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NRF2, cancer and calorie restriction

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Abstract

The transcription factor NF-E2-related factor (NRF2) is a key regulator of several enzymatic pathways, including cytoprotective enzymes in highly metabolic organs. In this review, we summarize the ongoing research related to NRF2 activity in cancer development, focusing on *in vivo* studies using NRF2 knockout (KO) mice, which have helped in defining the crucial role of NRF2 in chemoprevention. The lower cancer protection observed in NRF2 KO mice under calorie restriction (CR) suggests that most of the beneficial effects of CR on the carcinogenesis process are likely mediated by NRF2. We propose that future interventions in cancer treatment would be carried out through the activation of NRF2 in somatic cells, which will lead to a delay or prevention of the onset of some forms of human cancers, and subsequently an extension of health- and lifespan.

Keywords

calorie restriction; carcinogenesis; NRF2; phytochemicals

Introduction

Cancer is a major health problem in developed and industrialized countries that dramatically diminishes the quality of life and life expectancy. Cancer incidence has been increasing, particularly, during the last decades, becoming the second most common cause of death after heart disease and first in several subgroups of the population. For instance, in the United States of America, the lifetime probability of cancer diagnosis is about 44% for men and 37% for women. Moreover, cancer is the first cause of death among women aged 40–79 years and among men aged 60–79 years (Jemal *et al.*, 2009). Despite the immense efforts

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Conflict of interest

The authors declare no conflict of interest.

that have been made in early diagnosis and in the improvement of treatment modalities, the mortality rates from most cancers have not significantly decreased in the past 30 years.

The tumour suppressor gene *NRF2*

The transcription factor NF-E2-related factor (NRF2) mediates the antioxidant response and decreases tumour susceptibility in most carcinogenesis models. It acts against spontaneous and induced carcinogenesis through the modulation of insulin/insulin-like growth factor-1 (IGF-1) signalling pathway, promoting survival (Henderson *et al.*, 1998; Ramos-Gomez *et al.*, 2001, 2003; Pearson *et al.*, 2008). Moreover, during the last years NRF2 action has been suggested to be involved in many aged-related diseases, summarized in Table 1.

NRF2 belongs to a basic region-leucine zipper (bZip)-type transcription factor family that shares a conserved structural 'cap n collar' domain (Moi *et al.*, 1994; Itoh *et al.*, 1995; Kensler *et al.*, 2007). NRF2 was originally identified as an erythroid-restricted DNA-binding activity and is evolutionarily conserved (Kobayashi *et al.*, 2002; Suzuki *et al.*, 2005). One example is *Caenorhabditis elegans*, which has the NRF2 homologue SKN-1. This transcription factor controls the response of the worms to oxidative stress. Interestingly, there are some differences in the functioning of SKN-1 protein, working as a homodimer in this model. SKN-1 is expressed in the intestines and ASi neurons, where it acts in the upregulation of the calorie restriction (CR) metabolism and oxidative stress resistance metabolism, respectively (Onken and Driscoll, 2010). At least the role of NRF2 in glutathione synthesis, quinone reduction and protection from reactive oxygen species (ROS) is mediated by SKN-1 in *C. elegans*. Downstream targets of SKN-1 overlap with pathways regulated by CR and lifespan promoting an increased longevity and healthspan under SKN-1 overexpression in worms (Tullet *et al.*, 2008). The homologue of NRF2 in *Drosophila melanogaster* has been proposed recently (Sykiotis and Bohmann, 2008). The gene *CncC* binds to the small musculoaponeurotic fibrosarcoma proteins and protects under oxidative treatments. The homologues in vertebrates have also been determined in several models such as *Gallus gallus* and *Danio rerio* (Maher and Yamamoto, 2010). Interestingly, to date there has not been any NRF2 homologues proposed in bacteria or yeast.

In mammals, this family is composed of four proteins, p45-NFE2, NRF1, NRF2 and NRF3, as well as two distantly related proteins termed Bach1 and Bach2 (Chan *et al.*, 1993a, 1993b; Moi *et al.*, 1994; Itoh *et al.*, 1995; Oyake *et al.*, 1996; Muto *et al.*, 1998; Kobayashi *et al.*, 1999). Interestingly, the 'cap n collar' genes knockout mice have provided an invaluable tool for studying the functions of these genes *in vivo*. Studies using NRF3 KO mice, the closest gene to NRF2, do not show phenotypical differences compared with control mice (Derjuga *et al.*, 2004; Kobayashi *et al.*, 2004b). NRF3 likely acts as a major regulator of phase 2 enzyme genes, as NRF3 is not expressed in most metabolic organs, such as the liver and intestine (Braun *et al.*, 2002; Derjuga *et al.*, 2004; Kobayashi *et al.*, 2004b).

NRF2 is the most potent transcription factor of the 'cap n collar' family, activating downstream targets about 100-fold (Toki *et al.*, 1997; Kobayashi *et al.*, 1999). NRF2 signalling is central to efficient detoxification of reactive metabolites and ROS. NRF2 is

induced by oxidative stress, which enhances protection against molecular damage that induces cancer development (Ingram *et al.*, 1990).

NRF2 is an unstable protein that has an estimated half-life time of 30 min (McMahon *et al.*, 2003). This protein is repressed in homeostatic conditions maintaining a low basal level of cytoprotective gene expression (Motohashi and Yamamoto, 2004; Motohashi *et al.*, 2004). A lower proteasome activity leads to increasing levels of NRF2 and its downstream targets, promoting a pathological status. Nuclear accumulation of NRF2 is abolished when protein synthesis is blocked by cycloheximide treatment, establishing that NRF2 activity is mainly regulated by its own stability.

Regulation of NRF2 transcriptional activity

NRF2 signalling is regulated by compartmental segregation from the cytoplasm to the nucleus. Under homeostatic conditions, NRF2 is bound to a Kelch-like ECH-associated protein 1 (Keap1) dimer in the cytoplasm (Itoh *et al.*, 1999; Tong *et al.*, 2006a). The interaction between NRF2 and Keap1 has been shown by yeast two-hybrid screening (Itoh *et al.*, 1999). Furthermore, immunohistochemical analyses have shown that Keap1 is associated with the actin cytoskeleton, which retains NRF2 in the cytoplasm (Kang *et al.*, 2004).

The Kelch domain of Keap1 interacts with two distinct amino-acid sequences present in the N-terminal of NRF2: ETGE and DLG (Tong *et al.*, 2006b; Hayes and McMahon, 2009). A sequential interaction process termed ‘hinge and latch’ mechanism has been hypothesized. The first interaction is through the ETGE, and subsequently the DLG docks onto the adjacent unoccupied Kelch-repeat domain. Keap1 sequesters NRF2 in the cytoplasm and acts as an adaptor enhancing the interaction of the Cullin 3-based E3-ubiquitin ligase complex 2 (Zhang and Hannink, 2003; Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004a; McMahon *et al.*, 2004; Furukawa and Xiong, 2005). This process leads to a continuous ubiquitination, proteasomal degradation and transcriptional repression of NRF2 by preventing its nuclear translocation (Itoh *et al.*, 1999; Kobayashi *et al.*, 2004a). Cul3-mediated NRF2 degradation has been shown in studies that show cytoplasmatic NRF2 accumulation in Cul3-silenced cells (Cullinan *et al.*, 2004; Furukawa and Xiong, 2005).

There are two independent hypotheses that explain different mechanisms responsible for dissociation of Nrf2 from Keap1. The first mechanism Keap1 acts as a primary redox sensor that contains reactive cysteines. All cysteine residues in Keap1 are found to be highly conserved across species (Itoh *et al.*, 1999; Eggler *et al.*, 2005). Nitric oxide (NO) is a multifunctional messenger that has been shown to induce the release NRF2 from Keap1. For instance, heme oxygenase-1 (HO-1) expression is increased after NO exposure in smooth muscle cells in an NRF2/antioxidant response element (ARE)-dependent manner (Liu *et al.*, 2007). NRF2 expression has shown an age-related decrease in rodent and human tissues (Suh *et al.*, 2004; Shih and Yen, 2007; Collins *et al.*, 2009; Duan *et al.*, 2009), possibly leading to higher levels of ROS and increased risk of cancer. This decrease on NRF2 activity has been suggested to be related to a decrease in NO-dependent regulation of NRF2 levels during senescence due to a functional inactivation of NO by high levels of superoxide

(Ungvari *et al.*, 2008). NO levels are increased under CR conditions, whereas superoxide is decreased in CR in rodents (Yang *et al.*, 2004). Moreover, high NO levels show cardioprotective effects in atherogenesis. Interestingly, NO mimetics have been proposed to ameliorate the age-related diseases such as Alzheimer's disease and are under clinical trials (Thatcher *et al.*, 2005). Oxidative modifications of these cysteines act as sensors for stresses, provoking the disruption of the Keap1-Nrf2 interaction and release of Nrf2, resulting in the stabilization and the accumulation of this protein into the nucleus (Dinkova-Kostova *et al.*, 2002; Wakabayashi *et al.*, 2004; Wilson *et al.*, 2005).

The second mechanism that regulates NRF2 activity is mediated through post-transcriptional modification on Keap1-Nrf2 complex by several classes of kinases. The phosphorylation state of Nrf2 enhances the stability and/or release of Nrf2 from Keap1 (Huang *et al.*, 2002). The specific kinases that are implicated in the regulation of Nrf2 activity include mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K), protein kinase R-like endoplasmic reticulum kinase (Cullinan *et al.*, 2003) and protein kinase C (PKC). *In vitro* studies have shown that phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1 (Huang *et al.*, 2002; Bloom and Jaiswal, 2003; Numazawa *et al.*, 2003). In this case, a mutation driving to a constitutively dephosphorylated NRF2 state (S40A) has been shown, which is the target site for PKC, and decreases Keap1 -NRF2 release. Then, inhibition of PI3K attenuates nuclear translocation of Nrf2 from the cytoplasm. Furthermore, Cullinan *et al.* (2003) showed that protein kinase R-like endoplasmic reticulum kinase phosphorylates Nrf2 and triggers dissociation from Keap1, resulting in increased nuclear translocation. Other studies showed that the phosphatase inhibitor okadaic acid increases Nrf2 accumulation and transcriptional activation, likely because phosphorylated proteins are less accessible to ubiquitin ligase (Nguyen *et al.*, 2003; Ramos-Gomez *et al.*, 2003).

Once NRF2 is released from Keap1 by any of these mechanisms, NRF2 can be imported into the nucleus (Dinkova-Kostova *et al.*, 2002; Wakabayashi *et al.*, 2004). On the basis of the repressive effect of Keap1 on NRF2 activity, loss of function of Keap1 KO mice was suggested to enhance a cellular cancer chemopreventive effect (Devling *et al.*, 2005). However, some studies have indicated that Keap1 KO mice resulted in constitutively hyperactive NRF2 signalling owing to its nuclear localization (Wakabayashi *et al.*, 2003, 2004; Okawa *et al.*, 2006). This overexpression is lethal owing to obstructive lesions mediated by hyperkeratotic outgrowth of the oesophageal and forestomach epithelial cells. In young Keap1 KO mice, nuclear levels of NRF2 as well as its downstream targets were substantially higher than control mice. Interestingly, this phenotype is reversed in Keap1::NRF2 double KO mice (Wakabayashi *et al.*, 2003). A specific conditional Keap1 KO in hepatocytes shows increased resistance against acute drug toxicity induced by acetaminophen and increased levels of NRF2-regulated antioxidative enzymes (Okawa *et al.*, 2006).

Nuclear NRF2 dimerizes with a group of nuclear bZip proteins termed small musculoaponeurotic fibrosarcoma proteins (Itoh *et al.*, 1995; Motohashi *et al.*, 2004). This dimerization strongly activates the transcription of downstream targets by enhancing the specificity to bind to a *cis*-acting enhancer of the ARE contained in the promoters of these genes (Friling *et al.*, 1990; Itoh *et al.*, 1997; Shou *et al.*, 2001; Yu and Kensler, 2005;

Yamamoto *et al.*, 2006). Interestingly, studies conducted *in vitro* indicate that the affinity of NRF2 heterodimeric complexes with small musculoaponeurotic fibrosarcomas to ARE sequences is similar, regardless of the phosphorylation state of NRF2 (Huang *et al.*, 2002).

Enzymes regulated by NRF2

Microarray results suggest that more than 200 gene products are under the transcriptional control of NRF2. Downstream targets include antioxidative enzymes, enzymes responsible for the production of antioxidants, reducing equivalents, cofactors and also genes that are classified into different categories, like 26S proteasome subunits, *PSMB5* subunit gene and some heat-shock proteins (Kwak *et al.*, 2003a, b). The main classes of NRF2-regulated genes include antioxidative enzymes like NAD(P)H:quinone oxidoreductase (NQO1), epoxide hydrolase, aldehyde dehydrogenase, aldoketo reductase, catalase, HO-1 (Favreau and Pickett, 1991; Li and Jaiswal, 1992; Prestera *et al.*, 1995; Thimmulappa *et al.*, 2002; Kwak *et al.*, 2003b; Leonard *et al.*, 2006). Another family of enzymes is involved in glutathione homeostasis, including glutathione reductases, peroxir-edoxin, thioredoxin and thioredoxin reductases and glutathione peroxidase (Friling *et al.*, 1990; Rushmore and Pickett, 1990; Reinhart and Pearson, 1993; Mulcahy and Gipp, 1995). It is well known that NRF2 also enhances toxin export through the multidrug response transporters, like the multiple drug resistance-associated protein, carboxyl esterase, esterase D, retinal oxidase/ aldehyde oxidase and carbonic anhydrase. Wassreman and others have shown the role of NRF2 in the upregulation of proteasome subunits and heat-shock proteins, such as heat-shock protein 40 and mitochondrial stress-70 protein, sequestosome 1 and ubiquitin C that recognizes and degrades damaged proteins (Wasserman and Fahl, 1997; Davies, 2001; Kwak *et al.*, 2003a; Rangasamy *et al.*, 2004). Even NRF2 appears to regulate the expression of other transcription factors, growth factors, receptors, molecular chaperons and its own expression, through two putative functional AREs identified in the NRF2 promoter (Kwak *et al.*, 2002).

The protein products of these genes provide multiple layers of protection during cellular insults, collectively favouring cell survival. For instance, these enzymes are essential for neuronal survival because they block neurotoxicity derived from glutathione depletion, lipid peroxidation, intracellular calcium overload, excitotoxins and disruption of the mitochondrial electron transport chain (Shih *et al.*, 2003; Lee *et al.*, 2003a, 2005a). Moreover, NRF2 activation leads to an increased cellular energetics and redox potential (Lee *et al.*, 2003b; Kraft *et al.*, 2004; Nguyen *et al.*, 2004). It is interesting to note that in the nervous system, NRF2-regulated genes are activated in astrocytes and also confer protection to neighbouring neurons (Calkins *et al.*, 2005; Jakel *et al.*, 2007). Also, induction of NRF2 expression in cultured endothelial cells results in a marked increase in NRF2-driven transcriptional activity leading to increased survival under oxidative stress treatments (Mostoslavsky *et al.*, 2006). Multiple studies have shown that NRF2 KO mice show a reduced constitutive expression of downstream targets in the main tissues that reach the electrophilic response, such as the liver, intestine and forestomach (McMahon *et al.*, 2001; Hayes *et al.*, 2000; Thimmulappa *et al.*, 2002; Wakabayashi *et al.*, 2003).

Some toxic compounds, for example, diesel exhaust particles, induce NRF2 expression and its nuclear accumulation. In turn, several antioxidant and phase 2 enzymes, like HO-1 and some glutathione *S*-transferase (GST) subunits, are significantly upregulated. Less well documented, but perhaps equally important, the activation of the NRF2 pathway also evokes the downregulation of many genes. Of note, NRF2 inhibits inflammation through decreasing expression of the pro-inflammatory mediators cyclooxygenase-2, interleukin-lβ, interleukin-6 and tumour necrosis factor-α (Khor *et al.*, 2006; Kensler *et al.*, 2007; Hayes and McMahon, 2009).

Importance of NRF2 activity in cancer prevention

NRF2 signalling is involved in the upregulation of enzymes that mediate the detoxification of reactive metabolites and ROS (see Table 2). These enzymes enhance the protection against molecular damage and eventually lead to a lower cancer development (Ingram *et al.*, 1990). Interestingly, there must be other different pathways that are not related to NRF2 activity that are able to regulate the longevity since CR increases longevity in NRF2 KO mice. To date, these specific pathways remain to be elucidated.

Overexpression of NRF2 or its downstream detoxification enzymes by transfection protects cells against carcinogen-induced DNA damage and/or cytotoxicity (Fields *et al.*, 1999). On the other hand, loss of expression of this gene or its targets induces sensitivity to DNA damage and carcinogenesis (Henderson *et al.*, 1998; Ramos-Gomez *et al.*, 2001, 2003). NRF2 KO mice are susceptible to a variety of oxidative insults, DNA adducts formation and cancer development, clearly indicating the critical contribution of NRF2 downstream targets to cellular protection. The potential of NRF2-regulated antioxidative response in protecting against two-stage induced cancer has been shown (Kwak *et al.*, 2002). NRF2 KO mice showed increased skin oxidative damage during 12-O-tetradecanoylphorbol-13-acetate promotion, leading to an increased multiplicity and incidence of skin tumours (Xu *et al.*, 2006). A decline in levels of NRF2 in aged organisms that promotes oxidative damage is well documented (Suh *et al.*, 2004). In a rat model, a decline in transcriptional activity of NRF2 in aged rats is responsible for the significant decline in glutathione levels in the liver. Furthermore, age-related NRF2 inhibition is observed in Parkinson, Alzheimer, Huntington's diseases and atherosclerosis models (Pratico and Delanty, 2000; Jenner, 2003). Genetic ablation of the *NRF2* gene increases the size of the lesions, whereas transplantation of NRF2-overexpressing astrocytes reduces it (Calkins *et al.*, 2005; Shih *et al.*, 2005). Some NRF2 activators of the triterpenoid family have been shown to improve the phenotype of these neurodegenerative diseases, such as 2-cyano-3,12-dioxooleana-1,9-dien-olic acid (CDDO), CDDO-ethyl amide and CDDO-trifluoroethyl amide (Stack *et al.*, 2010). Recently, the involvement of NRF2 in the pathogenesis of diabetes has also been shown. Hyper-glycaemic conditions in animals and human models are associated with an increased ROS production (Kiritoshi *et al.*, 2003; Ye *et al.*, 2004). NRF2 expression is decreased in athero-susceptible regions of the aorta (Zakkar *et al.*, 2009). NRF2 activation leads to an increased antioxidant battery that ameliorates the diabetic complications and diabetes itself through ROS scavenging by NRF2 downstream targets. The known decreased NRF2 expression in the elderly could lead to diabetes (Suh *et al.*, 2004). NRF2 activation by some phytochemicals, such as sulphoraphane and bardoxolone methyl, increase the expression on

antioxidant proteins conferring an increased protection to hyperglycaemia (Xue *et al.*, 2008). Furthermore, some studies have shown that NRF2 regulates inflammation process. NRF2-deficient mice show susceptibility to induced colitis, leading to a loss of colonic crypts, massive infiltration of inflammatory cells and anal bleeding (Khor *et al.*, 2008).

The dark side of NRF2

It has been documented that Keap1 KO mice, which constitutively express NRF2, died within 3 weeks of birth. Lethality has been attributed to hyperkeratosis of the oesophagus and forestomach cells and overexpression of keratins K1, K6 and loricrin, resulting in oesophageal occlusion and subsequent malnutrition (Wakabayashi *et al.*, 2003). However, hepatocyte-specific disruption of Keap1 does not seem to have any adverse effect in mice. These mice exhibit a normal phenotype and express high hepatic levels of NRF2 downstream targets conferring protection under acetaminophen and concanavalin A treatments (Okawa *et al.*, 2006; Osburn *et al.*, 2008).

Constitutive activation of NRF2 may have negative effects enhancing tumour cell protection against chemotherapy as shown in some non-synonymous polymorphisms that are determinant for susceptibility to cancer in humans (Palli *et al.*, 2000). For instance, non-synonymous Keap1 alleles afflicting Keap1 binding to NRF2 have been characteristically observed in human lung tumour and Keap1 mutations have also been found in breast and gall bladder cancers (Nioi and Nguyen, 2007; Shibata *et al.*, 2008). This fact promotes nuclear localization of NRF2 and constitutive expression of its downstream targets, which facilitates resistance of lung tumour cells to chemotherapeutic drugs (Padmanabhan *et al.*, 2006; Singh *et al.*, 2006; Tong *et al.*, 2006b; Pearson *et al.*, 2008).

CR induces NRF2 activity

It has been shown that NRF2 is responsible for most of the anticarcinogenic effects of CR (Pearson *et al.*, 2008). CR, reduced calorie intake without malnutrition, prevents carcinogenesis in spontaneous, chemically induced and radiation-induced cancer in experimental models (Tannenbaum and Silverstone, 1953; Andreou and Morgan, 1981; Kritchevsky *et al.*, 1984; Pollard *et al.*, 1984; Boissonneault *et al.*, 1986; Gross and Dreyfuss, 1986; Klurfeld *et al.*, 1987; Lagopoulos and Stalder, 1987; Birt *et al.*, 1991; Shimokawa *et al.*, 1991; Kritchevsky, 2001; Hursting *et al.*, 2003).

Early efforts to understand the interaction of reduced calorie intake and carcinogenesis have allowed researchers to begin making progress understanding the mechanisms behind these effects of CR. It is well known that CR decreases ROS production, enhances plasma membrane redox system, decreases inflammation process, induces modification in hormonal milieu and improves insulin signalling pathway, at least in part through the induction of NRF2 activity.

Given the importance of NRF2, our laboratory has focused on the study of this transcription factor in cancer and ageing research. We showed that NRF2 is responsible for most of the anticarcinogenic effects of CR in the two-stage carcinogenesis model. In our study, CR was not effective against chemically induced tumorigenesis in the NRF2 KO mice. Both *ad*

libitum-fed NRF2 KO and CR NRF2 KO mice developed tumours more readily and reached total tumour incidence at the age of 30 weeks, whereas 40% CR wt mice did not show any papilloma up to week 42. Even tumour multiplicity was not significantly different between the CR-fed KO mice and the *ad libitum*-fed wild-type mice, suggesting that the anticarcinogenic effect of CR solely depends on the activity of NRF2. However, we showed that CR was able to extend lifespan and increased insulin sensitivity similarly in NRF2 KO and in wild-type mice. Interestingly, we were able to identify a molecular pathway that dissociates the prolongevity and anticarcinogenic effects of CR in mice.

The reduction of NRF2 downstream targets in NRF2 KO mice lead to increased DNA damage. Moreover, in these mice NQO1 expression as well as its enzymatic activity was markedly reduced under CR compared with wild-type CR animals. It is possible that not only NRF2, but also other CR-induced pathways increase NQO1 gene expression in NRF2 non-dependent manner. We observed that NQO1 mRNA levels were increased in NRF2 KO mice under CR compared with their *ad libitum* counterparts, whereas other downstream targets such as HO-1, glutamatecysteine ligase, catalytic subunit, GST A1 and glutathione peroxidase-1 were not increased in NRF2 KO under CR conditions.

On the other hand, we showed that CR does not require NRF2 for insulin sensitivity as well as lifespan prolongation in mice. Proposed hallmarks of tumour prevention were measured and we found that insulin sensitivity and corticosterone levels were improved and increased, respectively, in CR NRF2 KO mice compared with *ad libitum*-fed ones. This fact could explain the delay in tumour incidence in CR groups compared with *ad libitum*. Interestingly, in longevity studies we observed similar increase in median lifespan in CR NRF2 KO and *ad libitum* mice, allowing us to separate the NRF2-mediated anticarcinogenic beneficial effect of CR from CR-induced longevity extension (Pearson *et al.*, 2008).

CR beneficial effects through the induction of NRF2 activity

During the early 1900s, some studies published the beneficial effects of underfeeding laboratory animals on transplanted and induced tumours (Moreschi, 1909; Rous, 1914). Since then, beneficial effects have been described on longevity, age-associated diseases, attenuation of functional declines, cognitive deterioration and carcinogenesis in many models (Hursting *et al.*, 2003; Pollak, 2009a). SKN-1 (NRF2 orthologue) is upregulated in the ASIs neurons in *C. elegans* under CR. Its activity increases the metabolic activity and is required for longevity extension in this model (Bishop and Guarente, 2007). Even CR shows lifespan extension when initiated later in life in rodents (Weindruch and Walford, 1982).

During the last decade, long-term studies have been examining the health benefits of CR in non-human primates. Monkeys under CR showed a delayed onset of age-associated pathologies, significantly better glucose tolerance, less muscle loss, no type 2 diabetes, cardiovascular disease, and brain atrophy and 50% lower cancer incidence compared with their *ad libitum* counterparts (Hansen *et al.*, 1995; Colman *et al.*, 2009). Final results from several ongoing non-human primate studies will be achieved over the next decade. Their results will give researchers more clues about the beneficial effects of CR on cancer and

ageing, and will allow them to perform future research (Ramsey *et al.*, 2000; Mattison *et al.*, 2007).

There are a few human studies suggesting beneficial effects of CR in humans. In the fifteenth century, Luigi Cornaro started a kind of CR when he was 40 years old. His diet was based on 400 g of food daily plus wine and daily exercise, he died at the age of 91, almost three times the average lifespan during this century in developed countries (Howell, 1987). Another documented case is the population of Okinawa during Second World War, who consumed fewer calories than their counterparts in the rest of Japan. During this time, they showed the lowest incidence of coronary heart disease, stroke, cancer and delayed ageing in the world (Suzuki, 2001; Willcox *et al.*, 2007). Interestingly, the subsequent diet normalization have raised the incidence of the mentioned diseases and ageing up to regular rates (Miyagi *et al.*, 2003). Furthermore, the beneficial effects in human studies are supported by studies of people on long-term CR, who show fewer signs of cardiovascular ageing (Holloszy and Fontana, 2007). Studies from Pennington Calerie team show that 6-month CR in humans decrease the levels of fasting insulin and core body temperature, two known biomarkers of longevity. Even more, it has been shown that energy expenditure is decreased and DNA fragmentation is lower owing to less damage to DNA. CR affects many pathways and leads to benefits for cancer development and other age-related diseases, summarized in Table 1. In the following sections, we will describe the present knowledge about molecular pathways improved by CR.

Decreased ROS production

CR decreases metabolic rate and oxidative damage. This effect is considered one of the major factors contributing to slowing the ageing process and preventing tumour formation. ROS production is achieved by products of metabolism mainly produced by mitochondrial oxidative phosphorylation as well as extracellular oxidant compounds. When their levels are exceeded, ROS modify cellular molecules, resulting in lipid peroxidation, DNA strand break, telomere shortening and protein carbonylation (Dexter *et al.*, 1989; Halliwell, 1992, 1996b; Djuric and Kritschevsky, 1993; Shaw *et al.*, 1995; Fitzmaurice *et al.*, 1996; Halliwell, 1996a, 2001; Alam *et al.*, 1997a,b; Stadtman and Berlett, 1998; Cakatay *et al.*, 2001; Warita *et al.*, 2001; Volchegorskii *et al.*, 2004).

Oxidative damage eventually produces DNA mutations commonly identified in age-related diseases. These mutations may confer growth advantage and eventually cancer development. In order to defend against ROS, cells under CR induce a coordinated expression of transcription factors, NRF2 among them, that increase antioxidant enzymes, including phase II detoxification enzymes and phase III efflux transporters (Motohashi and Yamamoto, 2004; Klaassen and Slitt, 2005; Mandlekar *et al.*, 2006).

Plasma membrane redox system improvement

One of the most important benefits of CR consists in the improvement of the plasma membrane redox system. CR enhances the activities and content of antioxidant compounds, which usually declines with age (Murakami and Johnson, 1996). Previous work carried out in our laboratory has shown that cytochrome b_5 reductase and NQO1 expression and

activities are increased in plasma membranes from rodent's tissues under long-term CR (Manjgaladze *et al.*, 1993; De Cabo *et al.*, 2004; Hyun *et al.*, 2006, 2007). Furthermore, the plasma membrane redox system also contributes to the regulation of the cellular redox homeostasis affecting $NADH/NAD^+$ ratio and contributing to regulate survival (Jimenez-Hidalgo *et al.*, 2009).

Improved apoptosis, inflammation and cell proliferation inhibition under CR

Cellular consequences of reduced energy intake in CR models are a decrease in inflammation, suppression of cell proliferation and encouraged apoptosis (Birt *et al.*, 1998). CR modulates a gene expression shift associated with inflammation, cellular stress, fibrosis, apoptosis, type I enzymes, cell division and DNA replication processes. Reduced nutrient availability is sensed by AMP kinase and protein kinase B/AKT, which are activated when AMP/ATP ratio increases. Another enzymatic pathway downregulated by CR is the mammalian target of rapamycin, which only transduces signals from upstream pathways when intracellular nutrients concentration is adequate.

In the early 1900s it was shown that chronic inflammation increases carcinogenesis. Inflammation is required to maintain integrity when cells are damaged by an infection in animals (Philip *et al.*, 2004). The inflammation process is initiated by a cascade of cytokines and chemokines that relay in the generation of oxidative stress that mutates DNA and promotes cancer (Lok *et al.*, 1988; Hursting *et al.*, 1994). Several studies have shown that CR promotes the reduction of inflammatory mediators, as tumour necrosis factor-α and interleukin-6 (Chandrasekar *et al.*, 1995). Alternative day fasting protects from the age-induced inflammation, resulting in less DNA damage and protein carbonylation in rats through a decreased nuclear factor-κB DNA-binding activity (Castello *et al.*, 2010). It has been shown that even short-term CR decrease pro-inflammatory gene expression in old rats (Jung *et al.*, 2009).

The decrease of the DNA replication rate under CR makes cells less susceptible to DNA damage induced by carcinogens, and decreases oncogenic cells proliferation. The modulation in the regulation of these pathways by CR leads to a lower energy expenditure, protein translation and proliferation, increased apoptosis, growth of mitochondria and promotion of autophagy process, which prevent cancer by eliminating damaged proteins and whole organelles. These processes promote cell survival in CR models, decreasing the opportunity for a damaged cell to survive, which makes the organisms less susceptible to cancer development (Franke, 2008).

Shift of the hormonal milieu

The protective properties of CR include the modulation of the hormonal content. A 10-fold higher level of plasma corticosterone at 0700 hours in CR mice compared with *ad libitum* counterparts has been published. The adrenal gland, which produces corticosterone and dehydroepiandrosterone, is necessary for beneficial effects of CR in the lung- and skin-induced cancer (Pashko and Schwartz, 1992). Adrenal hormones inhibit stimulated epidermal DNA synthesis and tumour formation in two-stage cancer skin model. Studies in mice showed less papilloma accumulation in control mice under CR compared with CR

adrenalectomyced ones (Stewart *et al.*, 2005). Interestingly, corticosterone supplementation in water resulted in cancer prevention in both mice strains, suggesting that elevation of corticosterone in CR mice mediates the prevention of skin cancer. Furthermore, *ad libitum* adrenalectomyced mice showed elevated lymphosarcoma incidence compared with corticosterone supplemented ones, indicating an anticarcinogenic role of this hormone (Birt *et al.*, 2004).

Another adrenal steroid, dehydroepiandrosterone, suppresses tumour formation and proliferation. Dehydroepiandrosterone administration in diet reproduces many of the beneficial effects of CR, including a repression of tumour development in several organs, and prolongs both mean and maximal lifespan of mice. It has been shown that dehydroepiandrosterone inhibits glucose-6-phosphate dehydrogenase, leading to a lower NADPH and ribose 5-phosphate levels with a consequent inhibition of deoxyribonucleotide synthesis (Gordon *et al.*, 1987; Garcea *et al.*, 1988; Shantz *et al.*, 1989; Pashko *et al.*, 1991).

The current hypothesis of glucocorticoid hormone function on cancer prevention is through the activation of the glucocorticoid receptor and increased glucocorticoid hormone, which drives to a decreased PKC activity (Birt *et al.*, 1999). Then, low levels of 12-O-tetradeca-noylphorbol-13-acetate-dependent PKC inhibits MAP-1/Raf-1 pathway, leading to an attenuation in the induction of the activator protein-1 transcription factor, which is essential for CR prevention of mouse skin carcinogenesis.

Improvement of insulin signalling pathways

CR improves markers of diabetes such as insulin sensitivity. In rodents and primates, CR causes a decline in insulin relative to glucose concentrations, circulating insulin, IGF-1 receptor, IGF-1 (which fall at first but rebound to normal levels) and increased concentrations of IGFBP3 (Cohen and Hilf, 1974; Taub *et al.*, 1987; Ruggeri *et al.*, 1989; Breese *et al.*, 1991; Masoro, 1995). The first evidence to support a role of insulin-like signals in the regulation of longevity and age-related diseases in mammals came from studies of mice with hereditary dwarfism (Ames dwarf) that showed low circulating IGF-1 (Brown-Borg *et al.*, 1996).

Lower levels of these proteins lead to a lower activation of downstream kinases PI3K/ protein kinase B, ultimately causing the dephosphorylation, nuclear translocation and activation of FOXO transcription factors. Moreover, high levels of IGFBP3 in CR animals induce pro-apoptotic and antiproliferative effects in cancer cells in an IGF-1 independent manner (Butt *et al.*, 2002; Lee *et al.*, 2005b; Kalaany and Sabatini, 2009). On the other hand, heightened activity of downstream insulin pathway in tumour cells drives a signal through receptors that reduces FOXO activity and promotes the growth and survival of cancer cells (Greer and Brunet, 2005; Pollak, 2009). There are some reported tumours that have developed escape mechanisms that allow them to evade to the beneficial effects of CR. Constitutively the deregulated activated insulin/IGF-1/IGF-1 receptor/PI3K signalling pathway is determinant of the sensitivity of tumours to CR (Tannenbaum and Silverstone, 1949; Weindruch and Walford, 1982; Cheney *et al.*, 1983; Pugh *et al.*, 1999; Kritchevsky, 2001; Sell, 2003; Thompson *et al.*, 2003). Activation of PI3K or inactivation of its

counterpart PTEN phosphatase results in the production of phosphorylated inositol lipids at the plasma membrane (Hennessy *et al.*, 2005). These lipids act as secondary messengers, and provide docking sites for many intracellular proteins, resulting in the activation of a variety of downstream signalling molecules.

The current hypothesis points towards a hallmark of CR-insensitive tumours that consists of increased PI3K/ protein kinase B activity and decreased FOXO-mediated transcription through phosphorylation, allowing tumour cells to proliferate in the absence of IGF-1 or insulin. Thus, PI3K-activating mutations are sufficient to induce resistance to CR benefits. Then, it is possible that insulin resistance in type 2 diabetes treatments might be beneficial in preventing tumours with activated PI3K/inactive PTEN pathway.

NRF2 activators

Owing to the difficult adaptation of humans to a CR diet similar to that performed under laboratory conditions, the upregulation of NRF2 has been proposed as a potential target to evaluate for CR mimetics. Then, activation of NRF2 and downstream targets by administration of some phytochemicals is a crucial target for tumour prevention. Epidemiological studies have clearly documented that some phytochemical's action is linked to a lower risk of many types of cancers (Chen *et al.*, 2000; Chan and Giovannucci, 2001). NRF2 signaling pathway has been a target for chemoprevention even before its molecular characterization by Wattenberg (1972), and several studies showed a markedly attenuated efficacy of various chemopreventive agents in NRF2 KO mice. Detoxification enzymes are expressed constitutively at low levels, but can be greatly enhanced in response to exposure to some phytochemical compounds that activate NRF2 transcription factor, summarized in Tables 2 and 3 (Hong and Sporn, 1997). Some NRF2 activators are currently under clinical trials in the study of aged-related diseases, such as CDDO-methyl ether and oltipraz (Kensler and Helzlsouer, 1995; Nagaraj *et al.*, 2010). Interesting data support these clinical trials in cancer studies; CDDO-methyl ether, also known as bardoxolone methyl, induces apoptosis in lung cancer cells (Lapillonne *et al.*, 2003; Iida *et al.*, 2004; Hyer *et al.*, 2005).

For example, dithiolethiones increase NRF2 activity and lead to the detoxification activity of GST, NQO1, ferritin H and L, as well as glutathione reductase downstream targets (Ansher *et al.*, 1986; Kwak *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Pietsch *et al.*, 2003). The most studied compound of this family, oltipraz, completely failed to protect the NRF2 KO mice, indicating the importance of NRF2 activity in chemo-protection. (Ramos-Gomez *et al.*, 2001; Iida *et al.*, 2004). An increase in glutathione levels in the liver, kidney and forestomach of mice was observed after oltipraz supplementation. Finally, the induced enzymes protect against cancer and reduce 10 times the volume of the liver occupied by preneoplastic foci at the same time that hepatic aflatoxin–DNA adduct formation is markedly reduced (Kensler *et al.*, 1987). Interestingly, there was no effect on tumour burden in NRF2-deficient mice. Clinical trials have shown that oltipraz modulates the activities of both conjugating/detoxication enzymes as well as cytochrome P450s.

Sulphoraphan is a potent isothiocyanate formed following myrosinase-catalyzed metabolism of glucosinolates and is present in high concentrations in broccoli sprouts and other crucifers. Isothiocyanates show a strong anti-inflammatory activity, probably achieved through inhibition of the nuclear factor-κB signalling pathway (Heiss *et al.*, 2001). It was found to be a potent activator of the NRF2-regulated response preventing tumorigenesis through improved activity of GSTs and NQO1 (Zhang *et al.*, 1994; Fahey *et al.*, 1997; Gerhauser *et al.*, 1997; Dinkova-Kostova *et al.*, 2004; Brigelius-Flohe and Banning, 2006; Hu *et al.*, 2006; Juge *et al.*, 2007). Dietary supplementation with sulphoraphan may be associated with a lower risk of prostate and colon cancer in mammals (Giovannucci *et al.*, 1995; Hecht *et al.*, 1995; Clinton *et al.*, 1996; Miller *et al.*, 2002; Campbell *et al.*, 2004; Chiao *et al.*, 2004).

Triterpenoids are also very potent activators of NRF2 and are able to activate NQO1 enzyme activity *in vitro* (Dinkova-Kostova *et al.*, 2001). Studies in transgenic reporter mice with the NQO1 ARE linked to a luciferase gene localized ARE activation in metabolic organs such as the kidney, salivary gland, liver and intestine (Yates *et al.*, 2007).

Curcumin, from turmeric, induces the expression of NRF2 downstream targets, like heme oxygenase-1 enzyme in human cells, and its anti-inflammatory activity has been shown to inhibit carcinogenesis in preclinical animal models (Shen *et al.*, 2006).

Resveratrol has been shown to reduce inflammation, possess cardioprotective and vasoprotective properties in a NRF2-dependent induction, which confers cancer prevention as shown in several preclinical animal models (Leifert and Abeywardena, 2008; Udenigwe *et al.*, 2008).

Ethoxyquindiet supplementation inhibited liver carcinogenesis in rats exposed to anatoxin Bl (Cabral and Neal, 1983). It has been subsequently shown that the induction of hepatic cytoprotective enzymes by these antioxidants is mediated by NRF2 signalling (Nair *et al.*, 2006).

The methyl ester and ethyl amide derivatives are less documented, but it has been shown that they are able to induce NRF2-regulated cytoprotective genes in the lung and have been studied in a post-initiation lung cancer mouse model (Yates *et al.*, 2007).

Metformin is a biguanide drug commonly used to treat type 2 diabetes that has been noted to extend healthspan of non-diabetic mice. In *C. elegans*, metformin dietary supplementation extends median lifespan through SKN-1 pathway in a conserved biochemical mechanism that acts like a CR mimetic (Onken and Driscoll, 2010).

Concluding remarks

During the last years, researchers have been trying to determine the mechanisms whereby dietary intake modulates cancer and ageing. The transcription factor NRF2 is activated under CR and induces cancer protection. Increased understanding of these physiological mechanisms would offer the potential to develop mechanism-based interventions to promote longevity, healthy ageing and lower cancer incidence (Figure 1). It has been shown that

longevity and cancer share some enzymatic pathways. Existing therapies designed to produce antiageing effects are likely also to have a cancer preventive effect and *vice versa*. In order to develop a preventive strategy for cancer treatment in humans a periodic fasting or intermittent CR has been proposed to be attainable and beneficial (Williams *et al.*, 1998; Johnson *et al.*, 2007)). In any case, the adaptation of humans to a CR pattern similar to that we performed with mice in our laboratory (ranging from 10 to 40% restriction in food daily intake) is difficult to obtain. We propose NRF2 as a potential target to evaluate and develop CR mimetics.

The activation of NRF2 and downstream targets by dietary factors is a crucial mechanism for tumour prevention owing to the potential to shift the metabolic balance, increasing NRF2 response and leading to the prevention of cancer development. Furthermore, it would be reasonable that the development of new nontoxic more potent NRF2-activating compounds will attenuate the carcinogenesis process. Although the NRF2-activating agents are present in short-time exposure, downstream induction is maintained for some days after exposure and cells respond to NRF2-activating agents repeatedly. This fact allows us to support a non-chronic NRF2 activator supplementation, which would improve cancer treatment and decrease possible phytochemical toxicity in organisms. A plausible cancer prevention strategy could be based on a similar approach that includes moderate CR, physical activity and cytoprotective supplementation. The next step in future investigations would be developing a mechanism to express NRF2 in non-cancer cells, while maintaining a lower NRF2 activation in preneoplastic or neoplastic cells, thus leading to selective toxicity in malignant cells.

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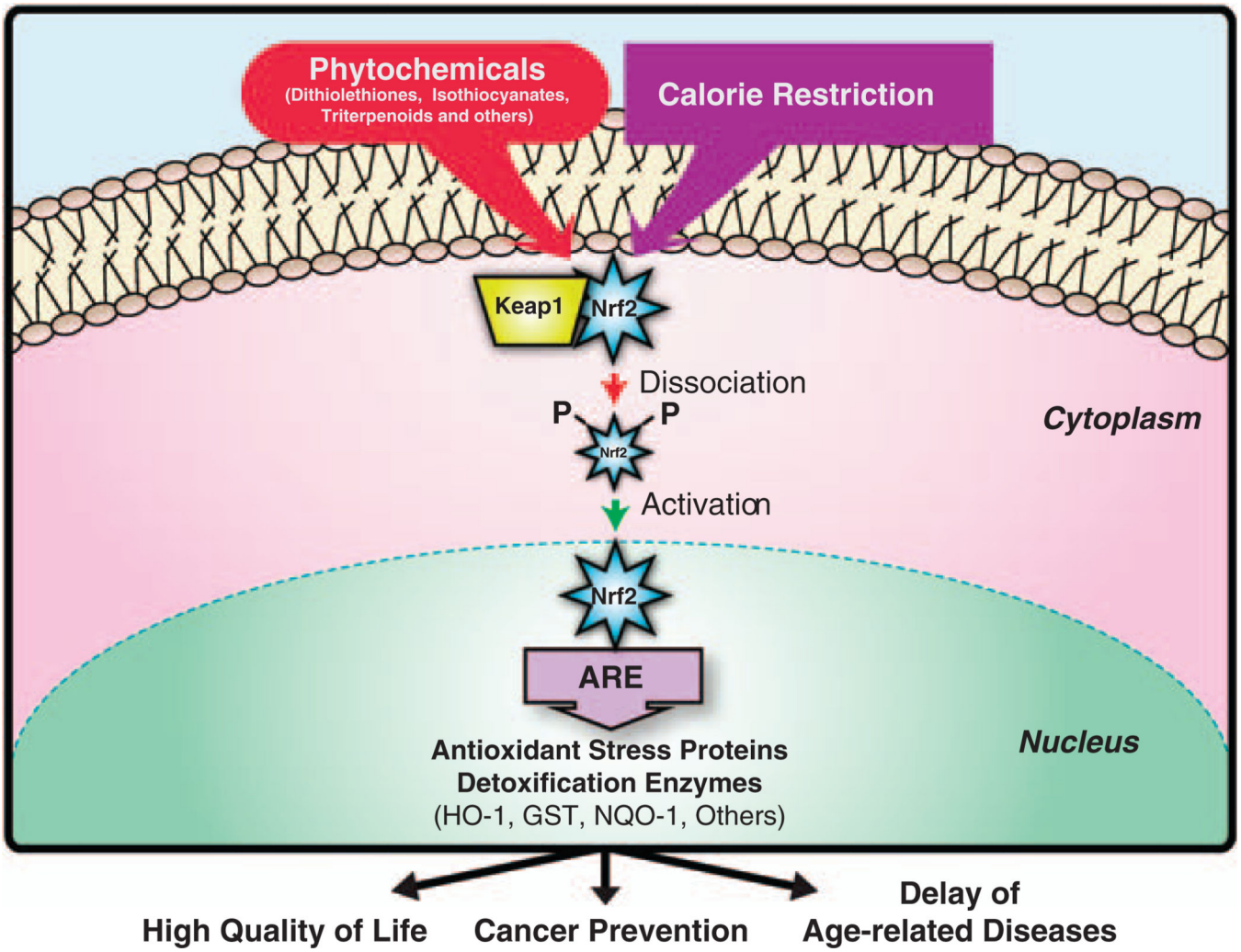

Figure 1.

Diagram of the activation of NRF2. Calorie restriction and a number of phytochemicals are able to induce the release of NRF2 from Keap1, allowing it to enter into the nucleus. Once in the nucleus, NRF2 binds to ARE sequences in the promoter of antioxidant and detoxifying enzymes, inducing their expression. Increased levels of these NRF2 downstream targets prevent age-related increases in cancer and ameliorates other age-related diseases, leading to an improved quality of life.

Table 1

NRF2 and CR involvement in aged-related diseases

Disease	NRF2	CR improvement
Cancer	Aoki *et al.*, 2001; auf dem Keller *et al.*, 2006; Kitamura *et al.*, 2007; Pearson *et al.*, 2008; Ramos-Gomez *et al.*, 2001	Birt *et al.*, 1991, 1993; Colman *et al.*, 2009; Tannenbaum and Silverstone 1953
Brain atrophy	Calkins *et al.*, 2005; Lee *et al.*, 2003a; Shih *et al.*, 2005	Colman *et al.*, 2009; Mattson *et al.*, 2001; Weed *et al.*, 1997
Parkinson	Burton *et al.*, 2006; Jakel *et al.*, 2007	Love, 2005; Mattson, 2003
Diabetes	Aleksunes *et al.*, 2010; Bartke *et al.*, 2007; Jiang *et al.*, 2010	Colman *et al.*, 2009; Hansen *et al.*, 1995
Cardiovascular	Chen *et al.*, 2003; Dai *et al.*, 2007; Mostoslavsky *et al.*, 2006; Warabi *et al.*, 2007	Colman *et al.*, 2009; Guo *et al.*, 2002
Muscle atrophy	Ding *et al.*, 2008	Marzetti *et al.*, 2009; McKiernan *et al.*, 2004
Bone health	Lee *et al.*, 2010b	Westerbeek *et al.*, 2008
Alzheimer	Kanninen *et al.*, 2009	Halagappa *et al.*, 2007; Qin *et al.*, 2008
Asthma	Rangasamy *et al.*, 2005	Johnson *et al.*, 2007

Abbreviations: CR, calorie restriction; NRF2, NF-E2-related factor.

Table 2

NRF2 downstream targets involved in cancer protection

Gene	Role in cancer prevention/cancer growth	References
GPX2	Inflammatory response	Banning *et al.*, 2008
PRDX1	Antioxidant	Cao *et al.*, 2009
PRDX61	Antioxidant	Chang *et al.*, 2007
NQO1	Antioxidant	Nolan *et al.*, 2010
CBR1	Antioxidant	La1 *et al.*, 2008
CBR3	Antioxidant	La1 *et al.*, 2008
CYP2B9	Drug metabolism	Muguruma *et al.*, 2006
FMO2	Drug metabolism	Fialka *et al.*, 2008
FMO3	Drug metabolism	Bae *et al.*, 2006
GSTA1	ROS protection	Nguyen *et al.*, 2010
GSTM1	ROS protection	Nguyen *et al.*, 2010
GSTP1	ROS protection	Nguyen *et al.*, 2010
GSTT1	ROS protection	Nguyen *et al.*, 2010
MGST3	ROS protection	Efferth and Volm, 2005
ALDH3A1	Metabolism	Patel *et al.*, 2008
GADD45G	DNA repair	Baguley, 2010
HSP40	Stress resistance	Mitra *et al.*, 2009
HSP70	Stress resistance	Wang *et al.*, 2010

Abbreviation: NRF2, NF-E2-related factor; ROS, reactive oxygen species.

Table 3

NRF2 activators

Compound	References
Dithiolethiones (oltipraz)	Ansher *et al.*, 1986; Kwak *et al.*, 2001; Pietsch *et al.*, 2003; Ramos-Gomez *et al.*, 2001; Iida *et al.*, 2004
Isothiocyanates (sulphoraphan)	Heiss *et al.*, 2001; Brigelius-Flohe and Banning, 2006; Dinkova-Kostova *et al.*, 2004; Fahey *et al.*, 1997; Gerhauser *et al.*, 1997; Hu *et al.*, 2006; Juge *et al.*, 2007; Zhang *et al.*, 1994; Campbell *et al.*, 2004; Clinton *et al.*, 1996; Chiao *et al.*, 2004; Giovannucci *et al.*, 1995; Hecht *et al.*, 1995; Lii *et al.*, 2010; Miller *et al.*, 2002
Triterpenoids (bardoxolone methyl)	Dinkova-Kostova *et al.*, 2001; Yates *et al.*, 2007
Curcumin	Shen *et al.*, 2006
Resveratrol	Leifert and Abeywardena, 2008; Udenigwe *et al.*, 2008
Ethoxyquindiet	Cabral and Neal, 1983; Nair *et al.*, 2006
Methyl ester and ethyl amide	Yates *et al.*, 2007
Piceatannol	Lee *et al.*, 2010a
Simvastatin	Chartoumpekis *et al.*, 2010
Metformin	Onken and Driscoll, 2010

Abbreviation: NRF2, NF-E2-related factor.