity.

IV. MULTIPLE MECHANISMS REGULATING NRF2 ACTIVITY

A. Cysteine Code and Two-Site Binding Mechanism

KEAP1 is a thiol-rich protein containing many cysteine residues, some of which are adjacent to basic amino acids that

foster reactive anionic forms of the sulfhydryl group. Covalent binding of electrophilic inducers to cysteine residues has been observed using mass spectrometry (42, 63). The significant contribution of the cysteine residues to the function of KEAP1 as a sensor has been demonstrated in cultured cells (53, 113, 137, 227, 265), zebrafish (113), and mice (191, 218, 260). These studies revealed three major cysteine residues of KEAP1 that are critical for modulating the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex. These critical cysteine residues are Cys151 in the BTB domain and Cys273 and Cys288 in the IVR **(FIGURE 8)**.

Substitution of Cys151 with serine did not affect the ubiquitin conjugating activity of the KEAP1-CUL3 complex. However, the KEAP1 C151S mutant did not respond to a large group of electrophiles, including diethylmaleate, *tert*-butylhydroquinone, dimethylfumarate, sulforaphane, and nitric oxide, which led to continuous ubiquitination and degradation of NRF2 (227). When Cys151 was substituted with tryptophane, the KEAP1 C151W mutant failed to associate with the CUL3 complex and did not support the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex, leading to the accumulation of NRF2 (47, 113). Considering the bulky nature of a tryptophane residue, KEAP1 C151W is likely to mimic an electrophile-conjugated inactive state, whereas KEAP1 C151S is likely to mimic a conjugation-free active state.

Derivatives of the triterpenoid compound 2-cyano-3,12-dioxooleana-1,9-dien-28-oic-acid (CDDO) are exceedingly potent inducers of the electrophile counterattack response. These derivatives bind to Cys151 and disrupt the interaction between KEAP1 and CUL3 (31, 76). Small molecules possessing similar backbone structures to CDDO (e.g., CDDO-Im) have been developed as stereoselective KEAP1 Cys151-binding reagents (66). Although KEAP1 C151W was shown to lose its association with CUL3 (47), most NRF2-inducing electrophilic chemicals do not directly disrupt the KEAP1-CUL3 interaction, with the only exceptions being CDDO-Im and possibly its derivatives (76). Irrespective of the precise structural mechanisms involved in the KEAP1-CUL3 interaction, these results strongly support the contention that Cys151 serves as a primary sensor cysteine by switching off KEAP1 activity on direct modification by electrophiles.

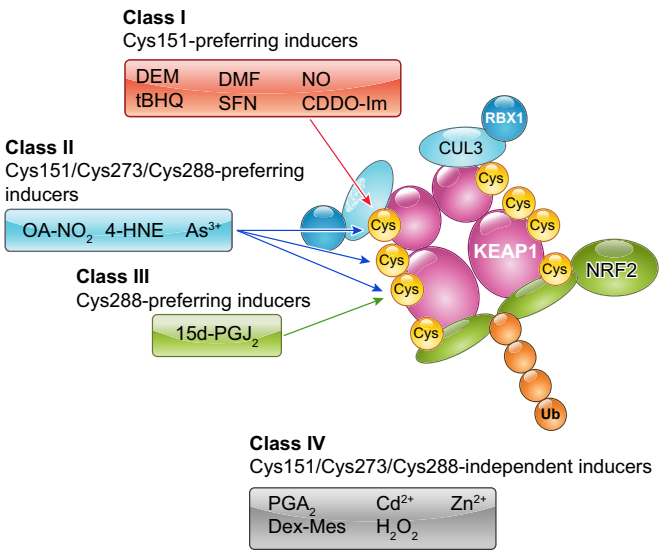

FIGURE 8. Cysteine codes of the KEAP1-based response to various redox disturbances. Three key cysteine residues, Cys151, Cys273, and Cys288, of KEAP1 are responsible primarily for the responses to a variety of electrophiles. OA-NO_2, nitro-fatty acids; 4HNE, 4-hydroxynonel.

Substitution of Cys273 and Cys288 with alanine or serine abrogated the ubiquitin E3 ligase activity of the KEAP1-CUL3 complex, leading to the accumulation of NRF2 (218). Notably, substitution of Cys273 with methionine or tryptophane retained the KEAP1 activity that represses NRF2. Similarly, substitution of Cys288 with glutamate, asparagine, or arginine retained KEAP1 activity (191). The KEAP1 C288E mutant molecule did not respond to 15d-PGJ2 (15-deoxy-Δ12,14-prostaglandin J_2), indicating that KEAP1 uses Cys288 for sensing 15d-PGJ2 (191, 227) **(FIGURE 8)**. For sensing nitro-fatty acids, 4-hydroxynonenal, and As^{3+}, all three major cysteine residues of KEAP1, i.e., Cys151, Cys273, and Cys288, are involved (191). All of the functionally substitutable amino acids for Cys273 and Cys288 required to maintain KEAP1 activity possess relatively bulky characteristics. This suggests that the mechanism by which KEAP1 responds to electrophiles through Cys273 and Cys288 differs from that of the Cys151-dependent response. The molecular basis for the Cys273- and Cys288-dependent response remains to be clarified.

In addition to Cys151, Cys273, and Cys288, a few other cysteine residues are suggested to participate in the sensor functions of KEAP1. Cys226 and Cys613, together with His-225, are required for sensing Cd^{2+}, As^{3+}, Se^{4+}, and Zn^{2+} (137). Cys226 and Cys613 formed intramolecular disulfide bonds when cells were treated with hydrogen peroxide, implying that Cys226 and Cys613 are located in close proximity in the fully folded state of KEAP1 (53). However, the cysteine residues responsible for sensing hydrogen peroxide also remain to be fully clarified.

Partial dissociation of NRF2 from KEAP1 appears to be an alternative mechanism for the inhibition of NRF2 ubiquitination. As described above, biochemical and biophysical analyses revealed that one molecule of NRF2 associates with a KEAP1 homodimer using two discrete motifs. The ETGE and DLG motifs utilize high-affinity and low-affinity binding sites, respectively, with almost two magnitudes of difference in their binding affinities (233). The KEAP1-NRF2 association is not completely disrupted following inactivation of KEAP1 by modification with electrophiles (109). Therefore, a fascinating model is that the ETGE and

DLG motifs serve as a hinge and latch, respectively, with NRF2 ubiquitination ceasing when just the latch is released or relaxed. Precisely how the latch is released is not clear and may require a complete crystallographic rendering of the complex for an answer.

B. KEAP1-Independent Mechanism of NRF2 Degradation

Although NRF2 activity is primarily regulated by KEAP1 in response to electrophiles, several investigators realized that NRF2 signaling can be activated through the PI3K-AKT signaling pathway (85, 124, 132). A key mediator in this action is GSK-3β, which is inhibited by AKT-mediated phosphorylation (192). NRF2 is phosphorylated by GSK-3β, enabling its recognition by β-transducin repeats-containing protein (β-TrCP) that in turn marks NRF2 for ubiquitination. Following its ubiquitination by the β-TrCP-CUL1 E3 ubiquitin ligase complex, NRF2 is degraded through the proteasome (174, 175). As already described, Neh2 serves as the degron for KEAP1-CUL3-dependent degradation of NRF2. By contrast, Neh6 is the degron exploited in β-TrCP-CUL1-dependent degradation of NRF2, as it contains serine residue targets for phosphorylation by GSK-3β (30) **(FIGURE 9)**.

The functional interaction and contributions of the two degradation mechanisms of NRF2 have been examined in the context of postnatal liver development in mice (140, 224). When murine *Keap1* and *Pten* (phosphatase and tensin homolog deleted on chromosome 10) genes are concomitantly disrupted in a hepatocyte-specific manner, the mice die within 3 wk after birth. PTEN is a phosphatase that antagonizes PI3K. In the event of *Pten* deficiency, inositol-3-phosphate levels increase and AKT is activated, while GSK-3β is inhibited. Inhibition of GSK-3β reduces NRF2 phosphorylation, such that NRF2 escapes KEAP1-independent β-TrCP-CUL1-dependent degradation in the nucleus. Thus a *Keap1*::*Pten* double-deficient condition is a state in which both pathways for NRF2 degradation are inactivated. Indeed, *Keap1*::*Pten* double-deficient mouse liver showed greater increases in NRF2 accumulation and upregulation of NRF2 target genes than seen in only *Keap1*-deficient mouse liver. Notably, during the first 3 wk after birth, *Pten* single-deficient liver did not show any clear increase in NRF2 accumulation, suggesting that KEAP1-dependent degradation of NRF2 in the cytoplasm is the primary mechanism to regulate levels of NRF2 (140, 224). Inactivation of KEAP1-independent degradation while preserving the KEAP1-dependent pathway led to only a marginal dif-

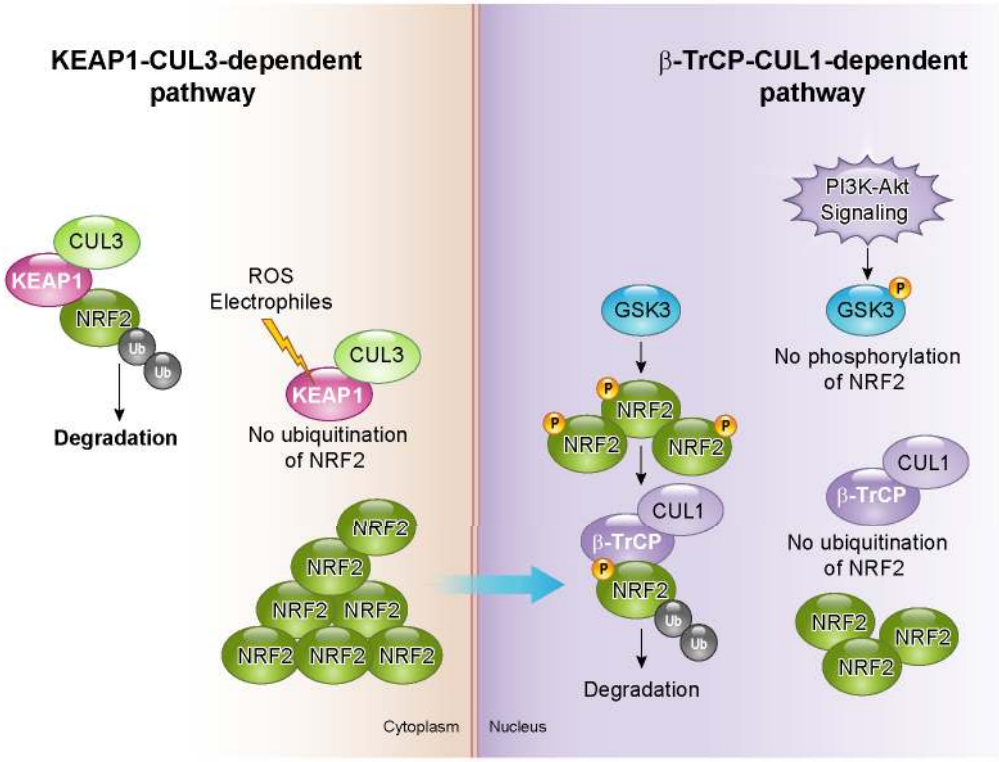

FIGURE 9. Two degradation pathways of NRF2. KEAP1-CUL3-mediated degradation and β-TrCP-CUL1-mediated degradation are considered to operate in the cytoplasm and nucleus, respectively. The latter is influenced by the activation of PI3K-AKT signaling.

ference in NRF2 protein levels in this experimental setting.

C. KEAP1-NRF2 System and Autophagy

In addition to cross talk with the PI3K-AKT signaling pathway, the KEAP1-NRF2 system has an intriguing functional interaction with autophagy (52, 82, 114, 120, 187). KEAP1 directly interacts with p62, which is a cargo receptor for selective autophagy. This interaction enables p62 to compete with NRF2 for KEAP1 binding and activation of the NRF2 pathway (114, 120). Of note, p62 binds to KEAP1 at the bottom of the DC domain and dislocates NRF2 from KEAP1, leading to the stabilization of NRF2. Defective autophagy in liver impairs the turnover of p62, causing severe liver injury. This is accompanied by the formation of inclusion bodies containing p62, KEAP1, and ubiquitinated proteins and leads to an elevation in the expression of NRF2 target genes (114, 187). This liver injury model can be rescued by NRF2 disruption, indicating that constitutive activation of NRF2 in the selective autophagy-defective condition is deleterious for the functional integrity of hepatocytes. Since p62 acts as an NRF2 target gene, a positive feedback loop between p62 and NRF2 operates: p62 accumulation promotes NRF2 activation, and in turn activated NRF2 further increases p62 (82).

Detailed analyses showed that a serine residue in the phylogenetically conserved "STGE" motif in p62 is phosphorylated, and that this phosphorylated STGE (pSTGE) motif has a higher affinity for the KEAP1 DC domain than unmodified STGE (67) **(FIGURE 10)**. The kinase mTOR complex 1 is responsible for phosphorylating p62, although the involvement of other kinases has also been suggested (67). When the pSTGE motif is compared with the ETGE and DLG motifs of NRF2 in relation to affinity for the KEAP1 DC domain, pSTGE is lower than the ETGE and higher than the DLG. Thus phosphorylated p62 may modulate the interaction between NRF2 and KEAP1.

Several stimuli promoting autophagy-related conditions enhance the phosphorylation of p62 (67). When mitophagy is induced by valinomycin, a mitochondrial uncoupling agent, p62 is phosphorylated in concert with an increased expression of NRF2 target genes. Similarly, p62 is phosphorylated and NRF2 is activated when xenophagy is induced on invasion by microbes. In both cases, mTOR complex 1 is

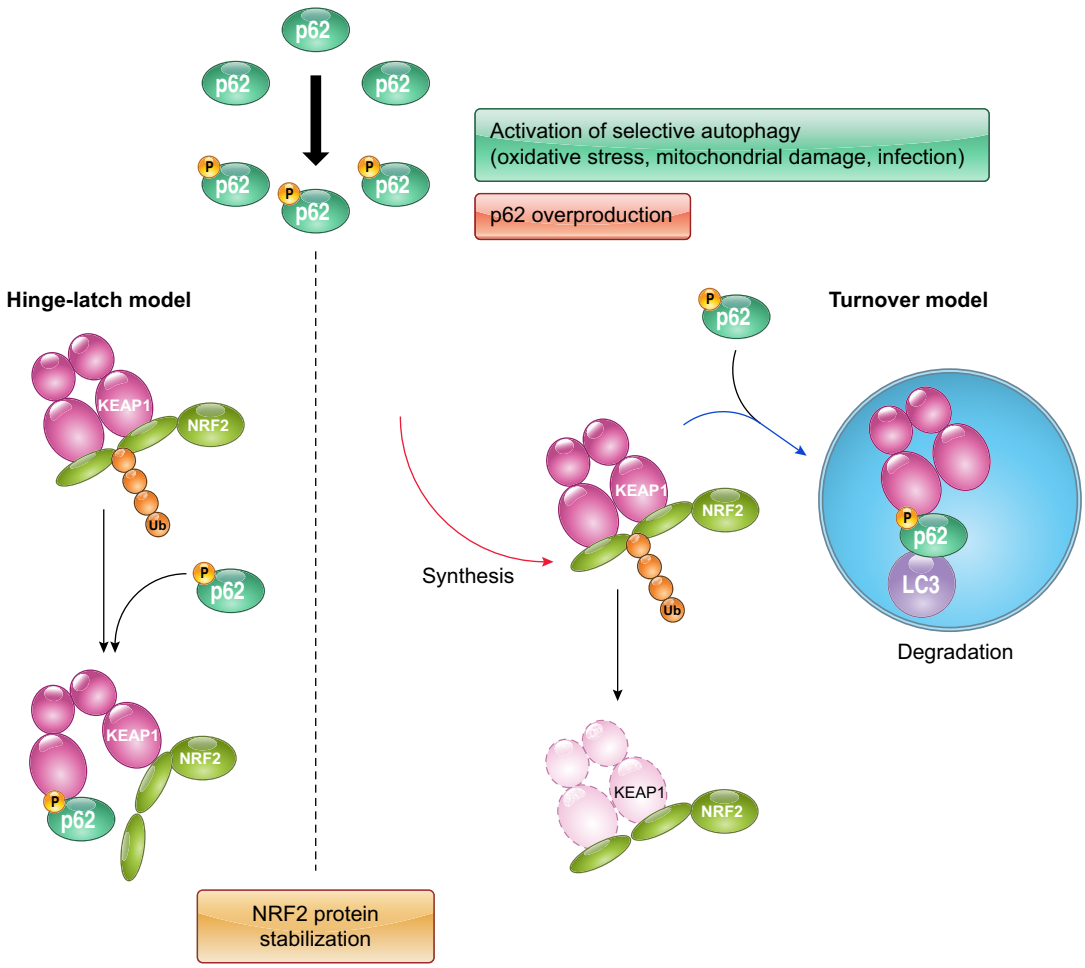

FIGURE 10. Cross talk between autophagy and the KEAP1-NRF2 pathway. Phosphorylated p62 possessing pSTGE has a high affinity for KEAP1, resulting in impaired ubiquitination and consequent stabilization of NRF2. Increased production of phosphorylated p62 disrupts the KEAP1-NRF2 interaction and sequesters KEAP1 to the autophagosome, resulting in the stabilization of NRF2.

responsible for phosphorylating p62, and the level of KEAP1 protein is reduced as a consequence of its autophagic degradation. This decline in KEAP1 protein level may also account for NRF2 stabilization, in addition to effects on the hinge and latch mechanism described above (sect. IV*A*).

The regulation of levels of NRF2 protein has been carefully analyzed; however, only recent studies have begun in the case of KEAP1. KEAP1 is degraded through autophagy in a p62-dependent manner (223). The half-lives of NRF2 and KEAP1 degradation are ~20 min and 12 h, respectively, indicating that the turnover rate of KEAP1 is considerably slower than for NRF2 (80, 223). However, treatment of cells with electrophiles such as *tert*-butylhydroquinone shortened the half-life of KEAP1, implying that electrophile-modified KEAP1 becomes a preferential substrate for autophagy with an accelerated turnover rate (223). As a consequence of KEAP1 degradation, NRF2 is stabilized and activates *Keap1* gene transcription, thereby enabling the replenishment of de novo synthesized KEAP1. The precise destiny of KEAP1 following modification by electrophiles, whether it is degraded or recycled, is currently not well elucidated. Therefore, the mechanism by which KEAP1 turnover is accelerated by electrophiles remains a potentially fascinating, but untold story.

D. Intranuclear Players for Regulating NRF2 Activity

NRF2 was validated as a potent activator of transcription compared with other members of the CNC family in a reporter cotransfection-transactivation assay using cultured cells (107, 149). The transactivation domains Neh4 and Neh5 of NRF2 interact with CBP and BRG1 (93, 267). CBP is a histone acetyltransferase, and BRG1 is a component of the SWI/SNF chromatin remodeling complex. The COOH-terminal domain Neh3 of NRF2 interacts with CHD6, which is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. CHD6 knockdown reduces the basal and induced expression of NRF2 target genes (157). Whereas these coactivators are important for transactivation, they may not explain fully the strong transactivation mediated by NRF2.

MED16, a subunit of Mediator complex, is a newly discovered member of coactivators that interact with the Neh4 and Neh5 transactivation domains (197). MED16 tethers Mediator complex to NRF2 binding sites through its interaction with NRF2 **(FIGURE 11)**. MED16 serves as a conduit for Mediator complex in a manner that is specific for NRF2-activating signals that include ROS and electrophiles. In the absence of MED16, the inducible expression of nearly three-fourths of NRF2 target genes is diminished (197). Consistent with this result, MED16-deficient cells are as susceptible to oxidative stress as are NRF2-deficient cells.

Comprehensive studies have defined the direct target genes of NRF2 (29, 62, 131), with much attention given to those with an expression that is elevated by NRF2. However, early gene expression studies using microarray analyses also indicated that many genes are repressed by NRF2 (119). It should be noted that several proinflammatory cytokine genes are repressed by NRF2 (111). In particular, the NRF2-mediated suppression of *IL6* and *IL1b* gene expression appears to be critical for the anti-inflammatory effect of NRF2. This observation reinforces the need to investigate this aspect of NRF2 function and to identify the additional factors involved. It is also necessary to determine whether or not NRF2 directly represses transcription in a locus-specific manner, and, if so, how NRF2 inhibits the recruitment of RNA polymerase II to the genes.

V. CONTRIBUTION OF NRF2 TO REDOX HOMEOSTASIS AND CYTOPROTECTION

A. Mouse Models for Analyzing the Contribution of the KEAP1-NRF2 Pathway to Various Diseases

The contribution of the KEAP1-NRF2 pathway to organismal homeostasis has been demonstrated in many studies using *Nrf2*-deficient and *Keap1*-deficient mice, either singly or combined with other transgenic and gene knockout mice. Studies representative of the pathological conditions that are alleviated by genetic or pharmacological means through NRF2 activation are shown in **TABLE 1**.

The first report on *Nrf2*-deficient mice in 1996 described no apparent abnormalities in the birth, growth, or fertility of the *Nrf2*-null mice (22). In 1997, a study using an independently generated line of *Nrf2*-null mice made the seminal discovery that NRF2 acts as a key regulator in the inducible expression of cytoprotective genes in vivo (78). Subsequently, these mice have been distributed to laboratories worldwide for further research. The 1997 pioneering discovery has served as the foundation for revealing the critical roles of NRF2 in stress defense mechanisms and remains the most highly cited paper in the field. The initial studies also instigated progress in the theory that a wide range of human diseases may be caused by dysregulation or impaired NRF2 function during times of oxidative stress. The model has been refined as conditional *Nrf2* knockout mice were generated. This feature enabled the examination of loss-of-function effects of NRF2 in a cell lineage-specific manner (182, 254).

Subsequently, *Keap1*-null mice were generated and reported in 2003 (240). Contrary to an initial expectation

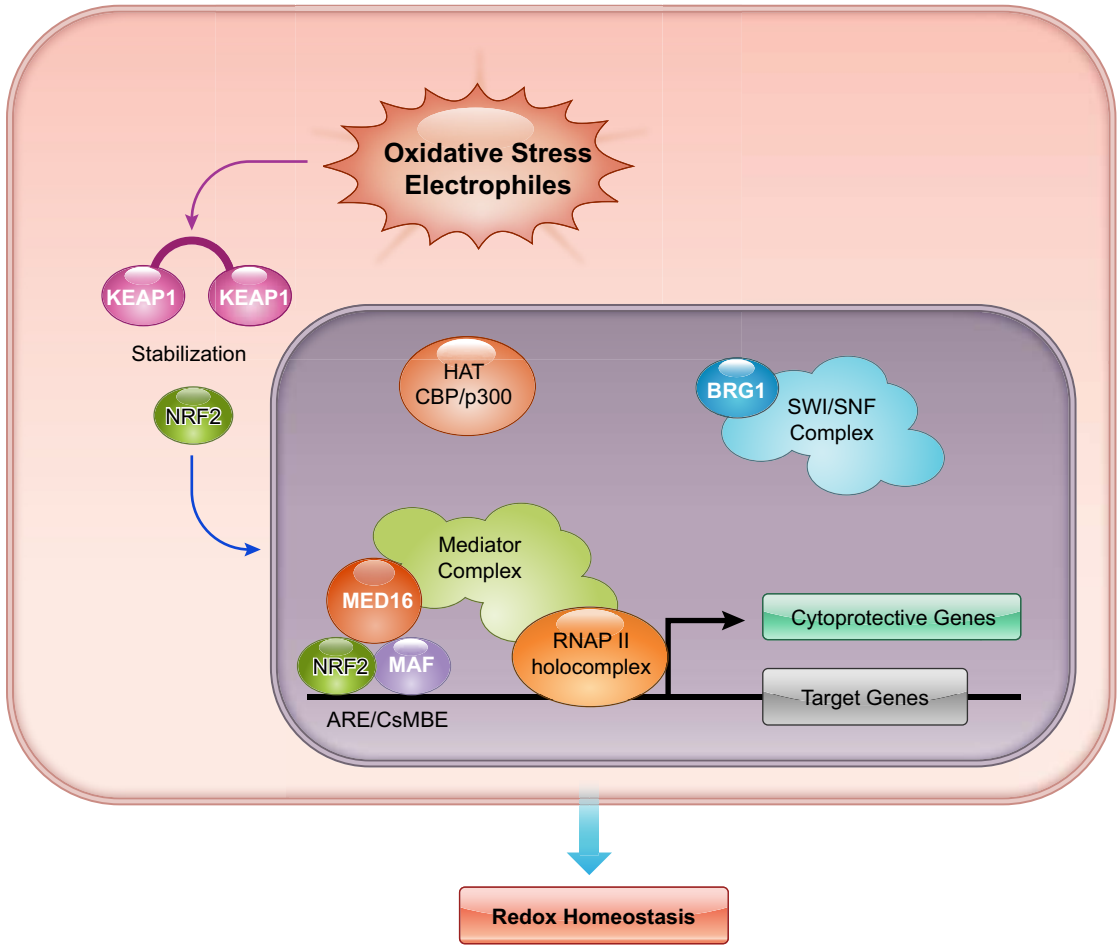

FIGURE 11. Intranuclear players cooperating with NRF2 in its potent transcriptional activity.

that *Keap1* disruption would render these mice as exceedingly robust due to constitutive stabilization of NRF2, *Keap1*-null mice died before weaning due to impaired feeding. The epithelial layers of the esophagus and forestomach of *Keap1*-null pups were dramatically thickened, resulting in stenosis of these tissues. Importantly, the abnormal thickening of the epithelial layers and lethality at the weaning stage were completely abrogated by simultaneous deletion of the *Nrf2* gene. This indicates that constitutive stabilization of NRF2 was responsible for the phenotypes.

Conditional *Keap1* knockout mice with a hepatocyte-specific *Keap1* disruption were generated in 2006 and demonstrated that the resultant increase in NRF2 signaling conferred resistance to acetaminophen hepatotoxicity (162). These mice turned out to be hypomorphic as the floxed allele led to a lower level of *Keap1* gene expression than seen with the wild-type allele (222). This knockdown allele $Keap1^{FA}$ (238), in combination with the knockout allele of *Keap1*, enabled the generation of a series of mice with graded expression levels of the *Keap1* gene (222). Since a single $Keap1^{FA}$ allele appears to express one-fifth of the Keap1 mRNA level of a single wild-type allele (10% and 50%, respectively), the bi-allelic *Keap1* expression levels are ~0, 10, 20, 50, and 60% of wild-type levels in $Keap1^{-/-}$, $Keap1^{FA/-}$, $Keap1^{FA/FA}$, $Keap1^{+/-}$, and $Keap1^{FA/+}$ mice, respectively **(FIGURE 12)**. Another conditional *Keap1* knockout mouse line was established in 2010 (14) with a floxed allele termed $Keap1^{FB}$ that does not exhibit knockdown phenotypes (238). The $Keap1^{FB}$ allele is useful for analysis of cell lineage-specific contributions of the *Keap1* gene, whereas $Keap1^{FA}$ is useful for changing *Keap1* levels in the whole body. In this regard, the $Keap1^{FA}$ knockdown mouse serves as a useful tool for modeling the pharmacological actions of pathway inducers in vivo.

B. Detoxification and Antioxidant Activities of NRF2 Alleviate Various Pathological Conditions

NRF2 was originally identified as a key regulator of phase II detoxication enzymes in response to electrophiles, so not surprisingly the first phenotypes found in *Nrf2*-null mice reflected enhanced susceptibility to xenobiotics **(FIGURE 13)**. *Nrf2*-null mice easily succumbed to acute respiratory

Table 1. *Pathological conditions that are alleviated by NRF2 activation or exacerbated by NRF2 disruption*

Disease	Species	Modifying Strategy of NRF2 Activity		Reference Nos.
		Pharmacological	Genetic	
Chronic kidney disease	Human	Bardoxolone methyl (CDDO-Me)		33, 171
Acute kidney disease	Mouse	Bardoxolone methyl (CDDO-Me)		251
Diabetic nephropathy	Mouse		*Nrf2*$^{-/-}$ mice	83
Hyperoxic acute lung injury	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	26, 181
Pleurisy	Mouse	15D-PGJ_2	*Nrf2*$^{-/-}$ mice	81
Emphysema	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	216
Allergic asthma	Mouse		*Keap1*$^{F/F}$:CAG-CreERT2 mice + tamoxifen	217
Lung carcinogenesis	Mouse	CDDO-Im, CDDO-Me		231
Pulmonary hypertension	Mouse	Oltipraz	*Nrf2*$^{-/-}$ mice, *Keap1*$^{F/F}$ (knockdown; KD) mice	46
Cardiac ischemia-reperfusion	Mouse	15D-PGJ_2	*Nrf2*$^{-/-}$ mice	95
Cardiac hypertrophy	Mouse	Dihydro-CDDO-TEA		252
Multiple sclerosis	Mouse	Fumaric acid esters	*Nrf2*$^{-/-}$ mice	127
Multiple sclerosis	Human	BG_2 (dimethyl fumarate)		54, 59
Alzheimer's disease	Mouse		Lentiviral expression of NRF2	91
Parkinson's disease	Mouse	Triterpenoids		86
Huntington's disease	Mouse	Triterpenoids		214
Amyotrophic lateral sclerosis	Mouse	Triterpenoids		155
Cerebral ischemia-reperfusion	Mouse	Neurite outgrowth-promoting prostaglandin (NEPPs)		196
Type 2 diabetes	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice, *Keap1*$^{F/-}$ (KD) mice	237
Type 1 diabetes	Mouse	Sulforaphane		212
Obesity	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	203
Acetaminophen hepatotoxicity	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	50, 183
Gastric carcinogenesis	Mouse	Oltipraz, sulforaphane	*Nrf2*$^{-/-}$ mice	51, 178
Aflatoxin-induced liver carcinogenesis	Rat	CDDO-Im	*Nrf2*$^{-/-}$ mice	84, 225, 263
Helicobacter pylori colonization	Mouse	Sulforaphane	*Nrf2*$^{-/-}$ mice	262
UV-induced dermatitis	Mouse	TBE-31	*Nrf2*$^{-/-}$ mice, *Keap1*$^{F/F}$ (KD) mice	106
X-ray irradiation-induced dermatitis	Mouse	Synthetic triterpenoid RTA 408		184
Diabetic retinopathy	Mouse		*Nrf2*$^{-/-}$ mice	253
Fuchs endothelial corneal dystrophy (FECD)	Human tissue culture	Sulforaphane		269
Noise-induced hearing loss	Human, mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	64
Rheumatoid arthritis	Mouse		*Nrf2*$^{-/-}$ mice	130
Sepsis	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice	230
Renal ischemia-reperfusion	Mouse	CDDO-Im	*Nrf2*$^{-/-}$ mice, *Keap1*$^{F/F}$ (KD) mice	156

distress syndrome after exposure to butylated hydroxytoluene (23) and suffered acute liver toxicity following acetaminophen administration (50). Pharmacological activation of NRF2 signaling in wild-type mice effectively attenuated acetaminophen hepatotoxicity (183) and cigarette smoke-induced emphysema (216). NRF2 plays a central role in cancer prevention, as indicated by an exacerbated susceptibility to chemical carcinogenesis and lost efficacy in chemoprevention in carcinogen-challenged *Nrf2*-null mice (178, 263).

Since NRF2 was also shown to activate oxidative stress inducible genes (74), *Nrf2*-null mice were used to analyze various pathological conditions related to oxidative stress.

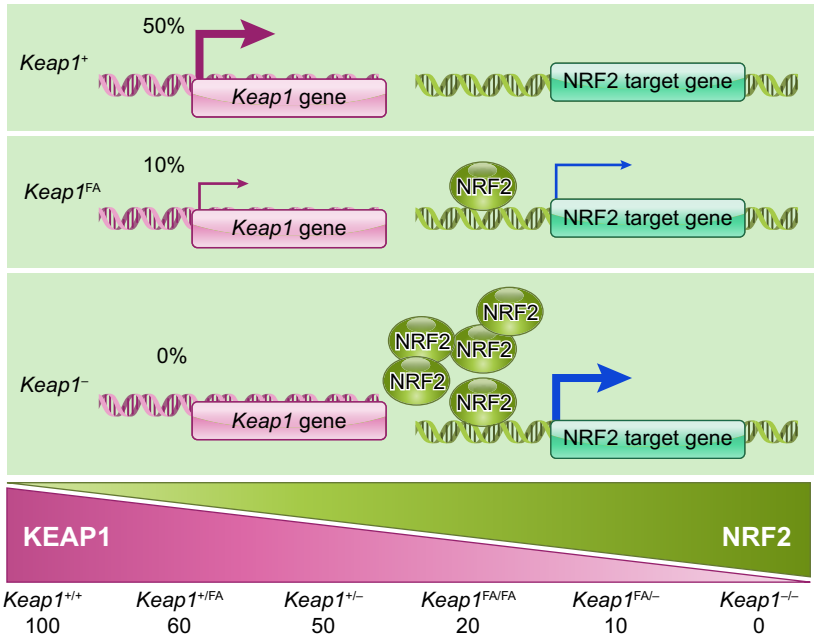

FIGURE 12. Graded expression of KEAP1 can be generated in mice by appropriate combinations of the *Keap1*-null allele and the *Keap1*-knockdown allele.

Typical instances of oxidative tissue damage are ischemia-reperfusion injuries of the brain, heart, and kidney. Exogenous and endogenous NRF2 inducers alleviate tissue damage following ischemia-reperfusion (95, 128, 156, 196, 198, 243). Noise-induced hearing loss is also considered an ischemia-reperfusion injury and similarly is effectively prevented by pretreatment with NRF2 inducers (64). Hyperoxic conditions give rise to oxidative tissue damage, particularly in the lung, and can be effectively prevented by NRF2 activation (26, 181). Ionizing radiation, including UV and X-rays, is another important cause of oxidative stress. NRF2 inducers protect skin from irradiation-induced dermatitis (106, 184).

Metabolic disorders, such as obesity and diabetes and its complications, are closely related to dysregulation of oxidative stress and are effectively treated with NRF2 inducers (83, 203, 212, 237, 253). NRF2 activation in multiple organs appears to orchestrate antidiabetic effects. For example, NRF2 induction in pancreatic β-cells suppresses oxidative damage of pancreatic islets and strongly restores insulin secretion in diabetic conditions (255). NRF2 induction in skeletal muscles alters glycogen metabolism and improves glucose tolerance (238). NRF2 induction in the hypothalamus reduces oxidative stress in astrocytes and protects leptin-secreting neurons, thereby improving control of systemic metabolism and obesity (256).

Neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, are another important category of diseases with etiopathogeneses likely linked to oxidative

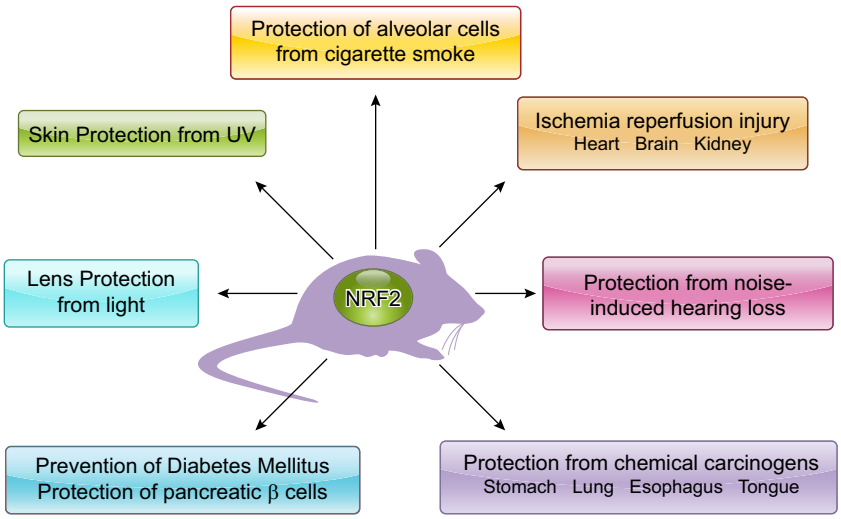

FIGURE 13. Beneficial effects of NRF2. Various pathological conditions are alleviated by NRF2 activation and often exacerbated in NRF2-null mice.

stress. NRF2 activation effectively alleviates the neuronal signs of these diseases in murine models (17, 86, 91, 155, 214). The administration of NRF2 inducers reduces oxidative damage in neural tissues in mouse models of Parkinson's disease and Huntington's disease (86, 214). However, because neuroinflammation underlies these neurodegenerative disorders, it is likely that the anti-inflammatory aspect of NRF2 contributes substantially to improving these pathological conditions.

C. Excessive Activation of NRF2 Causes Reductive Stress

G6PD is one of the major enzymes required for NADPH production, and an increase in its activity causes reductive stress. This reductive stress impairs cardiac function, as suggested by studies on inheritable human disorders linked to mutations in genes encoding αB-crystalline. The studies also suggest that constitutive activation of NRF2 in the myocardium is responsible for the reductive stress and consequent protein aggregation and cardiomyopathy (176, 177). Mutant αB-crystalline binds to KEAP1 and stabilizes NRF2. Transgenic mice expressing mutant αB-crystalline in heart exhibit ventricular dysfunction **(FIGURE 14)**. The cardiomyopathy and protein aggregation caused by the transgene is ameliorated by an *Nrf2*-null background (90). The human *LMNA* gene that causes muscular dystrophy increases cellular levels of p62/SQSTM1, activates the KEAP1-NRF2 pathway, and leads to reductive stress, as described in a recent report on myopathic lamin mutations (40). This implies that aberrant activation of NRF2 is also deleterious by causing proteotoxicity.

In contrast, transgenic mice with cardiomyocytic overexpression of NRF2 were resistant to myocardial oxidative stress, as well as cardiac apoptosis, fibrosis, hypertrophy, and dysfunction, under the sustained pressure overload induced by transverse aortic arch constriction (246). The study analyzed transgenic mice with a modest NRF2 expression, so their NRF2 accumulation levels might have been less than in mice with genetic mutations in αB-crystalline and LMNA. Alternatively, simple overexpression of NRF2 was not sufficient to cause myopathy, but NRF2 was required for the development of myopathy under specific conditions.

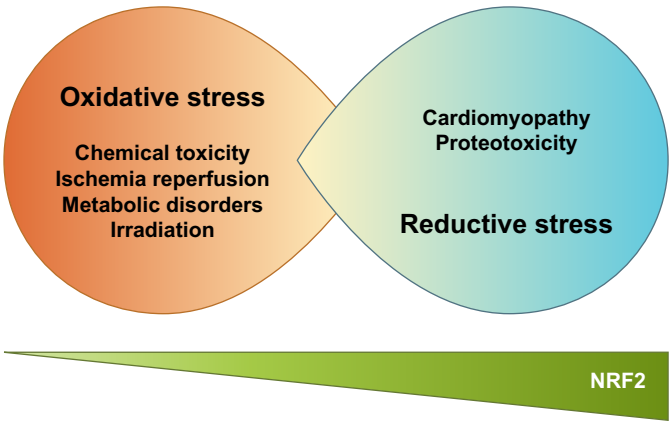

FIGURE 14. Impaired NRF2 function causes oxidative stress, whereas excessive NRF2 function generates reductive stress.

VI. NRF2 CONTRIBUTION TO ANTI-INFLAMMATORY EFFECTS

A. NRF2 Promotes the Resolution of Acute Inflammation

Besides increasing cellular antioxidant capacities, NRF2 evokes strong anti-inflammatory activity. Since NRF2 confers resistance to oxidative and electrophilic insults on cells, damage-associated molecular patterns are suppressed by NRF2 in affected tissues. This action appears to be one of the critical mechanisms behind the anti-inflammatory activity of NRF2 (**FIGURE 15**). In *Nrf2*-null mice, the following disease outcomes were exacerbated and protracted: pleurisy induced by carrageenan; emphysema induced by elastase and cigarette smoke; and pulmonary fibrosis induced by bleomycin (28, 75, 81, 180). *Nrf2*-null mice are also quite susceptible to lipopolysaccharide-induced acute inflammation, whereas NRF2 activation effectively protects against mortality after lipopolysaccharide challenge (230). When *Nrf2* or *Keap1* genes were disrupted specifically in mouse myeloid cells, the *Nrf2* mutants were susceptible to microbial infection and subsequent sepsis, whereas the *Keap1* mutants were resistant (115). These results indicate that NRF2 activation protects tissues by modulating innate immunity. The endogenous NRF2 inducer 15d-PGJ2 plays an important role in the anti-inflammatory function of NRF2 by binding to KEAP1 and activating NRF2 (141). In a model of concanavalin A-induced T-cell-mediated acute immune hepatitis, NRF2 disruption enhanced susceptibility, whereas KEAP1 disruption resulted in resistance. This is a similar pattern to that observed above in myeloid cells. Similarly, almost complete protection was achieved by pretreatment with the NRF2 inducer CDDO-Im in wild-type, but not in *Nrf2*-null mice (167).

B. NRF2 Ameliorates Chronic Inflammation

Tissue damage and inflammation are closely related to each other. When the root cause of tissue damage is persistent, inflammatory reactions can become chronic. An example is sickle cell disease, which is caused by a point mutation in the β-globin gene. Abnormally shaped red blood cells are prone to hemolysis and easily release heme into plasma, which provokes oxidative stress and inflammation. Systemic activation of NRF2 promotes degradation of the released heme and suppresses inflammation, which leads to the dramatic alleviation of organ damage in a sickle cell

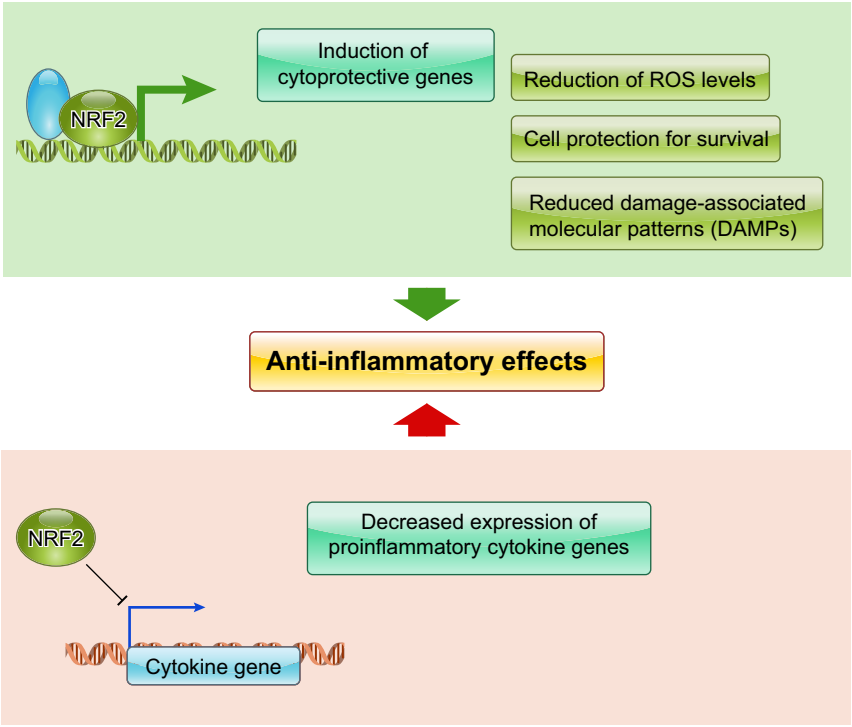

FIGURE 15. Two anti-inflammatory effects mediated by NRF2. NRF2 induces antioxidant response genes. In contrast, transcriptional expression of pro-inflammatory cytokine genes is efficiently inhibited by NRF2.

disease mouse model (98). In a mouse model of asthma, NRF2 protects airway epithelia and effectively reduces allergic inflammation and airway hyperresponsiveness (217).

The NRF2 inducer dimethyl fumarate exerts protective effects against neuroinflammation in a mouse model of chronic multiple sclerosis (127). Importantly, dimethyl fumarate became a Food and Drug Administration-approved drug after successful clinical trials for relapsing multiple sclerosis, which is an autoimmune-based inflammation of neural tissues (54, 59). Whether or not NRF2 inducers modulate adaptive immunity is unclear in these cases, although interesting studies have been published. T-cell-specific activation of NRF2 elevates the frequency of regulatory T cells (Tregs), which seems to be critical for protecting kidney after ischemia-reperfusion (160). Another report describes that systemic activation of NRF2 alleviates a lethal autoimmune condition due to Treg deficiency, suggesting that NRF2 suppresses effector T-cell activities independent of Tregs (220). The implication is that NRF2 influences the activity of adaptive immunity.

C. Molecular Basis of NRF2-Mediated Anti-Inflammatory Effects

Although NRF2-mediated tissue protection likely involves reducing damage-associated molecular patterns, which drive secondary inflammatory reactions, NRF2 directly antagonizes the induction of proinflammatory genes, such as *IL6* and *IL1b*, to exert some of its anti-inflammatory functions (111). When the cell-autonomous effects of NRF2 activation were examined in bone marrow-derived macrophages, NRF2 inhibited the expression of proinflammatory cytokines induced by polarization toward M1 type macrophages. NRF2 appears to interfere with the transcriptional activation machinery of proinflammatory genes in response to induction signals **(FIGURE 15)**. While the precise mechanisms as to how NRF2 inhibits the transcriptional activation of proinflammatory cytokine genes remain to be elucidated, this study clearly demonstrates that elements of the anti-inflammatory function of NRF2 can be distinguished from its classical antioxidant function.

VII. THE KEAP1-NRF2 SYSTEM IN CARCINOGENESIS

A. Cancer Chemoprevention Through Activation of NRF2

Many animal models and clinical trials have targeted the NRF2 pathway for preventing chemical carcinogenesis since the late 1990s, which was before NRF2 was identified as a master regulator of cellular defense mechanisms. In the early 1970s, phenolic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, were found to effectively suppress carcinogenesis in rodents (249). Many studies have shown that induction of cytoprotective enzymes is a critical and sufficient mechanism for protection against carcinogenesis provoked by exogenous and endogenous factors. Now a plethora of natural and synthetic compounds are known to activate NRF2 by inactivating

KEAP1. Clinically relevant classes of compounds include dithiolethiones (e.g., oltipraz; Ref. 48), isothiocyanates (e.g., sulforaphane; Ref. 43), and triterpenoids (e.g., CDDO-Im; Refs. 126 and 264). Oltipraz, sulforaphane, and CDDO-Im demonstrated a large dynamic range in potency, with a 1,000-fold difference in concentrations required for target gene induction in cell culture (100). This difference in potency may reflect in part their chemical mechanism of interaction with sensor cysteines, as all of these chemicals bind Cys151 of KEAP1 (44, 113). Among these chemicals, oltipraz and sulforaphane have been actively tested in clinical trials as chemoprevention agents. Triterpenoids are in the process of clinical development as therapeutic agents, but are also very promising agents for disease prevention, in part due to their remarkable potency.

NRF2 plays an important role in the detoxication of benzo[*a*]pyrene, a procarcinogen formed by the incomplete combustion of carbon. The preventive effects of NRF2 activation on benzo[*a*]pyrene-induced carcinogenesis have been demonstrated in mice treated with multiple NRF2 inducers, such as oltipraz (178, 179) and sulforaphane (51). NRF2 activation increases the efficiency of conjugation and excretion of benzo[*a*]pyrene, resulting in a reduced toxicological impact. Pretreatment with NRF2 inducers reduces tumor incidence in the forestomach. This protection is lost in the *Nrf2*-null background, indicating that the pharmacological activation of NRF2 is responsible. Moreover, wild-type mice treated with vehicle developed fewer forestomach tumors than *Nrf2*-null mice, highlighting their intrinsic sensitivity to carcinogenesis. NRF2 also modulates the pulmonary response to various toxicological insults, including exposures to diesel exhaust and cigarette smoke (69, 216). *Nrf2*-null mice are predisposed to oxidative DNA damage on exposure to diesel exhaust (2). NRF2 protects epithelial cells of the respiratory tract from the cytotoxicity and genotoxicity of cigarette smoke. *Nrf2*-null mice are significantly more susceptible to cigarette smoke-induced emphysema and pulmonary damage compared with wild-type mice (69, 180).

The mycotoxin aflatoxin B_1, produced by the fungus *Aspergillus flavus*, is a potent hepatic carcinogen in humans, rats, and infant mice (101). This carcinogen is often found as a contaminant in staple foods, especially corn and peanuts. Food contamination by aflatoxin is very difficult to eradicate, so methods for minimizing the detrimental health effects of aflatoxin is a critical issue in aflatoxin endemic areas of South Asia, sub-Saharan Africa, and Central America. Once ingested, aflatoxin is metabolically activated to aflatoxin-8–9-*exo* epoxide by cytochrome *P*-450. This reactive intermediate rapidly forms an N^7-guanine DNA adduct that leads to mutations within the cell. CDDO-Im, a potent NRF2-inducing triterpenoid, confers complete lifetime protection against aflatoxin-induced hepatocellular carcinogenesis in rats (84, 263). The protective actions of CDDO-Im against aflatoxin DNA adduct formation and acute toxicity are markedly attenuated in *Nrf2* knockout rat, which validates NRF2 as a key player in this protective action (225). Additional studies using carcinogens targeting other tissues further demonstrate the important role of NRF2 in cancer chemoprevention, as reviewed in Slocum and Kensler (211).

B. Somatic Mutations in the KEAP1-NRF2 System in Human Cancers

NRF2 activation is normally beneficial for the health and survival of organisms, including worms, flies, and mammals. However, cancer cells can acquire a similar advantage for their survival through mechanisms that lead to constitutive activation of NRF2 signaling. The first reports describing somatic mutations in *KEAP1* and *NRF2* genes in human non-small cell lung cancers were published in 2006 and 2008, respectively (170, 201, 206). Since then, somatic mutations involved in the KEAP1-NRF2 system have been described in other cancers, such as breast (158), gallbladder (200), esophagus, and skin cancers (103). Recent cancer genome analyses exploiting deep sequencing revealed that *KEAP1* and *NRF2* are frequently mutated in solid tumors arising from tissues susceptible to environmental exposures, such as head and neck, lung, liver, bladder, and the upper digestive tract (18, 70). Missense mutations in *CUL3* were found in sporadic papillary renal cell carcinoma and head and neck carcinoma, which also leads to activated NRF2 signaling (134, 166).

Mutations in the *KEAP1*, *NRF2*, and *CUL3* genes appear to be mutually exclusive (18, 122), suggesting that the consequences of these mutations converge on the same pathway. Indeed, according to the COSMIC (Catalogue of Somatic Mutations in Cancer) database **(FIGURE 16)** (7), somatic nonsynonymous mutations in *KEAP1* and *CUL3* were found throughout the coding region. These mutations are expected to abrogate the activity of the KEAP1-CUL3 complex, thus ameliorating the degradation of NRF2. In contrast, mutations in NRF2 are almost exclusively found in the ETGE and DLG motifs in the Neh2 domain, which are essential for the binding of NRF2 to KEAP1. The pattern of NRF2 mutations in human cancers serves as solid evidence for the importance of the ETGE and DLG motifs in regulating NRF2 stability through the two-site binding interaction for NRF2 with KEAP1, as described in the hinge-latch model for NRF2 activation introduced earlier in this review.

These mutations disrupt the interaction between NRF2 and KEAP1 without affecting the transcriptional activation function of NRF2, resulting in constitutive stabilization of NRF2 and persistent activation of its target genes. Therefore, mutations in either the *KEAP1*, *NRF2*, or *CUL3* gene result in the constitutive activation of NRF2 signaling in

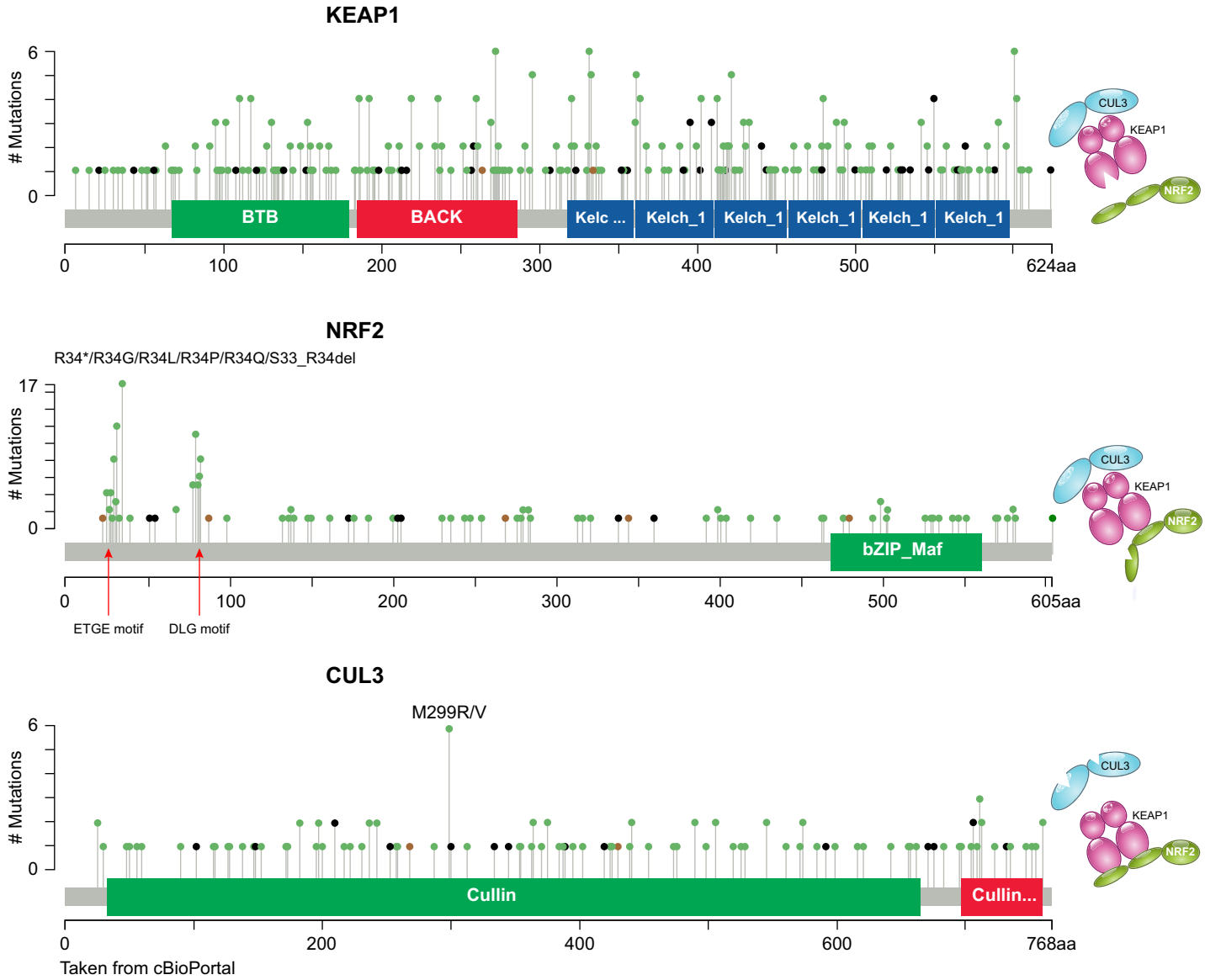

FIGURE 16. Somatic mutations found in *KEAP1*, *NRF2*, and *CUL3* genes in cancer. *KEAP1* and *CUL3* mutations are distributed in a wide range over the coding region, whereas *NRF2* mutations are mostly clustered in the ETGE and DLG motifs in Neh2.

cancers. Cancer cells with high levels of NRF2 activity undergo metabolic reprogramming that drives aggressive proliferation (140) as well as chemo- and radio-resistance to anticancer therapy (226), thereby becoming extremely malignant and more difficult to manage clinically. These cells are referred to as NRF2-addicted cancer cells (105, 226).

C. Pathways Leading to KEAP1-CUL3 Complex Dysfunction and NRF2 Induction in Cancers

Clinical studies of tumor tissues using histology revealed high levels of NRF2 accumulation in certain subsets of cancers (i.e., NRF2-addicted cancers) and found that NRF2 was persistently activated, as reflected in elevated target gene expression. These studies have been conducted in multiple research institutes around the world and consistently demonstrated that abundant and persistent activation of NRF2 was associated significantly with poor prognoses in various cancers, including lung, gallbladder, esophagus, breast, head and neck, and renal cancers (19, 72, 87, 134, 164, 201, 202, 244). This association was expected as NRF2 coordinately activates prosurvival genes through its detoxication and antioxidant functions, thereby conferring resistance to chemotherapy and radiotherapy in cancers.

Besides the somatic mutations in the *KEAP1* and *NRF2* genes, several other mechanisms have been described for the constitutive stabilization of NRF2 **(FIGURE 17)**. First, DNA hypermethylation at the promoter region of *KEAP1* in can-

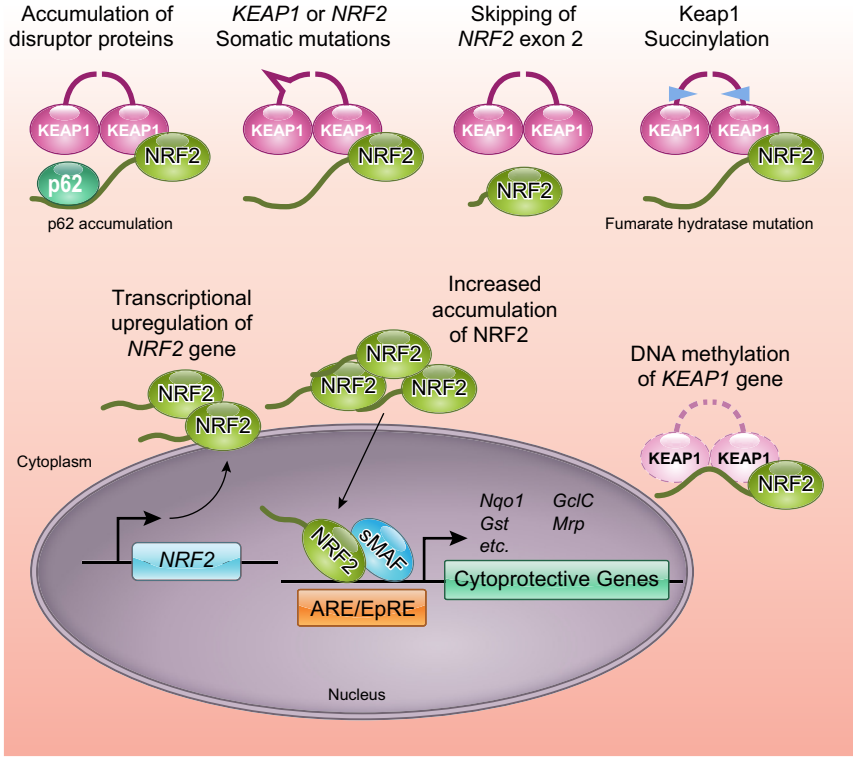

FIGURE 17. Multiple mechanisms lead to excessive activation of NRF2 in cancers.

cer cells decreases KEAP1 expression, leading to NRF2 stabilization. Such epigenetic abnormalities in the *KEAP1* gene in lung cancers and malignant gliomas are also associated with poor clinical outcomes (151, 152). In papillary thyroid carcinoma patients, promoter methylation of not only *KEAP1* but also *CUL3* has been observed, which increased the NRF2 signature of gene expression (133). Second, aberrant accumulation of p62/SQSTM1, which disrupts the KEAP1-NRF2 interaction, causes persistent activation of NRF2. The abnormal accumulation of p62/SQSTM1 is often observed in hepatocellular carcinoma (71, 129, 215), suggesting that increased NRF2 activity contributes to the malignant progression of this cancer. Third, loss of function of fumarate hydratase (FH) in cancers causes fumarate (an intermediate of the TCA cycle) to pool and leads to elevated accumulation of NRF2. Patients carrying heterozygous germline mutations in the *FH* gene exhibit elevated levels of fumarate and develop hereditary leiomyomatosis and renal cell cancer, a syndrome characterized by smooth muscle tumors and papillary renal cell carcinoma type 2 (232). Fumarate is weakly electrophilic and modifies the cysteine residues of KEAP1, thereby stabilizing NRF2 and leading to the elevated expression of NRF2 target genes (1, 165). This action offers an explanation, at least in part, for the highly malignant papillary renal cell carcinoma type 2 phenotype seen in patients.

Fourth, exon skipping of the NRF2 gene results in the production of NRF2 protein lacking a KEAP1-interaction domain (60). In lung cancers and head and neck cancers, which frequently express high levels of NRF2, the recurrent exclusion of exon 2 from NRF2 mRNA has been observed. This results in deletion of the stretch of amino acids D16-Q104 that span the DLG and ETGE motifs, as well as the lysine clusters targeted for ubiquitination. Fifth, transcriptional upregulation of the *NRF2* gene by KRAS or BRAF-driven oncogenic pathways involving MYC and JUN leads to increased NRF2 activity (35). To summarize, the six known mechanisms that lead to increased NRF2 activation and accelerated cancer progression are as follows: somatic mutation of *KEAP1* or *NRF2*; aberrant accumulation of p62/SQSTM1; epigenetic silencing of the *KEAP1* or *CUL3* gene; germline mutations in *FH*; exon skipping within the *NRF2* gene; and oncogene-mediated transcriptional upregulation of the *NRF2* gene.

D. Roles of NRF2 in Cancer Initiation, Progression, and Metastasis

NRF2 protects cells from DNA damaging insults, such as ROS and electrophilic toxicants, thereby inhibiting the initiation of cancer. However, once a cell is transformed by acquiring one or likely several oncogenic mutations, NRF2 in the pre-neoplastic or transformed cells contributes to their resistance to stress and survival, thereby supporting the establishment of cancers **(FIGURE 18)**. An important observation is that NRF2 activity is required for oncogenic KRAS ($KRAS^{G12D}$)-driven lung carcinogenesis in a mouse model (35). In the *Nrf2*-null background, neoplastic nodules in lung tissues were fewer in number and smaller in size, contained fewer Ki67-positive proliferating cells, and sur-

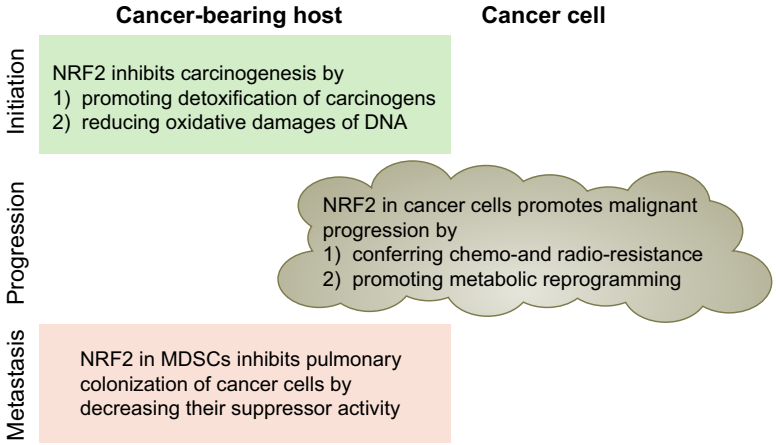

FIGURE 18. Roles of NRF2 in carcinogenesis highlighting the importance of context. NRF2 activation is beneficial and deleterious for the cancer-bearing host, depending on the time (initiation, promotion, and metastasis) and place (cancer cells or microenvironment). MDSCs, myeloid-derived suppressor cells.

vival of the mice was extended compared with wild type. Similar outcomes were observed in a urethane-induced lung carcinogenesis murine model in which KRAS mutations are frequent (194). *Nrf2*-null mice are prone to developing lung nodules after being treated with urethane, but the nodules do not acquire malignant characteristics. In contrast, wild-type mice are resistant to the initial development of lung nodules, but the nodules evolve into malignant adenocarcinoma. Consistent with this outcome, *Keap1* knockdown mice exhibited greater resistance to the initial development of nodules, but cells isolated from pulmonary nodules in *Keap1*-knockdown mice grew aggressively when engrafted subcutaneously into immuno-deficient nude mice (195). Similarly, activation of NRF2 by antioxidant antidiabetic agents accelerated tumor metastasis in a xenograft model using nude mice (245). These results indicate that NRF2 may play at least two roles during carcinogenesis: preventing cancer initiation and promoting malignant progression.

From the viewpoint of NRF2 function in the tumor microenvironment, NRF2 activation appears to be beneficial for the cancer-bearing host. When cancer cells are injected into tail veins of mice, *Nrf2*-null mice exhibit a higher number of lung metastatic nodules than wild-type mice (193). *Nrf2* deficiency, particularly in the myeloid cell linage, elevates the activity of myeloid-derived suppressor cells, which inhibits T-cell-mediated antitumor immunity (61). Therefore, it is plausible that systemic administration of NRF2 inducers to cancer-bearing hosts would effectively reinforce antitumor immunity to suppress cancer metastasis. However, administration of NRF2 inducers would need to be very carefully controlled in the case of cancer bearing hosts with immunosuppression (245).

E. NRF2 Contribution to Metabolic Reprogramming of Cancer Cells

The KRAS-driven carcinogenesis model revealed that NRF2 contributes to the establishment and progression of cancer cells (35). This effect differs from acquisition of elevated detoxication capacity (radio- and chemoresistance), as this phenomenon can be seen even in the absence of exogenous anticancer treatments. Cancer cells with high NRF2 activity aggressively proliferate, which undoubtedly contributes to their malignant nature (207, 268). The means by which NRF2 promotes the proliferation of cancer cells does not involve the canonical antioxidant and anti-inflammatory functions of NRF2.

In this regard, detailed analyses of NRF2 target genes in cancer cells identified metabolic enzymes that are involved in the pentose phosphate pathway and glutamine metabolism (140). In particular, in proliferating cells, NRF2 redirects glucose and glutamine into anabolic pathways and supports metabolic activities that are advantageous for proliferation (140, 208). Comprehensive metabolomic profiling of lung cancer cells revealed that the serine synthesis pathway is also facilitated by NRF2 through ATF-4 activation (36). CRISPR library screening identified glutamine transporter as a key factor supporting the growth of *Keap1*-mutant lung cancer cells, leading to the discovery of dependence on glutaminolysis of *Keap1*-mutant cancers (188). These findings are particularly intriguing, as the pentose phosphate pathway provides ribose for nucleotide synthesis and NADPH for lipid synthesis, whereas glutamine is important for glutathione synthesis and anaplerosis (replenishing) of TCA cycle intermediates that have been diverted for the synthesis of amino acids and other biomolecules. In addition, serine is an important one-carbon source for nucleotide synthesis and methylation reactions.

Notably, NRF2 plays a major role in these metabolic pathways in the presence of active proliferative signals rather than in quiescent cells. PI3K-AKT signaling is a major proliferative signal that inactivates GSK-3 by phosphorylation, resulting in NRF2 accumulation through inhibition of KEAP1-independent degradation (see **FIGURE 9**). As described above, an increased abundance in NRF2 enables activation of metabolic genes and promotes anabolic metabolism. This functional expansion of NRF2 is not limited

to cancer cells, but operates in normal cells within the transient and physiological magnitude of PI3K-AKT signal activation. Examples include reactive hypertrophy of liver following portal vein branch ligation (204) and possibly liver regeneration after partial hepatectomy (11, 241). In the latter setting, the NRF2-NOTCH axis, a reciprocal activation of NRF2 and NOTCH signaling pathways, plays an important role (241, 242). Still another intriguing recent discovery is a contribution of NRF2 to translation (25). NRF2 deficiency induces cysteine oxidation of subunits of the translational machinery, resulting in impaired mRNA translation. With these recent reports, it is clear that the regulatory layers for NRF2-mediated effects on cell proliferation have been expanded further than previously anticipated.

VIII. THERAPEUTIC APPLICATIONS OF NRF2 AND MODULATION OF ITS ACTIVITY

A. Development of NRF2 Inducers

Based on the profound contributions of NRF2 in the prevention and alleviation of a wide variety of pathological conditions in mouse models **(TABLE 1)**, worldwide efforts have been made to isolate from natural sources or develop potent and potentially specific NRF2-inducing chemicals. Among them, dimethyl fumarate (Tecfidera) has been approved by the Food and Drug Administration and Pharmaceuticals and Medical Devices Agency and is clinically prescribed to patients with multiple sclerosis. CDDO-Me, a member of the exceedingly potent class of oleanane triterpenoid NRF2 inducers, is undergoing clinical trials in Japan for diabetic nephropathy treatment, although its development was paused in the United States due to the occurrence of cardiac complications in patients with end-stage renal disease (33, 171). Inducers with less potency and specificity are also in clinical development, in particular nitro fatty acids (37) and the broccoli-derived isothiocyanate sulforaphane (45). It is possible that modification of additional cysteine residues beyond KEAP1 may trigger complementary signaling that enhances protective effects. Several small trials in autism (210) and air pollution (49) using sulforaphane in the form of broccoli extracts have shown promising effects likely to be NRF2 dependent, at least in part. Although the cysteine residues of KEAP1 are highly reactive and efficiently modified by these electrophiles, strategies that do not require specificity of electrophilic attack still need to be considered. Therefore, NRF2 inducers with higher specificity might be found in the form of non-electrophilic chemicals that disrupt the interaction between KEAP1 and NRF2 by filling a KEAP1 pocket that forms an interface with NRF2 (186).

B. Development of NRF2 Inhibitors

Cancers with persistent activation of NRF2 exhibit high dependency on NRF2 function for drug resistance and cell proliferation. In the treatment of these cancers, NRF2 inhibitors are expected to antagonize cancer progression and sensitize cancer cells to therapy. However, compared with NRF2 inducers, the development of NRF2 inhibitors is in its infancy, so very few NRF2 inhibitors have been reported. The plant-based product brusatol has been reported to inhibit NRF2 (185). Brusatol effectively decreases the protein levels of NRF2 and sensitizes cancer cells to chemotherapy and radiotherapy. Recently, another NRF2 inhibitor, halofuginone, was found to exert a chemosensitizing effect on cancer cells exhibiting constitutive NRF2 stabilization (235). Interestingly, brusatol inhibits global protein synthesis, possibly by targeting the ribosome (239), whereas halofuginone inhibits proline tRNA synthetase and activates the amino acid response pathway (99). This suggests that the inhibition of protein synthesis is a particularly effective means for antagonizing NRF2 activity. This approach is possible because of the short half-life of NRF2. In contrast, ML385 exploits an alternative mechanism by binding to the DNA binding domain of NRF2 and shows significant chemosensitizing activity, specifically in non-small cell lung cancer cells harboring KEAP1 mutations (209). An important point to consider when developing NRF2 inhibitors is the fact that systemic administration of NRF2 inhibitors would reduce both canonical detoxication and antioxidant defense responses, thereby changing the profiles or thresholds of dose-limiting toxicities associated with standard-of-care therapies. Therefore, a drug delivery system specific to cancerous tissue and/or a well-designed medication protocol are necessary to achieve the best efficacy. Alternatively, finding a therapeutic target that is selectively expressed in NRF2-addicted cancers is a new opportunity as represented by identification of an orphan nuclear receptor NR0B1 as a unique therapeutic target for *KEAP1*-mutant non-small cell lung cancers (8).

C. Diagnostic Use of Regulatory Single Nucleotide Polymorphisms in the NRF2 Promoter and NRF2 Binding Sites

NRF2 activity is regulated through protein stabilization, primarily by KEAP1, but is also regulated at the transcriptional level. Thus two layers of regulation exist that control the *NRF2* activity that impacts on the susceptibility of mice and humans to various pathological conditions (27, 64, 135, 219, 258) **(FIGURE 19)**. Importantly, NRF2 autoregulates transcription of the *Nrf2* gene (118).

A single nucleotide polymorphism (SNP) in the promoter region of the mouse *Nrf2* gene was first described in 2002 from a linkage analysis of susceptibility to hyperoxic challenges of different strains of mice (27). The susceptible

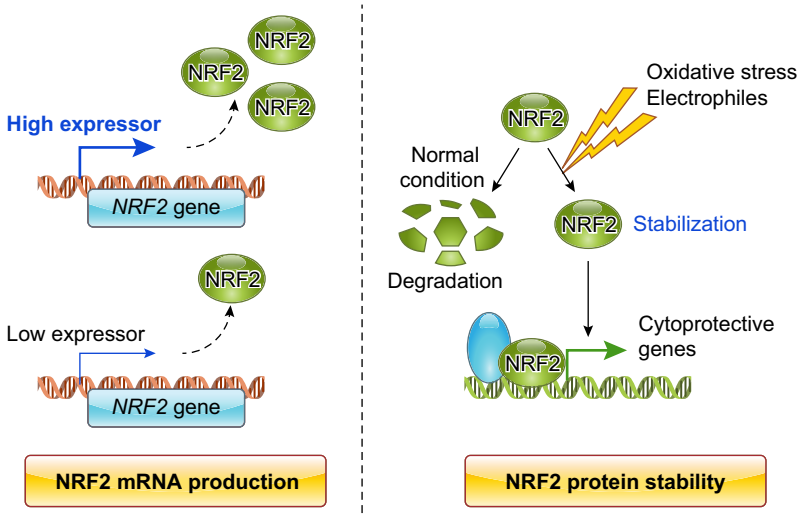

FIGURE 19. Transcriptional and posttranslational regulation of NRF2 activity. In addition to regulation of NRF2 protein stability, the transcription level of the *NRF2* gene is an important determinant of the overall activity of NRF2.

strain C57BL/6J and the resistant strain C3H/HeJ were compared in that study. A decreased expression of lung *Nrf2* mRNA and a T to C substitution in the promoter region of the *Nrf2* gene were found in C57BL/6J mice, which cosegregated with susceptibility phenotypes in the genome-wide linkage analysis. Indeed, *Nrf2*-null mice were susceptible to hyperoxia (26). Thus it is concluded that the *Nrf2* promoter SNP influences the transcription level of the *Nrf2* gene, which affects susceptibility to hyperoxia.

In humans, similar to the mouse *Nrf2* gene, a *NRF2* promoter SNP located 617 bp upstream from the transcription start site lowers the level of *NRF2* transcription (258) **(FIGURE 20)**. Of note, individuals who possess this SNP are more susceptible to acute lung injury and related diseases (135). The presence of this SNP was also found to significantly correlate with the incidence of non-small cell lung cancer (219). The NRF2 SNP is also associated with susceptibility to noise-induced hearing loss, for which oxidative damage is the major underlying cause (64).

In addition to the SNP in the *NRF2* promoter, those in NRF2 target sites (AREs/CsMBEs) may have substantial biological impacts **(FIGURE 20)**. Integration of genomewide maps of the NRF2 occupancy with disease-susceptibility loci revealed several associations between polymorphic AREs/CsMBEs and disease-risk SNPs (247). Bioinformatic analysis constructed an NRF2 binding prediction model that successfully identified polymorphisms in the AREs/CsMBE that affect NRF2 target gene expression (116). Thus the *NRF2* SNP and those in the NRF2 binding sites are likely to be used for personalized medicine in the future

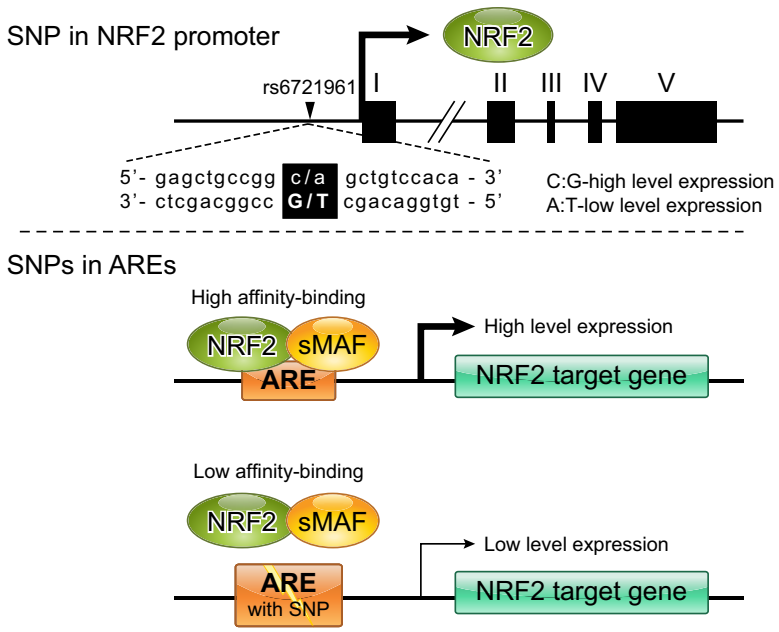

FIGURE 20. SNPs in the *NRF2* promoter and AREs. SNPs in the *NRF2* promoter alter the transcription level of the *NRF2* gene. SNPs in AREs alter the binding affinity of NRF2-sMAF heterodimers and transcription levels of NRF2 target genes.

as indicators for predicting disease susceptibility and drug toxicity.

IX. CONCLUSIONS: PERSPECTIVE ON THE KEAP1-NRF2 PATHWAY

Discovery of the Keap1-Nrf2 system revealed a revolutionary concept that has led to new approaches toward improving human health and combating diseases. One of the most salient questions regarding the molecular mechanism of the KEAP1-NRF2 system is how KEAP1 senses electrophilic and oxidative stimuli and transduces them into stabilized NRF2 and subsequent downstream signaling. To this end, elucidation of the overall structure of active and inactive CUL3-KEAP1-NRF2 complexes is underway. The specific positions and numbers of modified cysteine residues differ among chemical inducers, which likely leads to multiple means for structural alterations in the CUL3-KEAP1-NRF2 complex. We believe that full details on the structural features of inactivated or active KEAP1 are key to understanding the whole structure-function basis of this critical cysteine-based biosensor.

Other important questions focus on the roles that NRF2 plays in carcinogenesis. For instance, how and when in their etiology do cancers with constitutive NRF2 activation highjack and capitalize on the contributions of NRF2? Is NRF2 an actual oncogene or, as seems more likely, is it an important, facilitative susceptibility determinant? Long-term observations of *Keap1* knockdown mice and conditional *Keap1* knockout mice strongly suggest that simple *Keap1* deficiency is not sufficient for the establishment of cancers. In other words, NRF2 is not a driver of cancer nor a passive passenger, but rather a copilot and navigator. The presence of activated oncogenes in cells provides NRF2 a role for promoting the malignant evolution of cancers. An intriguing question here is, during the multistep carcinogenesis, when does NRF2 change its role from a faithful guardian to a betrayer? The answer to this question will provide a theoretical basis as to how NRF2 inducers could be used for cancer prevention and in which at-risk cohorts and when and in what settings should NRF2 inhibitors be applied for the treatment of cancer patients or prevention of recurrence. It is also necessary to recognize that there are settings of oncogene-driven carcinogenesis where constitutive activation of NRF2 signaling is protective. Thus context is the critical determinant of the risk and benefit mediated through NRF2 that must be considered (213).

From a clinical point of view, NRF2 inducers and inhibitors both have their own advantages and disadvantages. NRF2 inducers are expected to activate anticancer immunity, but confer resistance to anticancer therapy in cancers. NRF2 inhibitors are expected to reduce proliferation and increase the sensitivity of cancer cells to anticancer drugs. However, when they act within a cancer microenvironment, NRF2 inhibitors are expected to promote cancer metastasis by activating the repressor activity of myeloid-derived suppressor cells. We believe that the appropriate use of NRF2 inducers and inhibitors will greatly reduce the incidence of cancers and improve the prognosis of cancer patients. In addition to appreciating context, it is important to understand the nature of dose-response curves of such agents, whether they are linear or "S" or "U" shaped (100). Moreover, as NRF2 touches the etiopathogenesis of other acute and chronic diseases, ongoing clinical trials (https://clinicaltrials.gov/) using multiple classes of inducers should provide insights into the efficacy and optimization of interventions.

Health is a reflection of the ability of an organism to adapt to stress. How one responds to environmental and endogenous stress is inevitably a critical issue in maintaining health. Various diseases and aging processes must be analyzed and evaluated under the influence of these stress factors. The KEAP1-NRF2 system, a thiol-based sensor-effector apparatus, plays a central role in responses for better adaptation. Thus informed harnessing of this pathway is key to the prosperity and continuation of the human species.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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