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The complexity of the Nrf2 pathway: Beyond the antioxidant response

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Abstract

The NF-E2-related factor 2 (Nrf2)-mediated signaling pathway provides living organisms an efficient and pivotal line of defensive to counteract environmental insults and endogenous stressors. Nrf2 coordinates the basal and inducible expression of antioxidant and phase II detoxification enzymes to adapt to different stress conditions. The stability and cellular distribution of Nrf2 is tightly controlled by its inhibitory binding protein Kelch-like ECH-associated protein 1 (Keap1). Nrf2 signaling is also regulated by post-translational, transcriptional, translational and epigenetic mechanisms, as well as by other protein partners, including p62, p21 and IQ motif-containing GTPase activating protein 1 (IQGAP1). Many studies have demonstrated that Nrf2 is a promising target for preventing carcinogenesis and other chronic diseases, including cardiovascular diseases, neurodegenerative diseases, and pulmonary injury. However, constitutive activation of Nrf2 in advanced cancer cells may confer drug resistance. Here, we review the molecular mechanisms of Nrf2 signaling, the diverse classes of Nrf2 activators, including bioactive nutrients and other chemicals and the cellular functions and disease relevance of Nrf2 and discuss the dual role of Nrf2 in different contexts.

Keywords

NF-E2-related factor 2 (Nrf2); Kelch-like ECH-associated protein 1 (Keap1); nutrients; activator; antioxidant; ROS

1. Introduction

Understanding of the NF-E2-related factor 2 (Nrf2) pathway has been greatly advanced in the last two decades (Figure 1). Nrf2 is a master regulator of the antioxidant response and

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Conflict of interest statement

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xenobiotic metabolism through the regulation of a wide range of antioxidant and phase II detoxification genes [1, 2]. Nrf2 protects cells from stressors, including endogenous substances, reactive oxygen species, radiation, environmental toxins, and xenobiotics from food. Therefore, activation of the Nrf2 pathway might be a promising strategy for chemoprevention. This view is supported by a number of studies demonstrating that Nrf2 is essential for chemopreventive agents, such as sulforaphane and oltipraz, to block carcinogenesis and that Nrf2-deficient mice are more prone to chemical-induced cancer development [3–5]. Recent studies have demonstrated that Nrf2 also protects against chronic diseases such as cardiovascular disease, neurodegenerative diseases, and pulmonary injury [6–8]. However, the role of Nrf2 in human diseases is more complicated than originally thought. For example, Nrf2 promotes cancer cell survival and confers resistance to chemotherapeutics and radiotherapy, which has been coined as the “dark side of Nrf2” [9]. The paradox is not yet resolved and stimulates more research to rationalize the dual role of Nrf2 at different contexts.

Many structurally distinct chemicals are inducers of antioxidant and phase II detoxification enzymes (Table 1). Their common features include their electrophilicity and reactivity to sulfhydryl groups [10]. With the discovery of the antioxidant response element (ARE), Nrf2 and Kelch-like ECH-associated protein 1 (Keap1), the molecular mechanisms of Nrf2 activation have been unraveled [1, 11–14]. Some Nrf2 activators exhibit promising efficacy in preventing or treating chronic diseases, including cancer. However, the “dark side” of Nrf2 and off-target side effects of Nrf2 inducers must be taken into consideration when applying them in clinical trials.

2. Regulation of the Nrf2 pathway

2.1 The classical Nrf2-Keap1 signaling pathway

As depicted in Figure 2, Keap1 is a key Nrf2 repressor and plays a pivotal role in regulating the Nrf2 signaling pathway [12, 13, 45, 46]. Nrf2 has two binding motifs in the Neh2 domain, the ETGE and DLG motifs, and recruits two molecules of Keap1 in the absence of stimuli [47, 48]. Keap1 serves as a bridge between Nrf2 and ubiquitination ligase Cullin-3 (Cul-3), which is required for the ubiquitination of lysines in the Neh2 domain and subsequent proteasomal degradation [45, 46, 49, 50]. The negative regulation of Nrf2 by Keap1 has been confirmed in mouse models. *Keap1*-knockout mice express constitutively high levels of Nrf2, and select heterozygous *Keap1* mutations abrogate the repressive effects of wild-type Keap1 on Nrf2 [51, 52].

Oxidative stressors or electrophiles inhibit the ubiquitination-dependent degradation and increase nuclear accumulation of Nrf2. As a cysteine-rich protein, Keap1 is an excellent sensor for chemical inducers [53]. Accumulating evidence lead to the “cysteine code” hypothesis, which proposes that different classes of Nrf2 activators have unique preferences in modifying specific cysteines (reviewed in [54]). Sulforaphane and tBHQ activate Nrf2 in a Cys151-dependent manner, whereas endogenous alkenal metabolites prefer Cys273/288 [17, 34, 55]. Cysteine modifications alter the proper conformation of the Keap1-Nrf2-Cul3 complex but do not dissociate Keap1 from the Neh2 domain of Nrf2 [56]. In the “hinge & latch” model, Nrf2 activators disrupt the relatively weak interaction between Keap1 and the

DLG motif, but not the one between Keap1 and the ETGE motif [47, 48]. The switch from two-site to one-site binding inhibits Nrf2 ubiquitination, thereby rescuing Nrf2 from degradation. Nrf2 accumulates in the nucleus, where it dimerizes with small Maf proteins and binds to the ARE *cis*-regulatory sequences to trigger transcriptional expression [1, 57]. A large number of genes have been identified as downstream targets of Nrf2, including NAD(P)H:Quinone Oxidoreductase 1 (NQO1) and certain glutathione S-transferases (GSTs) (reviewed in [58]).

2.2 Posttranslational regulation of Nrf2

Phosphorylation of Nrf2 can be detected by radioactive ^{32}P labeling, phosphorylation-specific antibodies and mass spectrometry [59, 60]. A number of studies have examined the upstream regulators of Nrf2 phosphorylation, including protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), PKR-like endoplasmic reticulum kinase (PERK), phosphatidylinositol 3-kinase (PI3K), and glycogen synthase kinase-3 (GSK-3). PKC phosphorylates Nrf2 at Ser40, a residue located in the Neh2 domain that binds Keap1. PKC activates the Nrf2 pathway, likely through disturbing the Nrf2-Keap1 interaction [60–62]. The effects of MAPKs on Nrf2 signaling appear to depend on the specific MAPK. In general, activation of JNK1 and ERK2 promotes the Nrf2 pathway, whereas activation of p38 is inhibitory [63]. Multiple sites in Nrf2 are phosphorylated by various forms of MAPKs (JNK1/2, ERK2, p38, and MEKK3/4) in HEK293T cells, but the Nrf2-mediated ARE response is unaffected by mutation of these sites [60]. This finding raises the question whether MAPK-mediated activation of Nrf2 occurs through direct phosphorylation. PERK enhances the nuclear accumulation of Nrf2 under endoplasmic reticulum stress [64]. PI3K also promotes the nuclear translocation of Nrf2 and induces the expression of *ARE*-containing genes [65]. By contrast, GSK-3, negatively regulated by PI3K, inhibits Nrf2 by promoting its degradation [66–69]. GSK-3 catalyzes the phosphorylation of Nrf2 at the Neh6 domain and decreases its stability independent of Keap1-mediated degradation [68]. Taken together, protein kinases play a crucial role in Nrf2 signaling. However, it remains unclear whether the phosphorylation of critical Nrf2 residues directly affects Nrf2 signaling. Protein kinases might regulate the Nrf2 pathway through direct phosphorylation or indirect signaling cascades, which need to be dissected in the future.

2.3 Regulation of Nrf2 by p62, p21 and IQGAP1

Several studies have revealed the important role of p62 in the Nrf2-Keap1 pathway. The p62 protein, also named as sequestosome 1 (SQSTM1), is involved in autophagy by regulating the formation of protein aggregates [70]. p62 competes with Nrf2 for binding with Keap1 through its STGE motif [71–74]. p62 also sequesters Keap1 in the autophagosome, leading to the autophagy-dependent degradation of Keap1 [71–75]. Consequently, Keap1-mediated Nrf2 ubiquitination is attenuated, and Nrf2 stability is increased. Overexpression of p62 in HEK293 cells increases both the protein level of Nrf2 and the mRNA expression of its target genes HO-1 and NQO1 [73]. In the livers of autophagy-deficient mice where p62 levels are high, the expression of Nrf2 and its downstream genes are markedly elevated [71, 76]. Persistent activation of p62 and Nrf2 contributes to diseases such as liver dysfunction and hepatocellular carcinoma, possibly through abnormal autophagy [71, 75–77]. Interestingly, arsenic induces Nrf2 in a p62-dependent manner, whereas sulforaphane and tBHQ are

independent of p62 [44, 72]. The crosstalk between p62 and the Nrf2-Keap1 pathway provides an emerging pathway to examine the role of Nrf2 in carcinogenesis.

Direct interaction between Nrf2 and $p21^{Cip1/AF1}$ contributes to the basal and inducible antioxidant response [78]. Chen *et al*. revealed the binding of p21 to Nrf2 at the DLG and ETGE domains, both of which are required for Keap1-mediated ubiquitination and degradation [78]. In addition, the authors reported that ubiquitination of Nrf2 is enhanced in p21-deficient HCT116 cells under both basal and tBHQ treated conditions, leading to decreased stability of Nrf2. It is likely that p21 alters the conformational structure of the Keap1-Nrf2 complex. Loss of p21 substantially reduces the basal and induced levels of Nrf2 protein and its target genes in HCT116 cells and mouse livers.

We recently identified the IQ motif-containing GTPase-activating protein 1 (IQGAP1) as a binding partner of Nrf2 through the IQGAP1 IQ domain (amino acids 699–905) [79, 80]. IQGAP1 is a scaffold protein that interacts with proteins such as actin, E-cadherin, β-cadherin, and calmodulin [81]. IQGAP1 is an important component in maintaining cytoskeletal architecture, cell-cell adhesion, and Ca^{2+} signaling [81]. We demonstrated that ectopically expressed IQGAP1 enhances the stability of GFP-Nrf2, and conversely, IQGAP-1 deficiency decreases Nrf2 stability [79]. Treatment with Ca^{2+}, an agonist for IQGAP1 signaling, promotes the nuclear accumulation of the IQGAP1-Nrf2 complex and induces the expression Nrf2 target genes, such as GSTpi, GCLC and NQO1. We also demonstrated that MEK-ERK-mediated Nrf2 phosphorylation is attenuated in IQGAP1 knockdown cells [80]. Given that a direct interaction between IQGAP1 and both MEK and ERK has been reported [82], we speculate that IQGAP1 might provide a platform for the crosstalk between MAPK and Nrf2 signaling.

2.4 Transcriptional regulation

Regulation of Nrf2 at the transcriptional level is far less studied compared to its regulation at the protein level. DeNicola *et al*. demonstrated that oncogenic alleles of *Kras*, *Braf* and *Myc* induce mRNA expression of Nrf2 and its target genes [83]. The transcriptional upregulation may involve the binding of Myc and Jun to the *Nrf2* promoter, although detailed molecular mechanisms are not clear. Enhanced Nrf2 expression leads to the reduction of intracellular ROS and may provide a more favorable environment for cancer cell proliferation.

2.5 Translational regulation

We have reported the translational machinery that controls protein synthesis of Nrf2 [84]. Human Nrf2 mRNA contains an internal ribosomal entry site (IRES) at the 5’ untranslated region that is required to initiate the internalization of Nrf2 mRNA into ribosomes for protein synthesis. In addition, an inhibitory element (IE) exists upstream of the IRES, blocking ribosomal internalization of mRNA. Hydrogen peroxide and sulforaphane treatment induces the entry of Nrf2 mRNA into polysomal fractions and augments its translation in an IRES-dependent manner. These findings suggest that the Nrf2 translation efficiency is low in normal conditions and markedly increases under stress such that cells can consume energy efficiently for protein synthesis and degradation.

2.6 Epigenetic regulation

Accumulating research demonstrates that the transcriptional expression of some specific genes is controlled by epigenetic modification involving chromatin structural alterations. Epigenetic modulations can be caused by DNA methylation, histone modifications, and microRNAs [85]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group to the 5′ position of the cytosine residue within CpG dinucleotides [86, 87]. Inappropriate DNA methylation of CpG islands in tumor suppressor genes and oncogenes has been observed in many cancer cells and is one of the potential carcinogenic mechanisms during the development of human cancers [85, 88–91].

Our group has reported that the transcription of Nrf2 is suppressed in prostate tumors of TRAMP mice and tumorigenic TRAMP-C1 cells due to the hypermethylation of select CpGs in the promoter of Nrf2 [92]. Repressive proteins, such as methyl-CpG-binding protein 2 (MBD2) and trimethyl-histone H3 (Lys9), are enriched in this region. Because DNA methylation is reversible, combined treatment with 5-aza-29-deoxycytidine (a DNMT inhibitor) and trichostatin A (a histone deacetylase inhibitor) restores Nrf2 expression in TRAMP-C1 cells [92]. Recently, a variety of bioactive nutrients, e.g. curcumin [21], tocopherols [93], sulforaphane [19, 20], 3,3′-diindolylmethane (DIM) [94] were found to modulate DNA methylation and/or histone modification, effectively restoring Nrf2 expression.

Recently, several microRNAs have been found to regulate the Nrf2-Keap1 pathway. MicroRNAs, transcribed from genetic loci, are small (20–22 nucleotides) non-protein-coding RNAs [95]. MicroRNAs regulate gene expression by inhibiting translation or inducing degradation of their target mRNAs [95]. Overexpression of four microRNAs, including miR-144, miR-153, miR-27a, and miR-142-5, either individually or as a group, can reduce Nrf2 mRNA and protein levels, leading to reduced glutathione production in neuronal SH-SY5Y cells [96]. MiR-144 and miR-28 mediate the degradation of Nrf2 mRNA by targeting the 3’-untranslated region (3’-UTR) [97, 98]. Moreover, miR-34a is elevated in the livers of aged rats, which is associated with suppressed expression of Nrf2 [99]. The negative regulation of miR-34a on Nrf2 was further confirmed by the transfection of miR-34a in HEK293 cells. In addition, miR-200a mediates the degradation of Keap1 mRNA in breast cancer cells, and the reduction of Keap1 activates the Nrf2 pathway [100].

3. Nrf2 activators: diverse structures and distinct mechanisms

Nrf2 activators comprise a range of structurally diverse chemicals, classified as isothiocyanates, Michael reaction acceptors, dithiolethiones, oxidizable diphenols/phenylenediamines/quinones, thiocarbamates, polyenes, hydroperoxides, trivalent arsenicals, heavy metals and dimercaptans (Table 1) [10, 101]. Keap1 is the primary target of Nrf2 activators, most of which are electrophilic or reactive to thiol groups. Select Keap1 cysteine residues undergo covalent modifications depending on the nature of the Nrf2 inducers [15, 36, 37, 53, 56]. It is intriguing whether the position of the modified cysteine affects the subsequent biological effects. In addition to Keap1, some Nrf2 activators also modulate other machinery in Nrf2 signaling, as discussed in the preceding section.

3.1 Nrf2 activation and bioactive nutrients

The human diet provide a wide variety of bioactive nutrients that posses health beneficial effects and able to activate the Nrf2 signaling pathway. Isothiocyanates (cruciferous vegetables) [102], organosulfur compounds (garlic and onions) [103], polyphenols (green tea and spice turmeric) [28], and isoflavones (soy beans) [104] have been characterized as potent Nrf2 activators. In general, these naturally occurring compounds can stimulate various upstream kinases, interfere the Keap1-Nrf2 interaction, and/or disturb cellular redox balance, resulting in the activation of the Nrf2 pathway. Administration of these compounds, i.e., sulforaphane [105], curcumin [106, 107], daillyl trisulfide (DATS) [24], epigallocatechin-3-gallate(EGCG) [108, 109] and genistein [31], have been reported to be protective against carcinogenesis, neurodegeneration, cardiovascular disease and diabetic neuropathy in rodent models in part through activation of the Nrf2 pathway. Consuming sufficient amount of fruits and vegetables is not only to meet the nutritional needs but also to boost defense capacity against many oxidative stress and inflammation-associated disease.

Sulforaphane, an isothiocyanate, can be digested from cooked cruciferous vegetables and a variety of oral supplements containing purified sulforaphane or broccoli sprout extract [82–86]. Sulforaphane activates the Nrf2-Keap1 pathway through direct modifications of critical Keap1 cysteines, especially Cys151, as evidenced in mass spectrometry analysis, site-directed mutagenesis and *in vivo* experiments [15–17, 34, 110]. Sulforaphane treatment also promotes the ribosomal internalization of Nrf2 mRNA for protein synthesis [84]. Recently, we demonstrated that sulforaphane restores Nrf2 mRNA expression epigenetically through the demethylation of promoter CpGs in TRAMP-C1 and JB6 cells [19, 20]. Following acute or long-term administration of sulforaphane, antioxidant and phase II drug metabolizing enzymes are induced in the liver, intestines, skin, prostate, and blood lymphocytes [111–117]. Sulforaphane has been shown to be protective against carcinogenesis in various types of carcinogen-induced and transgenic cancer models (reviewed [58, 118]). Clinical studies have demonstrated that the consumption of broccoli sprout extract changes the disposition of aflatoxin-DNA adducts and is well-tolerated in humans [119–121].

Curcumin, a polyphenolic phytochemical, is one of the most active components of Curcuma longa (turmeric or curcuma), which is a rhizomatus monocotyledonous perennial herbaceous plant member of the ginger family (Zingiberaceae) [122]. Evidence has demonstrated that curcumin could strongly induce HO-1 and other protein members of Phase II detoxification through the activation of Nrf2/ARE pathway in different tissues [123]. In a long term in vivo study, curcumin showed cancer prevention effect by inducing phase-II antioxidant enzymes via activation of Nrf2 signalling, restoration of tumour suppressor p53 and modulation of inflammatory mediators[106]. After intraperitoneal injection of curcumin to mice, it highly induced Nrf2/ARE activity in intestine, liver, kidney and spleen [107]. Curcumin was found to induce HO-1 expression via activation of Nrf2 by binding to cysteine residue of Keap1 [22]. Curcumin were also found to increase NQO1 expression and the binding activity of Nrf2 to antioxidant response element (ARE) [23]. Our recent epigenetics study found that curcumin restores the expression of Nrf2 via demethylation of its CpGs promoter region [21].

DATS, an organosulfur, is one of the major constituents in garlic oil. In in vitro and in vivo experiments, DATS activates Nrf2 and induces HO-1 and NQO1 expression which appears to be mediated through modification of the Keap1 Cys288 residue [24]. DATS-induced ROS production and the subsequent activation of upstream kinases may also be related to the activation of Nrf2 [24]. When cardiomyocytes exposed to high glucose were treated with DATS, it showed protection against hyperglycemia-induced ROS-mediated apoptosis by increase of HO-1 and Nrf2 expression via upregulating the PI3K/Akt/Nrf2 pathway [25].

EGCG, a polyphenol found abundantly in green tea possesses anti-oxidative stress and chemopreventive activities [124]. Using in vitro and in vivo studies, EGCG has demonstrated its effect in increasing NRF2 expression as a possible chemopreventive agent for cancer [109] or lupus nephritis [108]. Many publications have revealed the mechanism of EGCG on Nrf2 pathway. EGCG has demonstrated its protective effect on human umbilical vein endothelial cells from PM2.5-induced oxidative stress injury by upregulating Nrf2/HO-1 via activation of the p38 MAPK and the ERK1/2 signaling pathways [28]. Na et.al. found that EGCG induces Nrf2-mediated expression of MnSOD and HO-1 and activation of ERK1/2 and PI3K/Akt in MCF10A cells [30]. EGCG could promote the nuclear translocation of Nrf2 and EGCG were found to promote the dissociation of Nrf2 from Keap1 [29]. Kanzaki et.al. reported that SFN and EGCG augmented the nuclear translocation of Nrf2 and the expression of HO-1 in mouse monocytic cell line [26]. Wang et.al. also found EGCG promotes Nrf2 nuclear translocation in normal rat kidney proximal tubular epithelial cell line NRK-52E [27].

Genistein is a major soy isoflavone in soy products. Low dose genistein was demonstrated to exert profound neuroprotection, antioxidant and cognitive function preservation effects in rats via enhanced eNOS activation and upregulation of the Nrf2/HO-1 via increaseing keap1 S-nitrosylation, nuclear accumulation of Nrf2 and enhanced DNA binding activity of Nrf2 [31]. Genistein was found to protect cerebrovascular endothelial cells from oxidative damage by activation of Nrf2 signaling pathway via modulating PI3K activity [32].

3.2 Nrf2 activation and other compounds

Synthetic oleanane triterpenoids such as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid **(CDDO)** and its derivatives are the most potent Nrf2 inducers ever discovered, activating Nrf2 signaling at nanomolar concentrations [33, 125]. These compounds belong to the Michael reaction acceptors and are reactive to nucleophiles containing –SH groups when incubated with dithiothreitol (DTT), cysteine, or reduced glutathione [126]. Given the chemical properties of these compounds, they very likely modify one or more Keap1 cysteine residues, although the exact ‘cysteine code’ remains to be elucidated [34, 35]. Oral administration of CDDO-Im, an imidazole derivative of CDDO, elevates the expression of HO-1 and NQO1 in the liver and other organs in mice in an Nrf2-dependent manner [33, 127]. A variety of CDDO derivatives are effective for preventing or treating cancer in preclinical models (reviewed in [128]). In addition, CDDO-Im also provides protection against kidney injury, emphysema and cardiac dysfunctions through the Nrf2 pathway [129, 130].

Oltipraz is a synthetic dithiolethione, which is a class of organosulfur compounds. Dithiolethiones are reactive to thiols and potentially modify Keap1 cysteine residues [131, 132]. 3H-1,2-dithiole-3-thione, a dithiolethione structurally similar to oltipraz, induces intermolecular disulfide bonds between two Keap1 molecules at Cys273 and Cys288 [36]. Other studies suggest that dithiolethiones activate the Nrf2 pathway through the generation of H_2O_2 or other superoxides, which are another class of Nrf2 inducers [132–134]. Oltipraz stimulates a battery of antioxidant and phase II detoxification enzymes, such as NQO1, GSTs and UGTs, and elevates GSH *in vitro* and *in vivo* [3, 4, 135, 136]. Oltipraz, an ancient compound used in early chemoprevention studies in the 1980s [137–140], suppresses tumorigenesis induced by a broad range of carcinogens (reviewed in [131, 141]). Its chemopreventive efficacy in carcinogen-induced models appears to depend on Nrf2, as its efficacy is lost in Nrf2 knockout (KO) mice [3, 4, 135]. A clinical study reported that oltipraz increased aflatoxin-mercapturic acid conjugates in urine in subjects with high exposure to aflatoxin B1, indicating faster excretion of aflatoxin B1 [142].

tBHQ and tBQ are oxidized products from butylated hydroxyanisole (BHA), which is an oxidizable diphenol and commonly used as an antioxidant food preservative. Cys151 of Keap1 is a specific sensor for tBHQ. Nrf2 induction by tBHQ is diminished in cell culture and zebrafish by the ectopic expression of Keap1-C151S mutants, as well as in mouse embryonic fibroblasts (MEFs) and primary peritoneal macrophages derived from transgenic mice expressing the Keap1-C151S mutant [17, 34, 38, 110]. Although tBHQ-Cys151 adduct has not been detected by mass spectrometry, the oxidized quinone metabolite of tBQ forms a covalent adduct with Keap1 at Cys151 in a cell-free system [37]. Furthermore, tBHQ enhances the ubiquitination of Keap1, possibly from the switch of Cul3-mediated ubiquitination from Nrf2 to Keap1, leading to the stabilization of Nrf2 protein [39]. Nrf2 induction by BHA and tBHQ is associated with the activation of JNK1 and ERK2, respectively [40]. BHA regulates a wide array of genes involved in phase II detoxification, ubiquitination, transporters and protein kinases in the small intestine and liver in mice through an Nrf2-dependent pathway [143]. Notably, the *in vivo* effects of BHA might be due to the combined action of BHA itself and its oxidized metabolites such as tBHQ and tBQ, which are more potent Nrf2 activators [144, 145]. The health concerns of the use of BHA as a food additive have been debated since the 1960s. One early study reported that BHA feeding (0.5% w/w) for 60 weeks inhibited ciprofibrate-induced hepatic tumorigenesis in rats [146]. By contrast, other studies found that BHA increased the toxicity of other chemicals or radiation, but BHA feeding (0.4%) alone for 104 weeks did not lead to carcinogenesis in rats [147, 148].

Arsenic is a ubiquitous environmental toxin, found in ground water, soil, and air. Arsenic exposure leads to prolonged Nrf2 activation through unique mechanisms that are not observed in sulforaphane or tBHQ treatment [41, 44]. Nrf2 induction by arsenic is associated with elevated p62 and the accumulation of autophagosomes, and knockdown of p62 diminishes the induction [44]. The proper interaction of Nrf2 and Keap1 is likely to be disordered due to the Keap1-p62 interaction and the sequestration of Keap1 in autophagosomes during arsenic-induced autophagy deficiency [44, 73]. The half-life of Nrf2 is markedly extended by approximately 10-fold when Nrf2 ubiquitination is inhibited by

arsenic [41]. Although Nrf2 deletion rescues liver dysfunction in autophagy-deficient mice, it is not clear whether prolonged Nrf2 activation contributes to arsenic toxicity. By contrast, Nrf2 deficiency exacerbates arsenic toxicity in liver and bladder [149].

4. Nrf2 and cellular functions

4.1 Redox balance and xenobiotic metabolism

Reactive oxygen species (ROS), such as superoxides, hydrogen peroxide, and hydroxyl free radicals, are constantly produced in aerobic organisms. Aerobic respiration, during which some electrons leak from the electron transport chain and activate oxygen molecules, is the major source of ROS. Exposure to radiation, ultraviolet light, tobacco smoke, or metal can also trigger ROS production. Nitric oxide (NO), a reactive nitrogen species (RNS) generated by a group of NO synthases, has important physiological functions in regulating muscle contraction, inflammatory response, vasodilation, and platelet aggregation. Balanced cellular ROS/RNS is essential to maintain normal physiological processes, whereas excess amounts of ROS/RNS are harmful to intracellular macromolecules and are associated with chronic diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), diabetes, cardiovascular disease, inflammatory diseases, and cancer [150]. Mammalian cells have evolved with complicated antioxidant systems for survival, consisting of non-enzymatic antioxidants such as vitamin C and E and inducible antioxidant enzymes. Nrf2 coordinates the expression of ARE-containing genes, including superoxide dismutases (SODs), glutathione peroxidase (GPx), NQO1, heme oxygenase-1 (HO-1), and many enzymes involved in glutathione production, which can respond quickly to oxidative stress and maintain a balanced redox state in cells [151].

Humans also face carcinogenic and mutagenic stresses caused by xenobiotics from environmental pollutants. In general, xenobiotics undergo metabolism through two distinct phases: phase I metabolism that includes oxidation, reduction and hydrolyses, and phase II metabolism that comprises conjugation reactions such as glucuronidation, glutathione conjugation and sulfation [152]. Phase I metabolism of xenobiotics often produces carcinogenic intermediates, as exemplified by benzo[*a*]pyrene and aflatoxin [153, 154]. Phase II metabolism often yields more water-soluble and fewer toxic metabolites. Nrf2 controls many phase II metabolizing enzymes, such as the GST family, the sulfotransferase 3A family, and the UDP-glucuronosyl transferase (UGT) family [58, 151]. Indeed, Nrf2 deficiency predisposes the toxicity of various carcinogens such as benzo[*a*]pyrene and aflatoxin [5, 135].

4.2 Inflammation

Excessive and chronic inflammation contributes to many acute and chronic diseases, including autoimmune, neurological and cardiovascular diseases and cancer [155, 156]. Increasing evidence suggests that Nrf2 may also protect against inflammation as well as oxidative stress [157–163]. Nrf2 mitigates chemical-induced pulmonary injury and inflammation [164, 165]. Severe tobacco smoke-induced emphysema, airway inflammation and asthma in mice with genetic ablation of Nrf2 may be caused by the reduction of antioxidant gene expression and the induction of interleukin (IL)-4 and IL-13 in

bronchoalveolar lavage fluid and in splenocytes [166, 167]. Nrf2 is also involved in the modulation of the innate immune response, as demonstrated in Nrf2-deficient MEFs [168]. Nrf2 may block lipopolysaccharide (LPS)-induced ROS generation of tumor necrosis factor alpha (TNF-α), IL-6 and chemokines (Mip2 and Mcp-1) in mice peritoneal neutrophils [169]. Compared with wild-type mice, LPS stimulates a high level of inflammatory-related signals, such as TNF-α, IL-1, cyclooxygenase 2 (COX-2), and iNOS, in primary peritoneal macrophages in Nrf2-KO mice [170, 171]. More severe DSS-induced colitis was observed in the colon tissues of Nrf2-KO mice than in wild-type mice, and a lower induction of phase II antioxidant and detoxification enzymes and a higher induction of pro-inflammatory biomarkers were observed in Nrf2-KO mice [160]. This finding suggests that Nrf2 may indirectly protect cells from inflammatory damage through antioxidant activation [169, 172]. NF-κB is a key regulator in the innate immune/inflammatory pathway, and the activation of NF-κB has been demonstrated in many cancer types [173, 174]. When cells are exposed to various stimuli, such as TNF-α, IL-1, H_2O_2, LPS, or microbial infection, the induced proteasome-mediated degradation of IκB proteins leads to the translocation of NF-κB from the cytoplasm to the nucleus [175, 176]. Activated NF-κB may further trigger the expression of downstream target genes, including various inflammatory cytokines and chemokines, adhesion molecules, COX-2, and NO synthase as well as other stress response genes [176–179]. This suggests that there is cross-talk among Nrf2, NF-κB and inflammation [180]. The Nrf2-ARE signaling pathway may be downregulated by pro-inflammatory signaling, as NF-κB could block the binding between CREB-binding protein (CBP) and Nrf2 or promote the interaction of HDAC3 with either CBP or MafK [181]. Additionally, higher activation of NF-κB in response to LPS and TNF-α has been observed in Nrf2-deficient MEFs compared with wild type MEFs [168]. NF-κB activation can also be affected by Nrf2 target genes such as HO-1, NQO1 and thioredoxin (TRX) [182–185].

4.3 Transporters and drug resistance

Transporters mediate the deposition of endogenous substances and xenobiotics, including drugs. For example, efflux transporters in the intestine and brain limit the permeability across the gastrointestinal tract and blood-brain barrier [186]. Overexpression of efflux transporters is a common mechanism of drug resistance, causing failure of cancer chemotherapy (reviewed in [187–189]). One superfamily of efflux transporters is the ATP-binding cassette (ABC) family, which uses the energy of ATP hydrolysis to pump xenobiotics out of cells against a concentration gradient.

A growing body of evidence suggests that Nrf2 is involved in regulating the expression of efflux transporters, especially those belonging to the ABC family. ARE-like sequences are identified in the promoters of genes encoding multidrug resistance-associated proteins (MRP), including Mrp1, Mrp2, Mrp3, Mrp4, and Abcg2 [190–193]. The binding of Nrf2 to AREs has been confirmed in chromatin immunoprecipitation (ChIP) or ChIP-seq experiments [190–194]. In addition, the Nrf2 activators tBHQ, butylated hydroxyanisole (BHA), oltipraz, and ethoxyquin induce the expression of MRPS in cultured cells as well as in mouse and rat livers [190, 193, 195–197]. Nrf2-knockout mice exhibit lower basal expression of Mrp3 and Mrp4 and are less sensitive to BHA and oltipraz-induced expression of Mrp2, Mrp3 and Mrp4 compared with Nrf2 wild-type mice [193, 198]. Constitutive

overexpression of Nrf2 confers chemoresistance in some advanced cancer cells, including human lung adenocarcinoma A549 cells and pancreatic carcinoma Panc1 and Colo357 cells. The knockdown of Nrf2 using siRNA or Nrf2 inhibitors sensitizes cellular responses to common chemotherapeutic drugs or natural compounds [199–202]. Collectively, Nrf2 is a potential target to circumvent chemoresistance.

4.4 Metabolic reprogramming

The distinct patterns of energy metabolism in normal and cancer cells have long been observed, but until recently, very little was known regarding the role of Nrf2 in this metabolic switch. Cancer cells tend to generate ATP through anabolic glycolysis of glucose rather than mitochondrial oxidative phosphorylation, a phenomena known as the Warburg effect [203]. Despite the reduced efficiency of energy production through glycolysis, the Warburg effect provides a variety of glycolytic intermediates that are needed to synthesize nucleosides and amino acids for cell proliferation and division [204, 205]. Recently, Mitsuishi *et al*. found that Nrf2 redirects glucose and glutamine metabolism in favor of the production of ribose-5-phosphate and NADPH through anabolic pathways [206]. In their study, several metabolic genes were identified as Nrf2 targets. Nrf2 activates glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), transketolase (TKT), transaldolase 1 (TALDO1), and malic enzyme 1 (ME-1) by binding their AREs. These findings provide mechanistic understanding of how Nrf2 supports cancer proliferation.

Substantial evidence has linked the impaired Krebs cycle (tricarboxylic acid cycle) to Nrf2 activation via the metabolite fumarate [207–209]. In the normal Krebs cycle, fumarate is converted to malate by fumarate hydratase (FH) and remains at a low cellular concentration. Accumulation of fumarate can form an adduct with Keap1 on its cysteine residues and provoke Nrf2 activation [207, 208]. In hereditary type 2 papillary renal cell carcinoma, the high level of fumarate caused by a loss-of-function mutation in FH is associated with sustained activation of Nrf2 [207, 208]. The disrupted Krebs cycle triggers metabolic reprogramming toward glycolysis [210]. It would be interesting to elucidate the detailed molecular mechanisms of Nrf2 in the metabolic switch.

5. Nrf2 and diseases

5.1 The protective role of Nrf2 in chronic diseases

The protective role of Nrf2 in carcinogenesis has been demonstrated in animal models of different types of cancer. In early chemoprevention studies, it was observed that chemopreventive agents modulate the disposition of carcinogens and inhibit carcinogenesis through induction of phase II metabolizing enzymes [138, 211, 212]. Nrf2 is required for sulforaphane and oltipraz to exert chemopreventive effects, as shown by comparing their efficacy in Nrf2 WT and KO mice [3, 4, 213]. Furthermore, Nrf2 deletion markedly increases susceptibility to carcinogen-induced tumor development in various organs, including colon, skin, breast, bladder and liver [5]. Nrf2-KO mice exhibit more pronounced inflammation in a dextran sulfate sodium-induced colitis model [160, 214, 215]. These

results strongly suggest that Nrf2 protects against tumorigenesis by modulating the disposition of carcinogens and inflammatory response.

The impact of Nrf2 in neurodegenerative diseases has been evaluated using mouse models of PD and AD. Nrf2-KO mice were more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of dopaminergic neurons, which is a key characteristic of PD [6, 216, 217]. Similar results were observed in the 6-hydroxydopamine-induced PD model [218]. Conversely, astrocyte-specific overexpression of Nrf2 abolished MPTP-induced neurotoxicity [6]. In a chronic neuroinflammation mouse model induced by long-term administration of MPTP, the levels inflammatory makers such as COX-2, iNOS, IL-6, and TNF-α were higher in the brains of Nrf2-KO mice than WT mice [219]. In transgenic APP/PS1 AD mice, the overexpression of Nrf2 in the hippocampus significantly improved learning and memory ability [220].

A number of studies have demonstrated that Nrf2 protects against cardiovascular diseases. Compared with WT mice, Nrf2-KO mice exhibit exaggerated cardiac hypertrophy, overt heart failure, and increased mortality caused by transverse aortic constriction [221]. Nrf2-KO mice exhibit more severe cardiac oxidative stress and cardiac hypertrophy after sustained administration of angiotensin II [7]. CDDO-Im treatment relieved smoke-induced cardiac dysfunction in Nrf2-WT mice, but the beneficial effects were not observed in Nrf2-KO mice [129]. Enhancing Nrf2 is a promising strategy to prevent the exacerbation of chronic obstructive pulmonary disease (COPD). Features of COPD include persistent inflammation and oxidative damage [8]. Nrf2-KO mice are more susceptible to cigarette smoke-induced emphysema [222]. Genetic ablation of Nrf2 enhances bronchoalveolar and airway inflammation in cigarette smoke-and ovalbumin-induced mouse models [215, 222]. Nrf2-KO mice also exhibit more severe pulmonary inflammation and lung tissue injury in mouse models of COPD exacerbation triggered by bacterial or viral infection [223–225]. Furthermore, sulforaphane enhances bacteria clearance and reduces pulmonary inflammation, which requires the involvement of Nrf2 [223].

5.2 The dark side of Nrf2 in carcinogenesis

Given the protective role of Nrf2 in counteracting different stressors and toxins, it is logical to speculate that it also provides protection for cancer cells. Indeed, an increasing body of evidence demonstrates that Nrf2 is constitutively elevated in many types of cancer cells or tumor samples from cancer patients [161, 226–232]. Overexpression of Nrf2 is associated with poor prognosis in cancer patients [233–235]. A recent study reported that urethane-induced lung tumors from Nrf2-KO mice failed to engraft in nude mice, whereas those from Nrf2 WT mice grew progressively [236]. Knockdown of Nrf2 led to cell cycle arrest at the G1 phase in A549 and NCI-H292 lung cancer cells [201]. Nrf2 might be required to sustain proliferative signaling and reprogram energy metabolism, which are hallmarks of cancer [201, 206, 237].

Several molecular mechanisms have been elucidated for the constitutive expression of Nrf2 in cancer. Somatic mutations in Keap1 or Nrf2 occur frequently in lung, ovarian, gallbladder, liver and gastric cancer (reviewed in [151, 238, 239]). Loss-of-function mutations in Keap1 weaken its binding affinity to Nrf2 and attenuate its repressive effect on

Nrf2 [226, 227]. It is noteworthy that mutations in Nrf2 occur predominantly in the DLG or ETGE motif in the Neh2 binding domain, thereby enabling Nrf2 to escape from Keap1-Cul3-mediated ubiquitination and degradation [202]. In addition, epigenetic silencing of Keap1 due to CpG hypermethylation in its promoter has been observed in some types of cancer [229, 240, 241]. Furthermore, defective autophagy in cancer causes abnormal autophagic degradation of Keap1 and impaired Keap1-Nrf2 interaction with the involvement of p62 [71–75]. Additionally, endogenous expression of the oncogenes Kras, Braf and Myc stimulates the transcriptional expression of Nrf2 in MEFs, which may also occur during tumorigenesis [83]. Collectively, the normal Keap1-Nrf2 axis is severely disturbed by multiple factors in cancer.

6. The complexity of the Nrf2 pathway: Strength and duration of activation, disease stages, and multiple targets of Nrf2 activators

Transcription factor activity is commonly a double-edged sword. The strength of its action is a crucial factor in determining which side is predominant. Kensler *et al*. proposed a U-shaped relationship of Nrf2 expression and cancer risk [242]. According this paradigm, extreme low or high Nrf2 levels increase disease risk. The U-shaped relationship effectively describes the observations that Nrf2 deletion increases susceptibility to carcinogens, neuronal toxins and bacterial and viral infection, whereas Nrf2 overexpression promotes drug resistance and cancer cell proliferation. Therefore, effective prevention occurs within a range between the biologically effective dose (BED) and the maximal-tolerated dose (MTD).

The duration of Nrf2 activation, which is determined by the underlying mechanism for activation, has profound biological consequences. For instance, chemical inducers and genetic modifications exhibit different response-time profiles for Nrf2 induction. Chemical inducers often exhibit a transient effect on Nrf2 pathway activation. We performed a pharmacokinetic-pharmacodynamic study of sulforaphane in rats and found that the expression of Nrf2-target genes peaks at 1–2 hours in blood lymphocytes after intravenous injection and returns to basal level after 24 hours [117]. By contrast, some chemical inducers have a prolonged effect on Nrf2 induction, likely through mechanisms other than the classical Nrf2-Keap1 interaction. One such example is arsenic, which recruits p62 and traps Keap1 in autophagosomes [44]. Furthermore, genetic factors such as Keap1 deletion, Keap1 mutation, Nrf2 mutation, and oncogenes generally lead to persistent activation of Nrf2, which is linked to the “dark side” of Nrf2 [239]. Transient Nrf2 activation through an intact Nrf2-Keap1 axis appears beneficial, whereas persistent Nrf2 activation might be harmful.

The dual role of Nrf2 is exemplified in different stages of tumorigenesis. Nrf2 blocks or delays tumorigenesis in normal and premalignant cells by relieving oxidative, mutagenic and inflammatory damage and by modulating carcinogen disposition. However, Nrf2 becomes undesirable when the defensive effects are hijacked by the malignant cells. The dual role is demonstrated in a recent study comparing WT and Nrf2-KO mice with urethane exposure [236]. Nrf2 prevents lung cancer initiation but enhances its progression in the late stage.

Many Nrf2 activators concomitantly exert multiple effects, such as inhibition of the NF-κB pathway and modulation of epigenetics, which appear to contribute to protective effects in addition to Nrf2 activation. For example, sulforaphane, 3H-1,2-dithiole-3-thione, and synthetic triterpenoid CDDO-Me repress the NF-κB mediated pro-inflammatory response by inhibiting the binding of NF-κB to DNA and the degradation of the NF-κB inhibitory protein IKK [171, 243–245]. In addition, several Nrf2 activators regulate epigenetic changes by affecting the enzymatic activity of HDACs and DNMTs. Class I, II and IV HDACs are zinc metalloproteins whose activity is dependent on Zn^{2+} [246]. Some Nrf2 activators interfere with HDAC activity, possibly through chemical characteristics (i.e., isothiocyanates, organosulfides and phenols) that may chelate Zn^{2+} [247]. Various Nrf2 activators inhibit the expression or the activity of DNMTs (i.e., sulforaphane and curcumin) or deplete the cellular pool of methyl donors (i.e., catechol polyphenols), resulting in altered DNA methylation [20, 21, 248]. The beneficial effects of Nrf2 activators may be due to a combination of Nrf2 activation, NF-κB inhibition and epigenetic regulation.

7. Conclusions and future prospects

Nrf2 plays a profound role in physiological and pathological processes. Nrf2 not only acts as a master regulator in the antioxidant response and xenobiotic disposition but also modulates the inflammatory response, metabolic programming, cell proliferation and survival. Nrf2 protects against many chronic diseases other than cancer, although the mechanism is not yet completely understood. Nutritional dietary phytochemicals such as sulforaphane, curcumin, DATS, EGCG and genistein have been reported to possess health beneficial effects and protective against carcinogenesis, neurodegeneration, cardiovascular disease and diabetic neuropathy through activation of the Nrf2 pathway. Future studies on the cross-talk between Nrf2 and other signaling pathways are needed. In addition, the dual role of Nrf2 in carcinogenesis raises concerns for the use of Nrf2 inducers in chemoprevention, although there is no evidence demonstrating that Nrf2 activation through an intact Nrf2-Keap1 axis initiates tumorigenesis. The difference between transient and constitutive activation of Nrf2 needs to be distinguished. Substantial evidence indicates that transient Nrf2 activation by pharmacological agents is beneficial. However, inhibition of the constitutive expression of Nrf2 may be a potential target to overcome drug resistance in cancer therapeutics. Furthermore, the structural information of Nrf2 remains to be unraveled, which will be helpful in designing better Nrf2 activators with high potency and reduced off-targets effects.

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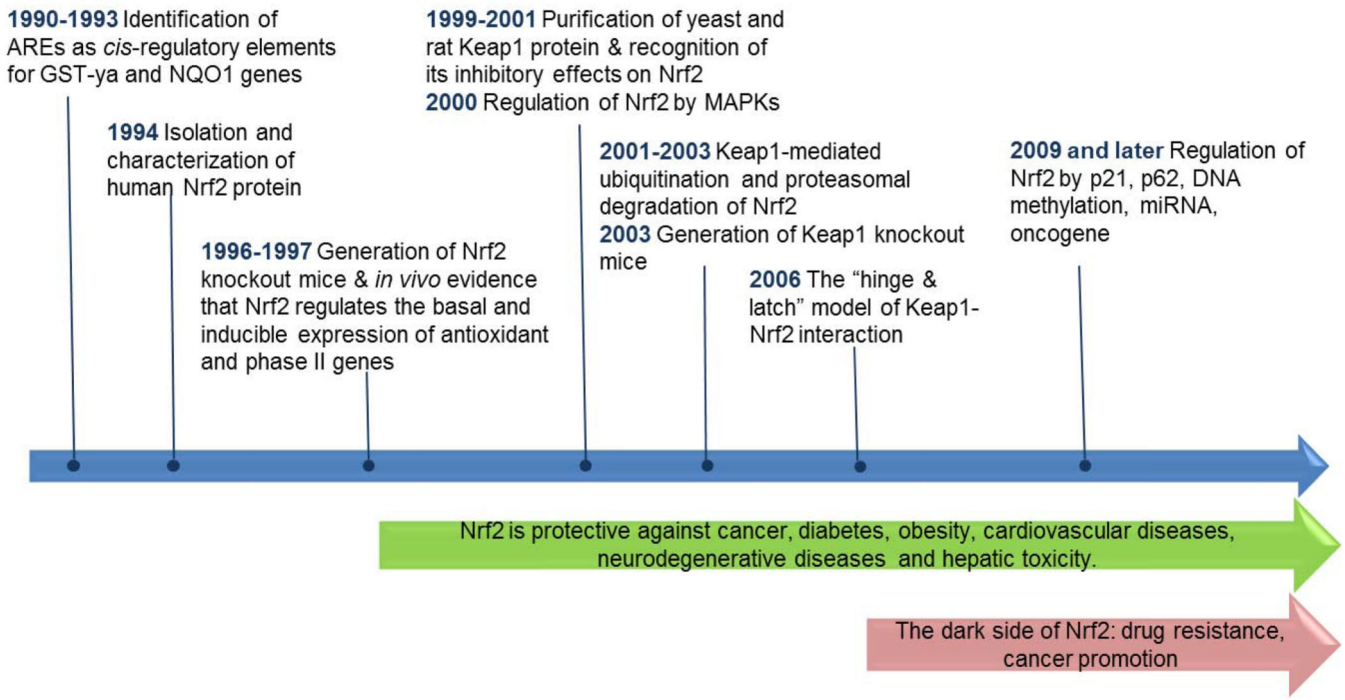


Figure 1.
Timeline of research advances in the Nrf2 pathway

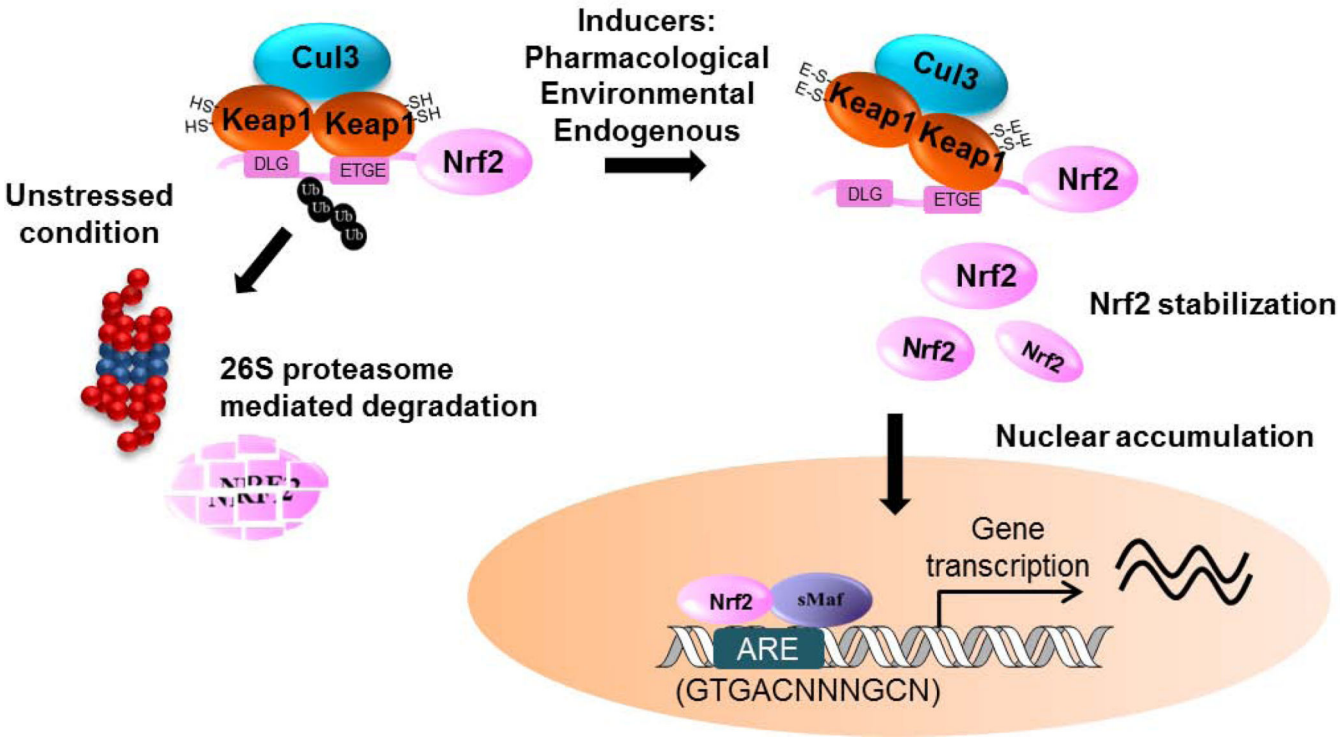
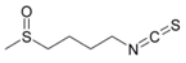
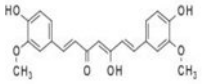
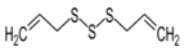
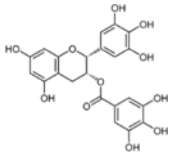
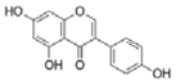
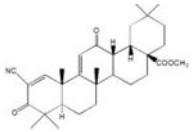
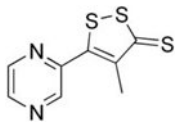
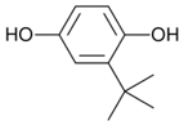


Figure 2.
Nrf2 signaling pathway schema diagram

Table 1

Selected Nrf2 activators

Compound	Structure	Class	Source	Mechanisms of Nrf2 induction	Reference
Sulforaphane		Isothiocyanates	Cruciferous vegetables	• Keap1-cys151 • Activation of ERK1/2 and Akt kinase • Acceleration of Nrf2 protein synthesis • De-methylation of Nrf2 promoter	[15–20]
Curcumin		phenols	ginger	• De-methylation of Nrf2 promoter • Binding with cysteine residue of Keap1	[21–23]
DATS		organosulfur	garlic	• Keap1-Cys288 • Inducing ROS production • Activation PI3K/Akt/Nrf 2	[24, 25]
EGCG		polyphenol	tea	• Activation of the p38 MAPK and the ERK1/2 signaling pathways • Dissociation of Nrf2 from Keap1 and Increasing the nuclear translocation Activation of ERK1/2 and PI3K/Akt	[26–30]
geneistein		isoflavone	lupin, fava beans, soybeans, kudzu, coffee and psoralea	• Increasing keap1 S-nitrosylation and enhancing DNA binding activity of Nrf2 • Increasing PI3K activity	[31, 32]
CDDO-Im		Michael reaction acceptors	Synthetic modification of natural product	• Keap1-cys(?)[1]	[33–35]
Oltipraz		Dithiolethiones	Synthetic	• Keap1-cys273, 288 • Generation of H_2O_2	[36]

Compound	Structure	Class	Source	Mechanisms of Nrf2 induction	Reference
tBHQ		Oxidizable diphenols	Synthetic	• Keap1-cys151 • Ubiquitination of Keap1 • Activation of JNK1 and ERK2	[17, 34, 37–40]
Arsenic trioxide	As^{3+}	Trivalent arsenicals	Environmental toxicant	• p62 dependent • Ubiquitination of Keap1 • Keap1-cys151[2] 273, 288	[41–44]

[1] Specific position of cysteine is not clear.

[2] Both dependence and independent on Keap1-cys151 were reported, probably due to different experimental conditions such as cell cultures, treatment time and choice of amino acid mutation.