ROS

REVIEW ARTICLE

Regulation of Nrf2 Signaling

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ABSTRACT | Regulation of antioxidant gene expression is essential for controlling oxidative stress and maintaining physiological homeostasis. In this context, the nuclear factor E2-related factor 2 (Nrf2) has been identified as the chief regulator of the transcription of diverse antioxidant genes as well as many other cytoprotective genes. Nrf2 activity is subjected to the regulation at various levels including protein stability, transcription, and post-transcription. Among the various regulatory pathways, the Keap1-Cul3-Rbx1 axis is the most prominent regulator of Nrf2 activity. Being a tightly controlled transcriptional activator of antioxidant genes, Nrf2 signaling is intimately involved in health and disease. While Nrf2 is a protector against oxidative and electrophilic tissue injury, persistent activation of Nrf2 signaling may also contribute to disease pathophysiology, such as cancer progression.

KEYWORDS | Antioxidant response element; Antioxidant; Aromatic hydrocarbon receptor; Gene regulation; Keap1; MicroRNA; NF-κB; Nrf2; p53; p62; Reactive oxygen species

ABBREVIATIONS | ARE, antioxidant response element; GSK-3β, glycogen synthase kinase-3beta; Hrd1, 3-hydroxy-3-methylglutaryl reductase degradation 1; NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor E2-related factor 2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; β-TrCP, beta-transducin repeats-containing protein

CONTENTS

1. OVERVIEW

Studies during the 1980s demonstrated that administration of chemoprotective agents to animals led to the simultaneous induction of multiple endogenous antioxidant enzymes including glutathione transferases (GST), and NAD(P)H:quinone oxidoreductase-1 (NQO1) [1–3]. The simultaneous induction of various antioxidants by chemical inducers suggested the existence of a central regulator for the above cellular defenses. Subsequent studies in early 1990s revealed an essential role for a cis-acting element, called antioxidant response element (ARE) [4] or electrophile response element (EpRE), in the regulation of antioxidant gene transcription [5]. In 1997, Itoh et al. discovered that the nuclear factor E2-related factor 2 (Nrf2) acts as an indispensable transcription factor that interacts with the ARE, leading to the increased transcription of various antioxidative and other cytoprotective genes [6].

Nrf2 is a member of the vertebrate Cap'n'Collar (CNC) transcription factor subfamily of basic leucine zipper (bZip) transcription factors. Other members of the CNC subfamily of transcription factors include nuclear factor E2-related factors 1 and 3 (Nrf1 and Nrf3), and p45 NF-E2. It is established that Nrf2 plays a central role in regulating both the constitutive and inducible expression of a wide variety of mammalian antioxidant genes. Nrf2 activation occurs under various stress conditions, such as exposure to mild oxidative or electrophilic stress. Multiple classes of chemical inducers are known to elevate endogenous antioxidants via activating Nrf2 [7]. Indeed, the most important feature of Nrf2 is the inducibility of its activity [7].

Activation of Nrf2 occurs primarily by increased stability of the Nrf2 protein and consequently more Nrf2 molecules are available for binding to the ARE to cause increased transcription of the antioxidant genes. In addition, Nrf2 activity can also be modulated at the transcriptional and post-transcriptional levels. Thus, this article first describes the various cellular factors and pathways regulating Nrf2 protein stability and then considers the regulation of Nrf2 gene expression at both transcriptional and post-transcriptional levels. The article concludes with a brief introduction to Nrf2 signaling in health and disease, emphasizing the notion that Nrf2 is a double-edged sword.

2. REGULATION OF NRF2 PROTEIN STABILITY

Several cellular factors are involved in the regulation of Nrf2 protein stability and consequently its nuclear translocation. Among them, Keap1 is the most prominent one.

2.1. Keap1 as the Chief Regulator of Nrf2 Protein Stability

Nrf2 normally resides in the cytosolic compartment through association with a cytosolic actin-binding protein, Keap1 (Kelch-like ECH-associated protein 1), which is also less commonly known as INrf2 (inhibitor of Nrf2). Keap1 plays a central role in the regulation of Nrf2 activity. Keap1 exists as dimers inside the cells. It functions as a substrate linker protein for the interaction of Cul3/Rbx1-based E3-

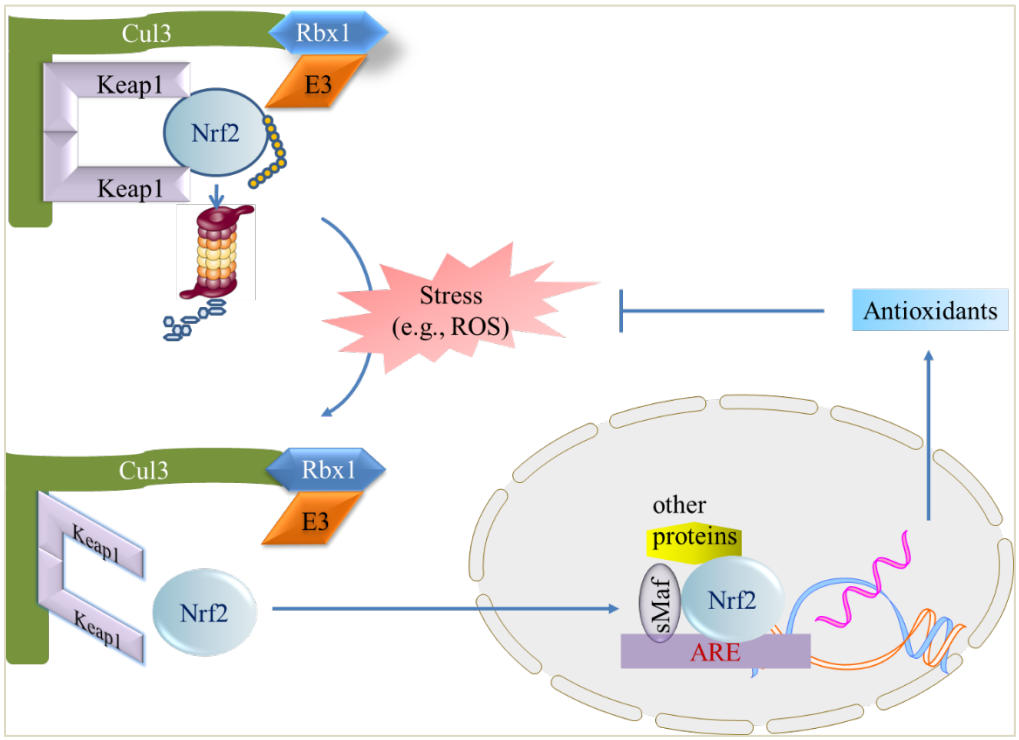

FIGURE 1. Keap1 as the chief regulator of Nrf2 activation and consequent ARE-driven antioxidant gene expression. As illustrated, under basal conditions, Keap1 functions as a substrate linker protein for interaction of Cul3/Rbx1-based E3-ubiquitin ligase complex with Nrf2, leading to continuous ubiquitination of Nrf2 and its proteasomal degradation. Under stress conditions, such as exposure to ROS, Nrf2 dissociates from Keap1(primarily as a result of oxidation of the cysteine residues of Keap1; see Figure 2) and becomes stabilized, and then translocates into the nucleus, activating ARE-driven antioxidant gene transcription. The increased antioxidants render the cells resistance to oxidative stress.

ubiquitin ligase complex with Nrf2, leading to the continuous ubiquitination of Nrf2 and its subsequent proteasomal degradation. Hence, the continuous degradation of Nrf2 under basal conditions keeps the Nrf2 level low and consequently the low basal levels of Nrf2-regulated antioxidants. When cells encounter stress, such as exposure to mild oxidative or electrophilic stress, or chemical inducers, Nrf2 dissociates from Keap1, becomes stabilized, and translocates into the nuclei. Inside the nuclei, Nrf2 interacts with other protein factors, including small Maf (sMaf), and binds to ARE, leading to increased transcription of antioxidant genes (**Figure 1**) [7]. In mammals, including humans, the Keap1-Cul3-Rbx1 axis is known as the most critical regulatory mechanism of Nrf2 activity.

2.2. Mechanisms Leading to Nrf2 Dissociation from Keap1

Several mechanisms have been proposed to explain the dissociation of Nrf2 from Keap1 under stress conditions. Among them, oxidation of the cysteine residues of Keap1 and the binding of p62 to Keap1 have received much attention (**Figure 2**).

2.2.1. Oxidation of the Cysteine Residues of Keap1

Keap1 is a cysteine-rich protein and some of the cysteine residues serve as redox sensors for electrophiles and reactive oxygen species (ROS). Modifications of these cysteine sulfhydryl groups, especially Cys151, Cys273, and Cys288, of the Keap1 protein cause dis-

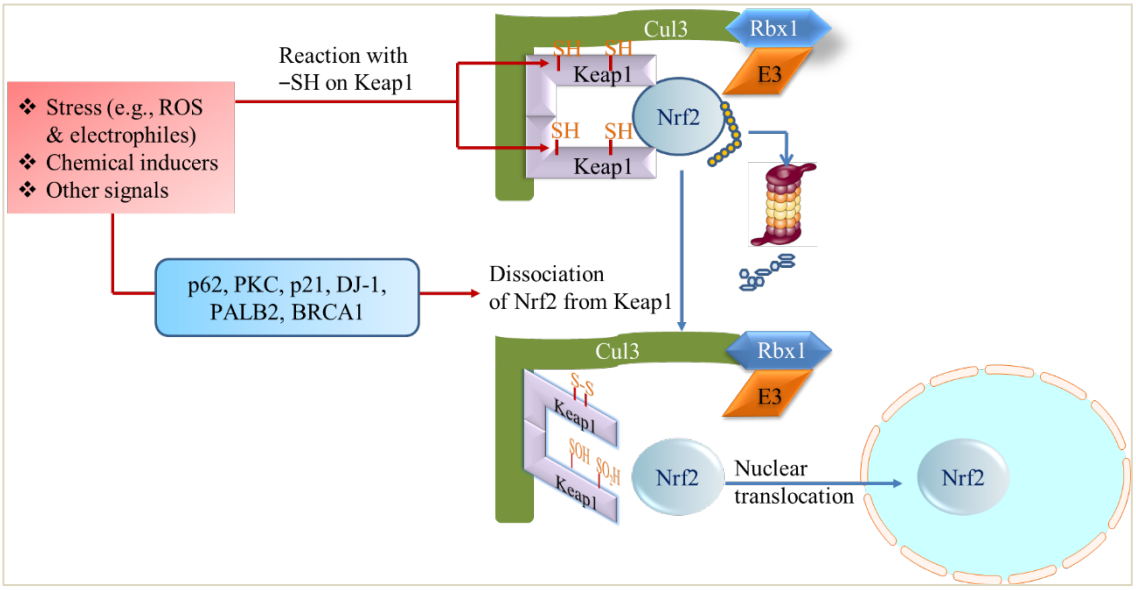

FIGURE 2. Mechanisms causing the dissociation of Nrf2 from Keap1. As illustrated, the predominant mechanisms by which stress conditions (e.g., oxidative and electrophilic stress) and chemical inducers cause the dissociation of Nrf2 from Keap1 is the oxidation of cysteine residues of Keap1. The cysteine sulfhydryl groups may be oxidized to form disulfide bridge, sulfenic acid (–SOH), or sulfinic acid (–SO_2H). These redox modifications cause the separation of Keap1 from Nrf2, leading to Nrf2 stabilization and nuclear translocation. In addition to the above major mechanism, protein kinase C may phosphorylate Nrf2, causing its dissociation from Keap1. Likewise, cellular factors such as p62, p21, DJ-1, PALB2, and BRCA1 may disrupt the binding between Nrf2 and Keap1 via direct protein-protein interactions.

sociation of Nrf2 from Keap1. As such, the Keap1-Nrf2 system is considered primarily a thiol-based sensor-effector apparatus for maintaining cellular redox homeostasis [7].

2.2.2. Binding of p62 to Keap1

p62, also known as sequestosome 1 (SQSTM1), is a ubiquitin-binding protein that targets protein aggregates for degradation via the autophagic pathway. p62 competes with Nrf2 for binding to Keap1, and binding of p62 to Keap1 leads to the degradation of Keap1 and the consequent Nrf2 stabilization [8, 9]. Notably, there is an ARE in the p62 gene promoter, and p62 is a target gene for Nrf2, thus creating a positive feedback loop by inducing ARE-driven gene transcription [10]. In other words, p62 increases Nrf2 protein stability, and Nrf2 activates p62 gene expression, and the increased p62 further increases Nrf2 protein stability, hence forming a positive feedback loop. As p62 is a cargo receptor for selective autophagy, Keap1-Nrf2 has an intriguing functional interaction with autophagy [11].

p62 may also link other cellular processes to Nrf2 activation. For instance, the stress-inducible antioxidant proteins sestrin 1 and sestrin 2 are shown to interact with Keap1, p62, and the ubiquitin ligase Rbx1. It is suggested that the antioxidant function of sestrins is mediated through the activation of Nrf2 in a manner reliant on p62-dependent autophagic degradation of Keap1 [12, 13].

2.2.3. Involvement of Other Factors

Protein kinase C (PKC) phosphorylates Nrf2 at Ser40, promoting the dissociation of Nrf2 from Keap1, thereby leading to increased transcription of ARE-driven antioxidant genes [14]. As PKC is redox-sensitive, ROS may cause activation of Nrf2 partly via redox modulation of PKC signaling.

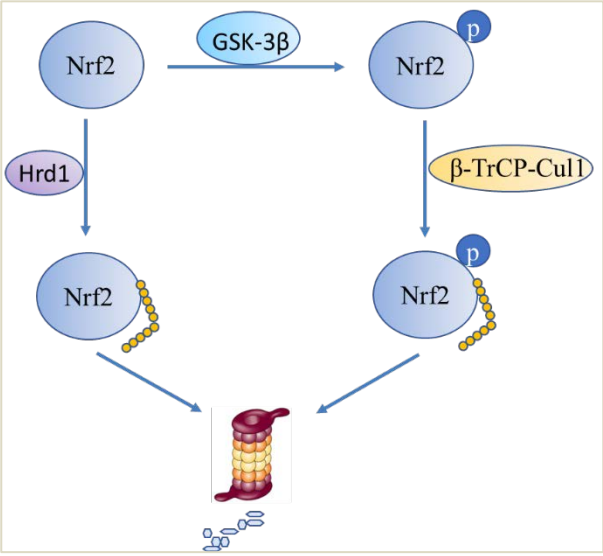

FIGURE 3. The Keap1-independent regulation of Nrf2 protein stability. As illustrated, the Keap1-independent regulation of Nrf2 protein stability may occur via two pathways. One pathway is that GSK-3β phosphorylates Nrf2 enabling it to be recognized by β-TrCP and ubiquitylated by the β-TrCP-Cul1 E3 ubiquitin ligase complex for the eventual proteasomal degradation. The other pathway is dependent on Hrd1, an E3 ligase that ubiquitylates Nrf2 for proteasomal degradation. Hence, both pathways are negative regulators of Nrf2 protein stability, and inhibition of these pathways would cause Nrf2 activation and increased antioxidant gene expression.

$p21^{Cip1/WAF1}$ and DJ-1 also stabilize Nrf2 by disrupting the Nrf2-Keap1 interaction [15, 16]. $p21^{Cip1/WAF1}$ is a cyclin-dependent kinase (cdk) inhibitor and is a key mediator of p53-dependent cell cycle arrest after DNA damage. DJ-1 is a protein deglycase, also known as Parkinson's disease protein 7, encoded by Park7 gene. It protects neurons from oxidative stress [17].

Proteins that stabilize Nrf2 via affecting the binding between Nrf2 and Keap1 also include PALB2 (partner and localizer of BRCA2) [18] and the tumor suppressor BRCA1 [19]. As the name indicates, the PALB2 protein binds to and colocalizes with the tumor suppressor BRCA2 in nuclear foci and likely permits the stable intranuclear localization and accumulation of BRCA2.

2.3. Keap1-Independent Regulation of Nrf2 Protein Stability

While Keap1 is the major cellular factor controlling Nrf2 activation, regulation of Nrf2 protein stability also occurs via Keap1-independent mechanisms. Among them, β-TrCP-Cul1- and Hrd1-dependent pathways are most notable (**Figure 3**).

2.3.1. β-TrCP-Cul1-Dependent Pathway

Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway causes Nrf2 activation, increasing ARE-driven antioxidant gene transcription [20]. A critical mediator in the PI3K/Akt-dependent pathway is glycogen synthase kinase-3beta (GSK-3β), which phosphorylates Nrf2 [21]. This phosphorylation enables Nrf2 to be recognized by beta-transducin repeat-containing protein (β-TrCP) that in turn marks Nrf2 for ubiquitination. Following ubiquitination by the β-TrCP-Cul1 E3 ubiquitin ligase complex, Nrf2 is degraded by the proteasome [22, 23]. On the other hand, PI3K/Akt phosphorylates GSK-3β, leading to its inhibition, thus linking PI3K/Akt activation to increased Nrf2 protein stability and augmented transcription of Nrf2/ARE-regulated genes [21].

2.3.2. Hrd1-Dependent Pathway

Hrd1 (3-hydroxy-3-methylglutaryl reductase degradation 1) is an E3 ligase known to play a role in the degradation of misfolded proteins in the endoplasmic reticulum. Hrd1 also causes ubiquitylation of Nrf2, leading to its degradation by the proteasome. Hrd1 is hence considered a negative regulator of Nrf2 activity [24]. Hrd1 is found to suppress Nrf2-mediated cellular protection during liver cirrhosis, and pharmacological inhibition of Hrd1 may prevent Nrf2 degradation and suppress liver cirrhosis [24].

3. REGULATION OF NRF2 GENE TRANSCRIPTION

In addition to the modulation of Nrf2 protein stability, regulation of Nrf2 signaling occurs also at the transcriptional level. The transcriptional factors involved include the aryl hydrocarbon receptor (AhR), NF-κB, and Nrf2 itself.

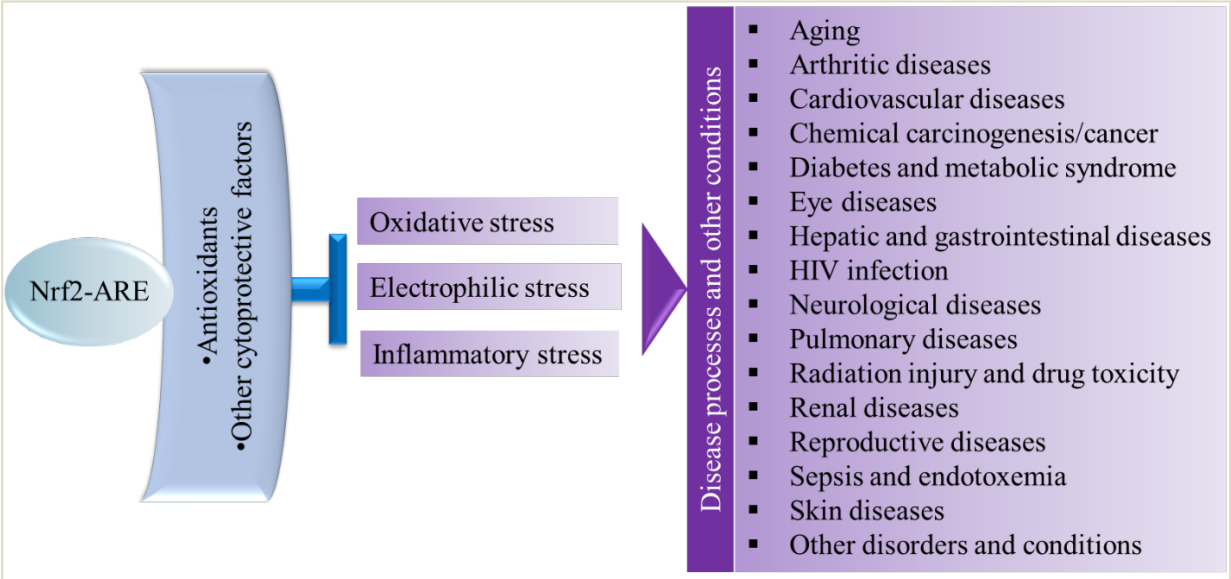

FIGURE 4. Nrf2 as a protector in disease processes and related conditions. Via regulating cellular antioxidants and other cytoprotective factors, the Nrf2-ARE pathway protects against oxidative stress, electrophilic stress, and inflammatory stress. Primarily based on studies with the Nrf2-knockout mouse model, maintaining and activating a functional Nrf2-ARE signaling has been found to play a protective role in a wide variety of disease processes and related conditions (e.g., aging) involving oxidative, electrophilic, and inflammatory stresses.

3.1. AhR

The transcription of Nrf2 gene is found to be activated by AhR. This is due to binding of the AhR (as a heterodimer with AhR nuclear translocator) to the xenobiotic response element-like sequences in the Nrf2 gene promoter and the consequent transactivation of the Nrf2 gene [25]. This pathway is responsible for the activation of Nrf2 by xenobiotics that are AhR ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [26].

3.2. NF-κB

Nrf2 gene promoter also contains a binding site for NF-κB, and NF-κB subunits p50 and p65 induce transactivation of Nrf2 gene [27]. This explains the activation of Nrf2 by NF-κB-activating inflammatory cytokines. Although NF-κB activates Nrf2, Nrf2 activation attenuates NF-κB signaling, suggesting a cross-talk between Nrf2 and NF-κB [28]. Inhibition of NF-κB signaling by Nrf2 may contribute, at least partly, to the anti-inflammatory function of Nrf2 activators, such as sulforaphane [29]. How Nrf2 suppresses NF-κB signaling remains elusive. It is suggested that Nrf2 activation may shift the cellular redox status to a more reducing state due to increased expression of antioxidants. Such a more reducing state attenuates NF-κB activation because NF-κB is less readily activated in a reducing environment [30, 31].

3.3. Nrf2 Autoregulation

There is evidence for the autoregulation of Nrf2 signaling. On the one hand, due to the presence of ARE-like sequences in the promoter region of the Nrf2 gene, Nrf2 may activate its own gene expression, leading to increased production of Nrf2 protein [32]. This represents a positive feedback mechanism. One the other hand, Nrf2 may stimulate Keap1 gene expression for its own degradation [33]. This negative feedback might limit the undue expression of Nrf2 and uncontrolled Nrf2 signaling.

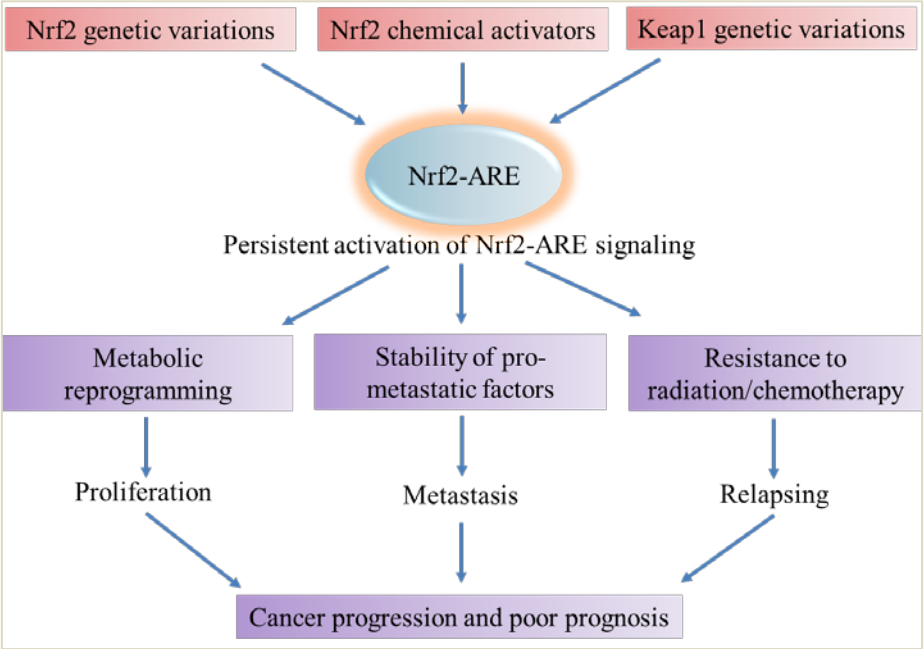

FIGURE 5. Nrf2 as a promoter in cancer development and progression. As illustrated, genetic variations in Nrf2 and Keap1 genes as well as chemical inducers may cause persistent activation of the Nrf2-ARE pathway leading to increased antioxidants and redox changes (not shown). Such changes may lead to (1) metabolic reprogramming of the cancer cells, promoting cancer cell proliferation, (2) increased stability of pro-metastasis factors, such as Bach1, promoting cancer metastasis, and (3) increased cellular defenses against cytotoxic anti-cancer drugs, causing drug resistance and cancer relapse. Collectively, these contribute to the progression of cancer and poor prognosis.

4. OTHER REGULATORY MECHANISMS OF NRF2 SIGNALING

4.1. MicroRNAs and Gene Splicing

Post-transcriptionally, Nrf2 is regulated by multiple miRNAs. Among them, miR-144 is the first microRNA shown to silence Nrf2 gene [34]. Nrf2 may also be regulated via alternative splicing in certain tumors; some splice variants lack the Keap1-interacting domain, thus resulting in increased Nrf2 protein stability and Nrf2-mediated antioxidant gene expression [35].

4.2. Interference with ARE

The tumor suppressor p53 is shown to suppress the Nrf2-dependent transcription of certain antioxidant genes possibly via direct interaction with the ARE-containing promoters, diminishing the promoter activity [36]. Likewise, activation of retinoic acid receptor alpha suppresses Nrf2-mediated antioxidant gene expression via forming a complex with Nrf2, causing decreased binding of Nrf2 to the ARE [37]. On the other hand, p300/CBP histone acetyltransferases are reported to directly bind and acetylate Nrf2 in the nuclei, leading to enhanced promoter-specific DNA binding of Nrf2 in response to arsenite-induced oxidative stress [38].

5. NRF2 SIGNALING IN HEALTH AND DISEASE

5.1. Nrf2 as a Disease Protector

The role of Nrf2 in health and disease has been extensively investigated primarily using the Nrf2-

knockout (also known as Nrf2-null or Nrf2-deficient) mouse model. With this animal model, Nrf2 has been found to play a protective role in disease conditions involving virtually all of the major organs or systems (**Figure 4**) [7].

The pathophysiological processes on which Nrf2 signaling provides protective effects involve, at least partially, the following: (1) oxidative and inflammatory stress; (2) electrophilic stress; or (3) chemical carcinogenesis. This is consistent with the ability of Nrf2 to regulate genes whose products play a major role in suppressing dysregulated inflammation and in the detoxification of ROS and electrophilic species [39, 40]. In this regard, oxidative and electrophilic stress is an important mechanism underlying chemical carcinogenesis [41, 42].

5.2. Nrf2 as a Disease Promoter

Although Nrf2 plays a critical role in protecting against chemical carcinogenesis in various animal models [43, 44], persistent activation of this transcription factor may play an unfavorable role in the development of cancer not immediately related to exposure to chemical carcinogens. Mutations or epigenetic modifications affecting the regulation or fate of Nrf2 can lead to constitutive hyperactivation of the Nrf2 signaling. Such a hyperactivation of Nrf2 signaling is found to preserve cancer phenotypes by mechanisms including: (1) metabolic reprogramming of the cancer cells [45–47]; (2) stabilizing pro-metastatic transcription factors, such as Bach1 [48]; and (3) providing selective resistance of the cancer cells to stresses, including the cytotoxicity of cancer chemotherapeutic drugs [27, 49] (**Figure 5**).

6. CONCLUSION

As an essential pathway regulating the transcription of a wide variety of antioxidative and other cytoprotective genes, Nrf2-ARE signaling is subjected to the regulation at the levels of protein stability, transcription, and post-transcription. Nrf2, however, is found to be a double-edged sword, meaning that activation of the Nrf2-ARE signaling can lead to either beneficial or detrimental consequences depending on the disease processes involved. It is imperative to take this notion into account when devising Nrf2-based strategies for disease intervention.

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