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The Nrf2-Keap1 Signalling Pathway: Mechanisms of ARE transcription regulation in antioxidant cellular defence

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Abstract : The Nrf2-Keap1 [Nuclear factor E2-related factor 2-Kelch like ECH-associated protein 1] pathway is the main regulator of cytoprotective responses to electrophilic and oxidative insults. Nrf2 regulates the expression of detoxifying enzymes by recognizing the human antioxidant response element (ARE) binding site and it can regulate antioxidant and anti-inflammatory cellular responses, playing an important protective role on the development of various diseases such as cancer. Briefly, under homeostatic conditions, Nrf2 is suppressed by association with its inhibitory partner keap1, but is stimulated upon exposure to oxidative orelectrophilic stress. Once activated, Nrf2 binds to ARE sites in the promoter regions of various detoxification and antioxidant genes, leading to the induction of multiple target cytoprotective genes that boost cellular detoxification processes and antioxidant potential. In this current review, we provide an overview on the Nrf2-Keap1-ARE transcription pathway. We also briefly summarize the mechanisms of Nrf2 transcription regulation and the involvement of multiple stress-mediated cell signaling kinase cascades with Keap1 in regulating Nrf2 activity.
Key Words: Nrf2, Keap1, ARE,Oxidative stress, Ubiquitination.

Introduction

Reactive oxygen species (ROS), chemically reactive molecules containing oxygen, play important roles in regulation of cell survival. Normally, moderate levels of ROS mayserve as signaling molecules in various pathways that regulates cell survival and death, whereas a sudden, prolonged exposure to ROS can cause cellular damage and mutagenesis, which leads to necrotic and apoptotic cell death[1]. Such toxic damages are usually detoxified by phase II detoxification enzymes and antioxidants. Thus, the AREwhich transcriptionally regulates genes encoding detoxification enzymes and antioxidant proteins play a crucial role in cellular defence system[2]. Nuclear factor E2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that binds to ARE leading to coordinated up-regulation of ARE driven detoxification and antioxidant genes[3]. Nrf2 signaling has additionally been shown to up-regulate the expression of proteasome catalytic subunits in several cell types.The proteasome system controls the degradation of cellular proteins that regulate cell cycle, transcription, apoptosis, and many other cellular processes[4].In homeostatic status, Nrf2 is constantly ubiquitinated through Keap1 in the cytoplasm through extensive hydrogen bonds. This interaction is between the double glycine

repeat (DGR) region of Keap1 and the Nrf2-ECH homology 2 (Neh2) domain on the N-terminal portion of Nrf2[5]. In response to electrophiles or ROSstress, cytosolic Nrf2 liberates itself from sequestration or negative regulation of Keap1 thereby releasing Nrf2 from proteasomal degradation and translocates into the nucleus[6]. Once in the nucleus, Nrf2 undergoes heterodimerization with various transcriptional regulatory proteins such as small Maf (sMaf) protein[7]. This protein complex then binds to the ARE, which is located in the upstream promoter regions, leading to the induction of a battery of target cytoprotective genes[8]. Since the expression of multiple antioxidant and detoxification genes are positively regulated by the ARE sequence, Nrf2 may serve as a master regulator of the ARE-driven cellular defence system against oxidative insults. How Nrf2 mediate these effects will be the interest of this topic review.

Components of Nrf2-Keap1-ARE transcription system

Nuclear factor E2-related factor 2 (Nrf2)

Nuclear factor E2-related factor 2 (Nrf2), also known as NFE2L2, has emerged as a transcription factor that plays an important part in the maintenance of cellular homeostasis[9]. Nrf2 originates from studies of β-globin gene expression with the description of locus control region as possessing substantial regulatory activities, and is related to transcription factor activating protein-1 (AP-1)[10]. Nrf2 belongs to the CNC ("cap 'n' collar") subfamily of the basic region leucine zipper transcription factors[3]. The CNC-basic leucine zipper family (CNC-bZIP), a subfamily of bZIP proteins, play essential roles in regulation of expression of genes involved in diverse biological activities such as; proliferation, apoptosis, differentiation, and stress responses[11]. Six members in this family have been reported: NF-E2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2, each of which has different biological roles[3].

The structure of the Nrf2 protein has been extensively examined. Nrf2 contains six NRF2-ECH homology domains designated as Neh1-Neh6[5]. Neh1 is located in the C-terminal half of the molecule and contains a CNC basic leucine zipper domains, which is necessary for DNA binding and dimerization with other b-Zip proteins e.g. sMaf proteins[7]. Neh2 domain is located in the proximal N-terminus region, and function as binding site for the Nrf2 inhibitor protein Keap1[12]. Nrf2 contains two Keap1 binding sites within the Neh2 domain: DLG motif and ETGE motif, which enable the formation of a complex of one molecule of Nrf2 and two molecules of Keap1[13]. Neh2 domain contains seven lysine residues for ubiquitin conjugation which confers negative regulation of the Nrf2 activity through proteasome-mediated degradation of Nrf2[14]. Neh2 domain has also a serine residue, Ser40, which is critical for Nrf2 release from Keap1, but not required for Nrf2 stabilization and accumulation in the nucleus[15]. It has been reported that electrophiles loose the interaction between Neh2 domain and Keap1 protein either by stimulating phosphorylation of Nrf2 or by directly modifying cysteine residues in Keap1[16]. Moreover, researchers have found that Keap1 may stay bound to the Neh2 domain during oxidative stress. This make stabilize the Nrf2 leading to an increased transcriptional response[14]. Neh3 domain,located at the C-terminal end of the protein, is necessary for transcriptional activity of Nrf2 by recruiting chromodomain helicase DNA-binding protein-6 (CHD6)[17]. Both Neh4 and Neh5 are considered as transactivation domains that rich in acidic residues and interact with cAMP response element-binding protein (CREB)-binding protein (CBP) to organize the start of transcription[18]. Neh6 domain lies in the central part of Nrf2 andcontributes to redox-independent negative control of Nrf2[19].

Kelch like ECH-associated protein-1 (Keap1)

As discussed, Nrf2 has six homologous regions named Neh1 to Neh6. The Neh2 domain located in the N-terminus of Nrf2 is known to have a negative regulatory role for the trans-activating activity of Nrf2. Neh2 interacts with cytosolic protein, Kelch like ECH-associated protein1 (Keap1),also known as inhibitor of Nrf2 (INrf2), and negatively controls Nrf2 function[5]. Keap1 which was cloned by Yamamoto and co-workers using the Neh2 domain of Nrf2 as bait in a yeast two-hybrid screening system, is an actin-binding protein that supress the activity of Nrf2 by simply sequestering the protein in the cytoplasm[20]. Studies have revealed that knockout of Keap1 will result in activation of Nrf2 signalling[21]. Itoh and his colleagueshave identified that the structure of murine Keap1 to be composed of 624 amino acids and there is around 95% homology between human and mouse[5]. Molecular analysis revealed that human Keap1 contains five major domains: an N-terminal region (NTR), broad complex, tramtrack, and bric-a-brac domain (BTB), a cysteine-rich intervening region (IVR), the double glycine repeat region (DGR) or Kelch domain, and a C terminal Kelch region (CTR) [22]. The N-terminal

and BTB regions were implicated in homodimerization and heterodimerization of the Keap1 protein[23]. The intervening region which is rich in cysteine residues was demonstrated to be essential for the activity of Keap1[24]. Both of BTB and IVR domains thought to be involved in proteosome-dependent Nrf2 degradation[25]. The DGR or Kelch domain binds to Neh2 domain of Nrf2, establishing Nrf2 onto actin cytoskeleton[26]. The C-terminal Kelch region of Keap1 was proved to bind to Neh2 domain of Nrf2[27].

Since keap1 was initially described as a cytoplasmic factor that sequester Nrf2 in the cytoplasm under unstimulated condition[23], it was believed that upon exposure of cells to oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus where it forms a heterodimer bound with its obligatory partner sMaf protein, and eventually activates ARE-dependent gene expression. Based on laboratory findings, Keap1 was demonstrated not just passively sequester Nrf2 in the cytoplasm, but also plays an active role in targeting Nrf2 for ubiquitination and proteasomal degradation[3]. Keap1 was also identified to be associated with Cul3, a scaffold protein responsible for formation of an ubiquitin ligase E3 complex[25]. Thus, Keap1 may not only affect Nrf2 localization, but also actively targets Nrf2 to degradation[28].

The antioxidant response element (ARE)

The antioxidant response elements (ARE) is a cis-acting enhancer sequence or regulatory element found in the promoter region of many genes encoding phase II detoxification enzymes and antioxidants[29]. Proteins that are members of the battery of ARE genes involve those associated with glutathione biosynthesis, redox proteins with active sulfhydryl moieties and drug metabolizing enzymes[8]. The existence of ARE was first observed in 1990 by Rushmore and Pickett as an enhancer sequence in the upstream region of the rat glutathione S-transferase Ya (GST-Ya) subunit[30] .Glutathione S-transferases (GSTs) are multifunctional family of enzymes that stimulate the conjugation of sulfhydryl moiety of glutathione (GSH) with xenobiotics and electrophilic compounds[31]. The 5′-flanking region of the rat GSTA2 subunit gene contains two distinguishable regulatory sites, and they are essential for the inducible expression of GSTA2 by its responsiveness to not only phenolic antioxidants and planar aromatic compounds, but also hydrogen peroxide and reactive oxygen species[8]. The most 5′ of the two sites contains a sequence identical to the xenobiotic response element (XRE) and therefore requires involvement of functional aromatic hydrocarbon receptor (AhR) induction. Consecutive deletion analyses of the promoter region identified a second, AhR-independent, regulatory region that is responsible for induction of GST-Ya by hydrogen peroxide and phenolic antioxidants. Further, mutational studies of a 41 bp region delineated that the core ARE sequence was defined as 5'-TGACnnnGC-3' or 3'-YCACTGnnnCG-5' which is responsible for transcriptional activation by β-napthoflavone (β-NF), t-butylhydroquinone (t-BHQ), and hydrogen peroxide[30]. At the same time, a similar enhancer sequence was also discovered in the mouse GST-Ya subunit gene, which is homologous of rat GSTA2 subunit gene, and was named as the electrophile response element (EpRE), because it was required for induction of GST-Ya by t-BHQ or electrophilic compounds that are easily oxidized to electrophiles[32].Moreover, Friling and his team have identified two nine base pair repeats, TGACATTGC and TGACAAAGC, these nucleotides were recognized as core sequences needed for transcriptional regulation. They postulated that sites composing two incomplete TPA (12-O-tetradecanoylphorbol-13-acetate)-response elements (TRE) and that binding of AP-1 was accountable for induction of the GST-Ya subunit[33].However, later analysis identified that the recognition site was not a TRE site, because the "GC" was required for transcriptional activation, but was not required for AP-1 binding[34,8]. In addition to genes that encode rat GSTA2 and mouse GSTA1 proteins, studies have demonstrated that ARE sequence was found in numerous genes, and contribute to the basal and inducible expression of downstream genes[25]. These genes includes, genes encoding rat GST-P1[35], rat and humanNAD(P)H quinine oxidoreductase(NQO1)[36,37], human glutamate cysteine ligase catalytic subunit (GCLC) and modulatory (GCLM) subunits[38], mouse ferritin-L, mouse metallothionein-1, and mouse UDP glucuronyl transferase (UGT)[29]. Itoh et al. have identified similarities between each of ARE, recognition sequences for Nrf2, and the sMaf family of proteins. They found that Nrf2 recognizes binding site involving TRE sequence, (A/G)TGA (C/G)TCAGC(A/G)[7]. Similar to the ARE, Nrf2 requiresGC(A/G) at the 3′-end of the recognition sequence and an "A" or "G" immediately preceding the TGAC/G sequence[39]. Additionally, it has been demonstrated that Nrf2 positively regulates NQO1 through its ARE[40]. Nrf2 is a critical regulator of AREs, and Nrf2-knockout mice had reduced levels of redox balancing proteins and detoxifying enzymes, resulting in knockout mice become more susceptible to carcinogenic compounds[41].

Mechanisms of Nrf2-Keap1-ARE transcription regulation

Role of Keap1 mediated-Nrf2 activation

Comprehensive studies have been devoted to elucidate molecular mechanisms responsible for activation of Nrf2. Under normal physiological conditions, Keap1 physically entrapsinactive Nrf2 in the cytoplasm, thereby repressing its translocation to the nucleus[5]. During electrophilic stress, Nrf2 translocates into the nucleus, thus initiating Nrf2-ARE transcriptional activation[42].While the molecular mechanisms involved in the Nrf2 transcriptional activation of antioxidant enzymes are still debated, studies have in consensus demonstrated that Keap1 is the major repressor of Nrf2 through various regulatory mechanisms. From *in vitro* experimentalstudies, Keap1 co-expression in cellshasbeen demonstrated toprevent Nrf2 transactivation activity[5]. The suppressive effect of Keap1 can be abolished significantly by antioxidant treatments, indicating that Keap1-Nrf2 complex may be destabilized by alterations in cellular redox state[43].

Keap1 as a substrate linker protein for Cul3-dependent ubiquitination complex

In the absence of cellular oxidative stress, Nrf2 is tethered within the cytoplasm by the inhibitory partner Keap1, which interacts with the actin cytoskeleton[26]. Keap1 serve as a substrate adaptor protein for ubiquitination (Ub) of Nrf2[44]. As a substrate linker protein, Keap1 by its two functional regions, DGR or Kelch domains, binds the substrate Nrf2, and by its N-terminal BTB region bind to the ubiquitination catalytic complex. Ubiquitination of a substrate protein is attained by consecutive reactions stimulated by ubiquitin activating enzyme (Ub-E1), ubiquitin conjugating enzyme (Ub-E2), and ubiquitin ligase (Ub-E3), leading to ubiquitination and proteasomal degradation of Nrf2 through the 26S proteosome[45,46]. Ub-E3 consists of a scaffold cullin protein binding to Ring box protein-1 (Rbx1) that recruits its relative E2 enzyme[47]. Cullin proteins are scaffold proteins that play major role in the post-translational modification of cellular proteins involving ubiquitin. There are eightcullin proteins that have been cloned [Cul-1 to Cul-7 and PARC (p53-associated, parkin-like cytoplasmic protein)], which are distinguished by a cullin homology domain[48]. Keap1 displays increasing selectivity to bind to Cul3 protein, weak binding to Cul2, and no binding activity to the remaining cullins[25,14]. During oxidative stress conditions, the ability of Ub-E3 to ubiquitinate Nrf2 is repressed and the degradation of Nrf2 is inhibited, leading to enhanced protein stability and activation of the antioxidant response. In response to Nrf2 activators, the activity of the Ub-E3 ligase complex is suppressed due to chemical modification of cysteine residues, especially modification at Cys151 in BTB domain of Keap1[49]. When Cys151 is modified, this modification leads to disruption between Keap1 and Cul3 binding, thus impairing the assembly of Nrf2 into the complex, which is required for Nrf2 ubiquitination[50,51]. However, Cys273 and Cys288 residues localized in the IVR domain of keap1were demonstrated to be essential for Keap1 to maintain Ub-E3 ligase activity and degrade Nrf2[52].

Electrophilic cysteine sensors of Keap1

The observation that Keap1 contains high density of cysteine residues suggests that it may be a sensor for recognizing the change in intracellular redox status stimulated by oxidative/electrophilic inducers[53] .These cysteine residues form protein-protein cross links after exposure to oxidative stress, leading to disturbance of Nrf2/Keap1 stabilization and dissociation of Nrf2[54]. Hayes et al. demonstrated that mouse and human Keap1 proteins contain 25 and 27 cysteine residues respectively,around half of which are likely to play critical roles in Keap1 function[55]. Numerous studies have elucidated that the sulfhydryl groups of various Keap1 cysteine residues can be directly altered by oxidation, reduction or alkylation. Of these residues, Cys151, 273, and 288 appear to be substantial for the dissociation of Nrf2 from Keap1/Nrf2 complex[54].Cys151 is critical for the release of Nrf2 suppression and ubiquitination induced by oxidative stress, via a conformational change in the Keap1-Nrf2-Cul3 complex leads to disruption between Keap1 and Cul3 binding and subsequently disruption of the Keap1-Nrf2-Cul3 complex itself[14,56]. However, modifications of Cys273 and 288 lead to a conformational change of Keap1 that disturb the interaction between the DLG of Nrf2 and the Kelch domain of Keap1[47].Mutation in the Cys151 residue of Keap1 to Ser151 did not affect Nrf2 degradation by Keap1, but abolished response to oxidative and electrophilic inducers. While, mutation of either Cys273 or Cys288 residues have been identified to block Keap1 from degrading Nrf2, indicating the critical role of these cysteine residues in Keap1 mediated Nrf2 ubiquitination[57]. Similar to Nrf2, Keap1 also undergo ubiquitination, but its

degradation was proteasome independent, which may contribute to the activation of Nrf2 signaling by ARE inducers[58]. However, various ARE inducers possess various effects on Keap1 stabilization and ubiquitination[28].

The ‘‘hinge and latch’’ two-site substrate recognition model

As discussed earlier, Keap1 serve as a substrate adaptor protein for Cul3-based ubiquitin E3 ligase and is important for ubiquitination of Nrf2. Based on mutation analyses assays, there are two Keap1 binding sites in the Neh2 domain of Nrf2; an ETGE motif and a DLG motif[59]. Both of ETGE motif and DLG motif can mediate a cooperative binding to Keap1. Mutations of the ETGE motifcan considerably disrupt Keap1 binding mediated by the DLG motif, therefore the ETGE motifappears to play a crucial role for the DLG motif[60]. In normal cells, Keap1 binds Nrf2 through a two-site recognition process, also called hinge and latch model[61,27]. In this model, each Kelch-repeat domain from a Keap1 homodimer binds to one Nrf2 protein through a strong binding ETGE motif (hinge) or through a weak-binding DLG motif (latch)[55].The high affinity binding mediated by the ETGE motif serves as a hinge to set Neh2 to Keap1, whereas the low affinity binding mediated by DLG motif works as a latch[27]. In addition, seven ubiquitin-accepting lysine residues of the Neh2 domain, which placed upstream the ETGE motif, have been demonstrated to be critical for Keap1-dependent poly-ubiquitination and degradation of Nrf2[15]. Binding the hinge and the latch motif is a lysine-rich central α-helix, in this nine-turn α-helix there are approximately 6 of 7 lysine residues are placed on the same side of the helical surface. Thus, when the “latch” is in position, it might aid to place the central α-helix in an appropriate direction in order to expose those lysine residues as the Ub acceptors[27] .Further, it has been suggested that the DLG motif may hold the signaling switch for the repressive regulation of Nrf2 through Keap1-dependent ubiquitination. The switch from two-site binding to one-site binding may clarify the observed facts that suppression of Nrf2 degradation is not at the expense of Keap1/Nrf2 binding[28].The hinge and latch model can also elucidate how thiol-modification disrupts the substrate presentation status and thus cause resistance of Nrf2 ubiquitination[62]. Other models that describe the interaction between Nrf2 and Keap1 have provided inconsistent data when compared with the “hinge and latch” model[63].

Role of stress-mediated cell signaling kinases in Nrf2-Keap1-ARE transcription regulation

Although Keap1 is the major regulator of Nrf2 activation, there are further evidences indicating multiple stress-mediated cellular signaling kinase cascades may participate with Keap1 in regulation of Nrf2 and ARE-mediated genes.Multiple cytosolic kinases, such as phosphatidylinositol 3-kinase (PI3K), protein kinase-C (PKC), and mitogen-activated protein kinases (MAPKs), can phosphorylate Nrf2 and modify transcription of Nrf2 target genes[64-66]. Previous studies have reported that protein kinase RNA-like endoplasmic reticulum kinase (PERK)-dependent phosphorylation of Nrf2 triggers dissociation of Nrf2–Keap1 complexes, which is essential for cell survival[67].However, phosphorylation of Nrf2 by PKC has been reported to bring about dissociation of Nrf2 from Keap1 without nuclear translocation of Nrf2 or induction of gene expression[68]. In addition, Nrf2 phosphorylation by MAPKs show limited contribution to the modulation of the Nrf2-dependent antioxidant response and mutation of all the MAPK sites in Nrf2 appears to have little impact on its activity[69]. Therefore, the exact role of Nrf2 phosphorylation in each of the steps of Nrf2-mediated gene induction is still debatable[64].

Phosphatidylinositol 3-kinase (PI3K)

Phosphatidylinositol 3-kinase and its downstream AKT (PI3K-AKT) have been reported to play a major role in Nrf2 activation[70]. Previous studies using multiple cancer cell lines revealed that PI3K/Akt pathway are required for Nrf2 activation by different inducers such as *t*-butyl hydroquinone (tBHQ), hemin, and peroxynitrite[71,72]. PI3K composed of 85-kDa and 110-kDa subunit; once activated, it phosphorylates phosphatidylinositol at the D-3 site of the inositol ring[73]. In response to oxidative stress, PI3K activation can trigger re-arrangement and depolymerization of actin microfilaments and cause Nrf2-actin complex to translocate into nuclear compartments. This suggests the involvement of cytoskeletal modifications which may destabilize the Nrf2-Keap1 complex[74]. PI3K has also been involved in the regulation of ARE and detoxifying enzymes. When PI3K is repressed, the expression of ARE reporter is also repressed. However, overexpression of PI3K leads to activation of ARE pathway in a dose dependent manner. All of these data suggest that PI3K/Akt pathway is essential in regulating Nrf2-ARE-dependent protection against oxidative stress[75].

Protein kinase C (PKC)

The significance of protein kinase C (PKC) is in phosphorylation of numerous target proteins that control cell growth, differentiation, and apoptosis[76]. PKC can immediately phosphorylate Nrf2 at Ser40[64], leading to its dissociation from Keap1-Nrf2 complex[77]. Ser40 is phylogenetically conserved site located in the Neh2 domain of Nrf2 to which Keap1 binds[16].PKC has eleven isoforms which are serine/threonine kinases, and play a major role in antioxidant induction of Nrf2 activation[78]. Particularly PKC-δ which have been demonstrated as the major isoform of PKC that phosphorylates Ser40, which leads to stabilization and nuclear localization of Nrf2[79]. Reports from several findings indicates the importance of PKC in ARE-mediated gene expression. For instance, Nrf2 nuclear translocation as well as activation of the ARE by tBHQ treatment have been found to be suppressed by PKC inhibitors. Moreover, Nrf2 phosphorylation in HepG2 cells is enhanced by tBHQ and phorbol 12-myristate 13-acetate (PMA), a potent PKC-activating phorbol ester, but abolished by PKC inhibitors. In addition, nuclear translocation of Nrf2 is induced by PMA but arrested by PKC inhibitors. Together, these findings imply that PKC phosphorylation of Nrf2 may be an important step in the signaling cascade to mediate ARE activation, which trigger the nuclear translocation of this transcription factor in response to oxidative stress[16].

Mitogen-activated protein kinases (MAPKs)

Involvement of the mitogen-activated protein kinases (MAPKs) cascade pathway in Nrf2-ARE-mediated regulation and transactivation has been clearly stated. MAPKs are prominent cellular signaling components that convert several extracellular signals into intracellular responses via multiple phosphorylation cascades[80]. Family of MAPKs include extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal protein kinase (JNK), and p38 MAPK[81]. All of these members have been involved in the regulation of proliferation, differentiation, and apoptosis[82]. It have been found that phosphorylation of Nrf2 by ERK2 and JNK1 pathway promotes Nrf2 signaling[83,80,65]. While, the activation of the p38 MAPK pathway seems to inhibit Nrf2 signaling[84,85]. Avariety of serine/threonine residues in Nrf2 have been recognized as targets of MAPK-mediated phosphorylation. Researchers have identified that multiple MAP kinases including ERK2, JNK1/2 and p38 MAPK could phosphorylate Nrf2 at Serine 215, 408, 558, 577 and T559.However, such phosphorylation may only moderately impact the activity of Nrf2. Thus, direct phosphorylations of Nrf2 slightly contribute to the regulation of Nrf2 activity[69].

ERK1/2 MAPK

MAPKs are characterized as proline directed serine/threonine kinases that participate in Nrf2-ARE-mediated transactivation[80]. ERK1/2, a downstream multi-target kinase effector of MAPK, is able to regulator of a diversity of transcription factors which is important in maintaining cell survival during oxidative stress[86]. In response to growth factors, tyrosine kinase receptors recruit guanine nucleotide transfer factor Son of Sevenless (SOS) and adapter molecule growth factor receptor-bound protein 2 (Grb2) complex. This complex stimulates the membrane bound small G-protein Rat sarcoma (Ras), which in turn recruits c-Raf (a MAP3K) to the membrane and activates it[87]. c-Raf phosphorylates MEK (a MAP2K), which leads to ERK activation[88]. ERK can also be activated via specific G-protein-coupled receptors and PKC[89]. Activation of the ERK pathway appears to enhance Nrf2 signaling[90] [90]. Nguyen et al. have revealed that ERK inhibitor, PD98059 or U0126 have weaken the stimulating effects of tBHQ on Nrf2 protein levels[46]. Inhibitors of the ERK signaling pathway have been found to abolish Nrf2 phosphorylation under hypoxic stimuli[91]. In addition, Zipper &Mulcahy have identified by using *in vitro* kinase assays that purified recombinant Nrf2 was a substrate of ERK. They reported that mutation at the phosphorylation sites of Nrf2 did not decrease its ability to respond to pyrrolidinedithiocarbamate (PDTC) treatment and to participate ARE mediated transactivation[65]. ERK-directed phosphorylation is desired for Nrf2 nuclear translocation during PDTC induced GCLM gene expression. Hence, these findings suggest the possibility that Nrf2 phosphorylation by the ERK1/2 MAPK pathway increases its stability which in turn contribute to the induction of ARE-regulated genes[24].

p38 MAPK

The role of p38 MAPK in Nrf2-ARE transcription has faced inconsistent results. Keum and co-workershave suggested in studies employing ectopic expression of dominant positive p38 MAPK isoforms that p38 cascades

are involved in the negative regulation of ARE-reporter gene expression[85]. On the contrary, negative involvement of the p38 MAPK in the regulation of the ARE was not consistently observed in other reports[92]. Nrf2 was also identified to be a substrate of p38 MAPK *in vitro* and the consequent phosphorylation could enhance the association of Nrf2 with Keap1. However, incompatible effects were demonstrated by Keum and co-workers, which may result from the prospective unspecific effects of the p38 inhibitors being applied which were thought to also interfere with the other kinase pathways involved in Nrf2 activation[85]. Giving that p38 MAPK is recognized to oppose effects of ERK activation, it is possibly that p38 MAPK serve as a mediator in the negative feedback loop against positive regulation of ERK MAPK[93].

JNK

c-JUN NH2-terminal protein kinase (JNK) are serine threonine protein kinases that belong to a family of mitogen-activated protein kinases[94]. JNK are encoded by three genes, MAPK8 (encodes JNK1), MAPK9 (encodes JNK2) and MAPK10 (encodes JNK3), which are subjected to alternative splicing resulting in at least ten isoforms[95]. JNK proteins are also called stress-activated protein kinases (SAPK), because it potently activated by various environmental stresses, such as UV light [94], γ-radiation[96], protein synthesis inhibitors[97], inflammatory cytokines[98], DNA-damaging drugs[99], and chemopreventive agents[100]. When SAPK/JNK active as a dimer, it can translocate into the nucleus and regulate transcription via its action on c-Jun and other transcription factors[101]. Participation of JNK pathway in promoting Nrf2-ARE transcription has been reported in many studies. For instance, isothiocyanate, a chemopreventive phytochemical, has been demonstrated to promote phosphorylation of Nrf2 subsequent to enhanced phosphorylation of JNK, which facilitated translocation of Nrf2 into the nucleus[101]. Similarly, Yu et al. have suggested that JNK MAPKcascade is implicated in the positive regulation of ARE-reporter gene expression in response to treatment with *t*-BHQ and sulforaphane in HepG2 cells[80]. In addition, activation of JNK pathway promotes recruitment of co-activator to the transcription initiation complex and up-regulate Nrf2 transcriptional activity[102].

Other proteins regulate Nrf2-Keap1-ARE transcription

Accumulating evidence suggests that phosphorylation of Nrf2 is a requirement for dissociation of Nrf2 from Keap1. Beside the above mentioned pathways that promote Nrf2-Keap1-ARE transcription machinery, there are more complex regulatory mechanisms involved in Nrf2-Keap1-ARE transcriptional activation. Various activator proteins have been elucidated to possess positive regulation towards Nrf2-ARE complex. Sequestosome-1 (SQSTM1), also called P62, a polyubiquitin binding protein, which targets various substrates for autophagy, was found to be an activator of Nrf2[103]. The STGE motif within SQSTM1 interacts with Keap1 with similar affinity to that of the DLG motif of Nrf2[104]. When autophagy is weakened, SQSTM1 within poly-ubiquitinated protein aggregates are not cleared and thus it remain within cells. Increased SQSTM1 regress the DLG motif out of the Keap1, represses Nrf2 ubiquitination, therefore stabilizes Nrf2 and increases the expression of cytoprotective genes[105]. Interestingly, SQSTM1 could enhance Nrf2-ARE transactivation through activation of a number of kinases such as PKC, PI3K and ERK MAPK signaling. These observations indicate potential participation of intermediate proteins as mediators of the stress responsive kinases in Nrf2-ARE transactivation[106]. Further, the DJ-1, a redox sensitive protein known to play protective roles in cancer and Parkinson's disease, was found to promote Nrf2-Keap1-ARE transcriptional activation. Distraction of DJ-1 protein was associated with decreased Nrf2 protein stability, while overexpression of DJ-1 retrieved protein stability by diminishing ubiquitination of Nrf2 in transformed and primary cell lines of both human and mouse species. These data suggest that DJ-1 stabilizes Nrf2 by disrupting Nrf2-Keap1 complex and subsequent Nrf2 degradation[107]. Tumor suppressors such as breast cancer type 2 susceptibility protein (BRCA2) and p21 have also been identified to be an activator of Nrf2. Studies reported that BRCA2 and p21 stabilize Nrf2 by preventing Keap1 interaction with Nrf2[108,109]. p21 through its KRR motif directly interacts with DLG and ETGE motifs in Nrf2, resulting in Nrf2 stabilization and accumulation[109]. In addition, CREB-binding protein, also called p300, a histone acetyltransferase, was shown to serves as a co-activator for Nrf2/MafK heterodimer complex in rat cells, allowing induction of expression of the Nrf2 target gene glutathione s-transferase pi (Gstp). The increase of this co-activator may provide a possible contributor factor for the increased Nrf2 mediated pathway reported in some cancerous cells[110].

By contrast, several proteins have been discovered to take part in negative regulation of the Nrf2-ARE complex. The role of transcription regulator protein BTB and CNC homology-1 (Bach1), a bZip protein, in competing

with Nrf2 for available binding site to ARE has been widely recognized to result in inhibition of Nrf2-mediated transcription[111].Studies have shown that Bach1 represses Nrf2 downstream genes, such as NQO1 and GST Ya, by binding to the ARE and inhibiting ARE-mediated gene expression[112]. Likewise, other bZIP proteins such as Nrf3 and p65 isoform of Nrf1 have also found to be as negative regulators of Nrf2 by competing with Nrf2 for ARE binding[113,114] .Furthermore, Fyn kinase, a member of Src subfamily, may act as negative regulator by competing with Nrf2 for binding to ARE resulting in suppression of ARE mediated gene expression. Fyn phosphorylates Nrf2 at tyrosine residue 568 which leads to a chromosomal region maintenance-1 (Crm-1) mediated nuclear export and degradation of Nrf2[115]. In addition, retinoic acid receptors (RARs), mostly RARα, which are activated by retinoic acids, were identified to be efficient inhibitors of Nrf2 transcription and ARE-mediated gene transactivation[116]. It has been demonstrated that the activation of RAR and its obligatory partner retinoid X receptor (RXR), which form RAR/RXR complex, was sensitive to cellular redox state. The activation was demonstrated to be stimulated by cellular reducing states, but an oxidative state inhibits it. These results point out that the interaction of RAR with Nrf2 may be affected by cellular redox balances[117].

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Abbreviations

AhR	aromatic hydrocarbon receptor
AP-1	activating protein-1
ARE	antioxidant response element
BTB	broad complex, tramtrack and bric-a-brac
bZIP	Basic Leucine Zipper Domain
CBP	CREB-binding protein
CHD6	chromodomain helicase DNA-binding protein 6
CNC	“cap ‘n’ collar”
Crm-1	chromosomal region maintenance-1
CTR	C terminal Kelch region
Cul	cullin
DGR	the double glycine repeat region
EpRE	electrophile response element
ERK	extracellular-regulated kinase
GSH	glutathione
GST	glutathione S-transferase
Gstp	glutathione s-transferase pi
IVR	intervening region
JNK	Jun N-terminal Kinase
Keap1	Kelch-like ECH-associated protein 1
Neh	Nrf2-Ech homology
NQO1	NAD(P)H quinoneoxidoreductase
Nrf2	Nuclear factor E2-related factor 2
NTR	N-terminal region
PDTC	pyrrolidinedithiocarbamate
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-kinasse
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RARs	retinoic acid receptors
Rbx1	ring-box protein 1
ROS	Reactive oxygen species

RXR	retinoid X receptor
SAPK	stress-activated protein kinases
SQSTM1	Sequestosome-1
t-BHQ	t-butylhydroquinone
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA-response elements
Ub	ubiquitination
UGT	UDP glucuronyl transferase
XRE	xenobiotic response element
β-NF	β-napthoflavone

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