

The Nrf2-Keap1-ARE Signaling Pathway: The Regulation and Dual Function of Nrf2 in Cancer

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Abstract

The NF-E2-related factor-2 (Nrf2) is a transcription factor that is ubiquitously expressed at low levels in all human organs. As Nrf2 regulates a major cellular defense mechanism, tight regulation is crucial to maintain cellular homeostasis. Activation of this pathway is important in preventing human diseases, such as cancer, neurodegenerative disease, cardiovascular diseases, ischemia, diabetes, pulmonary fibrosis, and inflammatory diseases. Conversely, high constitutive levels of Nrf2 occur in many tumors or cancer cell lines. Moreover, overexpression of Nrf2 in cancer cells protects them from the cytotoxic effects of anticancer therapies, resulting in chemo- and/or radioresistance. Therefore, understanding Nrf2 regulation and identifying Nrf2 activators or inhibitors for disease prevention or as sensitizing agents during cancer therapy, respectively, will have a significant impact on human health. The growing interest in Nrf2 is reflected by an exponentially increasing number of annual publications on this topic. The present forum provides a comprehensive review on research conducted since the initial discovery of the Nrf2-Keap1 pathway in 1999, provides readers with insight into contemporary research in this field, and assesses current known biological functions of this pathway. Additionally, the forum will serve as an important resource for both graduate students and active researchers who are interested in the Nrf2 field. *Antioxid. Redox Signal.* 13, 1623–1626.

Discovery and Regulation of the Nrf2-Keap1-ARE Signaling Pathway

CANCER CAN BE A PREVENTABLE DISEASE. For over half a century, scientists have engaged in identifying compounds, naturally occurring or synthetic, to block or suppress carcinogenesis. Most of these anticancer agents, collectively referred to as chemopreventive compounds, are able to up-regulate the expression of a broad array of detoxifying enzymes. In the early 1990s, careful analysis of the promoters of these genes led to the identification of a consensus sequence, termed the antioxidant response element (ARE). As more and more AREs were identified, it became apparent that the ARE sequences of different genes, and even of the same gene from different species, varied significantly. In this forum, John Hayes and colleagues have carefully compared AREs and categorized them into four classes: (i) 16-bp ARE with an embedded AP1-binding site, (ii) 16-bp ARE without an embedded AP1 site, (iii) 11-bp ARE with an embedded AP1-binding site, and (iv) 11-bp ARE without an embedded AP1 site (6).

NF-E2-related factor-2 (Nrf2), which belongs to the cap “n” collar family of transcription factors, was cloned independently in the laboratories of Y.W. Kan (human Nrf2) (23) and

Masayuki Yamamoto (ECH, chicken Nrf2) (9) in 1994 and 1995, respectively. Soon after, Jaiswal’s group provided evidence demonstrating that Nrf2 positively regulates NAD(P)H quinone oxidoreductase through its ARE (35). Nevertheless, the most convincing data demonstrating that Nrf2 is a critical regulator of AREs came from Tom Kensler’s laboratory. This group showed that Nrf2-knockout mice had reduced levels and impaired induction of detoxifying enzymes and redox-balancing proteins, rendering the knockout mice more susceptible to carcinogen-induced cancers (26). By transcriptional activation of an array of ARE-bearing genes, including detoxifying genes, drug transporters, and cellular redox regulators, Nrf2 has emerged as the master regulator of a cellular defense mechanism that elicits an adaptive response and promotes cell survival under stress. In this issue, Young-Joon Surh’s group provides an original research article demonstrating that this Nrf2-mediated adaptive response also provides protection against stress induced by glucose deprivation. In addition, Surh suggests that upregulation of heme oxygenase-1, an Nrf2 target gene, contributes largely to this adaptive response (19).

The year 1999 marked the discovery of the Nrf2-Keap1-ARE signaling pathway. Ken Itoh and colleagues from Masayuki Yamamoto’s group reported their seminal work on the

identification of a negative regulator of Nrf2, which they named Keap1 (11). In one of the forum's review articles, Ken Itoh highlights the historical perspectives of the pathway and how it has evolved into an important topic of research (10). Identification of Keap1 quickly directed the researchers in the field to focus on the molecular mechanisms of Nrf2 regulation. Two important mechanisms of Nrf2 activation were elucidated between 2002 and 2004. (i) Cysteine residues in Keap1 act as sensors of oxidative stress. Keap1 is a cysteine-rich protein containing 25 cysteine residues in mice or 27 residues in humans. Using purified murine Keap1, Dinkova-Kostova and colleagues from Paul Talalay's group provided the first *in vitro* evidence demonstrating that four specific cysteine residues, C257, C273, C288, and C297, were the most reactive toward dexamethasone, implicating the importance of these residues in redox sensing (4). Using a mutagenesis approach, Zhang *et al.* demonstrated *in vivo* that C151 was essential for activation of the Nrf2 pathway by *tert*-butylhydroquinone and sulforaphane, two well-studied inducers of Nrf2. However, C151 was not important for repressing Nrf2 under uninduced conditions. In contrast, mutation of C273 or C288 gave rise to a nonfunctional Keap1 that loses its inhibitory effect on Nrf2 (43). Similar conclusions were reached in regards to C273 and C288 by Paul Talalay and his collaborators and by Darley-Usmar's group (21, 38). Subsequently, a great deal of effort was invested in identifying the most important cysteine residues in Keap1 required for redox sensing and Nrf2 regulation. Holland and Fishbein have extensively summarized what is known and unknown regarding the chemistry of cysteine residues in Keap1 (7). (ii) Another significant achievement in the field was the identification of Keap1 as a substrate adaptor protein for the Cul3-E3 ligase, and the understanding of how Keap1 is able to repress the activity of Nrf2. In 2004, Keap1 was shown to be a substrate adaptor for a Cul3-containing E3 ligase by four independent groups (3, 5, 18, 44). The Cul3-Keap1 E3 ligase is one of many cullin-ring-ligases. Currently, it is well established that Keap1 is the major regulator of Nrf2. Keap1 controls the stability and thus the steady-state level of Nrf2 according to cellular redox conditions. Under redox-balanced conditions, Keap1 constantly targets Nrf2 for ubiquitination and subsequent degradation by the 26S proteasome. Under induced conditions, the activity of the Keap1-Cul3 E3 ligase is inhibited due to modification of cysteine residues in Keap1, resulting in stabilization of Nrf2 and activation of the Nrf2 pathway. A key addition to this model of Nrf2 regulation by Keap1 is the development of the "two-site substrate recognition," also known as the "hinge and latch" model (22, 34). It is thought that there are two binding sites within the Neh2 domain of Nrf2, a weak and a strong binding site, and each site binds to one Kelch domain in Keap1 in a heterotrimer complex consisting of two Keap1 molecules and one Nrf2 molecule. It was speculated that binding of both the "hinge" and "latch" sites to the two Kelch domains of the Keap1 homodimer locks the seven lysine residues within the Neh2 domain in a precise orientation for ubiquitin conjugation. In response to activation signals, modification of cysteine residues in Keap1 may cause a conformational change, which likely disrupts the weak latch binding site, thus preventing ubiquitin conjugation onto Nrf2. However, the precise mechanism by which the modification of cysteine residues in Keap1 leads to a conformational change remains elusive. For a more detailed overview on the regulation of Nrf2

by the Cul3-Keap1 E3 ligase, including comparison between different cullin-ring-ligases, their substrates, and how they are generally regulated, please see the review article contributed by my group (36).

Another recent development in the field is the recognition of crosstalk and integration between the Nrf2-Keap1-ARE pathway and other important pathways. In this issue, Tom Kensler and colleagues have provided a comprehensive review on the crosstalk between the Nrf2 signaling pathway and the consequence of such crosstalk with the arylhydrocarbon receptor, NF- κ B, p53, and Notch1 signaling pathways (37). The precise mechanisms and the functional importance by which these signaling pathways are integrated are still under intensive investigation. Identification of the crosstalk between the Nrf2-Keap1-ARE pathway and other pathways certainly demonstrates the existence of complex cellular networks.

The Dual Role of Nrf2 in Cancer

Nrf2 controls a critical cellular defense response by coordinated upregulation of many of its target genes, culminating in a cell survival response. Under stress conditions, prompt activation of the Nrf2-mediated defense response is important to maintain cellular homeostasis and to prevent the initiation of disease. A wealth of knowledge has been gained demonstrating that Nrf2 is essential in preventing many types of human diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, ischemia, diabetes, lung fibrosis, and inflammatory diseases (1, 13, 14, 16, 20, 27, 41, 42). For this reason, many researchers have focused on identifying potent Nrf2 inducers with low toxicity to enhance the Nrf2-mediated adaptive response for disease prevention. This has proven to be an effective approach, especially for chemoprevention. For example, human clinical trials, pioneered by Tom Kensler, were conducted to evaluate the efficacies of Nrf2 inducers such as oltipraz and broccoli sprouts. The results demonstrate the practical use of Nrf2 inducers to reduce carcinogenic metabolites of aflatoxin (15, 39). Mechanistic studies indicate that the chemopreventive activities of Nrf2 inducers lie in their ability to modulate absorption, distribution, metabolism, and excretion of carcinogens, as well as the anti-inflammatory response. As chronic inflammation has been recognized as a risk factor for cancer development, the anti-inflammatory activity of Nrf2 is considered a contributing factor to its role in chemoprevention. For more details regarding the association of the Nrf2-mediated antioxidant response and the anti-inflammatory response, please see the review article by Tony Kong and his collaborators (8).

Paradoxically, the role of Nrf2 in cancer promotion and in cancer resistance to therapeutic treatment, the "dark" side of Nrf2, has recently been revealed. Constitutive upregulation of Nrf2 is associated with many types of cancers, including skin, breast, prostate, lung, head/neck, and endometrium (12, 17, 24, 25, 28, 32, 33, 45). Sequence analysis of Nrf2 or Keap1 has identified many mutations within the Neh2 domain of Nrf2 or in the Kelch domain of Keap1, which result in the stabilization of Nrf2 due to the inability of Keap1 to target Nrf2 for ubiquitination and degradation (17, 24, 25, 28, 32). Clearly, overexpressed Nrf2 faithfully executes its protective role, without discriminating between normal cells and cancer cells. High levels of Nrf2 provide a growth advantage for

cancer cells. Further, Nrf2 promotes cancer cell survival under therapeutic regimens and thus contributes to chemoresistance, as demonstrated by several groups (2, 28, 29, 31, 40). In this issue, Shyam Biswal and colleagues reveal that Nrf2 also confers radioresistance to cancer cells (30). These results provide the molecular rationale for the identification and therapeutic development of Nrf2 inhibitors to enhance the efficacy of current cancer treatments. It is worth noting that the discovery of the dual function of Nrf2 in cancer has brought safety concerns with respect to the use of Nrf2 inducers for disease prevention; however, this is not an issue. In normal cells, Nrf2 is under tight regulation by the Keap1-Cul3 E3 ligase. In response to Nrf2 inducers, Nrf2 is only induced transiently, and functional Keap1 will reduce Nrf2 to low basal levels once redox balance is restored. However, high levels of Nrf2 are constitutively expressed in certain cancer cells, which creates an environment conducive to cell growth and also protects against oxidative stress and anticancer therapeutic agents. The collection of articles in this forum highlights the importance of understanding the molecular regulation of Nrf2, thereby providing a sound rationale for the development of therapeutic drugs targeting Nrf2 for disease prevention and treatment.

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Abbreviations Used

ARE = antioxidant response element
 Nrf2 = NF-E2-related factor-2

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