Title page

Mechanisms underlying chemopreventive effects of flavonoids *via* multiple signaling nodes within Nrf2-ARE and AhR-XRE gene regulatory networks

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Abstract

Flavonoids, a subclass of polyphenols, are abundant components of fruit and vegetables, high in the diet having an inverse association with the incidence of various degenerative diseases and cancer. Mechanisms underlying the beneficial effects of flavonoids on the human health are being investigated worldwide. Flavonoids have been found to reduce the risk of carcinogenesis by blocking the initiation and suppressing the promotion and progression of certain cancer cells. The chemopreventive effects of flavonoids are exerted through induction of cytoprotective mechanisms to prevent the activation of pro-carcinogens from attacking DNA and genome, and detoxify activated carcinogens by enhancing the conjugation and excretion. The balance of metabolic activation and detoxification of carcinogens is controlled through expression of drug-metabolizing Phase I and Phase II enzymes. If the detoxification pathway is saturated, the AhR-XRE-cytochrome P450s activation pathway produces arene oxides and additional damages to promote tumourigenesis. Fortunately, such oxidative damages can be prevented by CNC-bZIP transcription factors through differentially regulating antioxidant and detoxification genes, which contain ARE and its homologues in their promoters. Amongst CNC-bZIP family, Nrf2 is a master regulator of expression of drug-metabolizing enzymes, and its activity is negatively regulated by Keap1 and β-TrCP. Expression of *Nrf2* and downstream genes is tightly controlled by AhR and CNC-bZIP (e.g. Nrf1) family factors, whilst its negator *Keap1* is also regulated by Nrf1 and Nrf2. Such crosstalks between AhR-XRE and Nrf2-ARE regulatory networks indicate that flavonoids trigger multiple signaling pathways to integrally activate cytoprotective genes against cytotoxic insults and oxidative stress. However, it remains to determine the unique chemopreventive role of Nrf1 in regulating antioxidant, detoxification and cytoprotective genes.

1. Introduction

It is known that flavonoids belong to a subclass of polyphenols, which are abundant in our diet, and that evidence for their roles in the preventive medicine is emerging from research in cancer and other degenerative diseases, such as cardiovascular, Parkinson's and Alzhermer's diseases [1]. There are more than 4000 compounds that have been identified as distinct kinds of flavonoids, approximately 900 of which are consumed in the human diet. All flavonoids share a generic structure, consisting of two aromatic rings (A and B rings) that are linked by 3 carbons' atoms that are usually contained in an oxygenated heterocycle ring (C ring) (Fig. 1). Based on their differences in the C ring, flavonoids are further classified as flavonols, flavones, catechin-tanins, anthocyanidins, and isoflavones (Table 1). An additional number of different sugars, of which more than 80 kinds exist, also contribute to the chemical variety of flavonoids. Flavonoids are found in nearly all fruit and vegetables, existing in nature as conjugates in either gycosylated or esterified forms. The conjugates can be converted into aglycones by food-processing [2], and however, flavonoids also exist in nature as aglycones.

1.1 Bioavailability of flavonoids and their metabolisms

Amongst different flavonoids, the bioavailability varies depending on their chemical structures, sugar groups attached and their molecular weights. For instance, direct evidence has been obtained by measuring their concentrations in both the blood plasma and the urine [3, 4], after ingestion of either some pure compounds or food stuffs with known contents of the compounds of interest [5]. It is reported that the plasma concentrations of flavonoids are low, usually less than 1 μmol/L, but reaches to a certain maximum level 1 to 2 h after ingestion. Therefore, the maintenance of a high concentration in plasma requires repeated ingestion of the polyphenols over time [6]. Studies for investigating the extent of polyphenol absorption in humans, after the ingestion of a single dose of polyphenols that are provided as a pure compound, plant extract, whole food or beverage, have shown that the quantities of intact polyphenols in urine vary from one flavonoid to another. Amongst them, inter-individual variations have also been observed, probably due to differences in compositions of the colonic microflora that can affect their metabolisms differently [7].

The absorption and metabolism of polyphenols is routed from the stomach, passed through the gastrointestinal tract into the liver. After crossing those physiological barriers, polyphenols will be circulated in the blood plasma and then transported to various target tissues or excreted in the urine and/or the bile. Flavonoids in the aglycon form can be absorbed by the small intestine, but their most abundant forms as glycosides, esters or polymers in foods are hardly absorbed [8]. However, these conjugates of aglycones can be hydrolyzed by acids in the stomach and by microflora in the intestines, in order to convert to the forms that are readily bioavailable to the body. Only after being hydrolyzed in the gastrointestinal tract, the aglycones are absorbed by the intestinal enterocytes, where they undergo different conjugation reactions, including glucuronidation by UDP-glucuronyl transferase (UGT) and methylation by catechol-*O*-methyl transferase. Once flavonoids reach the liver, the remaining aglycone will further be glucuronidated or sulfated, whilst those methylated polyphenolics may be demethylated [9]. Intriguingly, flavonoids may undergo the oxidation reacted in their planar aromatic structures for the role as antioxidants to form quinine-like structures that are either detoxified by conjugation with reduced glutathiones or broken down to smaller phenolic compounds [10]. Finally, some of polyphenol metabolites enter the circulation in the blood, where the plasma albumin represents the primary protein responsible for binding and transporting polyphenols [6]. The affinity of polyphenols with the albumin varies according to their chemical structures, but it is not clear whether the binding to albumin affects their biological activities.

1.2 Beneficial properties of flavonoids

Collectively, dietary flavonoids have various beneficial properties, including antioxidant properties, chelation of

metals, and oestrogenic, anti-viral, anti-bacterial, anti-inflammatory and anti-mutagenic activities, along with a dual opposing role in either activation or inhibition of various enzymes. For the antioxidant activity possessed by flavonoids, there are three basic requirements [11, 12]: i) free hydroxyl groups on the 5 and 7 positions of the A ring; ii) the presence of orthodihydoxyl (catechol) groups on the B ring; ii) the presence of a 2,3-double bond in the C ring (Table 1). It has been reported that quercetin, the anthocyanin aglycone and cyanidin have antioxidant potentials by 4-fold higher than that of trolox, an analogue of vitamin E [13], and that their antioxidant activities of flavonoids are suggested to be responsible for their roles in cancer prevention [14, 15]. Quercetin, cyanidin and procyanidin are identified as good chelators of metals, such as iron, zinc and copper [16, 17]. As these flavonoids could inhibit platelet aggregation and leukocyte adhesion by chelating iron and scavenging of the relevant radicals, they can thus contribute to the prevention of cardiovascular disease [18]. Furthermore, quercetin and kaempferol have been reported to increase the activity of thioredoxin reductase in the normal human keratinocytes [19].

Epidemiologically, the increase in the intake of flavonoids helps decreasing the risk of developing cardiovascular disease, age-related disease such as Alzheimer's disease, and various types of cancer [9]. However, the mechanisms responsible for their beneficial effect are still under intensive investigation. One of the mechanisms that have been proposed is that flavonoids are protective through their antioxidant properties. Since that sustaining elevated levels of reactive oxygen species are clearly associated with various neoplastic diseases, the antioxidant property, together with the ability of flavonoids to induce cytoprotective enzymes and regulatory proteins, can hence contribute to their chemopreventive effects. Another possible mechanism is anti-inflammation, because flavonoids extracted from fruit and vegetables can inhibit the NF-κB signaling pathway that is involved in the induction of inflammation [20]; this process contributes to the initiation and progression of neoplastic tumours [21]. Moreover, An additional number of cellular response signaling pathways towards regulation of cell cycle, proliferation and apoptosis [22, 23], are induced by flavonoids, which are responsible for their chemopreventive effects.

2. Roles of flavonoids in cancer chemoprevention

Collectively, numerous mechanisms have been implicated in the development of cancer [24]. The carcinogenesis is a polygenic-evolved pathological process complex with multifactorial, multievent, and multistep, hierarchically from initiation, promotion, progression and angiogenesis to invasion and metastasis. By blocking the initiation of carcinogenesis and/or suppressing the later stages, flavonoids can reduce the risk of carcinogenesis and thus severs as chemopreventive agents. Regarding the initiation of carcinogenesis, it always starts with DNA adducts, gene mutation and other genetic alterations. In order to avoid this initiation and deteriorative consequence, a number of direct and indirect intrinsic strategies can be evolutionally developed for the host to prevent DNA attack from electrophiles, free radicals, reactive oxygen, nitrogen and sulphur species, to enhance the repair of damaged DNA, to inhibit the uptake of pro-carcinogens into cells, and to reduce the toxicity of activated carcinogens in cells by enhancing their biotransformation, conjugation and excretion [25]. For instance, quercetin has been reported to protect the cell and DNA from being damaged by hydrogen peroxide and benzo[a]pyrene (BaP) [26, 27].

The progression of cancer could also be halted by activation of cell cycle arrest or apoptosis. A number of flavonoids either as individuals or in combination, which have been found to suppress cell proliferation or induce apoptosis of carcinoma cells, include quercetin [28], epigallocatechin gallate (EGCG), resveratrol [29, 30], kaempferol [31], procyanidin and pomegranate extracted ellagitannins [32]. Also, cell growth has been inhibited by EGCG through induced cell cycle arrest in the G_0/G_1 phase [33]. Furthermore, another flavonol found in rice bran, tricin, was shown to inhibit the growth of breast tumour cells through the G_2/M arrest [34]. Therefore, distinct flavonoids have various potentials to exert chemopreventive effects through different mechanisms. In addition, isoflavone genistein has also an inhibitory effect on the growth of human ovarian cancer cells (OcC1 and SKOV3) and prostate cancer cells (LNCaP) through up-regulating antioxidant and detoxification genes [35, 36].

3. Inducible expression of drug-metabolizing enzymes by flavonoids

The drug-metabolizing enzymes were also designated as xenobiotic transformation enzymes. Xenobiotics include a broad spectrum of chemicals: manufactured or natural drugs (e.g. flavonoids and isoflavone genistein), pollutants, alkaloids and pyrolysis products found in food or environments. Most of such xenobiotics are toxic and, if accumulated in the body, they may cause cell damage and eventually kill an organism. To defend against those xenobiotics to which the human are constantly exposed, as well as endobiotics in the body and even toxic products from the cell metabolisms, the human body system has evolutionally developed a large number of drug-metabolizing enzymes with various functional specificities, which enable to biotransform, detoxify and eliminate potential exogenous and endogenous toxicants. The following examples of reactions and relevant enzymes involved in the detoxification include: i) oxidation reaction catalyzed by cytochrome P450 (CYP) enzymes, alcohol dehydrogenase, aldehyde dehydrogenase and glutathione peroxidase; ii) reduction reaction catalyzed by aldo-keto reductases (AKR), short chain dehydrogenase and/or reductase, and NAD(P)H:quinone oxidoreductase 1 (NQO1); iii) hyrdolysis catalyzed by epoxide hydrolase; iv) conjugation reactions catalyzed by glutathione transferase (GST), sulfotransferase (SULT), UGT, methyl transferase and N-acetyl transferase (NAT) [37].

Generally, the above first three reactions (i.e. oxidation, reduction and hydrolysis) can introduce a functional group to the substrate, such as -OH, -NH_2, -SH or -COOH, leading to a modest increase in the hydrophilicity of the end products. By contrast, glutathionylation glucuronidation, sulfonation, acetylation, methylation, and other conjugations require cofactors, such as glutathione, other amino acids or sugars, in the reaction with cognate functional groups in the substrates originally, or introduced through the other types of detoxification reactions. As compared with other reactions, such conjugation reactions can result in a significant increase in the hydrophilicity of the substrates, therefore promoting the excretion of foreign chemicals and metabolites from the host cells and the organism [38]. Based on these classic biochemical reactions, the concept of Phase I and Phase II drug metabolism was proposed early in the 1970's [39]. The Phase I enzymes include those responsible for hydrolysis, oxidation, and reduction of xenobiotics, whilst the Phase II enzymes catalyze the conjugation of xenobiotics with sugars, glutathione and other amino acids. As such Phases I and II enzymes are likely up-regulated by pretreatment with flavonoids, this class of xenobiotics can therefore be preventive or therapeutic beneficial in the case of drugs. In the other hand, modification of xenobiotics by drug-metabolizing enzymes can also change their biological effects by either lessening or worsening their cytotoxicity. Overall, drug-metabolizing enzymes play a vital role in determining the intensity and duration of action of drugs, their chemical toxicity and chemical tumourigenesis [40].

Take the drug-metabolizing gene *Nqo1* as an example, its chemical inducers are collectively classified into nine diverse classes (Fig. 2) [41, 42]: i) diphenols, phenylenediamines and quinones; ii) michael reaction acceptors; iii) isothiocyanates, dithiocarbamates and related sulfur compounds; iv) 1,2-dithiole-3-thiones, oxathiolene oxides, and other organosulfur compounds; v) hydroperoxide; vi) trivalent arsenicals; vii) heavy metals; viii) vicinal dimercaptans, and ix) carotenoids and related polyenes. Although these chemicals are structurally distinct, they share common properties of electrophilicity and the capacity to modify sulfydryl groups. Notably, it has been shown that certain of these inducers are administrated to up-regulate *NQO1* responsible for the detoxification of electrophilic toxicants, in order to block the initiation of tumours in various tissues, such as liver, colon, mammary gland and pancreas [43].

4. Mechanism for induction of drug-metabolizing enzymes by Nrf2 binding to the ARE

The mounting substantial evidence has revealed that drug-metabolizing enzymes (e.g. NAT, GST, SULT, UGT and NQO1) play important roles in the detoxification of electrophilic toxicants, and induction of these genes can protect the cells against carcinogenesis and mutagenesis. Such genes encoding these drug-metabolizing enzymes

are regulated by the family of cap'n'collar (CNC) basic-region leucine zipper (bZIP) transcription factors (Fig. 3) through differentially binding to antioxidant response elements (AREs) and its homologous consensus sequences in their promoter regions (Fig. 4) [44]. The first isolated CNC-bZIP protein in mammals was designated the nuclear factor-erythroid 2 (NF-E2) p45-subunit [45]. Subsequently, three closely related transcriptional activators Nrf1 (including a long form TCF11 and a short LCR-F1 isoform) [46-48], Nrf2 [49] and Nrf3 [50]were cloned in succession, along with two distantly related repressors Bach1 and Bach2 [51]. The vertebrate members of this family share two highly conserved structural domains, i.e. the 'CNC' domain and bZIP domain (Fig. 3), with the *Drosophila* Cnc proteins [44, 46, 49, 52]. The *Caenorhabditis elegans* protein skinhead-1 (Skn-1) also belongs to this superfamily due to its CNC domain situated just N-terminal to the basic region [53-55], but it lacks the leucine zipper subdomain. Beyond Skn-1, all other CNC-bZIP proteins form a functional heterodimer with one of small Maf proteins (e.g. MafK, MafF and MafG) or another bZIP protein (e.g. c-Jun), and thus can differentially bind to various ARE/AP-1-like DNA consensus sequences (Fig. 4), which are contained in distinct subsets of target genes [56]. Therefore, both CNC and bZIP domains determine the property of the family proteins to differentially bind ARE-driven genes with specificity.

4.1 The ARE and its homologues confer differential gene regulation by Nrf2 and other CNC-bZIP transcription factors

The ARE was also designated as electrophile response element, which thus represents a *cis*-acting enhancer sequence that mediates transcriptional activation of those genes in the intracellular responses to electrophiles and oxidative stress. Such proteins that are members of the ARE-gene battery include those associated with glutathione biosynthesis, redox proteins with active sulfydryl moieties and drug-metabolizing enzymes [57, 58] (Tables 2 and 3). This regulatory element was first identified within the 5′-flanking region of the rat *GSTA2* containing a 41-bp DNA motif, and later was designated as the ARE based on its responsiveness to phenolic antioxidants [59]. Deletion and mutational analysis defined that the core nucleotide sequence, 5′-TGACnnnGC-3′ (Fig. 4), is essential for the response to these chemicals [59]. Furthermore, the nucleotides situated at 5′-end immediately to the core ARE is also required for both the basal and inducible expression of the gene regulated, but was not sufficient for induction when its upstream TCA sequence or downstream A/T-rich region was mutated [60]. Consistent with this finding, 5′-TMAnnRTGAYnnnnGCR wwww-3′ (M=A/C, R=A/G, Y=C/T, W=A/T) is the extended ARE core sequence, demonstrating the importance of the flanking sequences for the context-specific regulation of gene transcription [60]. However, the 3′-flanking 'wwww' tetra-nucleotide is not required for basal and inducible gene expression; this was found in experiments of a series of point mutations across the whole ARE in the mouse *Nqo1* promoter [61]. In addition, this study also revealed that the nucleotides that had previously been suggested to be redundant (which was shown as 'n' in the sequence mentioned above) are considered as a requirement for the gene induction; by contrast, the core sequence that had been shown essential before was found dispensable in the case of mouse *Nqo1* [61]. Taken together, these studies indicate that there are distinct ARE sequences in the promoter regions of different genes. In addition to genes that encode the rat *GSTA2* and mouse *GstA1*, genes encoding the rat and human *NQO1* [62, 63], γ-glutamyl cysteine ligase catalytic (*GCLC*) and modifier (*GCLM*) subunits [64-66], and haeme oxygenase 1 (*HO-1*) [67] were transcriptionally regulated *via* the ARE sites (Table 2). By contrast, an ARE-like sequence was found in some antioxidant and detoxifying gene promoters (e.g., 5′-TGCCattGC-3′ in rat *GstA2*, Fig. 4) [68], but its function has not clearly been characterized.

The core ARE shares a striking sequence similarity with the recognition sites for either NF-E2 or small Maf family factors [69, 70]. The antisense sequence of the NF-E2 binding site is likely to be considered as a type of ARE. Both include either the TPA-response element (TRE, 5′-TGAC/GTC/AA-3′, also called AP-1 binding site), or its 5′-TGAC-3′ tetranucleotide motif. In addition, the ARE requires a 5′-GC(A/G)-3′ trinucleotide at its 3′-end. Both of these motifs also exist in the MARE (Maf recognition element) [71, 72]. Within the 'core' ARE sequences

the 5′-TGAC-3′ motif is represented in other *cis*-regulatory DNA consensus sequences, that are recognized by members of the AP-1, the ATF, and the cAMP-response element binding protein (CREB) families (Fig. 4). The motif is also present in the unfolded protein response element (UPRE) [73] and the recognition site of the Skn-1, a *Caenorhabditis elegans* transcription factor with a C-terminal CNC-basic region [54, 74, 75]. The 5′-GC-3′ motif has been shown to be critical for the ARE-mediated inducibility [59-61], but it is also embedded in other consensus sequences, such as the amino acid response element and the p53 binding site [76]. In addition, the 5′-GAC-3′ motif is present in the consensus binding sites of transcription factors p53 and NF-kB [77, 78] . Transcription of *GCLC* can occur indirectly through an NF-kB-recognized site [79, 80], as well as through the ARE. Collectively, the evolutionary conservation of these consensus sequences suggests that the ARE and the *cis*-elements with homologous sequences, together with their cognate DNA-binding transcription factors, produce the contexture of a large gene regulatory network. A possibility cannot therefore be ruled out that, under certain pathophysiological conditions, there may be some promiscuity between *trans*-acting factors and *cis*-elements or dual regulation of certain genes. This suggests that exposure of cells to a variety of severe distinct stresses could activate an overlapping spectrum of genes controlling redox homeostasis and relevant physiologies. Conversely, if this gene regulatory network is out of control, it would turn on to enter a pathological response process.

The observation that the ARE sequence resembles that of the TRE has raised the possibility that members of the AP-1 family regulate certain ARE-driven genes. Despite their similarities, the ARE has unique features that sets it apart from AP-1 binding sites. For example, there exists a GC dinucleotide motif at the 3′ end of the ARE core sequence. This difference suggests that activation of gene expression via the ARE and the TRE are mediated through different signaling pathways. It appears that AP-1 makes little contribution to ARE-driven gene expression as a reporter construct based on the mouse gsta1-ARE was active in mouse F9 embryonal carcinoma cells which lack significant TRE-binding activity [81]. However, it should be noted that the ARE found in some genes contains an embedded TRE sequence [68, 82-84], suggesting that such genes may be controlled through both the ARE and AP-1 binding sites. In fact, supershift assays have shown that a number of transcription factors can bind to the ARE, notably the CNC/bZIP family including Nrf1, Nrf2, Nrf3, NF-E2 p45, Bach1 and Bach 2 (Fig. 3), the AP-1 family such as c-Jun, c-Fos and ATF4, and the small Maf proteins [85-90]. This is explained by the fact that many of these bZIP proteins can potentially dimerize with each other to generate a diverse array of functional protein complexes that bind to DNA with unique and/or overlapping specificity [91, 92].

Collectively, CNC-bZIP family transcription factors play important roles in development and the regulation of expression of cytoprotective genes involved in various biological processes, including proliferation, apoptosis, differentiation, and stress responses. Amongst this family, Nrf2 is thought as a master regulator of the basal and inducible expression of ARE-driven genes through its functional heterodimer with small Maf proteins. The most compelling evidence that Nrf2 makes a major contribution to the regulation of ARE-driven genes has been obtained from the study of *Nrf2* knockout mice. In particular, the basal and inducible expression of *Gst* and *Nqo1* is substantially reduced in *Nrf2*$^{-/-}$ mice, when compared with their wild-type counterparts [71, 93]. Besides Nrf2 and small Maf proteins, other CNC-bZIP transcription factors may influence ARE-driven gene expression [44, 76].

In vitro DNA-binding studies using antibody supershift assays have shown that Nrf1 and the AP-1 family members can bind the ARE [68]. In fact, Nrf2 is a dispensable factor and cannot compensate the loss of Nrf1 function, because the *Nrf2* knockout mice exhibited normal growth and development, without the spontaneous development of cancer [94]. By contrast, global disruption of *Nrf1* leads to mouse embryonic lethality [52, 95], and conditional knockout of *Nrf1* specifically in the liver, bone and brain of neonatal mice results in non-alcoholic steatohepatitis and hepatic neoplasia [96, 97], reduced bone size [98] and neurodegenerative disease [99, 100], respectively. This obvious discrepancy between the phenotype of *Nrf1*$^{-/-}$ and *Nrf2*$^{-/-}$ mice clearly indicates that the two CNC/bZIP proteins are functionally distinct: Nrf1 fulfils a unique indispensable function, that cannot be substituted compensatively by Nrf2 and other CNC-bZIP factors, in regulating a subset of ARE-battery genes

responsible for cellular homeostasis and organ integrity during normal development and health growth,

In addition, induction of those Phase II enzymes by flavanoids occurs upstream through several intracellular signal transduction pathways, involving mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), or phosphatidylinositol-3 kinase [101-103]. These signaling pathways are integrated to activate antioxidant, detoxification and cytoprotective genes regulated by Nrf2 and other transcription factors.

4.2 The structure of Nrf2 with its biological and physiological functions

Among the CNC/bZIP family members, Nrf2 acts as a central transcription factor in the ARE-driven gene response [68, 104], and regulates the expression of those genes encoding antioxidant enzymes, metal-binding and detoxification proteins (Table 2), as well as drug-metabolizing enzymes (Table 3). The function of Nrf2 is determined by its six structural domains, namely Neh1 to Neh6, which are conserved amongst species [105]. The Neh1 domain comprises the CNC region fused to bZIP region and confers its ability to dimerize with small Maf proteins and its ability to bind DNA as an obligate heterodimer. The N-terminal Neh2 domain is required for redox-sensitive negative control of the CNC-bZIP factor [105], whilst the C-terminal Neh3 domain interacts with chromodomain helicase DNA-binding protein 6 and therefore might associate with the transcriptional apparatus [106]. Both the central Neh4 and Neh5 are two transactivation domains that interact with CREB-binding protein [107]. The following Neh6 domain contributes to redox-independent negative control of Nrf2 [108]. Some of these domains are also homologous with the equivalents of other CNC-bZIP factors (Fig. 3).

Nrf2 enables the cellular adaptation to oxidants and electrophiles by stimulating the transcriptional activation of around 100 cytoprotective genes [82, 109-114], each containing at least one ARE in their promoters [59, 61]. Such genes whose expression is regulated by Nrf2 include those encoding: i) antioxidant and redox buffer proteins, and other oxidoreductases; ii) enzymes involved in regeneration of NADPH, synthesis of glutathione and other cofactors and their modulation; iii) enzymes for DNA repair to remove oxidative damage; iv) drug-metabolizing enzymes responsible for the Phases I and II detoxification; v) drug-efflux pumps (e.g. multidrug resistance associated proteins) required for the Phase III drug metabolism; vi) heat shock proteins and other molecular chaperones; vii) the 26S proteasomal α- and β-subunits for proteolytic degradation; viii) some growth factors, growth factor receptors, and various transcription factors involved in cell survival, anti-inflammatory and other protective responses. It has been shown that the up-regulated expression of these ARE-battery genes can increase the capacity of cells to scavenge electrophiles, free radicals, and reactive oxygen, nitrogen and sulphur species, and thus this protective effect enables the cells to defend against oxidative damage to lipids, DNAs and RNAs so as to prevent the initiation of tumouriogenesis. The increased levels of drug-metabolizing enzymes and drug-efflux pumps allows the detoxification of a wide variety of toxic compounds, including those containing α, β-unsaturated carbonyl, epoxide, halide, hydroperoxide and quinone moieties, and further removal of their inactive conjugated metabolites from cells [115]. Overall, up-regulation of ARE-driven genes by activated Nrf2 enables the cells to adapt to the increased concentration of electrophiles, free radicals, and reactive oxygen, nitrogen and sulphur species. Conversely, knockout of *Nrf2* in the mouse markedly increased the hypersensitivity to hyperoxia [116], and the susceptibility to various forms of chronic lung diseases produced by exposure to cigarette smoke [117, 118]. Importantly, it has been shown that Nrf2 can protect the cells against the formation of DNA adducts and/or gene mutations resulted from aflatoxin B_1, BaP and diesel exhaust fumes [119-121]. Thereby, Nrf2 has been considered as a target of chemopreventive agents against carcinogenesis.

Notably, studies have also revealed that activation of Nrf2 and its downstream ARE-driven genes potentiates the prevention of neurodegenerative, neovascular, cardiovascular diseases and diabetes [122-125]. In the pathogenesis of all these diseases, oxidative stress is a common etiological factor, as implicated in the development of cancer. These and other studies have proved that the cytoprotection exerted by up-regulation of Nrf2 is ultimately due to an increase in the expression of ARE-driven genes transactivated by Nrf2 in the antioxidant

responses to combat oxidative insults.

4.3 Negative regulation of Nrf2 by Keap1

The activity of Nrf2 is negatively regulated by kelch-like ECH-associated protein 1 (Keap1) through binding to its Neh2 domain [105], and thus Keap1 was also designated as an inhibitor of Nrf2 (iNrf2) [126], which retains this CNC-bZIP protein in the cytoplasm under normal conditions (Fig. 5). Clearly, Nrf2 is a highly unstable protein with a short half-life ($t_{1/2}$ ~15 min), subject to the proteolytic degradation catalyzed by the 26S proteasomal complex *via* the ubiquitin-dependent pathway [127, 128]. Studies by different groups have showed that the association of Keap1 with Nrf2 promotes ubiquitylation of this CNC-bZIP protein in a normal constitutive manner [129, 130] through the cullin 3 (Cul3)-dependent pathway [131, 132]. Keap1 is thereby identified as an adaptor protein of the ubiquitin E3 ligase Cul3 complex with ring-box protein 1 (Rbx1). This adaptor protein is composed of three main structure domains: i) the Broad-complex, Tramtrack, Bric-à-brac (BTB) domain for its homodimerization; ii) the intervening region (IVR) for associated with the Cul3 ligase; and iii) the six Kelch repeats and its C-terminal region for docking the Neh2 of Nrf2 [133-137]}. Moreover, studies by genetic knockdown of the cellular Keap1 protein [114, 138] and using *Keap1* knockout animals [139-141] revealed that, upon interaction with Keap1/Cul3, Nrf2 is targeted directly for ubiquitylation and degradation.

Interestingly, Keap1 is also thought as a redox-sensing metalloprotein, because it is enriched with cysteine (Cys) residues; for example, 25 and 27 Cys residues are contained in the mouse and human Keap1 proteins, respectively[105]. Experimental evidence has shown that these Cys residues play a vital role in regulating the substrate adaptor function of Keap1, and approximately a half number of Cys residues are likely to be highly reactive, and hence are able to form thiolate anion under normal physiological conditions [113, 142-144]. These Cys residues present Keap1 as an attractive target for potential regulation by thiol-reactive chemical species and, hence, inhibitory modulation of its activity was suggested to be an important mechanism for Nrf2 activation [105, 130, 145, 146]. Ectopic over-expression of recombinant Keap1 in various cell lines has shown that Cys23, Cys273 and Cys288 are required for its repression of Nrf2 [130, 131, 147]. By contrast, Cys151 appeared to be required for inhibition of the substrate adaptor activity of Keap1 by inducing agents indicated [130]. Further detailed chemical and functional analyses, combined with molecular modeling and phylogenetic comparison, has showed that Keap1 can directly recognize NO, Zn^{2+}, and alkenals through three distinct Cys sensors, respectively [148]. The C288 alkenal sensor is of ancient origin, having evolved in a common ancestor of bilaterans. The Zn^{2+} sensor minimally comprises H225, C226, and C613. The NO sensor, emerged coincident with an expansion of the NOS gene family in vertebrates, comprises a cluster of basic amino acids (H129, K131, R135, K150, and H154) that facilitate *S*-nitrosation of C151. The authors suggest that Keap1 is a specialized sensor that quantifies stress by monitoring the intracellular concentrations of NO, Zn^{2+}, and alkenals, which collectively serve as second messengers that may signify danger and/or damage if organisms are to survive in harmful environmental conditions.

To explain how Keap1 recruits with Nrf2 and assists in ubiquitination of this CNC-bZIP protein by Cul3-Rbx1, a two-site substrate recognition model, also called the hinge and latch model, was presented [149-151]. In this proposed model, each of the Kelch-repeat domain from a Keap1 homodimer binds to one Nrf2 protein through either a weak-binding DLG motif (residues 29-31) or a strong-binding ETGE motif (residues 75-84), both located in the N-terminal Neh2 domain of Nrf2 (Fig. 5). The binding affinity of Kelch to the ETGE motif is approximately 100-fold higher than that of Kelch to the DLG motif [152, 153]. Structural biology studies [135, 151-154] suggest that the forked-stem homodimer of Keap1 binds both the DLG and ETGE motifs in Nrf2 to align the seven ubiquitin-accepting lysine residues between these two motifs into a conformation suitable for ubiquitin conjugation.

Consistent with the two-site structural model, stress-induced modification of Keap1 at the Cys residues, such as C151, C273, or C288 in the BTB and linker domain, imposes a conformational change that disrupts the weak

Kelch-DLG binding [105, 129-132]. The resulting dissociation of Nrf2 from Keap1 diminishes the CNC-bZIP protein ubiquitination and degradation in the cytoplasm, but increases the level of Nrf2 protein localized in the nucleus, resulting in the activation of Nrf2 signaling pathway. Besides the inhibition of Nrf2 ubiquitination [155], it can be stabilized through another model for ubiquitiylation of Keap1 triggered by its Cys modification or other induction mechanisms. It has been reported that certain xenobiotics can trigger the ubiquitylation of Keap1 [156, 157]. Besides Keap1, β-transducin repeat containing protein (β-TrCP) has also been identified to be involved in the 'Ying-Yang' regulation of Nrf2 protein stability through binding to the DSGxS motif in the Neh6 domain (Fig. 5) [158, 159].

4.4 Differential regulation of Nrf2 through distinct upstream signaling pathways

The above description has suggested that any mechanism that can disrupt the interaction between Keap1 and Nrf2 targeted for their ubiquitylation would lead to the activation of Nrf2-ARE gene regulatory network. For this reason, several upstream signaling protein kinases, such as PKC, MAPK, and PRKR-like endoplasmic reticulum (ER) kinase (PERK), have been implicated directly or indirectly in the modification of Nrf2, resulting in its activation. Upon oxidative stress, phosphorylation of Nrf2 at serine 40 by PKC has been reported to release this CNC-bZIP protein from Keap1 [102]. Additional study by Cullinan *et al* suggested that the membrane-bound ER stress sensor PERK can mediate phosphorylation of Nrf2, trigger dissociation of Nrf2 from the Keap1/Cul3/Rbx1 complex, and inhibit *in vitro* re-association of this complex with Nrf2 [160]. However, Nrf2 is not an ER-resident protein [161], and thus it is required to ascertain whether or how Nrf2 is recruited to the inner nuclear envelope membrane associated with PERK. Besides, activation of several upstream MAPKs, such as extracelluar signal-regulated kinase 2 (ERK2), ERK5, c-Jun NH_2-terminal kinase 1 (JNK1), can transduce differential signaling responses to the phosphorylation of Nrf2 and its transcriptional activation [162]. Further research found that phosphorylation of Nrf2 at serines 215, 408, 558, 577 and threonine 559 by MAPKs, including ERK2, JNK1/2 and p38 kinases, could moderately affect the activity of Nrf2. [163], and thus proposed that the direct phosphorylation of Nrf2 contributes limitedly to the regulation of Nrf2 activity. However, it was to the contrary that phosphorylation of Nrf2 by p38 kinase caused an increase in the interaction between Nrf2 and Keap1, which consequently attenuates both the constitutive and inducible Nrf2 activity [162, 164].

Recently, glycogen synthase kinase-3β (GSK-3β) was identified to catalyze phosphorylation of the DSGxS to become a DSGxpS phosphodegron for the binding to β-TrCP, enabling Nrf2 to be targeted for the ubiquitin E3 ligase Cul1/Rbx1 complex-mediated degradation pathway independent of Keap1 (Fig. 5) [158, 159, 165]. Additional study showed that, in response to oxidative stress, the cyclin-dependent kinase inhibitor p21 is regulated through its ^{154}KRR motif interacting directly with both the DLG and ETGE motifs in Nrf2, and that the interaction can competitively inhibit Keap1 for the binding to Nrf2, compromising its ubiquitnation [166]. This study, by using *p21*-deficient mice, demonstrated that p21 up-regulates Nrf2 under both basal and induced conditions.

4.5 Induction of the Nrf2 gene itself through both ARE and XRE

Besides the predominant regulation of Nrf2 by Keap1/Cul3-mediated ubiquitination and other various signaling pathways, it may also be regulated at its transcriptional level on the basis that within the *Nrf2* gene promoter region there contains two ARE sequences starting at -579 nt, 5′-TGACTCCGC-3′, and -317 nt, 5′-TGACTCCGC-3′ [111, 167], together with one xenobiotic response element (XRE) beginning at -712 nt, 5′-GCGTG-3′, and additional two XRE-like sequences starting at +755 nt, 5′-CACGC-3′ and +870 nt, 5′-CACGC-3′, respectively [168]. In fact, it has also been shown that treatments with either 3H-1,2-dithiole-3-thione, or isothiocyanate sulforaphane as an ARE inducer, can modestly increases expression of *Nrf2* mRNA in keratinocytes [111]. The presence of functional XRE in the gene promoter is also proved by the evidence that the XRE-specific inducer, 2,3,7,8-tetrachloro

dibenzo-p-dioxin (TCDD) increases the *Nrf2* mRNA levels in hepatoma 1c1c7 cells [168]. Furthermore, there exist multiple single nucleotide polymorphisms (SNPs) in the promoter of human *Nrf2*, and one of these SNPs (-617C/A) significantly reduces the gene expression [169]. However, it is not known whether such polymorphisms can prevent the variant allele from being transcriptionally activated by thiol-active agents. In addition, there contains functional ARE sequences in the *Keap1* gene promoter, and thus its transcriptional expression is finely tuned by an auto-regulatory feedback loop within Nrf2 [170] or another CNC-bZIP factor Nrf1 [171]. Together, the feedback controlling expression of both *Keap1* and *Nrf2* should be integrated with multiple signaling responses to the Keap1-Nrf2-ARE gene regulatory network, which talks with another XRE-pivoting network.

4.6 The dark side of Nrf2 involved in cancer promotion and drug resistance

The beneficial sides of Nrf2 are described above, but the CNC-bZIP factor also possesses several harmful properties on the opposing dark sides. The two-sided conclusion is drawn from several studies showing that Nrf2 can promote tumourigenesis and chemoresistance. The first evidence that Nrf2 was involved in cancer promotion was obtained from northern blotting and chromatin immunoprecipitation revealing that *Nrf2,* and placental *GSTP1* (that is not expressed in the normal liver) were specifically up-regulated in parallel with development of precancerous lesions and hepatocellular carcinoma [172]. Later studies have identified there exists the *Keap1* mutation or loss of heterozygosity in the *Keap1* locus in lung cancer cell lines or cancer tissues [152, 173], and the ultimate result of *Keap1* mutation is the increase in the constitutive activity of Nrf2 and the transactivation of its downstream genes. The investigation from 65 Japanese patients with lung cancer suggested that there was a high incidence of somatic mutations of *Keap1* with lung adenocarcinoma [174]. Consistently, another report indicated that Keap1 expression is reduced in lung cancer cell lines and tissues, compared to that expressed in normal bronchial epithelial cell line [175]. The reduced expression of keap1 is accompanied by Nrf2 over-expression at the later stage of lung cancer [176]. Moreover, the mutation of Keap1C23Y, leading to its inability to repress Nrf2, was also found in breast cancer [147]. Collectively, these findings suggest that loss of function of Keap1 results in the prolonged activation of Nrf2 activity. Such a consequence of prompting the survival of cancer cells is likely due to the up-regulation of a subset of the downstream genes, of which are involved in anti-apoptosis and/or anti-senescence. The permanently hyperactive Nrf2 can thus act as an unrecognized mediator of oncogenesis and promote tumourigenesis [174, 177, 178].

Besides cancer promotion, Nrf2 also contributes to the resistance of cancer cells to chemotherapy. It was indicated in a study showing that prognosis in patients with lung cancer that contain mutant *Keap1* or *Nrf2* was worse than that in patients with lung tumours lacking such mutations [179]. As the homeostatic activation of Nrf2 protects the normal cells against cytotoxic agents, it is possible that the malignant cells in human tumours are conferred by the permanently hyperactive Nrf2 to protectively resist against chemotherapeutic drugs. In fact, the *in vitro* studies by Wang *et al* investigated the role of Nrf2 in determining drug responses in lung carcinoma, breast adenocarcinoma and neuroblastoma, revealing that up-regulation of Nrf2 enhanced the chemoresistance whereas its down-regulation sensitizes cells to chemotherapeutic agents (e.g. cisplatin, doxorubicin and etoposide) [176]. It is therefore desirable for the strategy to overcome drug resistance caused by up-regulation of Nrf2. For this reason, Hayes *et al* have reviewed the means to solve this problem, either antagonizing Nrf2 directly or exploiting up-regulated ARE-drive genes to activate cytotoxic pro-drugs [113].

5. Mechanism for induction of drug-metabolizing genes by AhR binding to the XRE

Although carcinogenesis is a complex and protracted multistage process, the entire pathological course can indeed be initiated by a single event wherein a cellular macromolecule is damaged by one of endogenous or exogenous cytotoxic agents or carcinogens. Such initiatory events can be defended through cytoprotective strategies, such as up-regulation of drug-metabolizing enzymes that are involved in promoting the conjugation and excretion to

reduce the carcinogen toxicity. For example, reduction of electrophilic quinones by NQO1 has proved an important detoxification pathway, which converts quinones to hydroquinones and reduces the oxidative cycling. Such chemicals that can increase the expression of *NQO1* or its activity (Fig. 2) are helpful to prevent the initiation of cancer. Besides Nrf2, the aryl hydrocarbon receptor (AhR) also play a pivotal role in the transcriptional regulation of *NQO1* and other drug-metabolizing genes, e.g. those encoding cytochrome P450 (CYP) enzymes (Table 4) [180, 181].

5.1 Regulation of drug-metabolizing CYP genes by AhR through binding to the XRE

Collectively, CYP enzymes play important roles in drug, carcinogen, and steroid hormone metabolism [182]. It is identified that four (i.e. CYP1 to CYP4) of 18 mammalian *CYP* gene families are mainly responsible for metabolism of foreign compounds, including drugs, food additives and environmental pollutants [183]. Some of CYP enzymes are substrate inducible, a property that allows the cell to adapt to changing chemical environments. Induction of CYPs has advantages and yet disadvantages. On the one hand, the enzyme induction inhibits chemical carcinogenesis because it increases the rate of carcinogen detoxification to prevent the accumulation of lipophilic compounds so much as to reaching harmful levels. On the other hand, since CYP enzymes have broad substrate specificities, enzyme induction by one compound may lead to increased metabolism of another compound, leading to loss of the beneficial drug effects. It should seriously be taken into account that enzyme induction of CYPs produces an imbalance between bioactivation and detoxification, leading to adverse effects of drugs administrated on the organism. In the case of polycyclic aromatic hydrocarbons, found in cigarette smoke, the metabolism by cytochromes P450 can generate arene oxides, which are electrophiles binding covalently to cellular components. Besides arene oxides, other reactive species are also produced during the bioactivation process mediated by CYPs and other Phase I drug-metabolizing enzymes [184]. Therefore, at so high concentrations of drug compounds that biotransformation and detoxification pathways should become saturated, induction of CYPs can also increase the production of reactive metabolites beyond the capacity of cellular defenses, thereby producing potential toxicity or neoplasia [185, 186].

Expression of CYP1 to CYP4 families responsible for biotransformation of xenobiotics are tightly regulated through different mechanisms [183]. The expression of *CYP1* family members is principally regulated by AhR and its heterodimer partner called AhR nuclear translocator (ARNT) (Fig. 6) [187], whilst the expression of *CYP2, CYP3* and *CYP4* family enzymes is regulated by three distinct nuclear factors, i.e. constitutive androstane receptor, pregnane X receptor and peroxisome proliferator-activated receptor, respectively [188]. Typically, some inducers of *CYP1A1* include halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons and the environmental contaminant TCDD. To gain insights into the mechanism of *CYP1A1* induction, TCDD was employed as the xenobiotic inducer. Since CYP1A1 is clearly involved in both the metabolism of polycyclic aromatic hydrocarbons and the production of reactive genotoxic metabolites that may initiate carcinogenesis, it is important to understand the basis of *CYP1A1* induction. As expected, the study using *AhR*-defective and *ARNT*-defective cells revealed that induction of *CYP1A* is dependent on AhR/ARNT [189]. Later studies of the protein-DNA interaction showed an AhR/ARNT complex bind the *cis*-regulatory element 5′-TnGCGTG-3′, which is present in multiple copies within the enhancer of *CYP1A* [190]. This element was designated the XRE (Fig. 4), but it is also called the dioxin responsive element or the aryl hydrocarbon-responsive element [191]. Further mutational analysis of the core sequence indicates that 5′-CGTG-3′ is essential for the functional XREs [192].

Notably, the tetranucleotide 5′-CGTG-3′ from the XRE is embedded in either the hypoxia response element (HRE, 5′-T$^{A}/_{G}$CGTG-3′) or the UPRE (5′-TGACGTG$^{G}/_{A}$-3′) (Fig.4). This evolutionary conservation suggests possible crosstalks between XRE-, HRE- and UPRE-battery gene regulatory networks. On the other hand, since these three homologous *cis*-elements are so very much alike that it is hardly to distinguish from one after the other, they are likely to be recognized by cognate canonical and non-canonical transcription factors and partners. As a

consequence, the *cis*-element-specific binding of canonical factors could be either competitively inhibited or unexpectedly imposed by non-canonical misrecognized factors, in particular during pathological stress conditions.

5.2 The structure of AhR with its functional regulation

The AhR belongs to the family of eukaryotic Per-ARNT-Sim (PAS) domain proteins (Fig.6), that function as sensors of extracellular signals and environmental stresses affecting growth and development [193]. Amongst this family, AhR regulates adaptive and toxic responses to a variety of chemical pollutants, including polycyclic aromatic hydrocarbons and polychlorinated dioxins, and TCDD that serves as a classic inducer of the receptor. In the early 1990s, the mouse *AhR* cDNA was first cloned [194, 195], followed by the human and rat *AhR* cDNA cloned [196, 197]. Later, additional cDNA of *AhR* has also been isolated from other species such as birds, fish, amphibians, but the rodent and human *AhR* have been employed in the most extensive studies [198]. The comparative study demonstrated that AhR is highly evolutionarily conserved amongst distinct species [198]. The early studies to analyze the *AhR* cDNA revealed that the translated protein contains two structural domains, i.e. the basic helix-loop-helix (bHLH) and PAS domains, in the N-teriminal half of the molecule [194, 195]. The bHLH domain contributes to DNA binding and also to protein-protein dimerization through the HLH portion. It is important to note that just a nuclear localization signal is contained within bHLH, whilst one more nuclear export signals are present in both bHLH and PAS domains. The PAS domain is further divided into two subdomains PAS-A and PAS-B. A study using the yeast Gal4 fusion protein system provided evidence that the C-terminus of the AhR harbours a potent transactivation domain, consisting of proline/serine/thereonine (P/S/T)-rich, glutamine (Q-rich) and acidic subdomains, each of which exhibits varying levels of activation and functions independently [199-201]. In addition, the AhR shares structural similarity with its nucleus dimerization partner ARNT and its repressor AhRR (Fig. 6).

It is clear that the unliganded AhR is held in the cytoplasm as an inactive protein in a complex with the chaperone proteins HSP90, HSP23, and an immunophilin-like protein XAP or p23 (Fig. 7). The binding of HSP90 is essential to retain AhR in the cytoplasm, because this interaction can mask the nuclear localization signal of AhR. Upon ligand binding, the HSP90-bound AhR is released from the cytoplasmic complex before translocating into the nucleus, whereupon it heterodimerizes with another bHLH-PAS protein ARNT. This heterodimer subsequently binds to XREs in the regulatory region of target genes. [187]. It has been reported that a number of co-activators and various general components form the transcriptional complex with the AhR/ARNT heterodimer [202], but the specific interaction and order of the complex formation still needs to be fully elucidated.

After ligand binding, phosphorylation of both AhR itself and the HSP90 complex on several residues are required for transformation of the unliganded AhR into the fully functional form [203]. Subsequently, the fully functional AhR induces the expression of many detoxification genes, which contain XREs in their promoter regions. These genes include those CYP enzymes, e.g. *CYP1A1*, *CYP1A2, CYP1B1,* and *CYP2S1*, and other drug-metabolizing enzymes such as *UGT1A6*, *NQO1*, *ALDH3A1*, and several *GST* isoenzymes (Table 4) [203].

Collectively, distinct possible mechanisms by which AhR is down-regulated either before or after its activation, include the 26S proteasome-mediated degradation of AhR, competitive inhibition of AhR by its repressor AhRR, and binding to its antagonists. The *in vitro* experiment showed that AhR is rapidly depleted after exposure to its ligands [204-206]. This event is most likely to occur after the transcriptional activation of its target genes, but can be blocked by the proteasome inhibitior MG132. Such degradation occurs through the 26S proteasome complex present in both the cytoplasm and the nucleus. Further studies haave revealed that AhR degradation also occurs after the receptor translocates into nucleus, wherein it forms a complex with the Cul4B E3 ubiquitin ligase, damaged-DNA-binding 1, ransducin β-like 3 and Rbx1. The Cul4B E3 ligase can catalyze ubiquitylatin of AhR and other nuclear receptors, e.g. estrogen receptor α and β subunits, and androgen receptor [207]. The ubiquitin labeling targets AhR to the 26S proteasome-mediated degradation.

There exists a negative feedback loop of AhR signaling with its repressor AhRR, which can in turn be transcriptionally induced by activated AhR [208]. The promoter region of *AhRR* contains a functional XRE sequence, enabling the expression of *AhRR* gene upon ligand activation of AhR. As it contains two bHLH and PAS-A domains that are structurally similar with AhR, followed by a C-terminal transcription repression domain, AhRR also forms a heterodimer with ARNT [208]. This heterodimer binds competitively to the XRE sequence with the AhR/ARNT heterodimer and subsequently recruits co-repressors [209]. Overall, the ultimate activation of AhRR leads to the inhibition of AhR [208, 210]. In addition, it should be noted that hypoxia inducible factor 1α (Hif1α) is another bHLH-PAS transcription factor, and can also form a functional heterodimer with ARNT (also called Hif1β). They regulate target genes through the HRE, which contains 5′-CGTG-3′ identical with the essential XRE for binding to AhR or AhRR (Fig. 4). However, it is not determined whether the Hif1α-HRE gene regulatory pathway is involved in the drug metabolism or xenobiotic response or if AhR regulates some genes in the response to hypoxia.

5.3 Ligands of AhR regulate expression of XRE-driven genes

Transcription factor AhR acts as a soluble ligand-activated nuclear receptor. Such ligands of AhR include exogenous and endogenous compounds, and exhibit structural diversity, though their binding affinities differ to a great extent. Exogenous ligands consist of not only synthetic ones but also normal dietary components. Amongst those AhR ligands identified and characterized, exogenous synthetic ones that show the highest affinity include planar, hydrophobic halogenated aromatic hydrocarbons (e.g. polyhalogeneated dibenzo-p-dioxins, dibenzofurans, and biphenyls) and polycyclic aromatic hydrocarbons (e.g. 3-methylcholanthrene, BaP, benzanthracenes and benzoflavones), as well as related compounds. Between halogenated and polycyclic aromatic hydrocarbons, the former ligands are more metabolically stable and act as the most potent class of AhR inducers, within the pM to nM range of binding affinities, whereas the latter ligands are the more metabolically labile ones with the relatively lower binding affinity in the nM to μM range [211].

Dietary chemicals acting as ligands of AhR have been described in numerous studies, showing that those chemicals can either activate or inhibit the AhR signaling pathway. In 1978, Watternberg and Loub reported that indoles occurring in edible cruciferous vegetables can inhibit the formation of neoplsia induced by AhR in mice, indicating they can inhibit the activity of AhR [212]. In 1991, another group showed that indole-3-carbinolcan, one of the indoles, acts as AhR agonist and increases the *CYP1A1* activity [213]. Besides indole-3-carbinolcan, other dietary plant compounds such as curcumin [214], quercetin and keampferol [215], have been reported to be able to competitively bind to the AhR. On the other hand, some dietary plant chemicals, such as resveratrol [216], have also been identified as inhibitors of AhR. It is noteworthy to mention that many dietary chemicals themselves have no or little ligand-binding activity of AhR; however, once these chemicals entered the mammalian digestive tract, they may undergo the conversion into significantly more potent AhR ligands. Examples of such chemicals include indole-3-carbinolcan, which itself is a weak inducer of gene expression, whereas indole-[3,2-b]-carbazole, an acidic condensation product from indole-3-carbinolcan, has relatively high affinity of AhR (~0.2~3.6 nM) [211].

The evidence for endogenous ligands of AhR identified, in addition to exogenous ligands, has been provided in various studies. Firstly, the existence of endogenous ligands is postulated from the identification of the nuclear AhR complexes in unexposed cells in culture and tissue slices. Secondly, the effect of endogenous ligands is deduced from the fact that AhR-deficient cells had altered cell cycle progression [217, 218]. Thirdly, activation of AhR by endogenous ligands occurs in the *AhR* knockout animals, that exhibit numerous physiological changes and developmental abnormalities [219, 220]. It has been suggested that a number of the candidates for endogenous ligands of AhR, bearing various structures, include: indigoids, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, equilenin, arachidonic acid metabolites, heome metabolites, tryptophan metabolites, and ultraviolet photoproducts of tryptophan [221]. Taken together, these studies indicate that AhR can bind many

different chemicals, including environmental contaminants, therapeutic agents, naturally occurring chemicals and small molecules isolated from tissues. These chemicals have diverse structures and distinct affinities of ligand binding to the AhR.

5.4 Physiological functions of AhR

Some of the above-discussed AhR inducers are environmental pollutants that cause acute and chronic toxicity, a fraction of which themselves are carcinogens. They induce the AhR-mediated expression of genes responsible for xenobiotic-metabolizing enzymes, such as cytochrome P450 families. Besides its involvement in the xenobiotic metabolism, AhR plays crucial roles in distinct physiological processes [222], which range from reproduction, development, immunity, cell cycle, cell proliferation, to cell adhesion and migration [223]. Physiological functions of AhR are varying with its distinct expression in diverse cells, tissues and organs. The constitutive AhR is highly expressed in liver, but is also abundant in placenta, thymus, lung, kidney, small intestine, heart and pancreas [224].

The involvement of AhR in normal physiological processes has been proven by the evidence showing that it was activated in a xenobiotic-independent way [225-228]. The *AhR*-null mice also provided a deeper insight into the physiological process requiring for its transcriptional activity. These animal models not only demonstrated that this receptor is essential for dioxin-induced cytotoxicity [229] and carcinogenesis [230], but also revealed the existence of an *AhR*-deficient phenotype. Different studies showed that genetic deletion of the *AhR* in the mouse caused either early death or pathological changes by 13 months, which was accompanied by a wide variety of phenotypic alteration in major organ systems [231, 232]. These phenotypes include progressive cardiac hypertrophy, gastric hyperplasia that progressed into polyps with ages, T cell deficiency in spleens, and abnormalities in skin such as hyperkeratosis, and marked dermal fibrosis.

Besides the above effect on the cardiovascular system, the immune system and skin, the *AhR*-null females also showed difficulties in maintaining pregnancy, and their pups showed a poor survival during the lactation and weaning [233]. A significant impact of its function on the development of liver is supported by the facts that *AhR*-null mice exhibit smaller livers in size, and also show portal fibrosis and early lipid accumulation in this organ [219, 234]. Comparison of the liver mRNA profiles from between wild-type and *AhR*-null mice revealed that the expression patterns of 392 genes were changed due to the absence of AhR. The mechanisms underlying these physiological functions of AhR include its effect on the cell cycle, which can in turn affect the progress of cell proliferation, either inhibiting or promoting it depending on the cell phenotypes [223]. Also, the AhR is involved in cell adhesion and migration, in addition to developmental processes.

5.5 Dual apposing roles of AhR in the progress of tumourigenesis

As AhR can promote yet inhibit cell proliferation, there has been some discussion about whether it is a tumour promoter or tumour suppressor. The AhR cooperates with signaling molecules involved in cell survival pathways, which allow cells to sustain proliferation. An example is NF-κB [235], with which AhR can physically interact, leading to its activation in human breast cancer MCF-7 cells. Activation of NF-κB causes the transactivation of the *c-Myc* proto-oncongene. By this mechanism, the AhR may contribute to increased cell proliferation and carcinogenesis in the breast. Another study also showed that AhR induces the proliferation of human lung carcinoma A549 cells, due to the over-expression of the nuclear receptor. Transgenic mice expressing a constitutively active *AhR* have showed spontaneous tumours in the glandular stomach [236], and also increased frequency of the formation of hepatocarcinomas induced by N-ntrosodiethyl [237]. Such facts that over-expression and activation of AhR can stimulate cell proliferation and even promote carcinogenesis indicate that the receptor has oncogenic activity.

On the other hand, several studies found that activation of AhR can halt the cell cycle at different stages and also inhibit cell proliferation. In non-proliferating 5L-heptoma cells, induction of AhR by exogenous ligands

activates transcription of the *p27^{kip1}* tumour suppressor, and consistently induction of p27^{kip1} by dioxin in fetal thymus was accompanied by inhibition of cell proliferation [238]. Although AhR can stimulate proliferation of MCF-7 cells without exogenous ligands, the presence of exogenous ligands allows this receptor to synergize and interact with the Rb tumour suppresser, resulting in the inhibition of Rb-mediated E2F-dependent transcription and ultimately leading to cell cycle arrest [239]. Another study showed that cell cycle was blocked by dioxin at the G_1 in MCF-7 and mouse hepatoma Hepa-1 cells [240]. Such blockage was due to the fact that the interaction between activated AhR and the p300 co-activator leads to a displacement of p300 from the E2F-dependent promoter and proliferation arrest. The arrest of cell cycle by constitutively activated AhR has also been found in a few of other cell lines, transgenic mice, and mouse thymus in organ culture through different mechanism [223]. These findings suggest that constitutive or ligands-induced activation of AhR may act as a tumour suppressor by inhibiting cell proliferation. Overall, depending on the phenotypes of cells and inducers of the receptor, AhR can either inhibit or promote cell proliferation, and thus have dual tumour suppressor or oncogenic activity.

6. AhR crosstalks with multiple signaling pathways

The AhR mediates dioxin-induced toxicity and also influences many of physiological functions. The mechanisms that underlie the wide diversity of AhR activity are established by its cross talks with multiple signal transduction pathways (e.g. MAPKs), cell cycle progression and apoptosis, and transcriptional factors such as Nrf2 and Hif-1 [203, 241, 242]. The MAPKs, including three families: ERK1/2, JNK/SAPK and p38 kinases, mediate important intracellular signaling transduction [243]. MAPKs and their downstream protein kinases can phosphorylate a large panel of substrates (e.g. AhR and Nrf2) on serine and threonine residues, which enable them to regulate gene expression and protein functions. Generally, ERK1/2 are involved in regulating both mitogenic and developmental events, four p38 kinases play important roles in the inflammatory response, apoptosis and cell cycle, and three JNK isoforms play essential roles in multiple cellular signaling towards the immune system, stress-induced and developmentally programmed apoptosis, carcinogenesis, and pathogenesis of diabetes [244].

Although the well-known AhR ligand TCDD activates ERK and JNK, such activation occurred similarly in both the *AhR*-expressing and *AhR*-null cells, suggesting induction of MAPK by this ligand in an AhR-independent manner [245]. However, TCDD-stimulated MAPKs appear critical for the induction of AhR-dependent gene transcription and *CYP1A1* expression. TCDD and another ligand 3-methylcholanthrene induced morphological changes that modulate epithelial cell plasticity [246]. Such dioxin-induced events were mimicked by constitutive expression and activation of AhR. In addition, a correlated event was the activation of JNK, which is reversible using a JNK inhibitor, indicating the effect of AhR on cell plasticity is in an JNK dependent pathway. Therefore, these novel effects on cell plasticity support a mechanistic role for the AhR in cancer progression as mediated by many of its ligands. Activation of p38 kinases by the AhR ligand TCDD seems to be a cell-specific consequence [246], because p38 kinase activated by TCDD in an AhR-independent mechanism occurred in RAW 264.7 macrophages but not in embryonic fibroblasts.

Another signaling pathway AhR interacts with is the Rb-E2F axis, which is responsible for several cell cycle checkpoints at G1 and S phases. Direct interactions of ligand-activated AhR with either the hypophosphorylated Rb or E2F have been found [239, 240]. Such an interaction between AhR and Rb blocks the phosphorylation of Rb leading to the repression of S-phase specific gene transcription. Alternatively, AhR activation can induce CDK inhibitors that arrest the cell cycle at G1 phase. Additional study using *AhR*-expressing and *AhR-null* fibroblasts showed that the proliferation rate is faster in *AhR*-expressing fibroblasts compared with that in *AhR-null* fibroblast in a ligand independent manner [247]. Growth-promoting genes were significantly down-regulated in *AhR-null* fibroblast, whereas growth-arresting genes were up-regulated. These results suggested that AhR plays an intrinsic role in regulating cell proliferation independent of either exogenous or endogenous ligands. In contrast, AhR-dependent promotion of cell proliferation occurred through induction of JunD and cyclin A [248]. On the

other hand, activation of E2F1 can activate apoptosis, but there is also evidence suggesting that E2F1 acts as a tumour suppressor due to its ability to initiate apoptosis in cells that lose the normal cell cycle control [249]. It has been found that AhR and E2F1 can physically interact *in vitro* and *in vivo,* so as to result in the repression of the transcriptional activity of E2F, and ultimately the inhibition of apoptosis.

6.1 Cross talks between AhR and Nrf2

Besides the physical interaction, AhR can modulate expression of several key genes, which contain the XRE sequence in their promoter regions, at the transcriptional level (e.g. *Nqo1*, *Nrf2* and *AhRR*). Clearly, the AhR has been shown to be able to bind directly to the promoter region of *Nrf2* [168] and its target drug-metabolizing genes (e.g. *Nqo1*) through the XRE sequences (Fig. 7). As AhR and Nrf2 regulate expression of Phase I and Phase II detoxification enzymes, i.e. NQO1, an enzyme catalyzing BaP-quinone detoxification, knockdown of *AhR* by RNA interference (RNAi) diminished BaP-induced expression of *Nrf2* and *Nqo1*, and knockdown of Nrf2 significantly decreased *NQO1* mRNA and protein levels in cells treated with or without BaP [250]. Mutation of the Nrf2-binding ARE site abrogated the *Nqo1* promoter activity, but this activity was unaffected by mutation of the AhR-binding XRE site, suggesting a role for the signaling of AhR-XRE to activate Nrf2-ARE gene regulatory network in enhanced expression of *Nqo1*. The chemopreventive potential of functionalized aurones and related compounds as inducers of NQO1, in order to exploit the proposed crosstalk between the AhR and Nrf2 gene batteries [251]. Recently, the antifungal agent ketoconazole was identified as an inducer of AhR signaling and the Nrf2 antioxidant response in human keratinocytes [252]. Ketoconazole stimulated the nuclear translocation of Nrf2, and its cytoprotective effects against oxidative stress strongly depend on a functional AhR [252]. Sustained activation of the AhR induced by TCDD results in oxidative stress, DNA damage and subsequent steatohepatitis in *Nrf2*-null mice [253]. The aggravated hepatosteatosis is due to increased lipogenesis in the liver, as accompanied by higher expression of *Fgf21* and triglyceride-synthesis genes, and activation of *c-Jun* and *NF-κB*, but by down-regulation of bile-acid-synthesis genes and cholesterol-efflux transporters, and attenuated induction of phase-II enzymes *Nqo1*, *Gsta1/2*, and *Ugt2b35*. In additional low-glucose response, endogenous compounds are recruited as AhR ligands to induce various gene expression, of which *CYP1* and *Nrf2* induction was abolished by RNAi for *AhR* [254], suggesting a relationship between drug-metabolizing enzymes and mechanisms of the anti-stress response against tumor angiogenesis. These findings demonstrate that the AhR-Nrf2 pathway opens up new opportunities to prevent and treat cancer and other diseases [255].

As phytochemicals have the potential to counteract adverse effects of carcinogens, an impact of flavonoids on expression of AhR-Nrf2 pathway components in BaP-stimulated colon cancer Caco-2 cells were investigated [256]. In contrast to kaempferol, quercetin and BaP efficiently induced *CYP1A1*, *CYP1A2* and *CYP1B1* mRNA. The BaP up-regulated *AhR,* but down-regulated *AhRR*. By contrast, the flavonoids quercetin and kaempferol did not affect *AhR* expression but counteracted repression of *AhRR* induced by BaP. Only quercetin was found to induce *AhRR*, whilst *ARNT* appeared to be down-regulated by BaP, as well as flavonoids. Activation of the Nrf2 pathway by either BaP or the flavonoids was revealed by induction of *Nrf2* and target genes such as *NQO1*, *GSTP1*, *GSTA1* and *GCLC*. Importantly, the flavonoids can abolish the induction of *Nrf2*, *GSTP1* and *NQO1* by BaP. The authors suggested that quercetin acts a dual ARE-inducer and XRE-inducer, whilst kaempferol acts just an ARE-inducer (Fig. 7). Similarly, we also found that Quercetin and kaempferol up-regulated the Nrf2-ARE-*Nqo1* signaling pathway through stabilizing this CNC-bZIP protein (data unpublished). Furthermore, the ARE/XRE-driven reporter mutagenesis experiments showed that the ARE is required for both the basal and inducible expression of *Nqo1*, whereas the XRE is involved in the basal *Nqo1* expression but not in its induction by these two flavonoids, although they can acts as AhR agonists because expression *CYP1A1* is up-regulated in both experimental cells and the mouse small intestine. The chemopreventive effect of youngiasides, isolated from Crepidiastrum denticulatum, is elicited through induction of quinone reductase activity in hepatoma Hepa-1c1c7 cells, with a relatively high

chemoprevention index [257]. Youngiasides up-regulated the expression of *CYP1A1* and *quinone reductase* in Caco-2 cells through activation of both the Nrf2-ARE and AhR-XRE pathways, suggesting a bifunctional inducer of quinone reductase for potential chemopreventive agents. In addition, flavonoid-contained coffee induces expression of *UGTs*, e.g. *UGT1A8* to *UGT1A10*, in liver and stomach by the AhR-XRE and Nrf2-ARE [258, 259], in order to protect against the pathologies of chronic liver disease, hepatocellular carcinoma and diabetes. Both AhR and Nrf2 are also key regulator of human multidrug resistance protein 4 induced by TCDD, 3 methylcholanthrene or oltipraze [260]. It should be noted that Nrf2 can regulate expression of *AhR* and modulate its several downstream events [261]. In addition, other crosstalks between AhR and Nrf2 were reviewed by Hayes *et al* [242].

6.2 Cross talks between AhR and Hif1

The class of Hif1α, Hif2α, and Hif3α are also bHLH-PAS proteins that heterodimerize with ARNT; this complex preferentially binds to HRE and activates the transcription of genes, e.g. *erythropoietin* (*Epo*), that regulate adaptation to hypoxia [241]. The HRE are homologous with the XRE for binding of the AhR/ARNT complex (Fig. 4), and thus it is postulated that activation of one pathway would inhibit the other due to competition for ARNT or other limiting factors through binding to the HRE/XRE. For example, the promoter region of *Epo* also contains five functional XREs, besides HRE, immediately upstream of transcriptional start site [241]. Activation of the hypoxia response pathway inhibited up-regulation of *Cyp1a1*, but activation of the AhR actually enhanced the induction of *Epo* by hypoxia. This suggests crosstalks between Hif1α-HRE and AhR-XRE in response to hypoxia and xenobiotics. This is also supported by the evidence that hypoxia inhibited induction of AhR activity and also down-regulated expression of its target drug-metabolising enzymes in the ARENT-dependent manner [262, 263]. Activation Hif1α attenuated induction of AhR-regulated gene expression by BaP, leading to increased genetic instability and malignant progression in response to hypoxia and exogenous genotoxins [264]. However, an AhR ligand, aminoflavone (which is an active component of a novel anticancer agent AFP464 in phase I clinical trials) inhibited activity of Hif1α and protein accumulation in an AhR-independent pathway in MCF-7 cells [265].

Interestingly, expression of *Cyp2s1* highly in epithelial tissues is inducible by TCDD *via* the AhR pathway. Its promoter contains three overlapping HREs embedded within the trimeric XRE segment [266]. Each of the trimeric XRE sequence can bind the AhR/ARNT dimer and also mediate dioxin-dependent transcription of *Cyp2s1*, whilst each HRE within this segment can bind the Hif1α/ARNT dimer and contributes toward hypoxia inducibility. These two dimers differentially bind to the region containing the trimeric XRE segment of *Cyp2s1* in a dioxin- or hypoxia-dependent fashion. In addition to the HRE, UPRE also contains a portion essential for the functional XRE (Fig. 4). It was recently reported that activation of the AhR pathway and induction of the unfolded protein response are involved in suppression of adipocyte differentiation and adipogenesis by cigarette smoke, but AhR was neither activated by ER stressors and AhR agonists did nor induce ER stress response [267]. This case suggests no crosstalk between the XRE and UPRE gene regulatory networks, but it remains to be further identified using distinct experimental systems within other pathophysiological stress conditions.

7. Concluding remarks

Collectively, a number of cytoprotective mechanisms have been evolutionally developed to defense against toxic electrophiles, chemical carcinogens and oxidative stress. Some of these cytoprotective mechanisms have been portrayed as targets of cancer chemopreventive agents (e.g. phytochemicals)[268-270], of which much attention has been attracted with a particular focus on the intrinsic antioxidant and detoxification mechanisms [271-273]. Amongst the well studied are multiple signaling nodes and branches within both AhR-XRE and Nrf2-ARE gene regulatory networks (Fig. 7) [272, 274, 275]. These two reciprocally interactive signaling networks, along with their feedback regulatory loops, finely control expression of drug-metabolizing enzymes,

which are involved in biotransformation at Phase I (e.g. CYPs, NQO1), detoxification at Phase II (e.g. GSTs) and drug-efflux excretion at Phase III (MRPs). It has also been shown that Nrf1 and Nrf2 are two important CNC-bZIP proteins in regulating both the basal and inducible expression of antioxidant, detoxification and cytoprotective genes, in addition to those encoding drug-metabolising enzymes. Clearly, Nrf2 has been identified as a master regulator of drug-metabolizing enzymes and antioxidant cytoprotective proteins and also considered as a target for cancer chemoprevention [276-278]. The activity of Nrf2 is negatively regulated by Keap1 and β-TrCP in the different subcellular compartments (Fig. 5). These two adaptor proteins can respectively recruit the ubiquitin E3 ligases Cul3 and Cul1 complexes to the Neh2 and Neh6 domains of Nrf2 targeted for the 26S proteosome-mediated degradation. This negator *Keap1* gene expression is regulated by Nrf1 and Nrf2, whilst transcription of *Nrf2* and downstream genes (e.g. *Nqo1*), which contain two *cis*-elements XRE and ARE, is tightly controlled by AhR and CNC-bZIP (e.g. Nrf1) family factors. Such crosstalks between AhR-XRE and Nrf2-ARE regulatory networks indicate that multiple signaling pathways are integrated to activate antioxidant, detoxification and cytoprotective genes against cytotoxic insults and oxidative stress.

In targeted cells, toxic chemicals, drugs, pollutants, xenobiotics and pro-carcinogenes can be biotransformed to become reactive metabolites, electrophiles or activated carcinogens, as accompanied by free radicals and reactive oxygen species, produced in the activation by AhR-XRE-driven Phase I enzymes, in large part CYPs (on the right side of Fig. 7). At the same time, reactive metabolites and redox stress can also activate the Keap1-Nrf2-ARE-driven Phase II enzymes for the conjunction of those toxicants and carcinogens to be detoxifed and Phase III drug-efflux pumps for elimination of them. Based on these differences in activation of AhR-XRE and Nrf2-ARE alone or both, xenobiotics are classified into ARE-, XRE- and ARE/XRE-inducers (on the left side of Fig. 7). Therefore, it is critical to maintain the balance between the AhR-XRE-CYPs activation and Nrf2-ARE-Phase II detoxification in carcinogen-targeted cells. If the detoxification pathway is saturated, activated carcinogens, along with reactive electrophiles and reactive oxygen species produced in the Phase I activation, cannot completely detoxified and eliminated; the residuals of these insults may increase genomic instability and eventually initiate carcinogenesis.

In untargeted cells, nontoxic chemopreventive agents (e.g. flavanoids) can predominantly induce activation of AhR-XRE and Nrf2-ARE alone or both, and are thereby classified into monofunctional and bifunctional inducers (Fig. 7). Chemopreventive blocking agents either interact directly with reduced glutathione or acquire this ability indirectly as a consequence of biotransformation by Phase I enzymes, suggesting that these compounds produce a type of thiol/oxidative stress [279]. The redox stress may be caused by modification of cysteine residues in proteins [280-282] and also trigger redox signaling dependent on Keap1 [57, 283]. Therefore, up-regulated expression of drug-metabolising enzymes has been determined as targets of cancer chemoprevention against potential toxicants and carcinogens. Once the host cells are targeted by toxicants and carcinogens, the possible consequence is blocked by chemopreventively-enhanced antioxidant capacity by inducing GCLC, GCLM, glutathione synthase, peroxiredoxin, ferritins, and HO-1, and increased expression of drug-metabolising enzymes (e.g. NQO1, AKR, UGT and GST). Induction of these cytoprotective genes by chemoproventive agents has now been recognized primarily though the Keap1-Nrf2-ARE pathway. However, constitutive hyperactive Nrf2 has been shown to protect cancer cells against hypoxia stress and even therapeutic drugs, and as such a consequence, it promotes tumourigenesis and also increases drug resistance [174, 177, 178].

To date, there has been a disproportionate focus on Nrf2, but relatively less is known about the function of Nrf1. This consequence is due largely to the fact that *Nrf2* knockout mice are viable [94], whilst global knockout of *Nrf1* in the mouse leads to embryonic lethality and severe oxidative stress [52, 95, 284, 285]. Specifically, conditional knockout of *Nrf1* in the liver and brain of neonatal mice results in non-alcoholic steatohepatitis and hepatic neoplasia [96, 97] and neurodegenerative disease [99, 100], respectively. These facts demonstrate that Nrf1 fulfills an essential function, distinct from Nrf2, in regulating expression of antioxidant, detoxification and

cytoprotective genes responsible for maintaining cellular homeostasis and organ integrity. Molecular and cell biology studies have identified that Nrf1 is a membrane-bound glycoprotein spanning across the ER and nuclear envelope membranes [161, 286, 287] and it is activated by a redox inducer tBHQ [288]. It is therefore postulated that the Nrf1-ARE pathway is activated by ER-derived redox stress, in part produced in the ER-based Phase I enzymes-mediated bioactivation, and will be considered as a potential target of chemoprevention. Recently, two reports have showed that quercetin and genistein up-regulate Nrf1-mediated peroxiredoxins and glutathione peroxidase enables cytoprotection against oxidative stress-induced ocular disease and endothelial cell injury, respectively [289, 290]. However, it remains to determine the unique role of Nrf1 in chemoprevention from oxidative stress involved in tumour-promoting inflammation and in the ensuing course of carcinogenesis.

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Footnotes

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List of Abbreviations:

AhR = aryl hydrocarbon receptor; AhRR = AhR repressor; AKR = aldo-keto reductases; ARE = antioxidant response element; ARNT = AhR nuclear translocator; BaP = benzo[a]pyrene; bHLH = the basic helix-loop-helix domain; BTB = the Broad-complex, Tramtrack, Bric-à-brac domain; β-TrCP = β-transducin F-box/WD repeat containing protein; bZIP = basic-region leucine zipper; CREB = cAMP-response element binding protein; CNC = cap'n'collar; Cul = cullin; Cys = cysteine residue; CYP = cytochrome P450 enzyme; EGCG = epigallocatechin gallate; EPO = erythoropoietin; ER = endoplasmic reticulum; ERK = extracelluar signal-regulated kinase; GCL = γ-glutamyl cysteine ligase; GCLC = GCL catalytic subunit; GCLM = GCLmodifier subunt; GSK = glycogen synthase kinase; GST = glutathione *S*-transferase; JNK = c-Jun NH_2-terminal kinase; Hif1 = hypoxia inducible factor 1; HO-1 = haeme oxygenase 1; HRE = hypoxia response element; Keap1 = kelch-like ECH-associated protein 1; MAPKs = mitogen-activated protein kinases; NAT = N-acetyl transferase; NF-E2 = nuclear factor-erythroid 2; NQO1 = NAD(P)H:quinone oxidoreductase 1; Nrf = NF-E2 p45 subunit-related factor; PAS = eukaryotic Per-ARNT-Sim domain; PERK = PRKR-like endoplasmic reticulum kinase; PKC = protein kinase C; Rbx1 = ring-box protein 1; RNAi = RNA interference; Skn-1 = skinhead-1; SNP = single nucleotide polymorphism; SULT = sulfotransferase; TCDD = 2,3,7,8-tetrachloro dibenzo-p-dioxin; TRE = PA-response element for binding principally by AP-1; UGT = UDP-glucuronyl transferase; UPRE = unfolded protein response element; XRE = xenobiotic response element.

Tables

Table 1. Chemical characteristics of each subclass of flavonoids.

Class	Flavonols	Flavones	Isoflavones	Catechins	Anthocyanins
Carbon atom in ring C attacthed to B	2	2	3	2	2
C-ring unsaturation	2-3 double bond	2-3 double bond	2-3 double bond	None	1-2, 3-4 double bond
C-ring functional groups	3-hydroxy, 4-Oxo	4-Oxo	4-Oxo	3-hydroxy; 4-gallate	3-hydroxy

Table 2. A list of the ARE sequences in different gene promoters from different species.

The sequences shown are from the genes for antioxidant, metal-binding, and detoxification proteins from human, mouse and rat. As AP-1 binding site share some similarities in the sequence of ARE, their sequences in the genes were also shown in the table. The nucleotides in bold capital letters are those that share identity with the extended 16-bp ARE consensus sequences (5′-TMAnnRTGAYnnnGCR-3′, M=A/C; R=A/G; Y=C/G/T). The TGAC motif is identical with an equivalent portion of AP1-binding consensus site, whilst the GC motif is essential for the functioning of ARE. Both TGAC and GC motifs are placed in white bold letters on the black backgrounds. Some data are adapted from Hayes *et al* [57].

Function	Species	Gene	Element	Sequence
Antioxidant enzymes	Human	*GCLC*	ARE-4/AP1	**TC**CCC**GTGAC**TCA**GC**G
		GCLM	EpRE	AG**A**CAA**TGAC**TAA**GC**A
			ARE(var)	**TAA**CG**G**T**AC**GAA**GC**A
		GPX2	ARE-1	C**CA**GGA**TGAC**TTA**GC**A
			ARE-2	GT**A**CAG**TGA**GAGG**GC**A
		PRDX1	EpRE-1	**T**GTAAC**TGA**ATCA**GC**C
			EpRE-2	**T**TCTCC**TG**C**C**TCA**GC**C
		PRDX6	ARE	G**CA**AC**GTGAC**GA**GC**C
		TRX	ARE/AP1	**TCA**CG**T**T**AC**TCA**GC**A
		TXNRD1	ARE	**TCA**GAA**TGAC**AAA**GC**A
	Mouse	*Gsr1*	ARE-1	**TC**GCC**GTGAC**TAA**GC**A
			ARE-2	**TCA**CAG**TGAC**CAA**GC**G
		Slc7a11	EpRE-2	C**CA**GCT**TGA**GAAA**GC**G
	Rat	*SRXN1*	ARE-1/AP1	**TCA**CCC**TGA**GTCA**GC**G
Metal-binding proteins	Human	*FTL*	MARE/ARE	**TCA**GC**A**T**GAC**TCA**GC**A
		MT1B	ARE	G**A**GCAG**TGAC**CTG**GC**G
	Mouse	*Fth1*	FER1	C**C**TCC**A**T**GAC**AAA**GC**A
			AP1/NF-E2	C**C**ACC**G**T**GAC**TCA**GC**A
		Ftl1	EPRE	**TCA**GC**G**T**GAC**TCA**GC**A
		Mt1	ARE	GGCGC**G**T**GAC**CTG**GC**C
		Mt2	ARE/AP1	GGGGT**G**T**GAC**TCA**GC**G
	Mouse	*AKR1C2*	ARE	**TCA**GGG**TGAC**TCA**GC**A
		MGST1	EPRE	A**CA**TC**G**T**GAC**AAA**GC**A
		NQO1	ARE/AP1	**TCA**CAG**TGAC**TCA**GC**G
		UGT1A1	ARE	A**AA**CCC**G**GAC**T**TG**GC**C
Detoxification proteins	Mouse	*Akr1b3*	ARE-1	GG**A**GC**A**T**GAC**CCA**GC**A
		Gsta1	EpRE	**TAA**TGG**TGAC**TCA**GC**A
		Gsta3	EpRE	C**A**GGC**A**T**GAC**ATT**GC**A
		Mrp2	ARE	CTGGG**A**T**GAC**CTC**GC**A
		Nqo1	ARE	**TCA**CAG**TGA**GTCG**GC**A
	Rat	*Gsta2*	ARE	**TAA**TGG**TGAC**AAA**GC**A
		GStp1	GPE1/AP1	**TCA**CTA**TGA**T**T**CA**GC**A
		Nqo1	ARE	**TCA**CAG**TGAC**TTG**GC**A
			ARE'core'	**TGAC**NNN**GC**
			ARE concensus	**TMA**NNR**TGA**YNNN**GC**R

AP1-binding site TGASTCA

Table 3. A list of selected inducers to activate drug-metabolizing genes regulated by Nrf2.

Species	Gene	Selected inducer
Human	*NQO1*	B-NF, tBHQ
	GCL	BHA
	UGT1A6	tBHQ
	UGT1A8	EGCG
	UGT1A10	EGCG
Mouse	*GSTA1*	t-BHQ, SFN,3-MC, catechol
	GSTPi	
	GCL	
	NQO1	BHA,SFN,I3C
	UGT1A6	EQ,OTZ
	UGT2B5	Curcumin
Rat	*Nqo1*	B-NF, t-BHQ
	GSTA	
	GSTPi	
	UGT1A6	EQ, OTZ
	UGT1A7	EQ, OTZ
	UGT2B1	EQ,OTZ
	UGT2B3	EQ, OTZ
	UGT2B12	

Table 4. A list of drug-metablizing enzymes regulated by AhR. Such a list of genes regulated by AhR through the XRE is still growing, some of which were described [180, 181].

Species	Gene
Mouse	*Cyp1a1*
	Cyp1a2
	Cyp1b1
Rat	*Cyp1a1*
	Aldh3a1
	Ugt1a1
	GSTya
	Nqo1

Figures and legends:

Fig. (1) General structures of common food flavanoids.

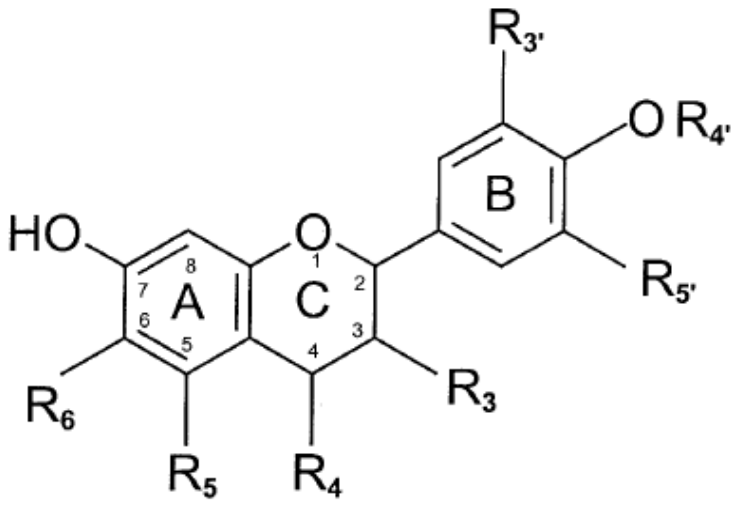

Fig. (2) Structures of typical Nqo1 inducers selected from each class.

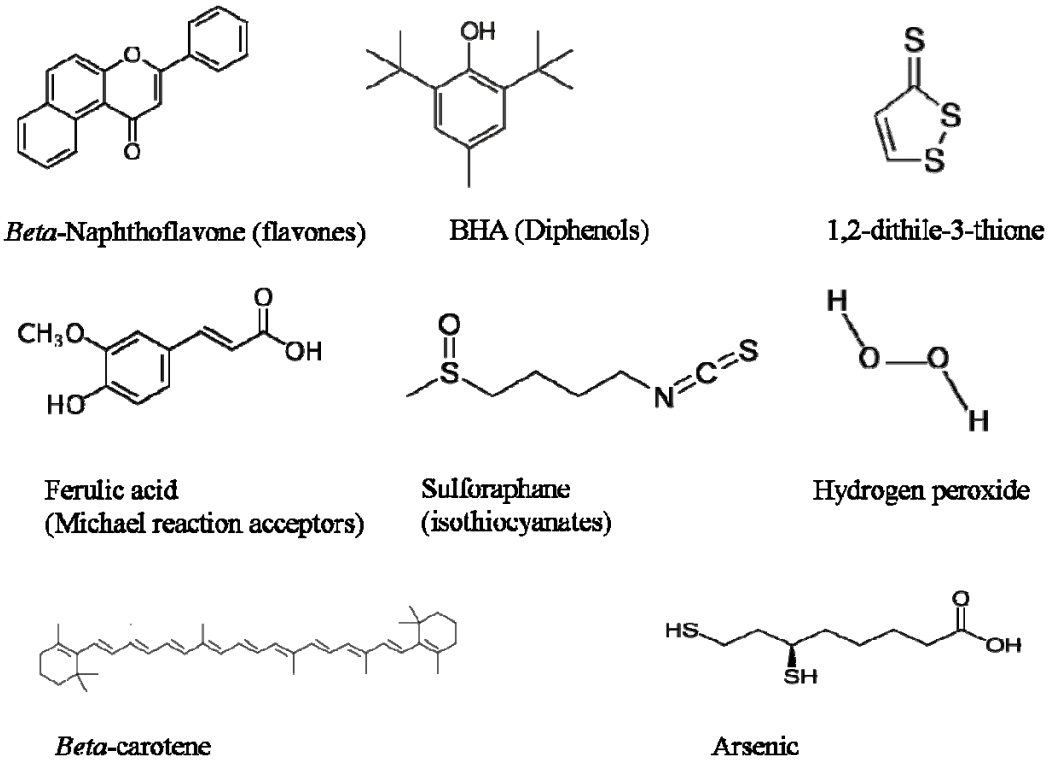

Fig. (3) Schematic diagrams of structural domains of Nrf2 and other CNC-bZIP proteins

Amongst the family members, the CNC and bZIP domains are highly conserved, and thus integrally called the Neh1 domain in Nrf2. The term Neh indicates Nrf2-ECH homology. The Neh2 domain of Nrf2 is represented by the Neh2L in Nrf1 and CNC proteins. Keap1 negatively regulates Nrf2, and possible CNC proteins, through binding to DLG and ETGE motifs. Although these two Keap1-binding motifs are contained in Nrf1, it is not regulated by Keap1. The Neh3 domain shares sequence similarity between the Nrf, Cnc and small Maf proteins, but it is absent in Bach1, Bach2 and Skn-1. The Neh4 and Neh5 domains, along with the DIDLID element, are responsible for transactivation (TA) of target genes. The Neh6 domain is homologous in all four Nrf proteins and is responsible for the β-TrCP-mediated degradation. The N-terminal domain (NTD) of Nrf1 contains N-terminal homolog box 1 (NHB1, which severs as an ER targeting signal) and NHB2, and shares conservation with Nrf3 and Cnc proteins. The BTB domain is responsible for dimerization in Bach1 and Bach2. Each member of the CNC-bZIP family can form a functional dimer with a small Maf or another bZIP protein (e.g c-Jun).

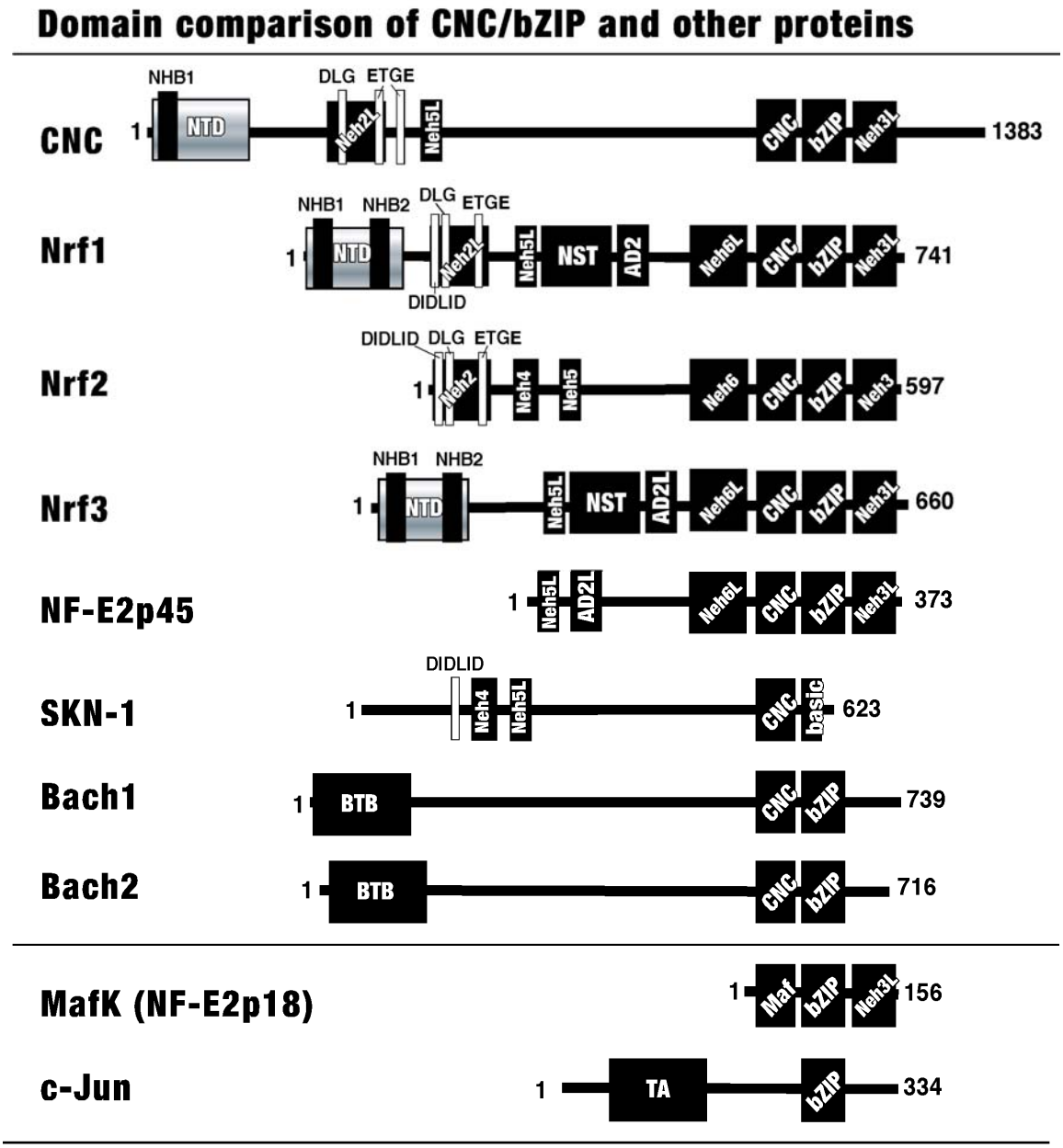

Fig. (4) Consensus sequences of ARE, XRE and other homologues found in the promoter regions of different stress responsive genes. The ARE and ARE-like elements share sequence conservation with the NF-E2, TCF11, AP-1 binding sites and T-MARE (TRE-type Maf recognition element). Amongst these sequences, these three motifs TGAC, GC and TCA are highly conserved. The functional dimers of CNC-bZIP proteins with small Maf (sMaf) or other bZIP proteins can differentially bind to the ARE, ARE-like, NF-E2, TCF11, MARE and AP-1 consensus sites, but it is not know whether they can bind to UPRE competitively with its canonical bZIP factors (XBP-1, ATF6 and HAC1). Expression of XRE-driven genes is regulated positively by AhR and negatively by its repressor AhRR. The CGTG motif essential for functioning of XRE is embodies in the HRE and UPRE response to hypoxia and ER stress, and both the XRE and UPRE also contain the TGAC motif that shares identity with the equivalent of ARE/AP-1 site. These homologous *cis*-elements and their cognate canonical and non-canonical transcription factors can form an all-potent gene regulatory network, which can integrate from multiple signaling towards distinct response gene expression.

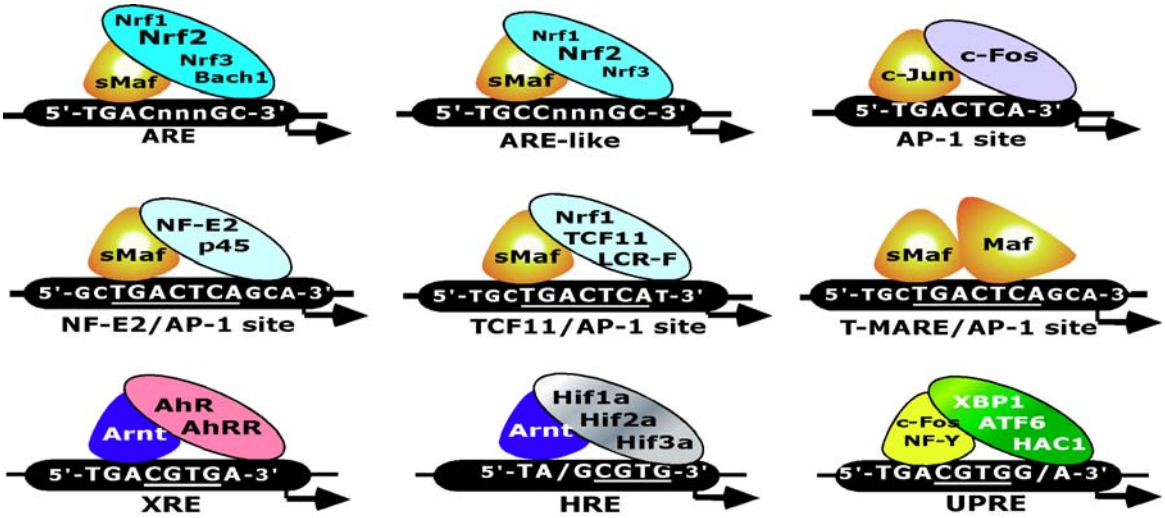

Fig. (5) Nrf2 is negatively regulated by Keap1 and β-TrCP within distinct signaling pathways. (**A**) Shows structural domains of Nrf2, Keap1 and β-TrCP. Nrf2 contains two Keap1-binding DLG and ETGE motifs in its Neh2 domain, and also two β-TrCP-binding DSGxS and DSAxxS degrons in the Neh6 domain. The adaptor Keap1 is homodimerized through its BTB domain, and binds Cul3 E3 ubiquitin ligase and Nrf2 through its IVR and doubleglycine-repeat (DGR) domains, respectively. The β-TrCP dimer formed through its D-domain (DD) binds Cul1 E3 ubiquitin ligase and Nrf2 through the F-box and WD40 domains, respectively. (**B**) Shows a model for negative regulation of Nrf2 by Keap1 or β-TrCP. Under normal redox conditions, a homodimer of Keap1 can bind to the DLG and ETGE motifs of Nrf2 through its six-bladed β-propeller structure formed by both the DGR and the CTR (C-terminal region). In addition interacting with Nrf2, Keap1 also binds the Cul3- Rbx1 complex and this association allows ubiquitination of Nrf2 to target for proteosomal degradation. Upon oxidative stress, repression of Nrf2 by Keap1 is antagonized when reactive cystines of this adaptor protein, particularly within its IVR are modified, and/or Nrf2 at Ser^{40} located between the DLG and ETGE motifs is phosphorylated (P) by protein kinase C (PKC) or other kinases (e.g. PERK). Then phosphorylated Nrf2 is translocated into then nucleus through its nuclear localization signal within the basic region. In the nucleus Nrf2 binds to the ARE-driven genes after it forms a functional heterodimer with a sMaf protein through the interaction between their ZIP regions. The β-TrCP dimer can bind Nrf2 through the DSGxS and DSAxxS degrons, which is phosphorylated by GSK3, and this also allows the associate of this CNC-bZIP protein with the β-TrCP-mediated Cul1-Rbx1 complex targeted for the nuclear degradation.

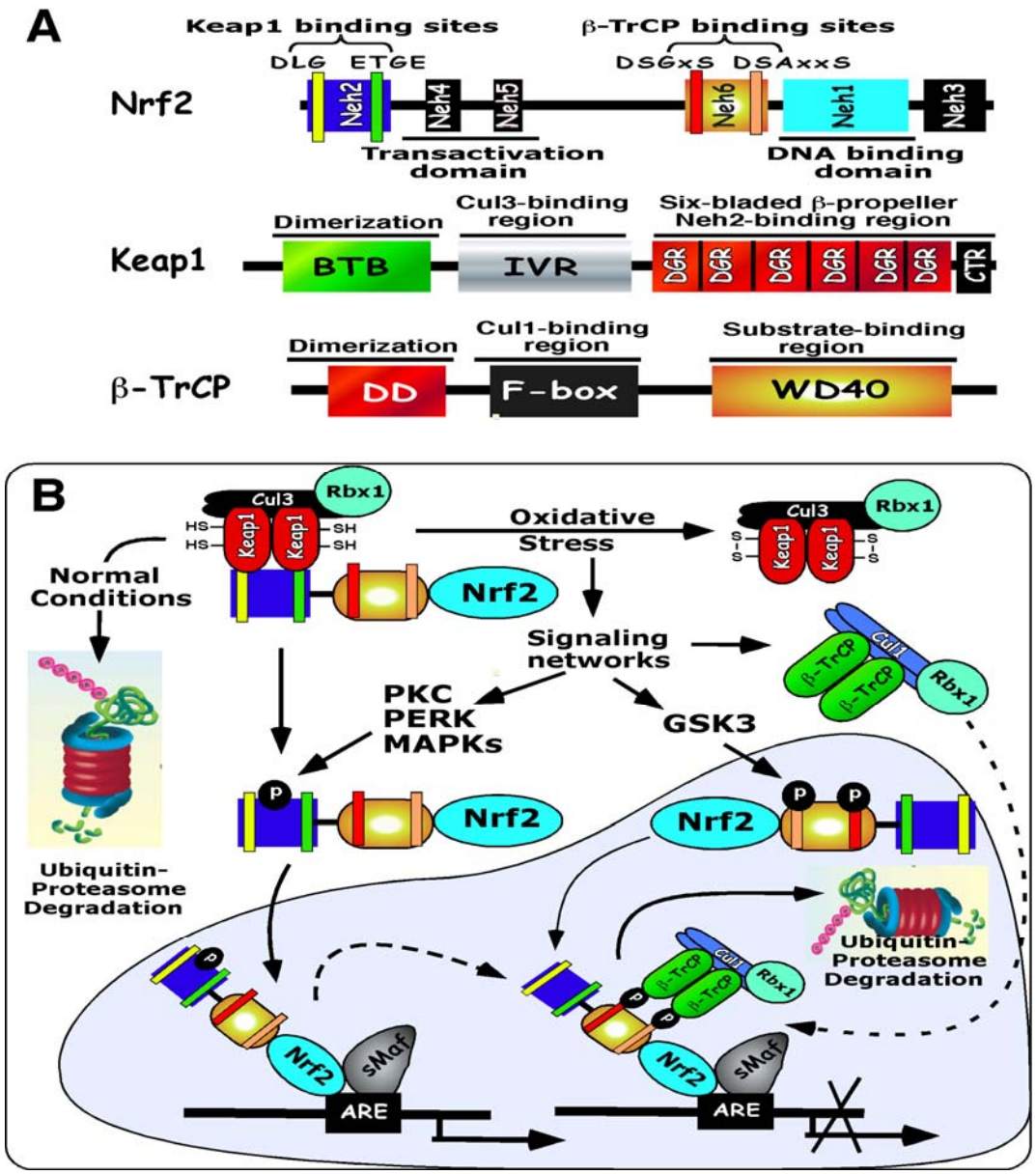

Fig. (6) Schematic representation of the mouse bHLH-PAS domain proteins AhR, AhRR, ARNT and Hif1α. Their structural domains are characterized as the basic helix-loop-helix (bHLH), Per-Arnt-sim (PAS), transactivation (TAD) or trans-repression (TRD). The basic region of bHLH contributes primarily to its DNA binding activity and the nuclear localization signal, whereas the HLH portion is also responsible for binding to target genes and dimerization with its partner, The PAS is a signal-sensing domain, and also contributes to binding for cofactors and other proteins. In addition to TAD, Hif1α contains a VHL-recognized ODDD that mediates the oxygen-regulated stability.

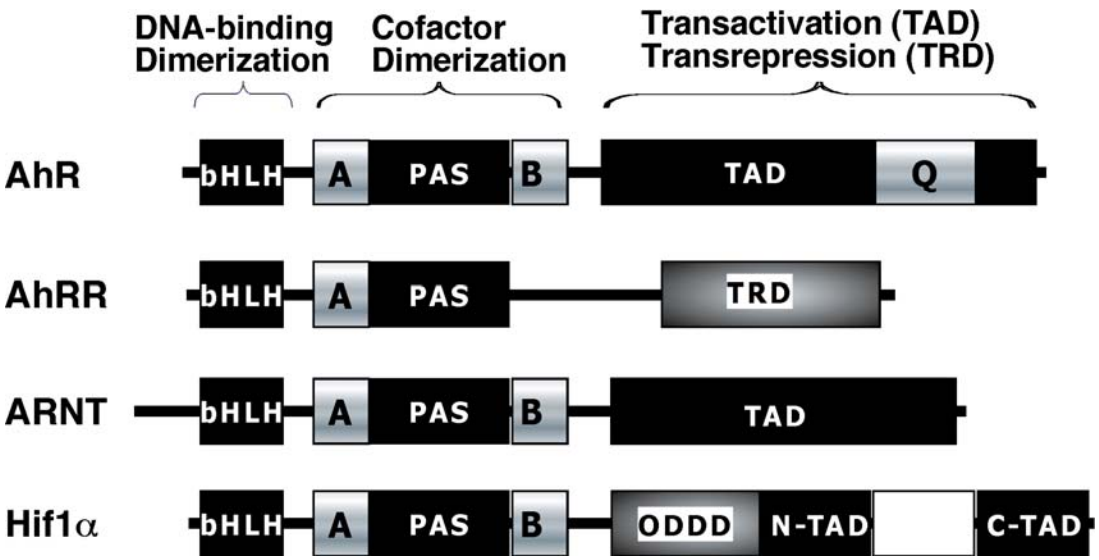

Fig. (7) Multiple signaling crosstalks between Nrf2-ARE and AhR-XRE gene regulatory networks
Based on induction of the responsive genes, xenobiotics including chemicals, pollutants and toxicants are divided into ARE-, XRE- and ARE/XRE-inducers. As ARE-inducers triggers redox stress response, the resulting Nrf2 will be released from Keap1, translocate into nucleus, heterdimerize with small Maf, and bind to ARE in the promoter region, leading to target gene activation. By contrast, AhR is kept inactive in cytoplasm by binding to a complex of Hsp90, XAP2 and p23 protein. Once XRE-inducers as ligands bind AhR, this receptor will be released from the complex and translocated into nucleus, wherein it heterodimerizes with ARNT. This dimer subsequently binds to XRE-driven genes; this binding activity can be inhibited competitively by AhRR, but its gene transcription is postitively regulated by AhR. The mouse *Nrf2* and *Nqo1* genes contains both XRE and ARE in their promoters, and thus they are regulated by AhR and CNC-bZIP family factors (e.g., Nrf1 and Nrf2 itself). The transcriptional activity of Nrf2 is negatively regulated by Keap1 and β-TrCP, and in turn the negator *Keap1* gene is positively regulated by Nrf1 and Nrf2. Such crosstalks between AhR-XRE and Nrf2-ARE networks, along with their respective negative feedback loops, finely control antioxidant, detoxification and drug-metabolizing genes. These two signaling response networks have been portrayed as targets of chemopreventive blocking agents (e.g. flavonoids), The bifunctional inducers activate transcription of ARE-driven genes after they are biotransformed by CYPs largely in the ER into reactive intermediate metabolites, that have characteristic of the monofunctional inducers. Such antioxidant, detoxification and cytoprotective genes are induced by nontoxic chemopreventive agents in order to block Phase I enzymes-mediated bioactivation toxicants and pro-carcinogens (RH) into reative intermediates (e.g. RO, $R^{\bullet}$, $ROO^{\bullet}$ and ROOH represent redical, alkoxyl, peroxyl and hydroperoxide, respectively). These possible intermediates can further be detoxified by Phase II enzymes to become glutathione-conjuncted compounds (RO-G) and then be excreted by Phase III efflux pumps.