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Nrf2:INrf2(Keap1) Signaling in Oxidative Stress

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Abstract

Nrf2:INrf2(Keap1) are cellular sensors of chemical and radiation induced oxidative and electrophilic stress. Nrf2 is a nuclear transcription factor that controls the expression and coordinated induction of a battery of defensive genes encoding detoxifying enzymes and antioxidant proteins. This is a mechanism of critical importance for cellular protection and cell survival. Nrf2 is retained in the cytoplasm by an inhibitor INrf2. INrf2 functions as an adapter for Cul3/Rbx1 mediated degradation of Nrf2. In response to oxidative/electrophilic stress, Nrf2 is switched on and then off by distinct early and delayed mechanisms. Oxidative/electrophilic modification of INrf2cysteine151 and/or PKC phosphorylation of Nrf2serine40 results in the escape or release of Nrf2 from INrf2. Nrf2 is stabilized and translocates to the nucleus, forms heterodimers with unknown proteins, and binds antioxidant response element (ARE) that leads to coordinated activation of gene expression. It takes less than fifteen minutes from the time of exposure to switch on nuclear import of Nrf2. This is followed by activation of a delayed mechanism that controls switching off of Nrf2 activation of gene expression. GSK3β phosphorylates Fyn at unknown threonine residue(s) leading to nuclear localization of Fyn. Fyn phosphorylates Nrf2tyrosine568 resulting in nuclear export of Nrf2, binding with INrf2 and degradation of Nrf2. The switching on and off of Nrf2 protect cells against free radical damage, prevents apoptosis and promotes cell survival.

Introduction

Oxidative stress is induced by a vast range of factors including xenobiotics, drugs, heavy metals and ionizing radiation. Oxidative stress leads to the generation of Reactive Oxygen Species (ROS) and electrophiles. ROS and electrophiles generated can have a profound impact on survival, growth development and evolution of all living organisms [1,2] ROS include both free radicals, such as the superoxide anion and the hydroxyl radical, and oxidants such as hydrogen peroxide [3]. ROS and electrophiles can cause diseases such as cancer, cardiovascular complications, acute and chronic inflammation, and neurodegenerative diseases [1]. Therefore, it is obvious that cells must constantly labor to control levels of ROS, preventing them from accumulation.

Much of what we know about the mechanisms of protection against oxidative stress has come from the study of prokaryotic cells [4,5]. Prokaryotic cells utilize transcription factors OxyR and SoxRS to sense the redox state of the cell, and during oxidative stress these factors induce the expression of nearly eighty defensive genes [5]. Eukaryotic cells have similar mechanisms to protect against oxidative stress [Fig. 1; ref. 3,6–9]. Initial effect of oxidative/electrophilic

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stress leads to activation of a battery of defensive gene expression that leads to detoxification of chemicals and ROS and prevention of free radical generation and cell survival [Fig. 1]. Of these genes, some are enzymes such as NAD(P)H:quinine oxidoreductase 1 (NQO1), NRH:quinone oxidoreductase 2 (NQO2), glutathione *S*-transferase Ya subunit (GST Ya Subunit), heme oxygenase 1 (HO-1), and γ-glutamylcysteine synthetase (γ-GCS), also known as glutamate cysteine ligase (GCL). Other genes have end products that regulate a wide variety of cellular activities including signal transduction, proliferation, and immunologic defense reactions. There is a wide variety of factors associated with the cellular response to oxidative stress. For example, NF-E2 related factor 2 (Nrf2), heat shock response activator protein 1, and NF-kappaB promote cell survival, where as activation of c-jun, N-terminal kinases (JNK), p38 kinase and TP53 may lead to cell cycle arrest and apoptosis [10]. The Nrf2 pathway is regarded as the most important in the cell to protect against oxidative stress. [3,6–9]. It is noteworthy that accumulation of ROS and/or electrophiles leads to oxidative/electrophile stress, membrane damage, DNA adducts formation and mutagenicity [Fig. 1]. These changes lead to degeneration of tissues and premature aging, apoptotic cell death, cellular transformation and cancer.

Antioxidant Response Element and Nrf2

Promoter analysis identified a cis-acting enhancer sequence designated as the antioxidant response element (ARE) that controls the basal and inducible expression of antioxidant genes in response to xenobiotics, antioxidants, heavy metals and UV light [11]. The ARE sequence is responsive to a broad range of structurally diverse chemicals apart from β-nafthoflavone and phenolic antioxidants [12]. Mutational analysis revealed GTGACA***GC to be the core sequence of the ARE [11,13–14]. This core sequence is present in all Nrf2 downstream genes that respond to antioxidants and xenobiotics [3,6–9]. Nrf2 binds to the ARE and regulates ARE-mediated antioxidant enzyme genes expression and induction in response to a variety of stimuli including antioxidants, xenobiotics, metals, and UV irradiation [6,15–21].

Nrf2 is ubiquitously expressed in a wide range of tissue and cell types [22–24] and belongs to a subset of basic leucine zipper genes (bZIP) sharing a conserved structural domain designated as a cap'n'collar domain which is highly conserved in Drosphila transcription factor CNC (Fig. 2; ref. 25]. The basic region, just upstream of the leucine zipper region, is responsible for DNA binding [3] and the acidic region is required for transcriptional activation. ARE-mediated transcriptional activation requires heterodimerization of Nrf2 with other bZIP proteins including Jun (c-Jun, Jun-D, and Jun-B) and small Maf (MafG, MafK, MafF) proteins [18–20,26–27].

Initial evidence demonstrating the role of Nrf2 in antioxidant-induction of detoxifying enzymes came from studies on the role of Nrf2 in ARE-mediated regulation of NQO1 gene expression [17]. Nrf2 was subsequently shown to be involved in the transcriptional activation of other ARE-responsive genes such as GST Ya, γ-GCS, HO-1, antioxidants, proteasomes, and drug transporters [3,6–9,28–33]. Overexpression of Nrf2 cDNA was shown to upregulate the expression and induction of the NQO1 gene in response to antioxidants and xenobiotics [17]. In addition, Nrf2-null mice exhibited a marked decrease in the expression and induction of NQO1, indicating that Nrf2 plays an essential role in the *in vivo* regulation of NQO1 in response to oxidative stress [26]. The importance of this transcription factor in upregulating ARE-mediated gene expression has been demonstrated by several *in vivo and in vitro* studies [reviewed in ref. 3]. The results indicate that Nrf2 is an important activator of phase II antioxidant genes [3,8].

Negative Regulation of Nrf2 mediated by INrf2

A cytosolic inhibitor (INrf2), also known as Keap1 (Kelch-like ECH-associating protein 1), of Nrf2 was identified and reported [Fig. 2; ref. 34–35]. INrf2, existing as a dimer [36], retains Nrf2 in the cytoplasm. Analysis of the INrf2 amino acid sequence and domain structure-function analyses have revealed that INrf2 has a BTB(broad complex, tramtrack, bric-a-brac)/POZ (poxvirus, zinc finger) domain and a Kelch domain [34–35] also known as the DGR domain (Double glycine repeat) [37]. Keap1 has three additional domains/regions: the N-terminal region (NTR), the invervening region (IVR), and the C-terminal region (CTR) [8]. The BTB/POZ domain has been shown to be a protein-protein interaction domain. In the Drosophila Kelch protein, and in IPP, the Kelch domain binds to actin [38–39] allowing the scaffolding of INrf2 to the actin cytoskeleton which plays an important role in Nrf2 retention in the cytosol [40]. The main function of INrf2 is to serve as an adapter for the Cullin3/Ring Box 1 (Cul3/Rbx1) E3 ubiquitin ligase complex [41–43]. Cul3 serves as a scaffold protein that forms the E3 ligase complex with Rbx1 and recruits a cognate E2 enzyme [8]. INrf2 via its N-terminal BTB/POZ domain binds to Cul3 [44] and via its C-terminal Kelch domain binds to the substrate Nrf2 leading to the ubiquitination and degradation of Nrf2 through the 26S proteasome [45–49]. Under normal cellular conditions, the cytosolic INrf2/Cul3-Rbx1 complex is constantly degrading Nrf2. When a cell is exposed to oxidative stress Nrf2 dissociates from the INrf2 complex, stabilizes and translocates into the nucleus leading to activation of ARE-mediated gene expression [3,6–9]. An alternative theory is that Nrf2 in response to oxidative stress escapes INrf2 degradation, stabilizes and translocates in the nucleus [49–50]. We suggested the theory of escape of Nrf2 from INrf2 [49] and similar suggestion was also made in another report [50]. However, the follow up studies in our laboratory could not support the escape theory. Escape theory is a possibility but has to be proven by experiments before it can be adapted. Therefore, we will use the release of Nrf2 from INrf2 in the rest of this review.

Numerous reports have suggested that any mechanism that modifies INrf2 and/or Nrf2 disrupting the Nrf2:INrf2 interaction will result in the upregulation of ARE-mediated gene expression. A model Nrf2:INrf2 signaling from antioxidant and xenobiotic to activation of ARE-mediated defensive gene expression is shown in Fig. 3. Since the metabolism of antioxidants and xenobiotics results in the generation of ROS and electrophiles [51], it is thought that these molecules might act as second messengers, activating ARE-mediated gene expression. Several protein kinases including PKC, ERK, MAPK, p38, and PERK [49,52–56] are known to modify Nrf2 and activate its release from INrf2. Among these mechanisms, oxidative/electrophilic stress mediated phosphorylation of Nrf2 at serine40 by PKC is necessary for Nrf2 release from INrf2, but is not required for Nrf2 accumulation in the nucleus [49,52–53]. In addition to post-translational modification in Nrf2, several crucial residues in INrf2 have also been proposed to be important for activation of Nrf2. Studies based on the electrophile mediated modification, location and mutational analyses revealed that three cysteine residues, Cys151, Cys273 and Cys288 are crucial for INrf2 activity [50]. INrf2 itself undergoes ubiquitination by the Cul3 complex, via a proteasomal independent pathway, which was markedly increased in response to phase II inducers such as antioxidants [57]. It has been suggested that normally INrf2 targets Nrf2 for ubiquitin mediated degradation but electrophiles may trigger a switch of Cul3 dependent ubiquitination from Nrf2 to INrf2 resulting in ARE gene induction. The redox modulation of cysteines in INrf2 might be a mechanism redundant to the phosphorylation of Nrf2 by PKC, or that the two mechanisms work in concert. In addition to cysteine151 modification, phosphorylation of Nrf2 has also been shown to play a role in INrf2 retention and release of Nrf2. Serine104 of INrf2 is required for dimerization of INrf2, and mutations of serine104 led to the disruption of the INrf2 dimer leading to the release of Nrf2 [36]. Recently, Eggler at al. demonstrated that modifying specific cysteines of the electrophile-sensing human INrf2 protein is insufficient to disrupt binding to the Nrf2 domain

Neh2 (58). Upon introduction of electrophiles, modification of INrf2C151 leads to a change in the conformation of the BTB domain by means of perturbing the homodimerization site, disrupting Neh2 ubiquitination, and causing ubiquitination of INrf2. Modification of INrf2 cysteines by electrophiles does not lead to disruption of the INrf2–Nrf2 complex. Rather, the switch of ubiquitination from Nrf2 to INrf2 leads to Nrf2 nuclear accumulation.

More recently, our laboratory demonstrated that phosphorylation and de-phosphorylation of tyrosine141 in INrf2 regulates its stability and degradation, respectively [59]. The de-phosphorylation of tyrosine141 caused destabilization and degradation of INrf2 leading to the release of Nrf2. Furthermore, we showed that prothymosin-α mediates nuclear import of the INrf2/Cul3-Rbx1 complex [60]. The INrf2/Cul3-Rbx1 complex inside the nucleus exchanges prothymosin-α with Nrf2 resulting in degradation of Nrf2. These results led to the conclusion that prothymosin-α mediated nuclear import of INrf2/Cul3-Rbx1 complex leads to ubiquitination and degradation of nuclear Nrf2 presumably to regulate nuclear level of Nrf2 and rapidly switch off the activation of Nrf2 downstream gene expression. An auto-regulatory loop also exists within the Nrf2 pathway [61]. An ARE was identified in the INrf2 promoter that facilitates Nrf2 binding causing induction of the INrf2 gene. Nrf2 regulates INrf2 by controlling its transcription, and INrf2 controls Nrf2 by serving as an adaptor for degradation.

Other Regulatory Mediators of Nrf2

Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) is a transcription repressor [62] that is ubiquitously expressed in tissues [63–64] and distantly related to Nrf2 [8]. In the absence of cellular stress, Bach1 heterodimers with small Maf proteins [65] that bind to the (ARE) [66] repressing gene expression. In the presence of oxidative stress, Bach1 releases from the ARE and is replaced by Nrf2. Bach1 competes with Nrf2 for binding to the ARE leading to suppression of Nrf2 downstream genes [66].

Nuclear import of Nrf2, from time of exposure to stabilization, takes roughly two hours [67]. This is followed by activation of a delayed mechanism involving Glycogen synthase kinase 3 beta (GSK3β) that controls switching off of Nrf2 activation of gene expression (Fig. 3). GSK3β is a multifunctional serine/threonine kinase, which plays a major role in various signaling pathways [68]. GSK3β phosphorylates Fyn, a tyrosine kinase, at unknown threonine residue(s) leading to nuclear localization of Fyn [69]. Fyn phosphorylates Nrf2 tyrosine 568 resulting in nuclear export of Nrf2, binding with INrf2 and degradation of Nrf2 [70].

The negative regulation of Nrf2 by Bach1 and GSK3β/Fyn are important in repressing Nrf2 downstream genes that were induced in response to oxidative/electrophilic stress. The tight control of Nrf2 is vital for the cells against free radical damage, prevention of apoptosis and cell survival [3,6–9,70].

Nrf2 in Cytoprotection, Cancer and Drug Resistance

Nrf2 is a major protective mechanism against xenobiotics capable of damaging DNA and initiating carcinogenesis [71]. Inducers of Nrf2 function as blocking agents that prevents carcinogens from reaching target sites, inhibits parent molecules undergoing metabolic activation, or subsequently preventing carcinogenic species from interacting with crucial cellular macromolecules, such as DNA, RNA, and proteins [72]. A plausible mechanism by which blocking agents impart their chemopreventive activity is the induction of detoxification and antioxidant enzymes [73]. Oltipraz, 3*H*-1,2,-dithiole-3-thione (D3T), Sulforaphane, and Curcumin can be considered potential chemopreventive agents because these compounds have all been shown to induce Nrf2 [74–81].

Studies have shown a role of Nrf2 in protection against cadmium and manganese toxicity [82]. Nrf2 also plays an important role in reduction of methyl mercury toxicity [83]. Methylmercury activates Nrf2 and the activation of Nrf2 is essential for reduction of methylmercury by facilitating its excretion into extracellular space. *In vitro* and *in vivo* studies have shown a role of Nrf2 in neuroprotection and protection against Parkinson's disease [84–86]. Disruption of Nrf2 impairs the resolution of hyperoxia-induced acute lung injury and inflammation in mice [87]. Nrf2-knockout mice were more prone to tumor growth when exposed to carcinogens such as benzo[a]pyrene, diesel exhaust, and N-nitrosobutyl (4-hydroxybutyl) amine [88–90]. INrf2/Nrf2 signaling is also shown to regulate oxidative stress tolerance and lifespan in Drosophila [91].

A role of Nrf2 in drug resistance is suggested based on its property to induce detoxifying and antioxidant enzymes (92–97). The loss of INrf2 (Keap1) function is shown to lead to nuclear accumulation of Nrf2, activation of metabolizing enzymes and drug resistance (95). Studies have reported mutations resulting in dysfunctional INrf2 in lung, breast and bladder cancers (96–100). A recent study reported that somatic mutations also occur in the coding region of Nrf2, especially in cancer patients with a history of smoking or suffering from squamous cell carcinoma (101). These mutations abrogate its interaction with INrf2 and nuclear accumulation of Nrf2. This gives advantage to cancer cell survival and undue protection from anti-cancer treatments. However, the understanding of the mechanism of Nrf2 induced drug resistance remains in its infancy. In addition, the studies on Nrf2 regulated downstream pathways that contribute to drug resistance remain limited.

Future Perspectives

Nrf2 creates a new paradigm in cytoprotection, cancer prevention and drug resistance. Considerable progress has been made to better understand all mechanisms involved within the intracellular pathways regulating Nrf2 and its downstream genes. Preliminary studies demonstrate that deactivation of Nrf2 is as important as activation of Nrf2. Further studies are needed to better understand the negative regulation of Nrf2. Also better understanding of the negative regulation of Nrf2 could help design a new class of effective chemopreventive compounds not only targeting Nrf2 activation, but also targeting the negative regulators of Nrf2.

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Abbreviations

Nrf2
: NF-E2 related factor 2

INrf2
: Inhibitor of Nrf2 also known as Keap1

ROS
: Reactive oxygen species

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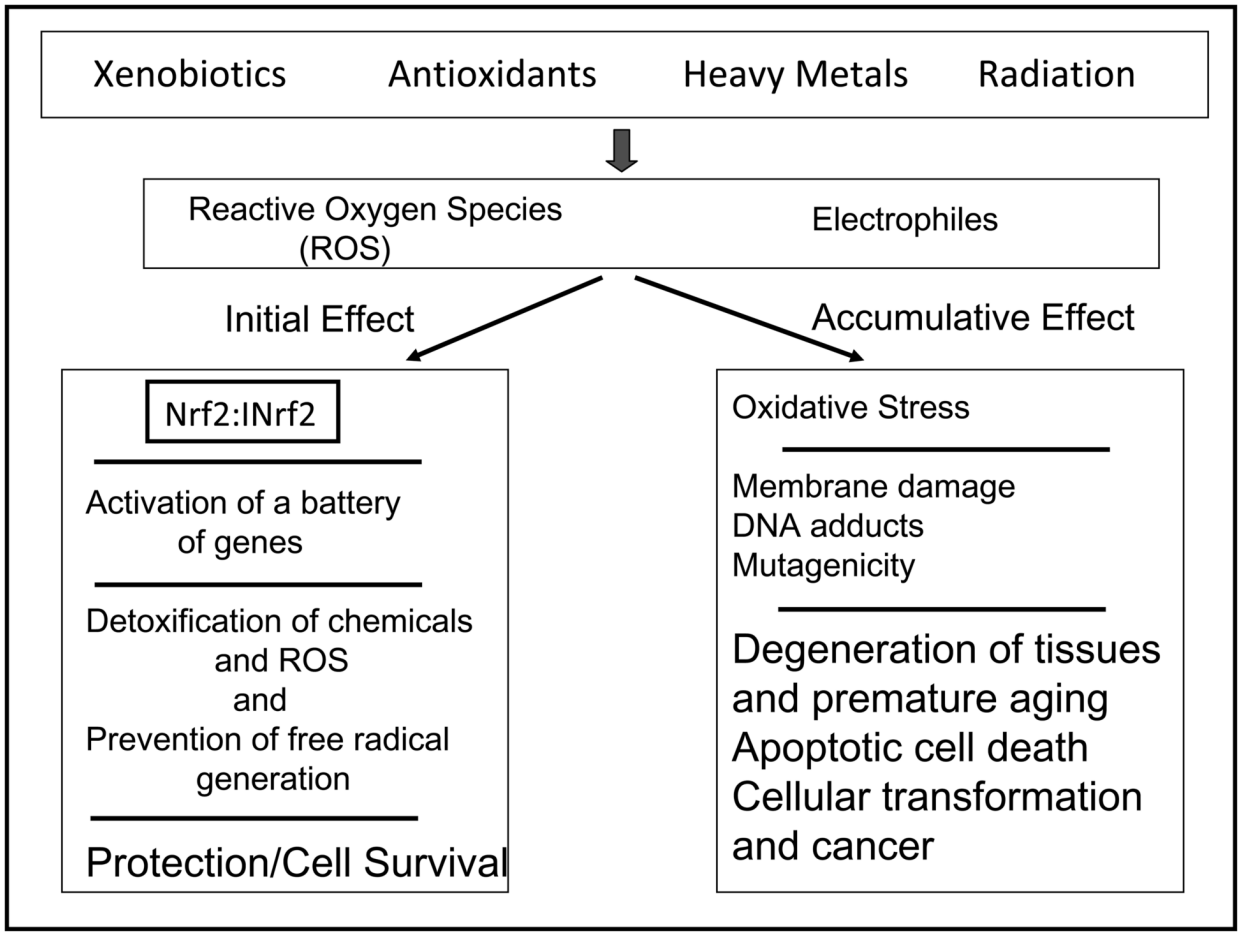

Fig. 1.
Chemical and radiation exposure and coordinated induction of defensive genes.

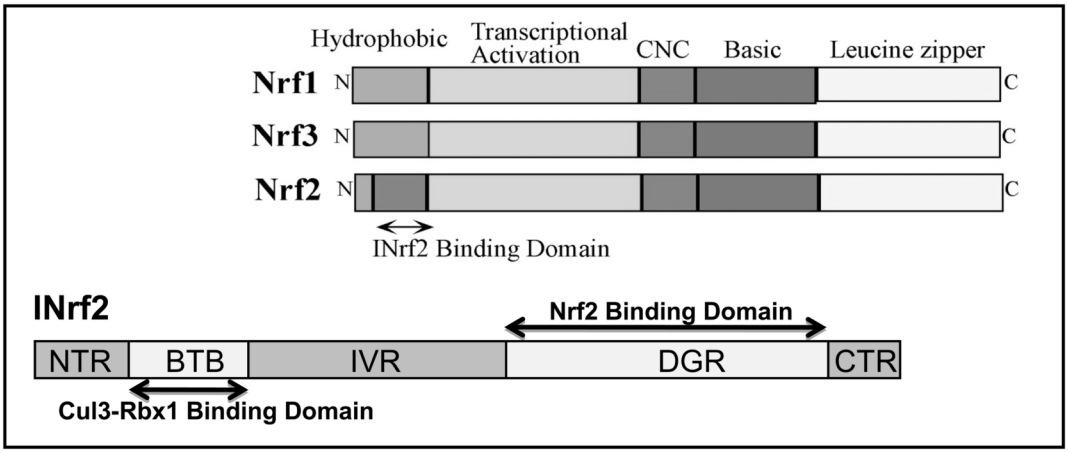

Fig. 2. Schematic Presentation of Various Domains of Nrf (Nrf1, Nrf2, Nrf3) and INrf2

Nrf, NF-E2 Related Factor; INrf2, Inhibitor of Nrf2; NTR, N-Terminal Region; BTB, Broad complex, Tramtrack, Bric-a-brac; IVR, Intervening/linker Region; DGR, Kelch domain/ diglycine repeats; CTR, C-Terminal Region.

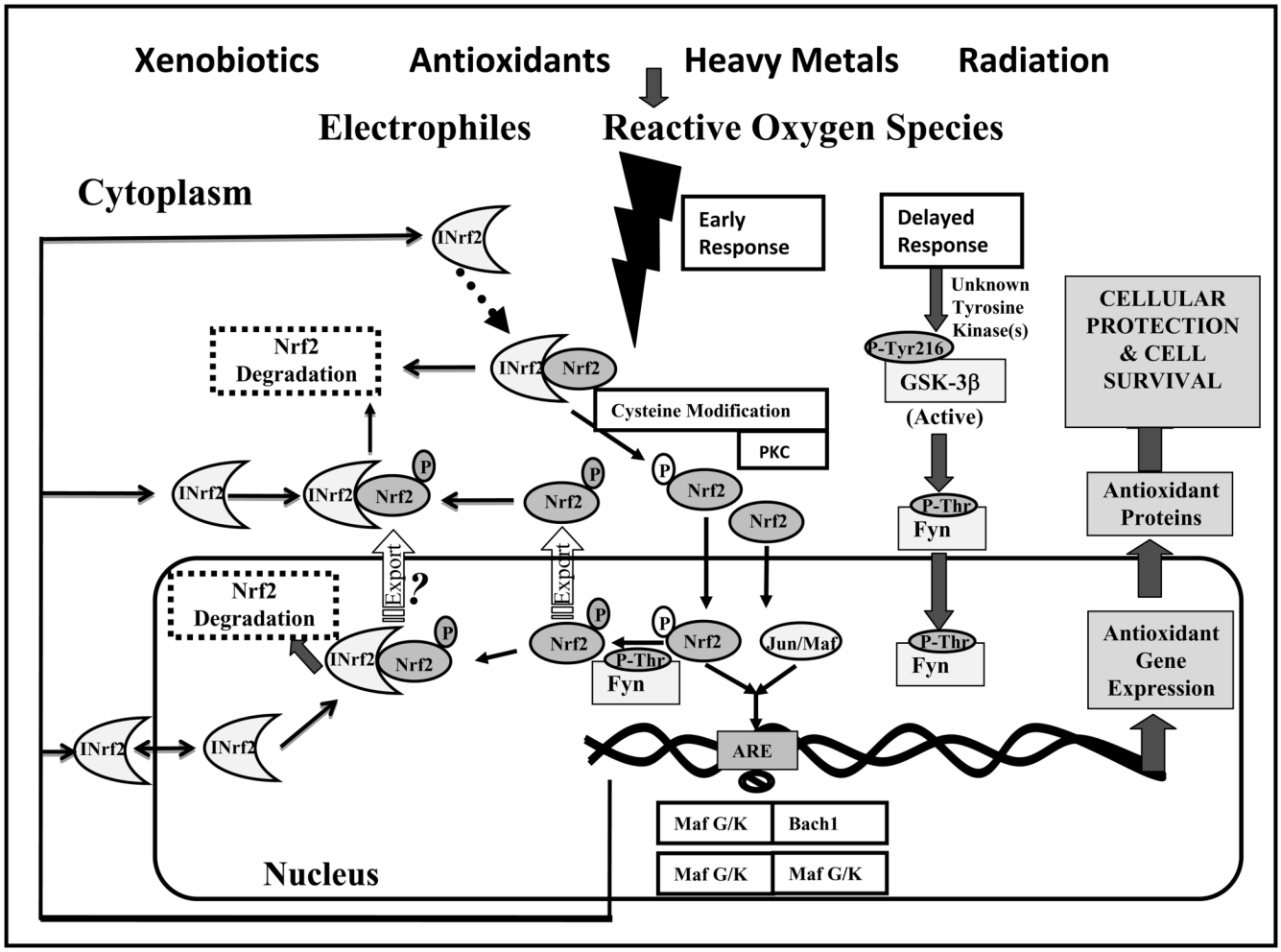

Fig. 3.
Nrf2 signaling in ARE-mediated coordinated activation of defensive genes.