Chapter 3

Multiple Modes of Nrf2 Regulation and Transcriptional Response

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Abstract

Cells have defense systems to deal with chemical insults from the environment. Some examples are chemical scavengers like glutathione and enzymes such as superoxide dismutase that inactivate radicals and other reactive chemicals in the cytoplasm. It is perhaps surprising that these protective systems are not maximally expressed in an unstressed cell. Rather, the ability to inactivate toxic chemicals is tightly regulated and only induced when needed. As a consequence, unstressed cells are usually very sensitive to radicals, but become more resistant as the cellular defense system has been appropriately upregulated after a few hours. The transcription factor Nrf2 is known to be a master regulator of many cytoprotective enzymes and proteins. Chemical inducers of Nrf2 inactivate its repressor, Keap1, when they react with critical cysteine residues in Keap1. The release of Nrf2 from Keap1 results in enhanced expression of genes involved in detoxification. This generates a feedback loop where Nrf2 induces protective enzymes capable of inactivating the chemical that reacted with Keap1. An unproven, but likely, scenario is that Nrf2 transcriptional response can vary depending on the nature of the chemical insult. The aim of this chapter is to examine the mechanisms by which the cell can sense different reactive chemicals and modulate protective responses. It is likely that this knowledge is of vital importance in the development of clinical Nrf2 activators in preventive medicine.

Keywords: Keap1, Nrf2, cysteine residues, transcriptional response, Nrf2 activator

1. Introduction

Cells in our bodies are constantly challenged by reactive substances such as radicals, in part due to the high oxygen content in the surrounding. To prevent unwanted chemical reactions,

cells have evolved sophisticated defense mechanisms. These systems allow cellular adaptation to toxins in our environment by regulating the expression of an elaborate network of cytoprotective proteins and chemical scavengers.

To a large extent, this cytoprotective system is regulated by the transcription factor nuclear E2-factor-related factor 2 (Nrf2) and its primary suppressor kelch-like ECH-associated protein 1 (Keap1). Under basal conditions, Keap1 decreases the Nrf2 protein levels through ubiquitination and proteasomal degradation [1, 2]. Upon activation, Nrf2 escapes Keap1 repression and Nrf2 protein levels get stabilized and translocate to the nucleus. In the nucleus, Nrf2-Maf heterodimer binds to the antioxidant response element (ARE) in the regulatory region of target genes and promotes transcription [3]. A wide array of genes including antioxidant proteins and detoxifying enzymes, transporter and metabolic enzymes, enzymes for glutathione biosynthesis, proteases, and chaperone are transcriptionally activated by Nrf2 [4]. It is likely that the cellular response to a chemical challenge will be different if the toxic substance is an oxidative or a reductive toxin.

An example of this diversification in response can be found in the bacteria *Escherichia coli* where the redox system is mainly regulated by transcription factors OxyR and SoxRS. The cysteine residues of OxyR sense elevated levels of hydrogen peroxide [5], whereas SoxRS iron-sulfur (2Fe-2S) clusters act as sensors of superoxide. When activated in this way, OxyR and SoxRS become active transcription factors that induce distinct but partially overlapping set of cytoprotective genes adapting the cell to deal with hydrogen peroxide or superoxide.

In a similar way, it is thought that our cells diversify the redox response depending on the nature of the chemical insult. As mentioned above, electrophiles and oxidants react with cysteine residues in Keap1 which blocks Nrf2 proteasomal degradation and thus mediates transcriptional activation of many genes [6]. However, little is known about how the Nrf2 system can differentiate the transcriptional response. Here, we review the most recent literature on how Nrf2 cross talks with multiple signaling pathways and evokes different signaling response including inflammation, metabolism, apoptosis, proliferation, and differentiation. This knowledge is likely of great importance when Nrf2-activating drugs are developed to boost our radical defense systems.

2. Redox regulation in bacteria

The bacterial redox system in *E. coli* is coordinately regulated by two transcription factors—SoxRS and OxyR. SoxRS is activated in response to stress induced by superoxide anion and OxyR responds to stress caused by hydrogen peroxide.

2.1. OxyR-hydrogen peroxide sensor

The transcription factor OxyR belongs to LysR family of transcriptional activators. In response to hydrogen peroxide stress, two cysteine residues, Cys-199 and Cys-208 oxidize leading to the formation of intramolecular disulfide bond [7]. Oxidized OxyR promotes transcription of

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genes including *katG* (a hydrogen peroxidase I), *ahpCF* (an alkylhydroperoxide reductase), *oxyS* (a regulatory RNA involved in DNA repair), *gorA* (a glutathione reductase), and glutaredoxin 1 (*grxA*). Enzymatic reduction of the disulfide bond switches off the OxyR function and the OxyR transcription factor therefore functions as an "on/off switch" with disulfide bond formation in response to oxidative stress [8–10]. Studies suggest that Cys-199 thiol activates OxyR through several redox-related modifications including S-OH, S-nitrosylation, and S-glutathione and the resulting OxyR differs in structure, properties, and genes activated [5].

2.2. SoxRS-superoxide sensor

The superoxide response system in *E. coli* regulates transcription of targets involved in detoxification (superoxide dismutase), DNA repair (endonuclease IV), and glucose-6-phosphate dehydrogenase. The iron-sulfur (2Fe-2S) clusters of the SoxR act as sensors of superoxide and undergo one-electron oxidation/reduction and induce the transcriptional activity of SoxR [11]. SoxR protein binds DNA to activate the expression of SoxS which in turn activates cell protection genes in response to superoxide and nitric oxide [12]. Site-specific mutation studies have shown that four conserved cysteine residues at the C-terminal domain of the SoxR polypeptide act as the ligands for the [2Fe-2S] clusters which has crucial role in transcriptional activity of SoxR [13].

2.3. OxyR-SoxR interaction

Both OxyR and SoxR proteins exist in oxidized and reduced forms but only the oxidized form of these proteins induces the expression of antioxidant defense system. Although the SoxR and SoxS proteins are mainly involved in response to superoxide, several studies have reported that SoxRS regulon may be activated by hydrogen peroxide indicating the overlapping between the specific response systems. SoxR protein senses the increased levels of hydrogen peroxide and activates the SoxRS system [14–16].

3. Keap1-mediated Nrf2 regulation

The critical importance of Keap1 as a negative regulator of Nrf2 is supported by the observation that the deletion of *Keap1* gene in mice causes constitutive activation of Nrf2. Keap1$^{-/-}$ knockout mice died shortly after birth due to hyperkeratosis in the upper digestive tract but the phenotype conditions were reversed when both Nrf2 and Keap1 were disrupted [17, 18]. The cysteine-rich protein Keap1 regulates active degradation of Nrf2 under basal conditions by functioning as an adaptor to cullin3 (Cul3)-ringbox1 (Rbx1) containing E3 ubiquitin ligase complex (**Figure 1**) [19]. The Neh2 domain of Nrf2 binds to Kelch domain of Keap1 through the "hinge-and-latch" mechanism [20]. Under basal conditions, Nrf2 ETGE motif acts as a hinge and forms an "open" conformation by binding to Kelch subunit of Keap1 and the DLG motif which acts as the latch binds to Keap1 subunit to form the "closed" conformation and targets Nrf2 for proteasomal degradation. Cysteine residues of Keap1 sense reactive oxygen species (ROS) or electrophiles in the cellular environment causing conformation changes in

Keap1. The modified Keap1 can disrupt its interaction with the low-affinity DLG motif, whereas the high-affinity ETGE motif remains associated with Keap1. As the DLG motif fails to bind to Keap1, it affects the orientation of lysine residues within the Neh2 domain of Nrf2 preventing its ubiquitination and degradation [21]. After redox homeostasis is restored, Keap1 moves into the nucleus and controls nuclear export of Nrf2 for subsequent proteasomal degradation in the cytoplasm (**Figure 1**) [22].

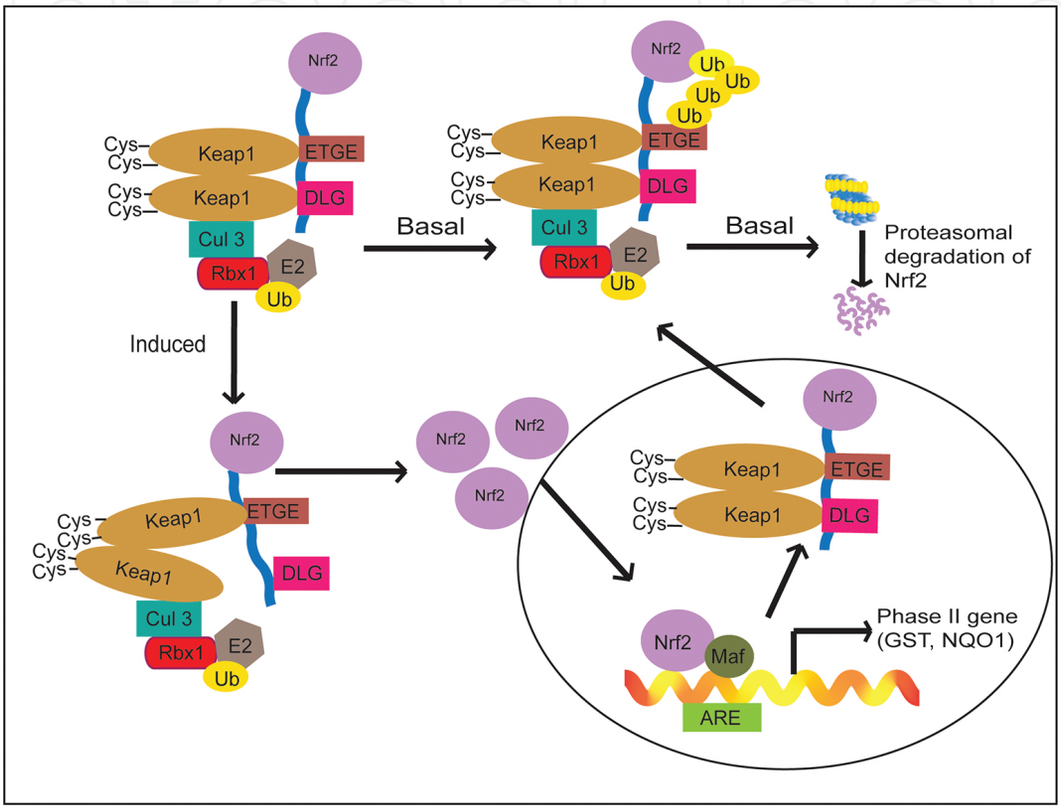

Figure 1. Keap1-mediated Nrf2 regulatory pathway. Under basal conditions, Nrf2 is bound to Keap1 and undergoes rapid degradation. Upon induction, cysteine residues in Keap1 are modified, the E3 ubiquitin ligase activity is suppressed, and Nrf2 levels increase. Activated Nrf2 enters the nucleus and dimerizes with Maf to promote transcription of ARE-dependent genes. Finally, Nrf2 is transported out of the nucleus by Keap1 for subsequent proteasomal degradation.

3.1. Distinct Keap1 cysteine modifications

Various synthetic and plant-derived phytochemicals including isothiocyanates, Michael acceptors, and coumarins are shown to activate Nrf2 system [23]. Many of these substances protect human cells and animals from a diverse array of toxins and radiation [24]. These structurally diverse Nrf2-inducing agents share a common property of reacting with sulfhydryl groups and cysteine residues in Keap1 [6, 25]. ROS or electrophilic reaction with specific cysteine residues causes conformational changes in Keap1 and prevents proteasomal degradation of Nrf2 [26]. Site-directed mutagenic studies have identified critical cysteine residues as important factors involved in Nrf2 regulation. Mutation in Cys273 or Cys288, located at

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intervening region of Keap1, blocked the Keap1-dependent ubiquitination and degradation of Nrf2 under basal conditions. Mutation in Cys151 at the BTB domain of Keap1 blocked Nrf2 release from Keap1 in response to sulforaphane and maintained Keap1 repression of Nrf2. Cys273 or Cys288 is required for Keap1 repression of Nrf2 under basal conditions and Cys151 is important for Nrf2 activation in response to electrophilic stress [27–29].

A "cysteine code" has been proposed for the Keap1-dependent Nrf2 regulation as cysteine modifications play crucial role in mediating Nrf2 activation [30]. Studies suggest that Nrf2-inducing agents such as diethylmaleate (DEM), dimethylfumarate (DMF), and sulforaphane prefer Cys151 residue for Nrf2 induction, whereas 2-cyano-3, 12 dioxooleana-1, 9d iene-28-imidazolide (CDDO-Im) and heavy metals such as cadmium chloride (CdCl2) and arsenic activate Nrf2 in a Cys151-independent manner. Another well-known Nrf2 inducer, tert-butyl hydroquinone (tBHQ), gets oxidized to the electrophilic metabolite tert-butyl benzoquinone and modifies the Cys151 cysteine residues [31, 32]. The differential reactivity of cysteines in Keap1, "the cysteine code," does not, however, explain how this translates into differential toxin-dependent activation of genes by Nrf2.

4. Nrf2 network

Recent advances have revealed that Nrf2 cross talks with different signaling pathways and influences the transcriptional response. Beyond cellular response against oxidative stress, Nrf2 is reported to be involved in inflammation, metabolism, apoptosis, proliferation, and differentiation.

4.1. Cross talk between Nrf2 and NF-κB pathway

Nuclear factor-κB (NF-κB) was first identified in David Baltimore's laboratory around 30 years ago as a transcription factor that activated the kB immunoglobulin promoter in B-cells [33]. Since then, NF-κB has been implemented in a diverse array of conditions mostly linked to acute and chronic inflammation.

NF-κB is normally sequestered in the cytoplasm by its negative regulator IkB-alpha;. Upon T-cell or B-cell receptor activation in response to infection or tumor necrosis factor (TNF)α receptor stimulation IkBα is phosphorylated by the IkB kinase (IKK) complex. Phosphorylation of IkBα is followed by ubiquitination and proteasomal degradation of IkBα and releases NF-κB. NF-κB translocates to the nucleus and activates target genes having the kB elements in their promoters [34]. It has been proposed that after simultaneous activation, NF-κB antagonizes Nrf2-mediated gene transcription. Conversely, some Nrf2 inducers suppress NF-κB signaling. Therefore, it seems that the inflammatory induction of NF-κB can be suppressed by Nrf2 activation and vice versa. It is possible that the inflammatory response that generates radicals to defeat bacteria must downregulate the radical scavenger function of the Nrf2 to function optimally [35] (**Figure 2**). Similarly, the radicals produced by the inflammatory response could activate the Nrf2 system in neighboring normal cells.

Therefore, the anti-inflammatory effects of Nrf2 can be due, in part, to its ability to act as a feedback regulator of NF-κB and the absence of Nrf2 can create a situation where NF-κB lacks a controller to turn off the inflammatory signal, resulting in chronic inflammatory conditions such as observed in arteries damaged by ionizing radiation [36]. For example, Nrf2 knockout mice are more susceptible to lipopolysaccharide (LPS)-induced neuroinflammation. Activation of the Nrf2 pathway in normal cells with sulforaphane decreased the production of inflammatory markers [37]. The Nrf2 target gene hemeoxygenase-1 (HO-1) inhibits NF-κB-mediated transcription of cellular adhesion molecules and could thereby block accumulation of inflammatory cells [38]. In addition, Keap1 downregulates NF-κB by promoting proteasomal degradation of its activator IKKβ [39].

Similarly, there are several examples of how NF-κB downregulates the Nrf2 response. Keap1 interacts with NF-κB, and thereby represses the Nrf2 transcriptional activity [40]. NF-κB blocks Nrf2 transcriptional activation of target genes [41]. This may result in increased oxidative stress which in turn further activates NF-κB [42]. In addition, NF-κB can also promote HDAC3 association with MafK and thus compete with Nrf2 heterodimer formation and transcription of Nrf2-dependent genes [43].

There are also examples where NF-κB promoting activation of Nrf2 and Nrf2-regulated genes. Functional NF-κB-binding sites have been found in the promoter of the NRF2 gene resulting in overexpression of Nrf2 in acute myeloid leukemia cells [44]. In another study, the activation of small GTPase protein RAC1 (Ras-related C3 botulinum toxin substrate 1) induced the expression of Nrf2 target gene HO-1 and caused the inhibition of NF-κB function [45].

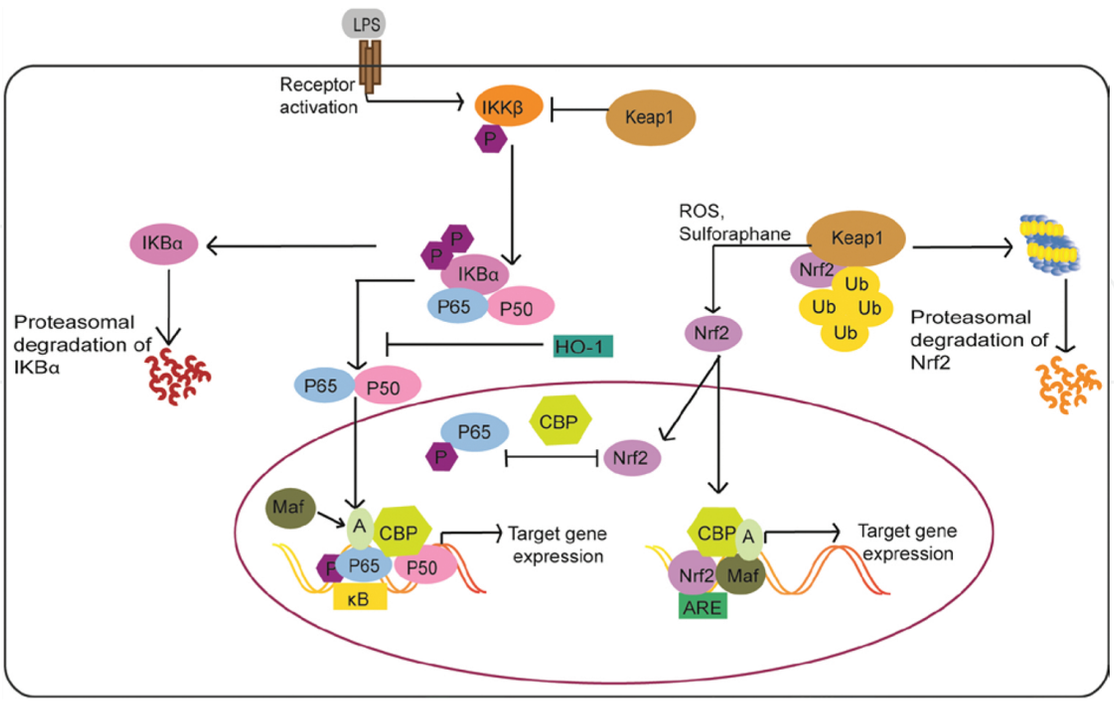

Figure 2. Different factors involved in the functional network between Nrf2 and NF-κB network.

Several other proteins are known to interact with both pathways during the signaling process. One example is p62 protein which accumulates due to autophagy deficiency, and activates Nrf2 through direct interaction with Keap1. P62 sequesters Keap1 into aggregates and inhibits Keap1-mediated ubiquitylation and degradation of Nrf2. This resulted in increased Nrf2 stabilization and activation of target genes [46, 47]. Similarly, p62 protein oligomerizes and promotes nerve growth factor (NGF)-mediated NF-κB signaling [48].

Another protein that interacts with both Nrf2 and NF-κB pathway is glycogen synthase kinase-3β (GSK-3β), a Ser/Thr kinase involved in glycogen metabolism and apoptosis. GSK-3β phosphorylates the Neh6 domain of Nrf2 and targets subsequent proteasomal degradation by β-TrCP (β-transducing repeat-containing protein)-Skp1(S-phase kinase-associated protein1)-Cul1-Rbx1 E3 ubiquitin ligase complex [49]. In NF-κB system, GSK-3β phosphorylates p65 subunit and increases its DNA-binding affinity and subsequent transcriptional response [50]. Moreover, β-TrCP mediates proteasomal degradation of the inhibitory protein, IκBα, and allows NF-κB release. Thus, β-TrCP functions as positive regulator of NF-κB activity and negative regulator of Nrf2-ARE activity (**Figure 2**).

4.2. Nrf2 and other signaling pathways

Phosphorylation of Nrf2 by several protein kinases can lead to stabilization and activation of Nrf2. For example, several studies have demonstrated the involvement of P13 kinase/AKT pathway in regulating Nrf2 nuclear translocation and ARE-dependent gene expression [51, 52]. P13K phosphorylation of PKB/Akt suppresses proteasomal degradation of Nrf2 by GSK-3β [53]. In another study, the biotinylated derivative of the triterpenoid CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) activated PI3K-PKB/Akt signaling through modification of Cys-124 in the active site of PTEN, causing inhibition of the lipid phosphatase function of PTEN [54]. Similarly, increased Nrf2 activity was observed in PTEN-mutant cells with increased activation of the PI3K-Akt signaling which in turn suppressed the GSK-3β-mediated Nrf2 repression [55]. But in Keap1-deficient cells, the deletion of PTEN increased Nrf2 accumulation to a greater extent and this supports the regulatory role of PTEN-GSK-3-β-TrCP signaling [56]. Under conditions of autophagy dysregulation, Nrf2 is activated by p62. In a recent study, Nrf2 inducer sulforaphane activated p62 through SPBP (stromelysin-1 platelet-derived growth factor-responsive element-binding protein), which acts as a coactivator of Nrf2 [57]. Another Nrf2 activator, arsenic, activates Nrf2 through p62-mediated Keap1 sequestration and this persistent activation of Nrf2 can be the reason for arsenic-induced toxicity [58]. The mitogen-activated protein kinase (MAPK)-signaling pathway is activated by Nrf2 inducers like tBHQ and sulforaphane [59, 60]. P38, a member of MAPK family, has been shown to influence Nrf2 activation both in positive and negative manner and this suggests the complex nature of Nrf2 regulation by MAPK [60]. During endoplasmic reticulum stress, protein kinase PERK activates Nrf2 and provides cell survival benefits. PERK-induced phosphorylation of Nrf2 allows its release from Keap1 and translocation into the nucleus for subsequent gene transcription [61].

Another signaling pathway involving protein kinase C has been shown to increase Nrf2-target gene expression while its inhibition caused significant decrease in tBHQ-induced Nrf2 nuclear

translocation [62]. PKC phosphorylates serine 40 of Nrf2 resulting in the Nrf2 release from Keap1 for subsequent gene expressions and mutation of specific residue reduced the gene expression level by 50% indicating that PKC functions along with Keap1 [63, 64].

5. Differential Nrf2 response

Based on the evidences, different inducers target specific or combination of different cysteine residues to activate Nrf2, suggesting the function of "cysteine codes" which converts the preferential target cysteine modifications into distinct biological effects. Understanding the cysteine code for each Nrf2-activating compound will help to increase the biological effects of different inducers. However, Nrf2 activation could not be solely responsible for the diverse biological effects caused by Nrf2 inducers. Most of the Nrf2-inducing agents have the inherent ability to react with cysteine residues and there is a possibility of interaction with other cellular proteins and thereby generating distinct cellular response. For example, a proteomics study of sulforaphane-derived sulfoxythiocarbamate analogs has identified different protein targets other than Keap1 [65]. Many Nrf2 inducers are well known for the prevention and treatment of several human disorders and some of them have been clinically investigated. For example, the methyl ester derivative of CDDO triterpenoid is a potent Nrf2 inducer and as low nanomolar concentrations of CDDO-Me (bardoxolone methyl) stimulate Nrf2-dependent gene expressions. The phase II clinical trials for the treatment of chronic kidney disease (BEAM study) in patient with Type 2 diabetes indicated that CDDO-Me could improve kidney function. However, the phase III trial (BEACON study) was terminated due to serious side effects and mortality observed in treated group [66]. However, the exact mechanism behind the adverse effects are not clear, the long-term drug exposure as well as administration of fixed dose of drug not adjusted for kidney function might have influenced the response. Nrf2 is overexpressed in many types of cancers and several oncogenes are reported to evoke Nrf2 expression in cancer cells [67]. As Nrf2 plays a key role in cytoprotection, cancer cells benefit the protective effect of Nrf2 to create a favorable microenvironment for tumor growth and drug resistance. Studies found that two different antioxidants, N-acetylcysteine (NAC) or vitamin E supplementation in mice with lung tumors substantially increased the number, size, and stage of the tumors. NAC and vitamin E reduced the ROS levels, DNA damage, and expression of *p53* tumor-suppressor gene [68]. Similarly, NAC and vitamin E supplementation increased metastasis in mice with malignant melanoma [69]. Consistent results were observed in another independent study where NAC administration increased metastasis of melanoma in mice [70]. It is notable that ROS plays both negative and positive roles in cellular signaling. In normal conditions, low levels of ROS may function as messengers in cell signaling, while excess ROS levels have adverse effect on cellular macromolecules and lead to cell death. Antioxidant agents protect cells against ROS by increasing the antioxidant potential through Nrf2 activation. However, suppressing normal physiological ROS may affect the cell communication and signal transduction. For example, exercise generates ROS but promotes health benefits, especially in increasing insulin sensitivity. Transient production of ROS during exercise induced signaling systems that activated molecular targets for insulin sensitivity;

however, antioxidant supplementation blocked the molecular signaling for cellular defense and insulin sensitivity mediated by exercise-induced ROS formation and thereby abrogates the health-promoting effects of exercise [71].

Several complex transcriptional and posttranslational networks are involved in mediating Nrf2 activation and thereby enabling diverse functional response. Moreover, networking with other signaling pathways expands the function of Nrf2 as a potent regulator of differential biological processes such as cell proliferation, apoptosis, angiogenesis, and metastasis. The cross-talks between different transcription factors may influence the outcome of therapeutic interventions. Understanding the molecular mechanisms involved in regulating Nrf2 can therefore provide insights that may benefit novel therapeutic manipulation of this pathway.

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