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REVIEW ARTICLE

Nrf2-ARE stress response mechanism: A control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases

SHRUTI SINGH[1], SONAL VRISHNI[1], BRIJESH K. SINGH[1], IRFAN RAHMAN[2] & POONAM KAKKAR[1]

[1]*Herbal Research Section, Indian Institute of Toxicology Research (CSIR), PO Box-80, Mahatma Gandhi Marg, Lucknow-226001, Uttar Pradesh, India, and* [2]*Department of Environmental Medicine, Lung Biology and Disease Program, University of Rochester Medical Center, Rochester, NY, USA*

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Abstract
Nrf2, a redox sensitive transcription factor, plays a pivotal role in redox homeostasis during oxidative stress. Nrf2 is sequestered in cytosol by an inhibitory protein Keap1 which causes its proteasomal degradation. In response to electrophilic and oxidative stress, Nrf2 is activated, translocates to nucleus, binds to antioxidant response element (ARE), thus upregulates a battery of antioxidant and detoxifying genes. This function of Nrf2 can be significant in the treatment of diseases, such as cancer, neurodegenerative, cardiovascular and pulmonary complications, where oxidative stress causes Nrf2 derangement. Nrf2 upregulating potential of phytochemicals has been explored, in facilitating cure for various ailments while, in cancer cells, Nrf2 upregulation causes chemoresistance. Therefore, Nrf2 emerges as a key regulator in oxidative stress-mediated diseases and Nrf2 silencing can open avenues in cancer treatment. This review summarizes Nrf2-ARE stress response mechanism and its role as a control point in oxidative stress-induced cellular dysfunctions including chronic inflammatory diseases.

Keywords: *Oxidative stress, nuclear factor erythroid 2-related factor, kelch-like ECH-associated protein1, antioxidant responsive element, redox homeostasis, inflammation*

Abbreviations: *ABC, ATP binding cassette; AD, Alzheimer's disease; AGE, Advanced glycation end products; AKR, Aldoketoreductase; ALI, Acute lung injury; APAP, Acetaminophen; ARE/EpRE, Antioxidant response element/Electrophilic response element; BSO, Buthionine-(S,R)-sulphoximine; BTB/POZ, Broad complex Tram-track brick-a-brack/poxivirus and zinc finger; CAPE, Caffeic acid phenyl ester; COPD, Chronic obstructive pulmonary disease; DATS, Diallyltrisulphide; DGR, Double glycine repeat; EMSA, Electrophoretic mobility shift assay; GM-CSF, Granulocyte Macrophage- Coloney stimulating factor; GPx, Glutathione peroxidase; GR, Glutathione reductase; GS, Glutathione synthetase; GST, Glutathione-S-transferase; HAK, Hydroxyalkenals; HAT, Histone acetyltransferase; HD, Huntington's disease; HO-1, Heme oxygenase-1; HSF1, Heat shock factor 1; IRE, Iron responsive element; IVR, Intervening region; Keap1, Kelch like ECH-associated protein; LOX, 5-Lipoxygenase; MARE, maf recognition element; MDA, Malondialdehyde; Mdr, Multidrug resistance protein; MEF, Mouse embryonic fibroblasts; Mrp, Multidrug resistence-associated protein; NAPQ1, N-acetyl p-benzoquinoneimine1; Neh, Nrf2-ECH homology; NES, Nuclear export signal; NGF, Nerve growth factor; NLS, Nuclear localization signal; NOX, NAD(P)H oxidase; NQO-1, NAD(P)H quinine oxido-reductase 1; Nrf2, Nuclear factor erythroid-2 related factor; PD, Parkinson's disease; PDGF, Platelet derived growth factor; PI3K, Phosphotidyl inositol 3-kinase; RSV, respiratory syncytial virus; SLE, Systemic lupus erythromatosus; TBI, Traumatic brain injury; TNF-α, Tumour necrosis factor α; v-maf, musculo-aponeurotic fibrosarcoma*

Correspondence: Poonam Kakkar, Herbal Research Section, Indian Institute of Toxicology Research (CSIR), PO Box-80, Mahatma Gandhi Marg, Lucknow-226001, Uttar Pradesh, India. Tel: +91-522-2627586 Ext.269. Fax: +91-522-2628227. Email: poonam_kakkar@yahoo.com

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Introduction

Exposure to various xenobiotics requires quick clearance as most of them are highly toxic. Biotransformation reactions are associated with detoxification of these xenobiotics, rendering them properly such as hydrophobicity and change in biological activity. However, sometimes this process leads to the generation of more chemically active electrophiles as well as potentially toxic metabolites that generate stress. Oxidative and electrophilic stresses are implicated in toxic manifestations and pathogenesis of various diseases. In order to combat these stresses, cells have evolved tightly regulated defense systems at the molecular level. At the forefront of this system are a specialized group of proteins known as transcription factors. These regulate basal and inducible expression of numerous phase II detoxification genes, through the recognition of specific DNA elements known as antioxidant responsive element (ARE) within their promoter region. Different hepatotoxicants lead to differential gene response largely reflected by activation of different transcription factors. For example, macrophage activators induce STAT3 and NF-κB, peroxisome proliferators activate PPARα, whereas oxidative stressors or reactive metabolites stimulate nuclear factor erythroid 2-related factor (Nrf2) [1]. Nrf2 is widely activated in response to stimuli, such as oxidative and reactive species. The activation of Nrf2 leads to upregulation of an entire array of genes that impart protection against many drug candidates and pharmaceuticals.

This review is an attempt to encompass a comprehensive view about the current knowledge of the regulation of redox signaling mediated by Nrf2 and upregulation of oxidative stress responsive genes. It also summarizes Nrf2-ARE stress response mechanism as a promising candidate for therapeutic intervention through the expression of endogenous cytoprotective proteins.

Reactive oxygen species generation and oxidative damage

Generation of reactive oxygen species (ROS) within cells is strictly regulated, as an increase in ROS has deleterious effects like damage to cell structures, nucleic acid, lipids and proteins [2]. Ironically, ROS also helps in maintaining redox balance known as 'redox homeostasis' via redox signaling by influencing the expression of a number of genes and signal transduction pathways [3–5].

Superoxide anion radical ($O_2^{\cdot -}$) is considered as the 'primary' ROS which can further react with other molecules to generate 'secondary' ROS via enzyme or metal catalysed processes [6]. It is majorly produced in mitochondria at Complex I (NADH ubiquinone oxidoreductase) and Complex III (ubiquinol/cytochrome c oxidoreductase). However, it has been reported that in mitochondrial fractions deprived of SOD, sub-mitochondrial particles generate superoxide at a rate of 4–7 nmol/min/mg protein, indicating that the mitochondrial membrane is the most quantitatively important physiological source of superoxide in higher organisms [7]. Phagocytic cells produce superoxide via NAD(P)H oxidase (NOX), whereas in non-phagocytic cells this activity is inducible as $O_2^{\cdot -}$ production is 1–10% of that of phagocytic cells [8]. Similarly membrane-bound 5-Lipoxygenase (LOX), a mixed function oxidase involved in leukotriene synthesis, generates O_2^{-} and H_2O_2 through the intervention of small GTPase Rac and SOD isoforms [9]. Endoplasmic reticulum (ER) and peroxisomes are also probable sites of ROS generation as a number of peroxidases, oxidases, monoxygenases, dioxygenases and isoforms of the Cyt P450 superfamily are found here. Hydroxyl ($OH^{\cdot}$) radical is another free radical which has high reactivity and a half-life of ~10^{-9} s [10]. Under stress, superoxide facilitates hydroxyl radical production via Fenton's reaction. Peroxisomes maintain a delicate balance between H_2O_2 production and its decomposition to ensure that there is no net production of ROS. Upon peroxisomal damage this H_2O_2 is released in cytosol and contributes to oxidative stress. Transition metal ions such as iron and copper are kept sequestered in cytosol by a number of chelating proteins like ceruloplasmin, ferritin, transferrin and metallothionein as they also aggravate ROS generation. Recent work of Uchiyama et al. [11] suggests that lysosomal/endosomal compartments in cultured hepatocytes store a large pool of chelatable iron which is released in response to oxidative stress in cytosol. It is then taken up by the mitochondria, thereby playing an important role in augmenting cytotoxicity.

Oxidative stress ensues when the balance between oxidants and antioxidants tips towards the former causing oxidation of lipids, proteins and DNA [8]. If irreversible, thise damage ultimately leads to mutagenesis, carcinogenesis and is also implicated in ageing. Pro-oxidants readily react to form adducts, e.g. 8-hydroxydeoxyguanosine (8-OHdG), which is a biomarker of DNA damage [12]. The attack of ROS on polyunsaturated fatty acids of phospholipids leads to the formation of peroxidation products such as malondialdehyde (MDA), hydroxyalkenals (HAKs) and isoprostanes, where MDA has mutagenic and HAK's have toxic tendencies [8]. This results in subsequent fragmentation and degradation of membranes with the loss of its integrity. The attack of ROS on protein leads to oxidation of critical amino acid residues such as cysteine and methionine, formation of intrasulphide bonds, homodimer and heterodimer formation, cross-linking and proteasomal degradation of target proteins [12]. Formation of protein carbonyl is another measure of protein oxidation [13]. Accumulation of advanced glycation end products (AGEs) is also a manifestation of ROS-induced damage [8].

Activation of Nrf2

The dual character of ROS is exhibited by its role as a critical second intracellular messenger in several signal transduction pathways at low concentrations [14], whereas at high concentrations cells try to confront stress by switching on antioxidant response through a change in gene expression [15]. Nrf2 is one of the redox sensitive transcription factors that gets activated in response to deranged antioxidant state and tries to restore the balance by transcribing antioxidant and phase II detoxifying genes.

Nrf2 structure

Nrf2 is found in most tissues [16] but is abundant in brain, liver, kidney and in systems that are exposed to external environmental stresses like gastrointestinal tract, skin, etc. It was first identified by Moi et al. [16] during the screening of the proteins that bind to the locus control region of the β-globin gene. When the novel protein was sequenced it was found to be homologous with an earlier known factor called p45-NF-E2 [17]. Later, it was found that p45–NF-E2 was only expressed in megakaryocytes, erythroid and mast cells, whereas Nrf2 is expressed in nearly all tissues [18]. Nrf2 belongs to the cap'n'collar (CNC) family of transcription factors which are homologous to Drosophila CNC proteins having distinct basic leucine-zipper motif. Nrf-1, Nrf-3, Bach1 and Bach3 are also members of this family [18]. It is kept in the cytosol tethered by inhibitory protein known as kelch-like ECH-associated protein (Keap1), which was discovered in 1999 and re-named in 2001 as inhibitory Nrf2, i.e. I-Nrf2 [19–21]. In situations where the redox balance of the cell tilts towards the oxidant side, Nrf2 translocates to the nucleus.

Nrf2 has a basic leucine zipper (bZIP) region in its C-terminal domain which acts as a DNA binding motif. During structural analysis of Nrf2, a 600 amino acid protein, six functional Nrf2-ECH (Erythroid cell derived protein with CNC homology) homology domains called Neh were identified (Figure 1A). Three Neh domains (Neh1, Neh2 and Neh3) are evolutionarily conserved while remaining domains are associated with its negative regulation, activation, as well as dimerization with small Maf proteins and DNA binding [17]. The Neh2 domain present on N-terminal is responsible for its binding to Keap1. Neh4 and Neh5 adjacent to Neh2 are capable of interacting with the co-activator cAMP responsive element binding protein (CBP) and is responsible for transcriptional activation of target genes by Nrf2 [17]. Neh 6 domain is redox insensitive and is responsible for degradation of Nrf2 in oxidatively stressed cells [22]. Neh1 has bZIP motif that dimerizes with small Maf proteins and helps in binding with the DNA. Nrf2 has a half-life of 13–20 min [23] which is extended under stress situations.

In quiescent conditions, Nrf2 is sequestered by Keap1 which is a cytoskeleton anchoring dimeric protein. Keap1 is a homologue of Drosophila actin binding protein Kelch [24]. This 624 amino acids long Keap1 has five domains: (i) the N-terminal region (NTR, from 1–60 amino acids); and (ii) the BTB/POZ domain (Broad complex Tram-track brick-a-brack/poxivirus and zinc finger) is found in actin binding proteins. It mediates Keap1 homodimerization [25], polyubiqutination of Nrf2 and its subsequent 26S proteasome-mediated degradation in basal conditions; (iii) Intervening region (IVR), from 180–314 amino acids containing eight cysteines out of 102 amino acid residues; (iv) Double glycine repeat (DGR) or Kelch repeats region (315–598 amino acids)

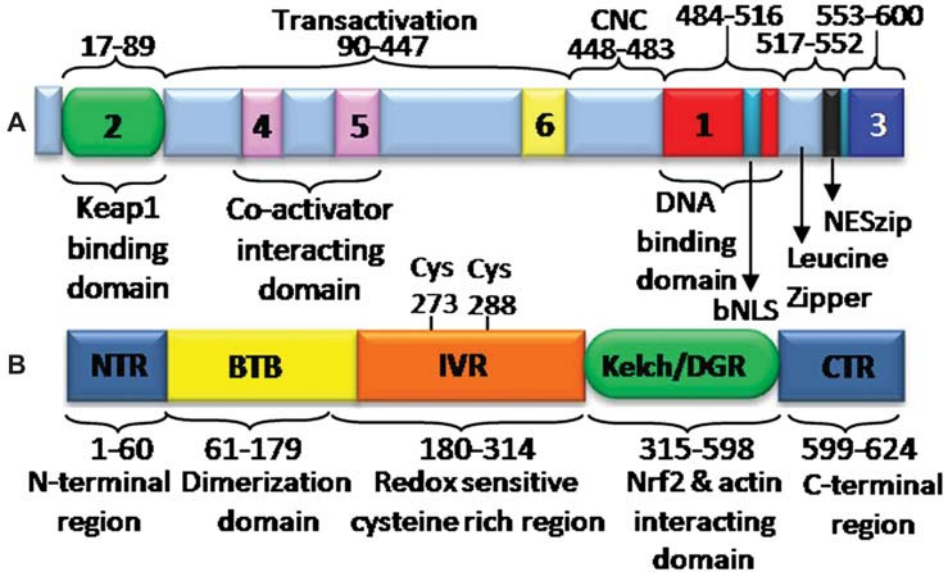

Figure 1. Schemetic structure of Nrf2-Keap1. (A) Domain structure of Nrf2: Neh-Nrf2-ECH homology, NLS-Nuclear Localization Sequence, NES-Nuclear Export Sequence. (B) Domain structure of Keap1: NTR-N- terminal region, BTB-Broad complex Tram-track brick-a-brack, IVR-Intervening region, DGR-double glycine repeat and CTR-C-terminal region.

containing multiple protein contact sites [21] and (v) C-terminal region (CTR), i.e. from 599–624 amino acids (Figure 1B). The DGR or Kelch domain has Nrf2 binding function along with its interaction with actin skeleton. Keap1 is sensitive to electrophilic and oxidative stimuli owing to presence of reactive cysteines. Keap1 forms a higher degree structural complex with Nrf2 and actin filaments that helps in its retention in cytosol via its network with cytoskeleton [26]. Keap1 holds back Nrf2 in cytosol, but this does not account for short life of Nrf2. The short half-life of Nrf2 is associated with its Keap1 mediated constitutive proteasomal degradation [27,28]. It is evident from the observation of Itoh et al. [23] that increased level of Nrf2 in cytosol is found when a proteasomal inhibitor MG132 is added. Keap1 also acts as an adaptor protein for ubiquitin proteasome complex named Cul3-based E3 ligase complex [29–32]. Nrf2 anchored to Keap1 is first ubiquitinated and then subjected to 26S proteasomal degradation. Ubiquitination involves three enzymes—E1-ubiquitin activating enzyme, E2-ubiquitin conjugating and E3-ubiquitin ligase enzyme. These enzymes act sequentially where E3 is bifunctional, i.e. (i) it targets substrate protein and (ii) catalyses ubiquitination. Keap1 recruits Cullin3 sub-type of E3 ligase to regulate Nrf2. 26S proteasome comprises of 20S and 19S sub-units whose expression is also transcriptionally induced in an Nrf2-dependent manner in mice liver [26]. Thus, Keap1 negatively regulates Nrf2 not only by its sequestration but also targeting it for proteasomal degradation.

Keap1-dependent Nrf2 activation

Interaction of Nrf2 with Keap1. In mammalian cells a single molecule of Nrf2 binds to the Keap1 dimer [33]. Nrf2 has two conserved motifs ^{29}DLG^{31} and ^{79}ETGE^{82} that interact with Arginine, Serine, Asparagine residues of DGR regions of Keap1 dimer in a non-overlapping manner [34]. The two ends of Keap1 dimer are hooked onto the two unique motifs of Nrf2 in a hinge and latch manner (Figure 2A). The hinge is provided by the high affinity ETGE motif and the latch by a low affinity DLG motif. These sites cause proper positioning of the target lysines of Nrf2 for ubiquitinization [34–36]. Under conditions of electrophilic and oxidative stress, ubiquitination is prevented most likely by loss of binding via DLG motif, as target Lys residues are displaced (Figure 2B) [37,38]. However, Nrf2 still clings to Keap1 dimer [34–36,39] via hinge motif. This further leads to saturation of Keap1 so that newly synthesized Nrf2 finally migrates to the nucleus to mount an antioxidant response [36–38], indicating that Keap1 negatively regulates Nrf2 not only by enhancing its rate of degradation but also by altering its subcellular localization. A completely different model proposed by Lee et al. [40] indicates an autoregulatory loop between Nrf2 and Keap1. According to this model, Nrf2 upon being activated also induces expression of Keap1. Studies reveal the presence of a functional ARE on the reverse strand of Keap1 promoter at position -46. Use of siRNA and mutations at -46 position have shown reduction in expression and induction of the Keap1 gene. This loop controls the cellular abundance of both Nrf2 and Keap1. Agents causing disruption of this loop have been found to increase Nrf2 activity and downstream gene expressions [40].

Keap1—a sensor of oxidative and electrophilic stress. Keap1 is found to be associated with two functions where on one side it acts as a sensor to oxidative and electrophilic stress and on the other it rapidly degrades Nrf2 via ubiquitination. Keap1 has 25 cysteine residues, all conserved in rats and humans [17]. Out of 25 cysteines, site-directed mutagenesis has highlighted the

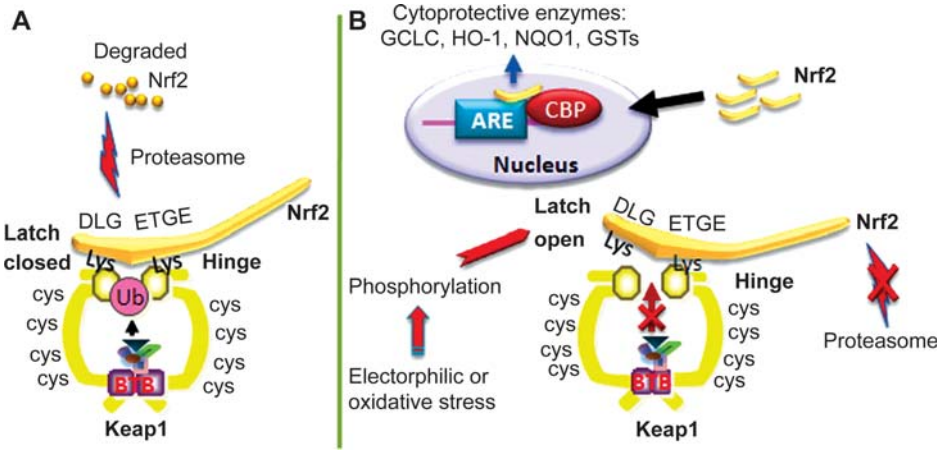

Figure 2. Hinge-Latch model for interaction between Nrf2-Keap1 and activation of Nrf2 under stress. (A) In quiescent state, Keap1 interacts with each other via BTB domain to dimerise while C-terminal localized Kelch/DGR domain of each interacts differentially with the N-terminal localized 'DLG' and 'ETGE' motifs of Nrf2. Here 'hinge' is provided by high affinity ETGE and 'latch' by low affinity DLG. (B) In case of high ROS levels, interaction between latch 'DLG' and DGR is disrupted causing displacement of Nrf2 and prevention of its ubiquitin-mediated proteasomal degradation leading to its nuclear accumulation and induction of cytoprotective genes.

role of three critical cysteine residues Cys-151, Cys-273 and Cys-288 [38]. Keap1 mutant lacking IVR domain has no Nrf2 repressive activity but it holds back Nrf2 in the cytosol. So, Cys-273 and Cys-288 do not control interaction of Nrf2 with Keap1. In a mutant study done to assess the effects of Cys-273 and 288 mutation on Keap1 and Nrf2 dissociation, the results revealed that no significant difference in the values of dissociation constant were observed (values were close to 10^{-9}). However, it does abolish Nrf2 repression activity and Nrf2 was found to be localized in the cytosol as it was not liberated from Keap1 owing to a lower nuclear entry. Thus, it could be concluded that Cys-273 and 288 are crucial for Nrf2 degradation and not dissociation [38]. Studies also reveal that oxidative stress and electrophilic stress do not affect the formation or degradation of Keap1-Nrf2 complex and that Nrf2 once trapped by Keap1 is never liberated. However, Cys-151 in BTB domain when substituted by serine failed to liberate Nrf2 even under stress conditions; hence, it may also act as an alternative oxidant sensor [37]. Therefore, it can be concluded that Cys-151 is highly reactive towards ARE inducers.

Nrf2 activation may also be a result of site-specific modification and ubiquitination of Keap1 by electrophiles [41] as a Nrf2-Keap1 complex is known to respond to oxidative stress in two ways. The first model suggests that electrophiles modulate the Keap1 structure leading to dissociation of Nrf2-Keap1 complex and subsequent nuclear localization of Nrf2 [17]. The second model suggests that modification of Keap1 Cys results in an altered E3 ubiquitin ligase function that causes reduction in Nrf2 degradation leading to compromised E3 activity and enhanced Nrf2 stability [42].

Post-translational modification-dependent Nrf2 activation

Phosphorylation of Nrf2. The exact role and underlying molecular mechanisms are poorly understood in the case of MAPK-induced Nrf2 activation as direct phosphorylation has been found to have a limited effect on Nrf2 signaling pathway [43]. *In vivo* studies revealed that Nrf2 is phosphorylated at multiple sites (S215, S408, S558, T559 and S577) by MAPKs but when all serines were substituted by alanine it caused a limited decrease in Nrf2 transcriptional activity [42]. S215, S408 and S577 formed consensus phosphorylational sites where S408 emerged to be the most important site. Complete abolition of Nrf2 activity was never observed focusing on phosphorylation redundancy. *In vitro* studies indicate an increased interaction between Nrf2 and Keap1 by p38 but under *in vivo* conditions phophorylation status of Nrf2 never affected Keap1-Nrf2 interaction.

Several studies propose that phosphorylation is carried out by multiple kinases (ERK, JNK, p38 and PI3K) on serine residue of Nrf2 N-terminal in response to oxidative stress and electrophiles [44]. The reports show use of various pharmacological inhibitors such as phosphatase inhibitor, okadaic acid. It is important to consider that these inhibitors may affect multiple signaling pathways which may integrate to affect Nrf2 activation. Hence, regulation of Nrf2 stability may present an important mechanism in the activation of ARE-dependent gene expression [45]. However, it still remains elusive whether a direct phosphorylation is involved in Nrf2 activation and whether *in vitro* results repeat under *in vivo* conditions as well. Additionally, over-expression of MAPKs may have a possible indirect role by controlling Nrf2 protein synthesis. As various components of translational assembly, e.g. eIF4E, eIF4B-BPI and eEF2 kinases are phosphorylated by MAP kinases [46–48], it may be possible that Nrf2 is regulated at the translational level where MAPK may have an indirect influence.

Nrf2 acetylation by p300/CBP. Recently a novel role of co-activators p300/CBP in Nrf2-dependent antioxidant gene regulation has been described. It has been reported that chromatin remodeling complex p300/CBP directly acetylates Nrf2 at specific Lysine residues. Any mutation in these residues does not affect the stability of Nrf2 but leads to a compromised transactivation and DNA binding capacity, suggesting the role of chromatin modification in Nrf2-dependent genes [49]. The Neh5 domain of Nrf2 contains a CBP-interacting motif that imparts them a considerable degree of transactivation while Neh4 transactivation increases Nrf2 affinity for ARE elements [50]. p300/CBP acts as a coactivator and helps in the recruitment of RNA Pol II, thus initiating transcription. p300/CBP has an intrinsic histone acetyl transferase (HAT) activity which makes them acetylate internal lysine residues of core histones. It acetylates all four core histones but preferentially acetylates histones H3 and H4 at their N-terminal tails. The hyperacetylated histones induce chromatin remodeling as the octamer after acetylation dissociates into tetramer and dimers. This causes decondensation of chromatin and recruitment of transcriptional regulators. Therefore, acetylation leads to activation of transcriptionally silenced domains [51]. Apart from histone acetylation, deletion analysis demonstrated that p300/CBP also acetylates internal lysines of Neh1 DNA-binding domain of Nrf2, thereby augmenting promoter-specific DNA binding. In mutant analysis a DY mutant of p300 failed to acetylate Nrf2 and studies also revealed that Nrf2 is a bonafide substrate of p300. However, a redundancy is observed when lysine to arginine substitution is done, indicating that partial acetylations are sufficient to make Nrf2 maximally active. Considering the fact that MafG forms a heterodimer with Nrf2 it may be possible that p300/CBP also

acetylates these small Mafs, thereby increasing synergistically their DNA binding ability. Therefore, it is concluded that acetylation fine tunes the transcriptional machinery [49]. Thus, in response to oxidative stress Nrf2 translocates to the nucleus where it is acetylated by p300/CBP, thus increasing its affinity for ARE, thereby initiating transcription. Hence, these residues may be considered as targets for therapeutic intervention via activation of Nrf2.

Nuclear-cytoplasmic shuttling of Nrf2. For Nrf2 to translocate to the nucleus a bipartite nuclear localization signal is present in its basic region (bNLS - ^{494}-RRRG-KQKVAANQCRKRK^{511}), while two nuclear export signals are found in leucine zipper (NES_{zip} ^{545}LKRRL-STLYL^{554}) and transactivation domains (NES_{TA} ^{175}LLSIPELQCLNI^{186}) [52]. In quiescent conditions, the bNLS causes translocation of Nrf2 into the nucleus while the two NES motifs (NES_{zip} and NES_{TA}) counter balance the driving force of bNLS motifs. This results in sequestration of Nrf2 in cytosol (Figure 3A), whereas under stress conditions redox sensitive NES_{TA} is disrupted, thereby causing its nuclear accumulation under the driving force of bNLS motif (Figure 3B) [53]. The above-stated mechanism fails to justify the constitutive transcription of drug metabolizing enzymes under homeostatic conditions. Therefore, an alternative hypothesis for Nrf2 signaling has been proposed suggesting constant shunting of *de novo* Nrf2 proteins into the nucleus [54] (Figure 4). In this hypothesis high transcription of ARE-regulated genes by Nrf2 is prevented by constitutive targeting of Keap1 into the nucleus for Nrf2 removal and its degradation. Keap1 enters into the nucleus via CRM1/exportin pathway and after degrading Nrf2 is translocated back to cytosol [54]. Keap1 is found to possess a nuclear exporting sequence in its linker region which is found to have leucines (at positions 308 and 310) that when mutated lead to nuclear accumulation of Keap1 [55]. In oxidative stress this shuttling is hampered leading to nuclear accumulation of Nrf2. Although, this mechanism has limited explanation but it is proposed that this may also exist. However, dissociation of Nrf2 from Keap1 in cytosol is mediated by PKC leading to the nuclear import of Nrf2 and Fyn kinase mediated Tyrosine-568 phosphorylation is essential for its Crm1 binding resulting in its nuclear export. Mutant Nrf2Y568A protein accumulates in the nucleus due to loss of nuclear export. Even in the case of H_2O_2 induced oxidative stress, Nrf2 accumulates in the nucleus to promote transcription of cytoprotective genes, but later Tyrosine 568 is reported to get phosphorylated for its nuclear export. In all probability phosphorylation exposes leucine rich export signals that interacts with Crm1. Mutant Nrf2 failed to exit the nucleus and degraded at a slower rate than wild type [56]. It has been shown that all Keap1 homozygous null mice died by day 21 although the embryonic fibroblast from these mice is viable [57]. In addition, the level of Nrf2 in liver nuclear extracts was significantly increased in Keap1-deficient mice as compared to wild type mice. Deficiency of Nrf2 rescued the Keap1 mutant mice from lethality and growth retardation. These findings suggest that continuous accumulation of Nrf2 may contribute to the lethality in Keap1 mutant mice. Once Nrf2 is exported from the nucleus, it is degraded by Keap1. Thus, based on the available information it can be suggested that, during oxidative stress, Nrf2 is synthesized at a normal rate, but its degradation rate decreases leading to its nuclear accumulation and ultimately enhanced transcriptional activity [28]. Some studies, however, point towards an increase of Nrf2 at transcriptional level as well [58].

Nrf2-mediated expression of antioxidant and detoxifying genes

To counteract insults caused by ROS and electrophiles, higher animals have developed a myriad of defense mechanisms which include phase II detoxification enzymes and antioxidant proteins [59]. The regulatory elements of these proteins show the presence of an antioxidant response element (ARE).

Antioxidant response element (ARE)/electrophilic response element (EpRE)

There are cis acting elements present in the promoter or enhancer region of antioxidant or electrophilic responsive genes [60,61]. The two terms ARE/EpRE

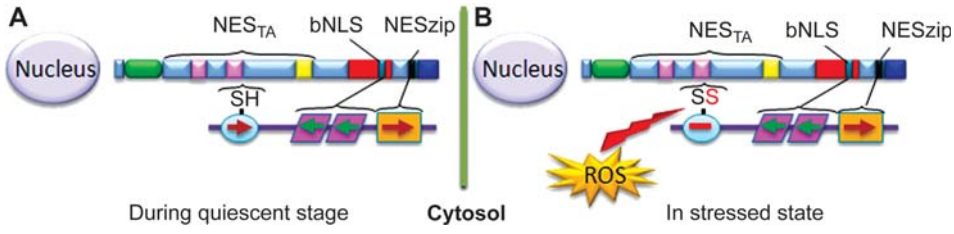

Figure 3. Hypothetical model of Nrf2 signaling. The identified NES and NLS motifs of Nrf2 are symbolized by filled circles and boxes, respectively. Their driving forces are designated by the direction and size of arrows. During the unstimulated condition (A) two NES motifs can counterbalance the driving force of the bNLS motif and sequester Nrf2 in the cytosol. During oxidative stress (B) the reactive cysteine in the NES_{TA} can detect the presence of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and disable the NES_{TA}. As a result, the driving force of bNLS motif prevails and causes Nrf2 nuclear translocation.

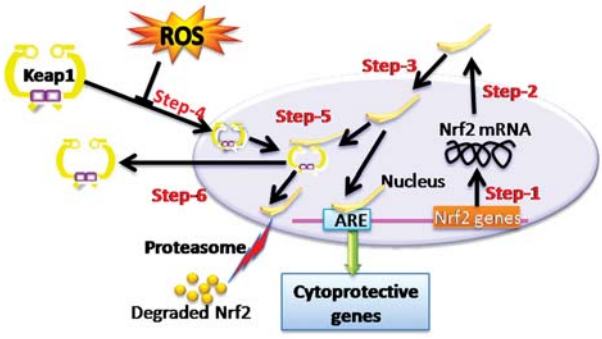

Figure 4. Model for localization and signaling of Nrf2. *De novo* Nrf2 (Steps 1 and 2) is shuttled to nucleus by the driving force of nuclear localization sequence (Step 3). Simultaneously Keap1 is also translocated to nucleus (Step 4) where it forms a complex with Nrf2 (Step 5). Nrf2 in the complex is then subjected to degradation in the nucleus (Step 6) and Keap1 translocates back to cytosol. Under stress conditions, redox-sensitive Keap1 is inactivated by ROS that hinders Steps 4, 5 and 6, resulting into nuclear accumulation of Nrf2 which causes up-regulation of ARE driven genes.

are used interchangeably because of homology found in their core region. The core region of ARE has 5'-(A/G)TGA(C/G)NNNGC(A/G)-3' where the 'C' at position 5' can be replaced by a 'G' without loss of function and the three nucleotides designated 'NNN' in the ARE can contribute to the activity of the cis-element [60,62] (Figure 5). However, variations in this core sequence have also been reported [63]. Genetic analysis has reflected that ARE sequence is found in numerous genes and plays an important role in their regulation. In mice and rats, Glutathione-S-transferase (GST) sub-unit A_1, sub-unit A_2 [60,64], NAD(P)H quinone oxido-reductase (NQO-1) [65], human γ-glutamylcysteine synthetase (hγGCS) heavy and light sub-units [66,67], heme oxygenase1 (HO-1) [68] and others are reported to be regulated by ARE.

As formation of heterodimer is a signature of Cap'n'Collar family proteins, Nrf2 also heterodimerizes with typical members of the AP-1 family (Jun and Fos) [69] or the small Maf proteins [70–72]. AP-1 might regulate the AREs containing and embedded TRE (e.g. TGACTCAGC), but not the AREs devoid of embedded TRE [73,74]. Maf proteins have homology to the avian transforming retroviral oncogene, *v-maf* (musculo-aponeurotic fibrosarcoma) [75]. There is an extensive similarity between the ARE and

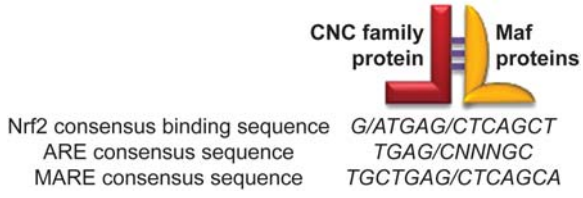

Figure 5. Interaction of Nrf2-Mafs heterodimer with the Antioxidant Response Elements (ARE) present in oxidative stress responsive genes.

maf recognition element (MARE). ARE have one half sequence similar to the core sequence of MARE while the opposite half is similar to its flanking sequence, where the latter is a GC rich region recognized specially by Maf protein-specific 'ancillary region'. The role of Maf protein in activation of ARE driven genes, however, remains controversial as *in vitro* data suggest that Maf suppresses Nrf2 activation [45,71,76]. On the other hand, *in vivo* data of rats indicate that Maf proteins such as MafF, MafG assist Nrf2 in the modulation of ARE driven genes [17]. In an attempt to identify transcription factors that could bind ARE, the role of AP-1 family has been extensively studied as its binding site has high sequence similarity with ARE. AP-1 family consists of Jun and Fos which are products of oncogenes *c-jun* and *c-fos* [77]. Over-expression analysis of Jun and Nrf2 in hepatoma cells have shown Jun acting as a positive regulator of ARE-mediated transcription. However, similar studies with Fos highlighted it as a negative regulator of transcriptional activity of Nrf2. The negative regulation by Fos can be explained on the basis of the fact that a small amount of ROS are consistently required for keeping cellular defenses active. Dysregulated activation of defensive and detoxifying genes can cause a significant reduction in ROS levels. So, to maintain certain critical levels of ROS, cells do require negative regulatory factors like c-Fos [78]. Hence, it can be stated that although AP-1 binding site resembles ARE and even AP-1 family of proteins bind to ARE in response to inducers, ARE does not serve as a high affinity site for Jun-Fos dimer. As the GC nucleotides present outside core element are critical for ARE driven expression, it is suggested that AP-1 proteins can differentially regulate gene expression because they fail to recognize this sequence [79].

Venugopal and Jaiswal [78] first reported the role of Nrf2 in ARE activation with the help of electrophoretic mobility shift assay (EMSA) and ARE reporter gene construct. They also noticed that tissue-specific expression of Nrf2 was identical to that of NQO-1 and DNA binding sequence for Nrf2 was similar to the ARE sequence. Although regulation of ARE driven genes by Nrf2 is a post-translational event, some evidence suggests that Nrf2 autoregulates its own expression [80]. Studies by Kwak et al. [80] reported two ARE elements present in the 5' region of mouse Nrf2 promoter regions which can be transcriptionally activated after chemoprotectant exposure. Interaction of ARE and Nrf2 contribute to elaborate defense against xenobiotics and oxidative stress by regulating an array of genes.

Genes upregulated by Nrf2 via ARE activation

Genes that are upregulated by Nrf2 after ARE activation are known as 'Nrf2 regulon' [81]. They perform a number of cellular functions that include drug

metabolism, ROS scavenging, glutathione homeostasis, stress response activated proteins and efflux transport pathways [45]. Expression of these genes is vital for the maintenance of redox homeostasis within the cell under stress conditions. Polymorphism in these genes enhances the risk of cancer as susceptibility to various chemicals increases [82–86]. On the contrary, certain carcinogens may activate the Nrf2 pathway leading to over-expression of ARE-driven genes that may prove advantageous to tumour cells as they render them with chemotherapeutic drug resistance. Nrf2 is also a key regulator of dysregulated inflammation. Since inflammation is a natural immune response to pathogenic infection leading to distruction of causal agents, its dysregulation leads to several pathologies, for example microcirculatory dysfunction, tissue damage, myocardial injury, multiorgan failure, acute respiratory trouble culminating into death [87]. During the last decade Nrf2 and its potential protective role in broad spectrum of toxic insults and disease pathogenesis have been extensively studied. These studies have conferred Nrf2 as a multi-organ protector [88]. The study of molecular mechanisms involved have gained momentum with the advent of technologies such as knockout or knock down mice, reporter constructs, transfection experiments and recently by DNA microarray analysis. For example, most of the current knowledge about Nrf2/Keap1 pathways has been obtained from the studies using global or tissue-specific Nrf2-deficient, Keap1-knockout as well as Nrf1/Nrf2 double knockout mice. Most of the current knowledge about the Nrf2/Keap1 pathway has been obtained from gene disrupted mouse models. These models include embryos and mice lacking Nrf2, Keap1, both Nrf2/Nrf1, liver-specific Keap1, Nrf1, etc. [89–92]. The gene knockout system in intact animals proves the role of Nrf2 in regulating antioxidant defense apparatus.

The Nrf2 regulon can be categorized under various classes on the basis of their functions—(a) glutathione homeostasis, (b) drug metabolism, (c) stress response protein or iron metabolism and (d) excretion/transporter.

Genes involved in glutathione homeostasis

GSH (γ-glutamyl-cysteinyl-glycine) is an important tripeptide which is imperative for the maintenance of redox status in cells during oxidative stress. It acts by detoxifying electrophiles, scavenging free radicals and also by enzymatic conjugation with the aid of enzyme Glutathione-S-Transferase (GST). The cell maintains a high level of GSH by controlling its rate of synthesis with combined rates of its utilization and loss through efflux. Synthesis of GSH is a two-step process—the first is catalysed by a rate-limiting enzyme called glutamate cysteine ligase (Gcl), also called glutamate cysteine synthetase (Gcs). The second step is catalysed by enzyme Glutathione synthetase (GS). Other enzymes involved in redox cycling are Glutathione peroxidases (GPx) and Glutathione reductase (GR).

Glutamate-cysteine ligase (Gcl)/glutathione synthetase (GS)

Gcl catalyses glutathione synthesis by the formation of γ-glutamyl cysteine from glutamate and cysteine, in the presence of ATP. Glycine is then added to this dipeptide by the enzyme glutathione synthetase to yield glutathione. In the case of Nrf2 knockout mice $Nrf2^{-/-}$ where GSH content is low, levels of both these enzymes were also found to be decreased [89]. Gcl is a holoenzyme comprising of a heavy catalytic subunit (Gclc) of molecular weight 73 kDa and a light modifier sub-unit (Gclm) of molecular weight 27.7 kDa. The genes encoding these enzymes have ARE sequence in their promoter regions [59,88]. In Nrf2 knockout studies, 4–5-fold lower expression of both Gclm and Gclc genes have been reported which lead to a reduction in GSH levels [93,94]. Similarly, ARE are also found in human GS, Gclm [93] and Gclc [95] promoters. Indirect upregulation of Gcl mRNA by Nrf2 has also been observed in rats via enhancement of transcription factors such as NF-κB and AP-1 families [96]. Recently, it has been demonstrated that Nrf2 expression is also regulated by GSH levels [97]. However, a study by Lee et al. [98] demonstrates that murine embryonic fibroblasts (MEFs) survived even when most intracellular GSH was depleted by buthionine-(S,R)-sulphoximine (BSO) treatment, because BSO treatment effectively upregulated Nrf2 mediated antioxidant enzymes via ERK cascade. Age-related depreciation in GSH and elevation of oxidative stress in hepatocytes has been reported to be associated with decreased or reduced transcriptional efficiency of Nrf2 [99]. Thus, Nrf2 can also counteract age-related hepatic oxidative stress.

Redox recycling enzymes

The complete system of GSH comprises a series of interactions involving redox cycling enzymes, prooxidants and antioxidants like ascorbate, α-Tocopherol, NAD(P)H, etc. The redox cycling enzymes attempt to replenish GSH as soon as it is consumed so that a high GSH/GSSG ratio is maintained. The two enzymes Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) form an integral part of this system. GPx is a selenium-dependent enzyme that catalyses reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohol using GSH. GR is a homodimeric flavoprotein that catalyses reduction of oxidized GSSG to GSH using NAD(P)H. The gastrointestinal isoform of GPx has been reported to contain an ARE sequence that binds Nrf2

[100] and upregulates GPx expression. Similarly upregulation of GR is also ARE driven [101].

Glutathione-S-transferase (GST)

GST belongs to a complex multigene family that detoxifies a large number of electrophiles by conjugating them with GSH. This conjugation reaction results in synthesis of mercapturic acid derivatives which are then excreted from the body [102]. These are dimeric cytosolic proteins with each sub-unit having an approximate molecular weight of 25 kDa. Liver, the target organ for detoxification of xenobiotics, contains 3.5% of soluble proteins as GSTs [103]. Mammalian cytosolic GST consists of α, μ, π, θ, ξ, ω and ζ based on substrate specificity, immunological identity, protein and DNA sequences [104–106]. The mitochondrial GST is called class κ. In the mouse, the cytosolic class α, μ, π and θ GST are subject to regulation by Nrf2 [107]. In the rat, the calss θ GST also seems to be regulated by Nrf2 [108]. The sequence similarity within members of each class is greater than 50% and less than 25% amongst members of different classes. The two structurally unrelated membrane-bound forms are microsomal GST and leukotriene C4 synthase [109]. The various forms of GSTs like GST A1, A2, A3, A4, M1, M3, M4 are induced by Nrf2 activating agents and are known to possess ARE in their promoter regions [94]. This is clearly demonstrated by the studies on knockout mice where the induction of hepatic and intestinal GST isoforms by BHA and ethoxyquin was impaired in the absence of Nrf2 [102].

Genes involved in drug metabolism

NAD(P)H quinone oxidoreductase-1 (NQO-1). It is a cytosolic dimeric flavoprotein existing ubiquitously in all tissues and catalyses two electron reduction of both endogenous and exogenous quinones [110]. Out of the two cytosolic isoforms, the role of NQO-1 is best studied in response to oxidative stress. It's activity differs in different individuals, different tissues of the same individual as well as between normal and tumour tissues [111]. Deletion mutagenesis experiments have shown several *cis* elements such as ARE, basal element and AP-1 element are essential for its expression [112]. Here, ARE is responsible for both basal and inducible expression of NQO-1 in response to xenobiotics. Mutation and deletion analysis in the promoter region of NQO-1 have lead to identification of core ARE sequence [62]. The studies conducted on $Nrf2^{-/-}$ mice have shown lack of induction in levels of NQO-1 by Nrf2 activators like BHA, in comparison to wild type where an overwhelming response is obtained [59].

UDP-glucuronosyltransferases (Ugt). These enzymes catalyse conjugation of exogenous and endogenous substances with glucuronic acid [113]. Among the many isoforms, Ugt1a6 has been found to be particularly associated with Nrf2. Basal expression of Ugt1a6 in the liver and lungs have been reported to be less than 56% in Nrf2-deficient mice as compared to wild type [114]. Munzel et al. [115] have suggested the presence of an ARE-like element in human Ugt1a6 promoter. Involvement of the Nrf2-ARE pathway in the induction of hepatic Ugt1a6 was further consolidated by studies with oltipraz in Nrf2 knockout mice [116].

Microsomal epoxide hydrolase (mEH). mEH are involved in the inactivation and hydrolysis of reactive epoxide intermediates by conversion of epoxide to vicinal dihydrodiol. This enzymatic hydration process produces metabolites that have lower reactivity and can easily be conjugated by GSTs facilitating their excretion. The substrate specificities of mEH range from simple aliphatic like octene oxide to large polycyclic aromatics like benzopyrene epoxides. Expression of mEH in multiple tissues have been implicated to be linked with Nrf2, based on the observation that the basal mRNA expression of mEH was reduced in $Nrf2^{-/-}$ mice [116]. However, in Keap1 knock down mice the transcriptional activation of mEH was not affected. This points towards involvement of other mechanisms apart from Nrf2 nuclear accumulation in the induction of mEH.

Genes involved in stress response proteins/iron metabolism

Ferritin. Ferritin is an intracellular iron binding protein comprising of 24 sub-units of heavy (H) and light (L) chains. It is highly conserved and ubiquitously expressed as it plays a major role in iron mediated oxidative stress. It sequesters excess iron present in cytosol and prevents formation of reactive $OH^{\cdot}$ radical through Fenton's reaction [117]. Post-transcriptional regulation of ferritin is iron-regulated interaction between transcription factors and iron-responsive elements (IRE) in its mRNA [118–120]. However, ferritin expression can also be regulated in an iron-independent manner during oxidative stress, as ARE is found in enhancer regions of mouse and human ferritin H genes. Similarly ARE was also identified in human ferritin L genes [121]. Pietsch et al. [122] proved that Nrf2 is one of the important transcriptional activators of ferritin H and L genes. Upregulation of ferritin during oxidative stress helps the cells to combat iron-mediated toxicity [123,124] by decreasing the iron pool that aggravates production of ROS.

Heme oxygenase-1 (HO-1). HO-1 catalyses oxidative cleavage of the mesocarbon of Fe-protoporphyrin-IX

as the first and rate limiting step to yield carbon monoxide (CO), biliverdin and free iron. Out of three isoforms only HO-1 can be induced by a variety of stimuli, particularly oxidative stress. It is also known as Hsp32 as it is transcriptionally upregulated following cellular injury [125]. HO-1 has *cis* regulatory ARE in its promoter region [126,127]. The products of heme catabolism have potential antioxidative effects as CO acts as a gaseous messenger in the vascular system improving nutritive perfusion. Biliverdin which is converted rapidly to bilirubin has antioxidant properties and free iron fosters synthesis of iron binding protein ferritin. On the contrary, high HO-1 also sensitizes cells to oxidative stress by release of reactive iron. Thus, transcriptional activation of HO-1 forms an integral part of antioxidant defense apparatus, although it cannot be seen as either exclusively cytoprotective or exclusively cytotoxic [128].

Efflux transporter genes. Elimination of toxic chemicals via plasma membrane bound efflux transporter protein also minimizes cellular injury. It can also be categorized as an important arm of the antioxidant defense apparatus. These include ATP-dependent efflux transporters like multi-drug resistance protein (Mdr) and multi-drug resistance-associated protein (Mrp) [129]. Mrp efflux activity is found to be reduced in human hepatoma cells exhibiting an overexpression of Keap1, suggesting that Nrf2 may be acting as a positive regulator of Mrp [130]. Out of nine isoforms of Mrp, Nrf2 activators induce the expression of Mrp2, 3, 4, 5 and 6. Most of these Mrps are localized in the basolateral membrane and transport toxic chemical intermediates from hepatocytes into the blood supply [131]. Among other transporter genes several ATP Binding cassette (ABC) family transporters such as Mdr1 and many solute carrier family members like organic/anionic/cationic transporters have been reported to be induced in an Nrf2-dependent manner [132]. This induction was found to be absent in Nrf2 knockout mice, which places these transporter genes as potential members of Nrf2 mediated oxidative stress responsive genes [133].

NF-κB-dependent pro-inflammatory genes. During inflammation several pro-inflammatory agents like cytokines, chemokines, adhesion molecules and receptors are found to be dysregulated in $Nrf2^{-/-}$ mice. TNF-α is considered to be one of the early pro-inflammatory cytokines responding to inflammation which then leads to increased level of IL-1β, IL-6 and ICAM-1, thereby augmenting inflammation [134]. All these pro-inflammatory agents respond to NF-κB signaling where a positive feedback loop exists. These factors enhance NF-κB activation which in turn enhances the level of these inflammatory products. Nrf2 modulates inflammation by inhibiting the NF-κB pathway. As an increase in ROS level activates an NF-κB signaling pathway, Nrf2 limits ROS level leading to inactivation of redox sensitive pro-inflammatory NF-κB pathway and thereby maintaining redox homeostasis [134,135]. The protective effect of Nrf2 via inactivation of NF-κB signaling is clearly manifested in the case of $Nrf2^{-/-}$ mice where increased NF-κB activation leads to pathogenic states like traumatic brain injury and septic shock.

Additional genes. Gene array studies evaluating expression analysis of knockout mice have led to documentation of numerous genes like peroxiredoxin1, thioredoxin1, thioredoxin reductase 1 and 3 as well as genes related to ubiquitin-proteasome system, protein synthesis and trafficking, cell cycle, fatty acid metabolism, immunity and phosphorylation-dephosphorylation cascades as Nrf2 responsive genes [59,133,136]. However, further genetic analysis is required to elucidate their direct involvement in antioxidant response.

The above discussion clearly suggests a cytoprotective role of Nrf2, but reports also indicate that continous accumulation of Nrf2 in the nucleus is harmful. In the case of Keap1 null mice continuous accumulation of Nrf2 led to post-natal death from malnutrition due to hyperkeratosis in the oesophagus and forestomach [40]. However, insufficient information is available about the biological effects of increased Nrf2 expression.

Role of Nrf2 in disease/toxicity

The role of Nrf2 has been found to be very effective in combating various oxidative stress mediated diseases (Table I). This is well manifested in the Nrf2 deficient animals where Nrf2 deficiency detiorates the diseased conditions, some times even culminating into death.

Nrf2 and neurodegenerative diseases

Neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) have been suggested to be an outcome of age-related increase in ROS production and a decreased ability to defend [137–139]. Besides mitochondria, activated microglia may also act as additional source of ROS [140]. Inflammation in the brain is a hallmark of neurodegenerative diseases that reduce neuronal viability [141]. It increases activation of brain immune cells, the microglia which in turn release ROS in extracellular melieu to eliminate pathogens. However, this ROS also acts on microglia itself which leads to induction of pro-inflammatory genes [142] culminating into the induction of Nrf2

Table I. Role of Nrf2 in major pathophysiological conditions.

Diseases	Role of Nrf2	References
Neurodegenerative diseases	Modulates microglial dynamics, protects astrocytes from insults and regulates inflammation.	[223–225]
Cardiovascular diseases	Target for cerebral ischemic therapies, Nrf2-deficiency increases susceptibility to both ischemic and nephrotoxic acute kidney injury; protects endothelial cells from oxidative and shear stress; replenishes endothelial function and NO availability.	[211,226–228]
Pulmonary diseases	Alters response of dendritic cells to allergens; prevents from emphysema/COPD; provides innate immunity against bacterial infection under hyperoxia; prevents from cystic fibrosis.	[185,198,229–231]
Diabetes	Replenishes NADPH level and protects from hyperglycaemic stress; inhibits cytokine-induced H_2O_2 production.	[169,232]
Auto-immune diseases	Prevents immune cell infiltration and glial activation; prevents multiple sclerosis lesions and immune-mediated hemolytic anaemia.	[197,233,234]
Cancer	Prevents carcinogenesis in normal cells, promotes chemo-resistance in cancer cells.	[189,190,235]

that down-modulates inflammation and protects from several neurodegenerative diseases.

Alzheimer's disease is a progressive neurodegenerative disease characterized by damage to neurons and consequent memory impairment [143]. AD brains are reported to show increased levels of neurotoxic trace elements like iron, aluminium, mercury and copper that generate ROS [144,145], oxidized misfolded proteins [146], lipid peroxidation products [146,147] and markers of DNA oxidation [148,149]. The Nrf2 expression profiles in AD brain show decreased nuclear Nrf2 levels [150], which suggest that Nrf2 signaling may be actively involved in early stages of AD pathology. Previous study showed that DJ-1 is a Parkinson's disease-associated protein [151–153], which can stabilize the antioxidant transcriptional master regulater Nrf2 [154]. Based on these findings it is summarized that a decreased level of Nrf2 in the brain in patients with AD is associated with the dysfunction of DJ-1, thereby leading to its proteasomal degradation. This may be a factor behind decreased activity of the Nrf2-ARE pathway.

Oxidative stress is also found to be critically involved in the etiology of Parkinson's disease [155] where loss of antioxidant GSH, reduction in mitochondrial complex I activity, increased lipid, protein and DNA oxidation/nitration, increased SOD activity and elevated levels of free iron is evident. However, much data is not available regarding the Nrf2-ARE defense system in PD but in cultured PC12 and glial C6 cells, flavonoid luteolin activates the Nrf2 pathway [156], while knockdown of Nrf2 nullified the protection pointing at its key role. In fact, in deltamethrin-induced toxicity in PC12 cells, t-BHQ, a known Nrf2 transcriptional upregulator incurred protection [157].

Huntington's disease is characterized by a cognitive decline in chloreiform movements and behavioural difficulties [158]. A conformational change in Huntington protein due to polyglutamine expansion in the Huntington gene [159] causes it to form aggregates in the nucleus and cytosol. HD brain is reported to show deficiencies of mitochondrial complex II and III that directly links to diminished ATP production [160,161]. Supplementation with a high energy doner increases production and ameliorates the disease severity [162,163] along with reduced levels of 8-OHdG [164]. The first study to show the involvement of Nrf2-responsive genes under oxidative stress in HD was by van Roon Mom et al. [165]. Most Nrf2-responsive transcripts involved in detoxification and antioxidant/reducing potential were found to be increased. Hence, the Nrf2 signaling pathway is a potential therapeutic target for neurodegenerative diseases where oxidative stress and neuro-inflammation occur. Acute lung injury (ALI) often follows traumatic brain injury (TBI) forming a critical factor affecting patient mortality. Oxidative stress is reported to be a factor in the pathogenesis of ALI following TBI. A study by Yan et al. [166] reveals activation of the Nrf2-ARE pathway which imparts protection to lungs against diffused inflammation and oxidative damage.

Nrf2 and cardiovascular diseases

Increased levels of ROS are found to be associated with cardiovascular diseases leading to impairment of endothelial function and lowered nitric oxide (NO) availability [167]. Upregulation of the Nrf2-Keap1 defense pathway has been reported to accord a protective effect on cardiovascular diseases. Diabetes, hypertension and atherosclerosis have been found to be associated with decreased vascular responses to insulin. This suggests that insulin has a key role in maintaining endothelial redox homeostasis. The study reveals that insulin-induced 2-fold increase in Gclc mRNA and protein on oxidative challenge, thereby recovering GSH levels and the role of Nrf2 is important in restoring these antioxidant defenses [168]. However, in the case of hyperglycaemia-induced diabetic complications, activation of NAD(P)H oxidase acts as an inducer of antioxidant defenses via the Nrf2-ARE pathway. Transient ROS production by

NAD(P)H oxidase induces the Nrf2-ARE pathway, while excess production leads to eNOS uncoupling, mitochondrial dysfunction and impaired antioxidant defenses [169]. Recently, a study done by Zakkar et al. [170] on endothelial cells suggests that activation of Nrf2 protects from development of atherosclerotic plaques and inflammation in response to low shear stress. Nrf2 suppresses endothelial cell activation by suppressing p38 signaling and VCAM-1 expression initially by inhibiting MKK3/6 signaling and by activating MKP-1, an anti-inflammatory molecule that inactivates p38 phosphorylation. Thus, more work in this direction can unravel approaches for prevention of atherosclerotic plaques [170]. Nrf2 is also found to be involved in lipid homeostasis where mice fed with a high fat diet were analysed. Results demonstrated mRNA of sterol regulatory element-binding proteins 1c and 2, fatty acid synthase, acetyl CoA carboxylase1, fatty acid elongase, 3-hydroxy-3methylglutaryl CoA synthase and reductase to be high in $Nrf2^{-/-}$ mice. So, the results suggest an anti-lipid accumulating property of Nrf2 by interfering with lipogenic and cholesterologenic pathways [171].

Nrf2 and pulmonary diseases

Oxidative stress is also found to be implicated in diseases like pulmonary distress syndrome, idiopathic pulmonary fibrosis, cancer and emphysema. In Nrf2-deficient lungs, environmental oxidants including hyperoxia, diesel exhaust particles, cigarette smoke and bleomycin cause severe injury. Microarray analyses by Cho et al. [172] highlighted the role of Nrf2-dependent genes that might be critical in pulmonary protection. Nrf2 deficient ($Nrf2^{-/-}$) mice have been found to be more susceptible to bleomycin caused airway inflammation [173], ovalbumin challenge [174] and hyperoxia [175]. A significant reduction in mRNA expression of ARE-responsive heme-oxygenase-1, GPx2 and NAD(P)H:quinone oxidoreductase was observed in $Nrf2^{-/-}$ mice as compared with the normal. However, in the case of bleomycin-induced fibrosis TGF-β enhances ROS production and suppresses Nrf2 responsive genes by mediating interaction of Smad3-ATF3 with Nrf2 [176,177]. Thus, by upregulation of lung antioxidant defense enzymes Nrf2 appears to protect against pulmonary hyperoxia/bleomycin injury and ovalbumin challenges. Recent studies by Rangasamy et al. [178] and Iizuka et al. [179] showed increased susceptibility of $Nrf2^{-/-}$ mice to Cigarette smoke (CS)-induced pulmonary emphysema as compared to wild-type mice. Furthermore, an Nrf2 activator CDDO-imidazolide exhibited a protective effect in mice with CS-induced emphysema [180], suggesting potential intervention of Nrf2 activation in COPD/emphysema. Loss of Gcl, DJ-1, a positive regulator of Nrf2 and post-translational modifications of Keap1-Bach1 equilibrium were found in pulmonary macrophages and in patients with COPD on Nrf2 down-regulation [181–184]. Here, Nrf2 exerts its protective effect by activating anti-proteases and antioxidants [185]. However, studies done by Biswas et al. [186] and Kode et al. [187] in human lung epithelial cells suggest that resveratrol, a red wine polyphenol, induces GSH synthesis via Nrf2.

In the case of hyperoxic lung injury, Nrf2-deficient mice manifest enhanced lung damage characterized by increased protein permeability, macrophage inflammation and epithelial injury [170]. Studies on Nrf2-knockout mice and Nrf2 transfected or deficient cell lines have proposed the essential role of Nrf2 in preventing carcinogenesis. Studies have also led to the hypothesis that activation of Nrf2 would attenuate the lung damage caused by cigarette smoke [178,180]. An anti-viral activity of Nrf2 has also been reported recently by Cho et al. [188] in case of respiratory syncytial virus (RSV) pathogenesis where RSV phenotypes were significantly enhanced in $Nrf2^{-/-}$ mice as compared to $Nrf2^{+/+}$.

Nrf2 and cancer

Studies done on Nrf2 null mice verify the pivotal role of Nrf2 in cancer prevention as these display increased sensitivity to chemical toxicants and carcinogens due to reduced basal levels of antioxidant and phase II detoxifying enzymes. Nrf2 prevents normal cells from malignancy, but also promotes survival of malignant cells by enhancing drug resistance leading to an increased risk of cancer. Nrf2 was found to offer resistance to chemotherapeutic drugs such as cisplatin, doxorubicin and etoposide [189]. However, this darker side of Nrf2 could be harvested and used as a potential therapeutic target where Nrf2 silencing could prevent cancer cell survival and its chemoresistant potential [190]. Lung carcinomas are often found to be associated with an aberrant Nrf2-Keap1 system, where there is a high incidence in loss of Keap1 function or a low level is found in lung cancer tissues [191,192]. However, a study done by Wang et al. [193] points towards induction of apoptosis in leukaemia-derived NB4 cells following fenretinide treatment. There is a sharp increase in ROS accumulation in response to fenretinide, thus activating transcription factors such as Nrf2 and HSF1 (Heat shock factor 1). Both factors act co-ordinately and convert oxidative signaling to pro-apoptotic and apoptotic events rather than survival. As cancer cells possess a pro-oxidant microenvironment, therefore strategies that promote oxidative stress selectively in cancer cells can be important [194,195].

Nrf2 and inflammation

Recent studies demonstrate that Nrf2-ARE signaling is also involved in resolving inflammation-associated

diseases such as autoimmune diseases, rheumatoid arthritis, asthma, emphysema, gastritis, colitis, sepsis, traumatic brain injury and atherosclerosis. Observations suggest an increase in inflammatory enzymes (iNOS, phox-47, gp91-phox and phox-67), cytokines (IFN-γ, IL1-β, TNF-α and IL-12) and chemokines (BLC, MIG) gene expression levels in the Nrf2-deficient mice compared to wild type, supporting the notion that Nrf2 modulates autoimmune neuroinflammatory responses [196]. Even defective Nrf2 mediated signaling mechanisms modulate the response of dendritic cells to a common environmental allergen, thus imparting susceptibility to allergic diseases [197] as Nrf2 has a role in anti-oxidative stress and pro-allergic Th2 mediated immunity orchestrated by dendritic cells. Sepsis is a bacterial infection following unregulated inflammation. Here, Nrf2 deficiency leads to enhanced inflammation and mortality against LPS. It exerts its effect via MyD88 dependent and independent signaling wherein $Nrf2^{-/-}$ mice NF-κB and interferon regulatory factor 3 are activated in response to LPS. Thus, Nrf2 modifies sepsis by raising appropriate innate immune response [185].

Nrf2 and environmental stress

It has been observed that constant exposure to the environment where several threats are present leads to Nrf2 upregulation. Particulate matter present in the environment has been found to promote asthma by inducing excessive ROS production. Consequently, phase II detoxification genes are upregulated by enhancing the half-life of Nrf2 and thus promoting its nuclear accumulation [198]. So, chemopreventive Nrf2 inducers may prove to have a protective role in particulate matter promoted asthma. A protective role of Nrf2 has been reported even in parthenoid pesticide Deltamethrin-induced neurotoxicity in PC12 cells [157]. Nrf2 can also be activated in cases of metal-induced toxicity. However, the molecular targets of Cd remain to be elucidated; Cd has been found to induce ROS production dramatically under basal conditions. Even in $Nrf2^{-/-}$ mouse embryonic fibroblasts (MEF), Cd at a concentration of 2 μM induced ROS production [199]. Cytoprotective genes HO-1 and NQO-1 were elevated in wild type mice, while induction was lost in $Nrf2^{-/-}$ mice. However, it is proposed that Cd induces Nrf2 via metal activated signaling pathways which employ interplay between ubiquitination/deubiquitination and Nrf2-Keap1 complex formation and dissociation [199]. In manganese intoxication also, increase in hepatic levels of Nrf2 and enhanced induction of phase II enzymes has been reported along with Nrf2 translocation to the nucleus [200]. However, mechanisms involved in lung injury and pulmonary diseases induced by inhaled hexavalent chromium Cr(VI) are not well known. O'Hara et al. [201] suggested that Cr(VI) attenuates induction of ARE-driven genes that help to counteract secondary insults.

Nrf2 and drug toxicity

The role of Nrf2 in protection against drugs like acetaminophen (APAP) induced toxicity is well worked out. APAP is one of the most widely used analgesic and anti-pyretic, whose biochemical properties have been extensively studied [114,202–204]. Excess dosage or prolonged intake may lead to liver failure resulting in death. In the case of over-dosing, reactive metabolite N-acetyl p-benzoquinoneimine (NAPQ1) formation increases, leading to depletion of GSH content. Furthermore, it can also bind to other biomolecules like important hepatic proteins causing liver injury. Although the exact mechanism of lethality caused by APAP is not known, it is likely due to the cumulative effect of oxidative stress, depressed mitochondrial functions, disruption of Ca^{+2} homeostasis and redox imbalance [203]. APAP was found to regulate expression of detoxifying enzymes like Gcl, GST and Ugt1a6 via Nrf2. When the studies were performed on wild type and Nrf2 knockout mice, it was observed that at low doses $Nrf2^{-/-}$ mice showed more mortality than wild type and the effect was more pronounced in males than females. The main reason attributed to this mortality was depletion of GSH due to decreased levels of GSH homeostasis maintaining enzymes resulting in inefficient protection [205]. It is also stated that Nrf2 knockout mice have low constitutive expression and an inability to induce cytoprotective genes.

Overall, Nrf2-Keap1 pathways have the potential to be used as a powerful protective strategy in different disease conditions. These responses have been shown to attenuate toxicity as well as protect against diseases [206]. Thus, Nrf2 is found to induce cellular rescue pathways against oxidative injury, abnormal inflammatory and immune responses, apoptosis and carcinogenesis [185].

Nrf2 activators/modulators: Implication in intervention of chronic inflammatory diseases

Emerging research findings suggest that there is a direct relationship between the food habits of people and their health conditions, as intake of several dietary phytochemicals have been known to reduce the risk and accord protection against various diseases such as cancers, cardiovascular abnormalities and neurodegeneration. The general mechanism of action involves activation of cellular stress response pathways through induction of kinases and transcription factors leading to expression of antioxidant and phase II enzymes. Amongst the various pathways Nrf2-ARE activation by these phytochemicals confers cytoprotection and

chemoprevention [80]. However, hormetic effects of these phytochemicals needs to be considered as at low doses they have stimulatory effects while at higher doses they may be toxic. This has increased the search for various substances that can act as modulators of Nrf2 and its activity. These therapeutic agents can be endogenous and exogenous substances which include phenolic antioxidants (α-napthaflavonone, butylated hydroxyanisole, tert-butyl hydroquinone), synthetic antioxidants (ethoxyquin, oltipraz, phorbol esters), triterpenoid analogue (olenolic acid derivatives, sisqueterpenes) and isothiocynates (sulforaphane). During oxidative stress, the gene expression profiles of these compounds have been studied in various genetic models.

The basic mechanism of these cytoprotectants involves direct or indirect alteration of Nrf2/Keap1 pathway that ultimately gives an adaptive response to metabolic stress. Among the inducers of antioxidant defense, various structurally-related plant polyphenolic compounds like curcumin, caffeic acid phenyl ester (CAPÉ) and carnosol have emerged as protective agents owing to their Michael reaction acceptor function [207–209] and electrophilic characteristics. In kidney epithelial cells, curcumin and CAPÉ mediate the antioxidant effect mainly through the induction of cytoprotective HO-1 gene by disruption of Nrf2/Keap1 pathway [210]. It appears that they react selectively with nucleophilic groups of Keap1 through their β-unsaturated carbonyl group to form Michael adduct [207]. This chemical modification may lead to activation of Nrf2, favouring its nuclear accumulation. Use of specific inhibitors elucidated stimulatory roles of p38 sub-families in signaling pathways during curcumin mediated HO-1 induction. Additionally, it can also form an adduct with GSH leading to aggravation in oxidative stress and triggering other stress response pathways. Recently curcumin has been reported to have anti-initiating actions as it decreases Ahr and CYP1A1 induction, thereby reducing ROS load with concomitant induction of Nrf2 [210]. Curcumin has also been found to up-regulate Nrf2 in rat brains against focal ischemia [211], retina cells from light and oxidant-induced cell deaths [212] as well as in chronic obstructive pulmonary diseases (COPD) [213]. Carnosol is another diterpine green tea polyphenol which exhibits antioxidant activity. The main effector function of this phytophenol is via its intrinsic antioxidant activity and induction of phase II enzymes which include GST, HO-1, NQO-1, Gcl, GS and aldoketoreductase (AKR) [208,214,215]. This regulation occurs uniquely by Nrf2 accumulation and increased transcription of ARE genes. Although the pathway remains elusive, a diverse assortment of roles to p38 and PI3K/Akt pathways is suggested [216]. This manifestation of antioxidant effect by protein kinases is suggested by either direct or indirect phosphorylation of Nrf2, leading to its stabilization. However, the possibility of phosphorylation of Nrf2-specific ubiquitin ligase leading to its persistent inhibition can not be ruled out.

One of the most potent naturally occurring inducers of phase II detoxifying enzymes is an isothiocyanate called sulforaphane (SF-R-1-isothiocynate 4 methyl sulphenyl butane) obtained from broccoli (a crucifer). The induction level and type of antioxidant enzyme regulated by SF vary with cell types both *in vitro* and *in vivo*. Enzymes reported to be affected are GST A, NQO-1, AKR, GR, Ugt1a6, Gcl, etc. As these enzymes are ARE-dependent, a common underlying mechanism of action affects their expression. SF forms a thioacyl adduct with reactive cysteines of Keap1 causing an alteration in antioxidant activity. PI3K, protein kinase C and MAP kinases are various signaling kinases reported to be involved. With the advent of microarray and knockouts, SF has been shown to exert its effect on diverse clusters of genes like ubiquitin proteasome, stress response proteins, kinases, phosphatases and transporter proteins [217]. Zakkar et al. [170] reported sulforaphane to have a suppressing activity on p38 and MKK3/6, thus suppressing VCAM-1 via Nrf2 at transcriptional level and regulating pro-inflammatory activation of endothelial cells. Sulforaphane also extends a protective role in RSV induced acute lung inflammation and pulmonary viral expression in $Nrf2^{+/+}$ mice [185]. Thus, action of SF on oxidative stress responsive genes, although vibrant, can occur through various pathways, but its mediation by Nrf2 is essential.

Similarly, organosulphurs like diallyltrisulphide (DATS) from garlic induces phase II enzymes such as NQO-1, GST, HO-1, GR, L & H ferritin while it has an inhibitory effect on phase I Cyp2E1 [218]. DATS induces ARE activity mainly via Nrf2 by altering intracellular redox balance as evident from studies, but the signaling pathway may have an overlying involvement of MAP kinase and Ca^{+2} dependent calmodulin kinase [219].

Quercetin and Brazilin have also been found to up-regulate antioxidant response. Quercetin has been reported to up-regulate not only expression of Nrf2 mRNA and protein, but also stabilizes Nrf2 by obstructing its ubiquitination and proteasomal turnover. It extends the half-life of Nrf2 from 20.3 min to 82.2 min. It even depreciated levels of Keap1 post-translationally by modifying Keap1 protein rather than via 26S proteasomal degradation pathways [220]. However, Brazilin induced HO-1 expression by Nrf2 via PI3K/Akt and ERK pathway in HEI-OCI cells. It induced HO-1 mRNA and protein expression in a dose- and time-dependent manner [221].

CDDO-Im a triterpinoid reduces oxidative stress, alveolar destruction, alveolar cell apoptosis and pulmonary hypertension in $Nrf2^{+/+}$ mice caused by exposure to cigarette smoke. Protection accorded

by CDDO-Im in emphysema is Nrf2-dependent as $Nrf2^{-/-}$ mice failed to show the repair of damage after CDDO-Im treatment. It not only reduced alveolar distruction but also improved a major cardiac determinant of COPD-related mortality [180]. In hepatic nuclear fractions a 380% increase in Nrf2 levels is found in wild type while no Nrf2 protein was found in Nrf2 null mice [81]. In the case of LPS induced inflammation CDDO-Im reduces the inflammatory response [135] in a variety of *in vivo* and *in vitro* models.

Apart from phytochemicals, drugs like lipid lowering statins have been found to elevate levels of antioxidant enzymes such as HO-1, GPx2 via Nrf2 both *in vitro* and *in vivo* [222]. Thus, it can be concluded that the activation mechanism of Nrf2 is induced by various compounds and is specific for cell types as well as the compound itself.

Nrf2, a probable answer to adverse drug reactions and cancer

The knowledge of Nrf2-ARE response pathway can be of immense use in handling adverse drug reactions as most of the drugs mediate damages by ensuing oxidative stress. Drugs administered along with a pharmacological activator that up-regulates Nrf2 can open new avenues for the usage of these drugs. Management of oxidative stress, which plays an important part in toxic manisfestations and disease conditions; can also be achieved with a better understanding of the Nrf2-ARE response mechanism. Although Nrf2 confers resistance against a wide array of stress, this property is aptly used by malignant cells to show chemo-resistance. So, the Nrf2-ARE response pathway can act as a critical point of modulation where drugs causing its silencing may prove a cure to the disease. Therefore, the basic challenge lies in selecting the drugs affecting Nrf2-ARE response that could selectively target only cancer cells without interfering with the normal cell metabolism as it is indespensible for counteracting day-to-day physiological challenges.

Conclusion and future directions

Nrf2 is an important redox-sensitive transcription factor which protects against oxidative stress by inducing the transcription of antioxidant and detoxifying genes through binding with ARE. It has the potential to be a target to intervene a variety of diseases, such as neurodegenerative, cardiovascular, pulmonary and chronic inflammatory diseases, where oxidative stress occurs and Nrf2 is deranged. Several approaches can be utilized to activate Nrf2 or reduce its degradation through modulating its post-translational modifications (e.g. phosphorylation and acetylation) or Keap1 stability. For example, administration of a potent Nrf2 small molecule activator (CDDO- imidazolide, sulforaphane, curcumin) was effective in attenuating cigarette smoke-induced emphysema in mice. Therefore, development of a specific Nrf2 activator to switch on its responsive genes would bring novel avenues for treatment of oxidative stress-induced disorder. It is interesting to note that Nrf2 itself is post-translationally modified, destabilized and degraded in development of the above diseases, such as COPD/emphysema. Hence, further studies are needed to investigate how effective Nrf2 activators or modulators and upstream signaling molecules are in interfering with difficult to manage disorders such as chronic inflammatory diseases and cancer.

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