New insights into nuclear factor erythroid 2-related factors in toxicology and pharmacology

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Published in:
Toxicology and Applied Pharmacology

DOI:
10.1016/j.taap.2019.01.014

Publication date:
2019

Document Version
Peer reviewed version

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The cap'n'collar-basic region leucine zipper (CNC-bZIP) family of transcription factors includes the founding member Nuclear factor-erythroid 2 (NF-E2) p45, NF-E2 p45-related factor 1 (Nrf1; also known as NFE2L1, LCRF1, TCF11, HBZ17 or FLJ00380), Nrf2 and Nrf3 (also abbreviated as NFE2L2 and NFE2L3, respectively), and the more distantly related members BTB (i.e., Broad complex, Tramtrack, and Bric-à-Brac) domain and CNC homolog 1 (BACH1) and BACH2 (Tebay *et al.*, 2015; Katsuoka and Yamamoto, 2016; Zhang and Xiang, 2016; Zhu *et al.*, 2016; Yamamoto *et al.*, 2018). In the last two decades, our understanding of CNC-bZIP proteins has advanced enormously, and our appreciation of their physiological significance has similarly increased. The primary objective of this special issue (SI) is to stimulate continuing effort to understand the toxicological and pharmacological roles of CNC-bZIP proteins, and that of Nrf2 and Nrf1 in particular.

Nrf2 is normally found in the cytoplasm of mammalian cells, where it associates with the redox-sensitive Kelch-like ECH-associated protein 1 (Keap1) E3 ubiquitin ligase substrate adaptor that polyubiquitinylates Nrf2 and targets it for proteolytic degradation by the 26S proteasome. This mechanism keeps cellular Nrf2 levels low and prevents Nrf2 accumulation in the nucleus where it would mediate signaling effects (Suzuki and Yamamoto, 2017; Yamamoto *et al.*, 2018). In response to a wide variety of oxidative and electrophilic insults, Nrf2 avoids Keap1-mediated proteolytic digestion and accumulates in the nucleus where it heterodimerizes with small musculoaponeurotic fibrosarcoma (MAF) proteins and binds to antioxidant response element (ARE; 5′-TGACNNNGC-3′) sequences within target genes, resulting in expression of that gene for a limited period (Suzuki and Yamamoto, 2017; Yamamoto *et al.*, 2018). The target

genes of Nrf2 include those that encode a variety of antioxidant and detoxification enzymes. Thus, the Keap1-Nrf2 system is recognized as a key player in controlling biochemical defense against exogenous and endogenous electrophilic and oxidative stressors. Importantly, accumulating evidence indicates that Nrf2 also plays critical roles in regulating expression of numerous genes involved in cell metabolism, proliferation and differentiation ((Pi *et al.*, 2010; Xue *et al.*, 2013; Murakami and Motohashi, 2015).

In the Review section of this SI, Ryoo and Kwak revisited recent experimental observations on the relationship between Nrf2 and mitochondria and discussed mechanisms by which Nrf2 controls mitochondria and metabolism in cancer cells. These authors report that Nrf2 is positively associated with mitochondrial biogenesis through direct upregulation of mitochondrial transcription factors and is involved in the mitochondrial quality control system via activation of mitophagy. Additionally, Nrf2 modulation in cancer cells leads to changes in the mitochondrial respiratory system and cancer bioenergetics that overall affect cancer metabolism. Ikehata and Yamamoto reviewed recent progress in the study of contributions by Nrf2 and related factors to protection against ultraviolet radiation (UVR). The Keap1-Nrf2 system is not always efficient in responding to UVR, especially to short wavelengths such as UVC and UVB, indicating that UVR is a poor activator of the Keap1-Nrf2 system. However, sustained activation of Nrf2 appears to suppress the harmful effects of chronic UVR exposure, such as photoaging and carcinogenesis in the skin, indicating that Nrf2 activation is beneficial for the protection of the skin from the harmful effects of UVR. However, sustained activation of Nrf2 may also adversely affect the skin, especially in the case of UVR-induced carcinogenesis. Sun *et al.* assessed the roles of Nrf2 in the development of alcoholic liver disease (ALD) and emphasized that Nrf2 in different cell types in the liver may play paradoxical roles in the progression of ALD. In the early stages of ALD, Nrf2 in hepatocytes plays a crucial role in regulating redox balance and lipid metabolism. With the

progression to steatohepatitis, the role of Nrf2 in Kupffer cells become evident, which alleviates the inflammatory response in the liver. During end-stage ALD, Nrf2 in hepatic stellate cells may be critical in modulating fibrogenesis. In light of the important protective roles of Nrf2 against oxidative damage, the study and validation of possible pharmacological targets that would restore the coordination of the networks in related pathologies has recently received particular attention. In the review by Yamawaki *et al.*, they summarized the current issues in the treatment of kidney diseases, Nrf2 activators as treatment options, and perspectives on pharmaceutical applications of Nrf2 activators.

In the Research Articles section of this SI, Raghunath *et al.* identified ARE sequences in all protein-coding genes in the zebrafish genome. They found multiple unique AREs that have not been reported previously in cytoprotective genes of this organism. In a detailed mechanistic study, McMahon *et al.* uncovered that Keap1 directly senses Zn^{2+} through a cluster of amino-acids that include His-225, Cys-226 and Cys-613. They presented evidence that binding of Zn^{2+} triggers a conformational switch in Keap1, which is envisaged to perturb the architecture of the cullin-3 RING ubiquitin ligase (CRL) complex CRL^{Keap1}, such that bound Nrf2 becomes mis-aligned with respect to the ubiquitin-charged E2 enzyme. The data are consistent with the notion that Keap1 possesses a Zn^{2+} sensor whose triggering distorts its structure in a fashion that inhibits ubiquitylation of Nrf2 upon the CRL^{Keap1} complex. Chen's group explored the role of Nrf2 in mediating aberrant hematopoiesis in response to low-dose benzene exposure in *Nrf2*-KO mice. They found that the hematotoxicity of low-dose benzene seems to decrease in *Nrf2*-KO mice based on peripheral blood cell counts, despite the fact that oxidative and DNA damage was significantly enhanced in the mutant mice. In addition, deficiency of Nrf2 triggered proliferation and differentiation of hematopoietic cells by accelerating cell cycle progression and induced a morphological abnormality in peripheral erythrocytes and bone marrow cells, implicating

compensatory changes that allow induction of dysfunctional defective blood cells. In an *in vitro* study, Meng's group reported that rat primary microglia and astrocytes display different responses to lead toxicity. In another *in vitro* study, Zhao's group examined the role of the Nrf2 signaling pathway in the cytotoxicity induced by the hypoxia mimetic cobalt chloride ($CoCl_2$) in human keratinocyte HaCaT cells. These workers found that stable knockdown (KD) of Nrf2 dramatically reduced expression of antioxidant enzymes and sensitized the cells to acute $CoCl_2$-induced oxidative stress and cytotoxicity, whereas *Keap1*-KD cells showed enhanced expression of ARE-driven genes and resistance to $CoCl_2$-induced cell damage. In addition, pretreatment of HaCaT cells with *tert*-butylhydroquinone protected these cells from $CoCl_2$-induced cell injury in an Nrf2-dependent fashion. In a systematic *in vivo* study, Cho *et al.* demonstrated that sulforaphane (SFN) significantly reduced acute lung injury-like phenotypes caused by subsequent hyperoxia exposure in an Nrf2-dependent manner. Differential lung transcriptome changes induced by SFN in wildtype and *Nrf2*-KO mice suggested that it acts through Nrf2 enhancing pulmonary mitochondrial dynamics and metabolism to maintain the bioenergetic demands of lung cells against oxidative stress. As a part of a series of studies on ARE inhibitors from Pi's laboratory, Zhu *et al.* identified a traditional Chinese medicine, triptolide, as an effective and potent Nrf2-ARE inhibitor. Importantly, triptolide, at non-toxic levels, markedly sensitized non-small-cell lung cancer cells to chemotherapeutic treatments *in vitro* and in a xenograft mouse tumor model.

Nrf1 serves as a unique vital player in maintaining cellular homeostasis and organ integrity during normal development and cell growth throughout life. Global loss of Nrf1 results in severe oxidative stress, genomic instability, embryonic lethality and developmental disorders. Conditional knockout of Nrf1 results in adult diseases such as non-alcoholic steatohepatitis, hepatocellular carcinoma, pancreatic β-cell and adipocyte dysfunction and neurogenerative

diseases (Pi *et al.*, 2010; Xue *et al.*, 2013; Murakami and Motohashi, 2015; Zheng *et al.*, 2015; Kim *et al.*, 2016; Fu *et al.*, 2018; Hou *et al.*, 2018; Wang *et al.*, 2018). Thus, Nrf1 is critically implicated in a variety of important physio-pathological processes by governing expression of crucial genes in order to reinforce antioxidant, detoxification and cytoprotective responses to cellular stress. Of clinical interest, Nrf1 mediates the proteasomal 'bounce-back' response, leading to drug resistance to proteasomal inhibitors for clinical treatment of neuroblastoma, multiple myeloma and triple-negative breast cancers (Steffen *et al.*, 2010; Bugno *et al.*, 2015; Sekine *et al.*, 2018). During its translation, Nrf1 is targeted to the endoplasmic reticulum (ER) and subject to extensive post-translational modification before it regulates its target genes. However, the mechanisms whereby Nrf1 is processed and topologically released from the ER before entering the nucleus is hotly debated. In this SI, a series of experiments from Zhang's laboratory demonstrate the maturation processing of Nrf1 to remove its N-terminal ~12.5-kDa and longer polypeptides. The authors have further elucidated topo-vectorial mechanisms that monitor dynamic movement of Nrf1 in and out of the ER lumen, as well as the selective proteolytic processing of the CNC-bZIP protein to remove distinct lengths of its NTD (most of which was refolded as a UBL module) and PEST-adjoining AD1 domains. More importantly, they have also established a general criterion acceptable for identification of the endogenous Nrf1α/TCF11 and derivative isoforms, with distinct molecular weights and half-lives determined in various experimental cellular settings. Furthermore, they propose that coupled positive and negative feedback circuits exist between Nrf1 and its target genes. These results suggest Nrf1 is subject to dual opposing control in which low doses of proteasomal inhibitors elicit a 'bounce-back' response but higher doses inhibit the transcription factor. Collectively, the findings from Zhang's group suggest the potential of Nrf1 to be developed as a new target for chemoprevention and therapy of cancers and other diseases.

Coordinately dealing with fluctuating levels of numerous metabolic and environmental stresses is critical for the survival of cells and whole organisms. Perturbations in redox balance may impair cellular homeostasis and trigger the onset of disease. Accordingly, cells have developed multiple and well-conserved mechanisms that regulate adaptive antioxidant responses. Oxidative stress, which is defined as a general response to internal and environmental oxidative challenges, is involved in triggering adaptation to oxidative damage. On the other hand, persistent oxidative stress may lead to disruption of redox signaling and loss of homeostatic mechanisms (Zhang *et al.*, 2010; Fu *et al.*, 2016). Thus, precise coordination of cellular adaptive responses to oxidative insults promotes stress resistance and recovery of homeostasis, whereas persistent adaptation would have a cost that may be involved in the pathogenesis of many chronic disorders (see Figure 1).

For many decades, ROS were considered cytotoxic waste products arising from cellular processes. Thus, antioxidant interventions were established in the settings of aging and chronic diseases. However, animal studies and epidemiological investigations on the therapeutic outcomes of antioxidant interventions have provided data that contradict the view that ROS are merely of toxicological significance, questioning long-standing beliefs of an ultimate beneficial role for antioxidant therapies in health and disease (Zhang *et al.*, 2010; Fu *et al.*, 2016). In agreement with the aforementioned accumulating evidence and in the context of major chronic diseases, we reasonably hypothesized that persistent activation of Nrfs-ARE caused by environmental stressors may be involved in the pathogenesis of various chronic diseases, such as Type 2 diabetes and malignant tumors (see Figure 1). Of course, coordinated efforts are needed to clarify the exact roles of various isoforms of Nrfs in the development and intervention of various chronic diseases.

In summary, while significant progress has been made in terms of elucidating how the different Nrf transcription factors (in particular Nrf2, and to a much lesser extent Nrf1) regulate the antioxidant response, critical questions still remain. Most obviously, we know relatively little about NF-E2 p45, Nrf3, BACH1 and BACH2. Other important open issues relating to Nrfs in toxicology and pharmacology may include, but are not limited to: (1) determining the spectrum of target genes of Nrfs in different cells under diverse stress challenges, and the potential involvement of Nrfs in regulating genes independently of ARE sequences; (2) the mechanistic aspects of the complex regulatory network of Nrfs-mediated transcription under basal physiological conditions and under adaptive response conditions; (3) the transcriptional regulation of Nrfs under a sustained stress challenge, in particular the involvement of non-coding RNAs, such as miRNAs; (4) the crosstalk between Nrfs and other stress response machinery in response to various internal and environmental challenges; (5) the toxicological significance and application of Nrfs network perturbation in toxicity testing; (6) the characterization of the cell-specific physiological functions of different isoforms of Nrf1; (7) identification and application of novel modulators targeting specifically the transcriptional activity of various isoforms of Nrfs; (8) precise phenotyping of cell-specific knockout or overexpression of Nrfs in a variety of disease models; and (9) identification and characterization of functional SNPs and epigenetic sites in the human genes of Nrfs.

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Figure legends :

Figure 1. Nrfs may play paradoxical *Yin*-and-*Yang* roles in the development of oxidative stress-related disorders. *Yin*-side of ROS: Prolonged overproduction of ROS may result in oxidative damage and even cell death, leading to impaired cell function; *Yang*-side of ROS: Transient ROS production in response to various stimuli may function as signals mediating cellular responses. *Yin*-side of antioxidants: Antioxidants may blunt ROS signaling in the cell; *Yang*-side of antioxidants: Antioxidants may generally protect cells against oxidative damage. *Yin*-side of Nrfs: Chronic activation of Nrfs-mediated antioxidant response under persistent oxidative stress may blunt normal ROS signaling in the cell; *Yang*-side of Nrfs: Nrfs activation and subsequent induction of antioxidants may protect cells from oxidative damage.