

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## The Dual Roles of NRF2 in Cancer

### This is the author's manuscript

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1589646> since 2016-08-29T12:26:38Z

*Published version:*

DOI:10.1016/j.molmed.2016.05.002

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***

*Questa è la versione dell'autore dell'opera:*

*[Trends Mol Med, volume 22, issue 7, 2016, doi: 10.1016/j.molmed.2016.05.002]*

***The definitive version is available at:***

*La versione definitiva è disponibile alla URL:*

*[<http://www.sciencedirect.com/science/article/pii/S1471491416300260>]*

# The Dual Roles of NRF2 in Cancer

Silvia Menegon<sup>1</sup>, Amedeo Columbano<sup>2</sup>, Silvia Giordano<sup>1</sup>

<sup>1</sup> University of Torino, Department of Oncology, Candiolo Cancer Institute-FPO, IRCCS, Strada Provinciale 142, 10060 Candiolo, Torino, Italy

<sup>2</sup> Department of Biomedical Sciences, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy

NRF2 has been traditionally considered as a tumor suppressor because its cytoprotective functions are deemed to be the main cellular defense mechanism against exogenous and endogenous insults, including xenobiotics and oxidative stress. However, several recent studies demonstrate that hyperactivation of the NRF2 pathway creates an environment that favors the survival of normal as well as malignant cells, protecting them against oxidative stress, chemotherapeutic agents, and radiotherapy. In a rapidly advancing field, this review summarizes some of the known mechanisms by which NRF2 can exert its oncogenic functions, and describes the current status of NRF2 inhibitors, providing a clear rationale for the consideration of NRF2 as a powerful putative therapeutic target in cancer treatment.

## Trends

Under homeostatic conditions, NRF2 activation prevents excessive cellular damage produced by metabolic, xenobiotic, and oxidative stress. NRF2 activation is thus important in cancer chemoprevention.

Nrf2-null mice are more prone to develop cancer in response to chemical and physical stimuli (nitrosamine, ultraviolet light, aflatoxin).

NRF2 hyperactivation confers several advantages to cancer cells, including protection from apoptosis and senescence, promotion of cell growth, and resistance to chemo- and radiotherapy.

In experimental models, Nrf2/Keap1 mutations are present at pre-neoplastic stages.

Distinct mechanisms are responsible for NRF2 activation in cancer, including (i) somatic mutations in NRF2 or KEAP1, (ii) epigenetic silencing of the KEAP1 promoter, (iii) microRNA-mediated regulation of NRF2 and KEAP1, (iv) aberrant accumulation of proteins that disrupt the interaction between NRF2 and KEAP1, (v) interaction with other 'cancer master players', and (vi) metabolic modifications.

## Keywords

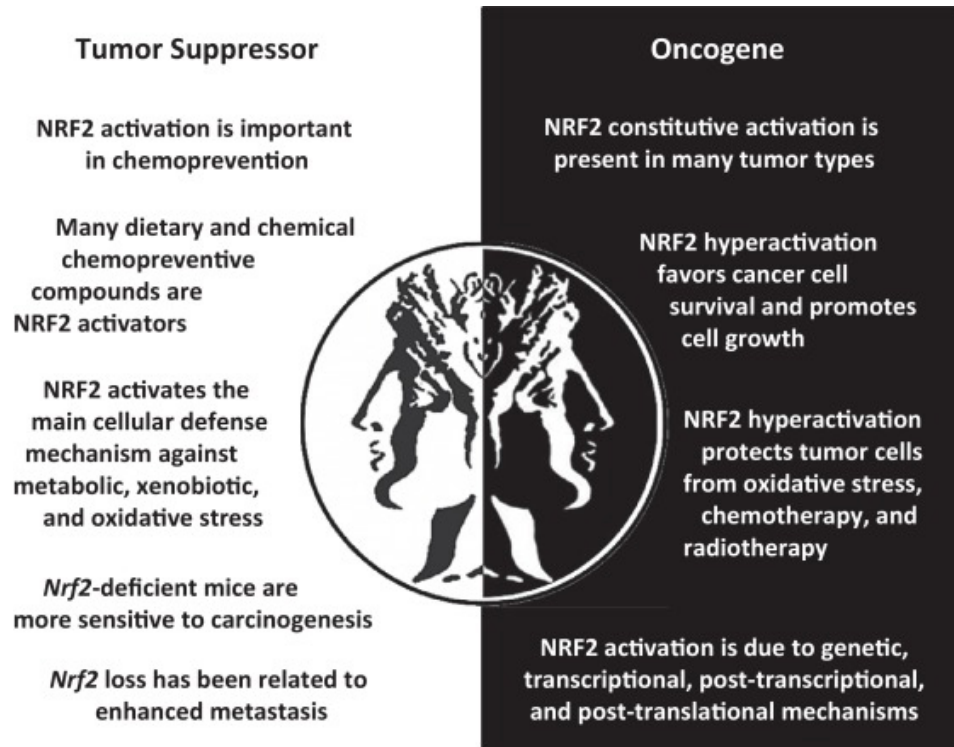
NRF2; KEAP1; antioxidant response; mutations

## NRF2: Tumor Suppressor or Oncogene?

The main function of the transcription factor NRF2 [also known as NFE2L2, nuclear factor (erythroid-derived-2)-like 2] is to activate the cellular antioxidant response by inducing the transcription of several genes to protect cells from the effects of exogenous and endogenous insults such as xenobiotics (see Glossary) and oxidative stress[1]. As a result, NRF2 has typically been regarded as a cytoprotective transcription factor and is considered as the main defense mechanism of the cell and a major regulator of cell survival. Thus, NRF2 has been traditionally deemed to be a tumor suppressor. Indeed, Nrf2-deficient mice are more sensitive to carcinogenesis 2 and 3, and Nrf2 loss has been linked to enhanced metastasis 4 and 5. Moreover, there are multiple reports describing the beneficial effects of NRF2 signaling in cancer chemoprevention[6].

However, in the past few years increasing evidence suggests that NRF2 activation might not be beneficial in all cancer types and stages. In fact, NRF2 promotes the survival not only of normal cells but also of cancer cells, supporting the hypothesis that NRF2 activation in malignant cells might sustain the evolution of the disease. The identification of a 'dark side of NRF2' 7 and 8 has generated controversy because it is still unclear whether NRF2 acts as a tumor suppressor or as an oncogene 7 and 9. Indeed, because oxidative stress has been implicated in the initiation of cancer, the anti-

oxidative role of NRF2 may play an anticancer role and has been implicated in chemoprevention. However, NRF2 hyperactivation in tumors creates an environment that may favor the survival of cancer cells by protecting them from excessive oxidative stress, chemotherapeutic agents, or radiotherapy 1, 6 and 10 (Figure 1, Key Figure). Therefore, there is a growing research spectrum geared towards defining the boundaries between NRF2 positive and negative effects in cancer, and to establish a precise rationale for undertaking NRF2 therapeutic targeting.

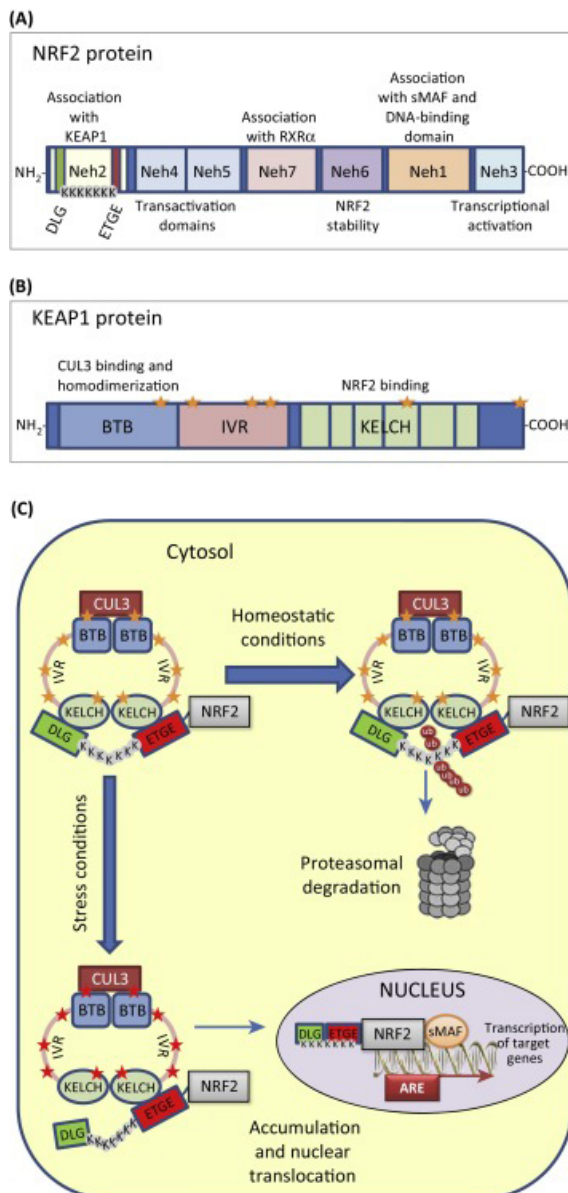


**Figure 1.** Key Figure: Schematic Representation of the Dual Role of NRF2 in Cancer Onset and Progression

The left side of the figure lists some of the main evidence supporting the tumor-suppressive role of NRF2; the right side of the figure summarizes the evidence to support the oncogenic role of NRF2. The bifrontal figure represents Janus, the Roman god with two faces.

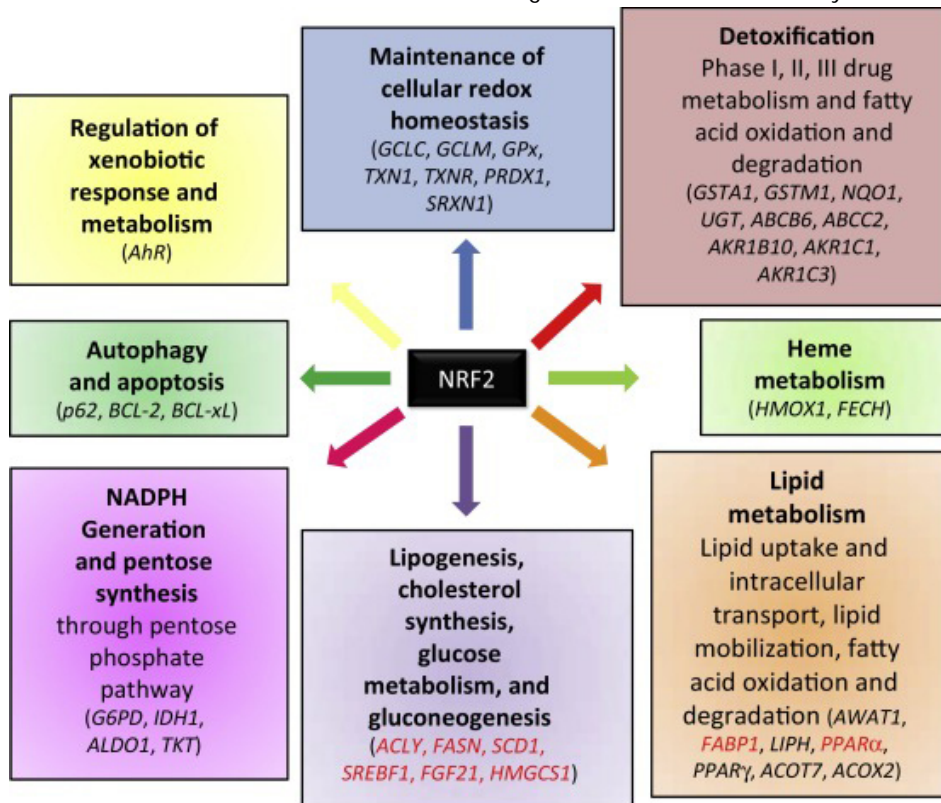
### The NRF2–KEAP1–ARE Signaling Pathway

NRF2, cloned and characterized on the basis of its ability to bind the NF-E2/AP-1 repeat in the promoter region of the  $\beta$ -globin gene [11], is ubiquitously expressed and dispensable for mouse development [12]. NRF2 is a member of the cap 'n' collar (CNC) subfamily of transcription factors and contains seven highly conserved domains, Neh1–7, distributed all along the gene (Figure 2A).



**Figure 2.** Domain Structure and Modality of NRF2–KEAP1 Interactions. (A) Organization and domain structure of NRF2. The NRF2 protein contains seven highly conserved regions known as NRF2–ECH homology (Neh) domains. The Neh1 domain is a basic region leucine-zipper motif necessary for DNA binding and dimerization with other transcription factors such as sMAF; Neh2 binds to the Kelch domain of KEAP1, and contains seven lysine residues responsible for ubiquitin conjugation, which leads to NRF2 proteasomal degradation; Neh3 is necessary for transcriptional activation; Neh4 and Neh5 are two independent transactivation domains; Neh6 is a serine-rich region that regulates NRF2 stability. Neh7 is involved in RXR $\alpha$  binding. (B) Organization and domain structure of KEAP1. KEAP1 has three discrete protein domains—the N-terminal Broad complex, Tramtrack, and Bric-à-Brac (BTB) domain; the intervening region (IVR) linker domain containing several cysteine residues that sense intracellular redox imbalance; and the Kelch domain that is required for homodimerization and contains several cysteine residues (orange stars) important in stress-sensing (the IVR domain also contains stress-sensing cysteine residues). The C-terminal KELCH domain forms a B-propeller structure and binds to the N-terminal Neh2 domain of NRF2. (C) The KEAP1–NRF2–ARE signaling pathway. Binding of KEAP1 to CUL3 leads to KEAP1 homodimerization. The high-affinity ETGE motif of NRF2 initially binds to the KELCH domain of KEAP1 and the lower-affinity DLG motif binds to the second KEAP1, closing the conformation of the complex. Under homeostatic conditions (right), NRF2 is polyubiquitinated at its lysine-rich (KKKKKKK) region and is then targeted to proteasome for degradation. Under increasing ROS levels conditions (left), the modification of cysteine residues on KEAP1 (red stars) imposes a conformational change that disrupts the weak KELCH–DLG binding, resulting in diminished NRF2 ubiquitination, without dissociation of NRF2 from KEAP1. NRF2 protein levels are thus increased and NRF2 translocates into the nucleus where it associates with sMAF (musculo-aponeurotic fibrosarcoma), subsequently binding ARE (antioxidant responsive element) sequences on target genes, regulating their transcription.

Upon cell exposure to oxidative stress, NRF2 translocates to the nucleus, forms a heterodimer with its partner sMAF (v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog), and binds to ARE (antioxidant responsive element) sequences to regulate the transcription of several types of genes [13] (Figure 2C). NRF2 downstream genes, illustrated in Figure 3, are involved in metabolism, intracellular redox-balancing, apoptosis, and autophagy. Based on the functions of these genes, it can be concluded that NRF2 activation protects cells from various stresses imposed by toxic exposure. Indeed, the NRF2-mediated antioxidant response is one of the major cellular defense mechanisms allowing cell survival in the face of toxic insults. This notion has been best demonstrated in animal models, as in the case of Nrf2-null mice, which are more sensitive to the toxic and carcinogenic effects of a wide variety of xenobiotics 14, 15, 16 and 17.



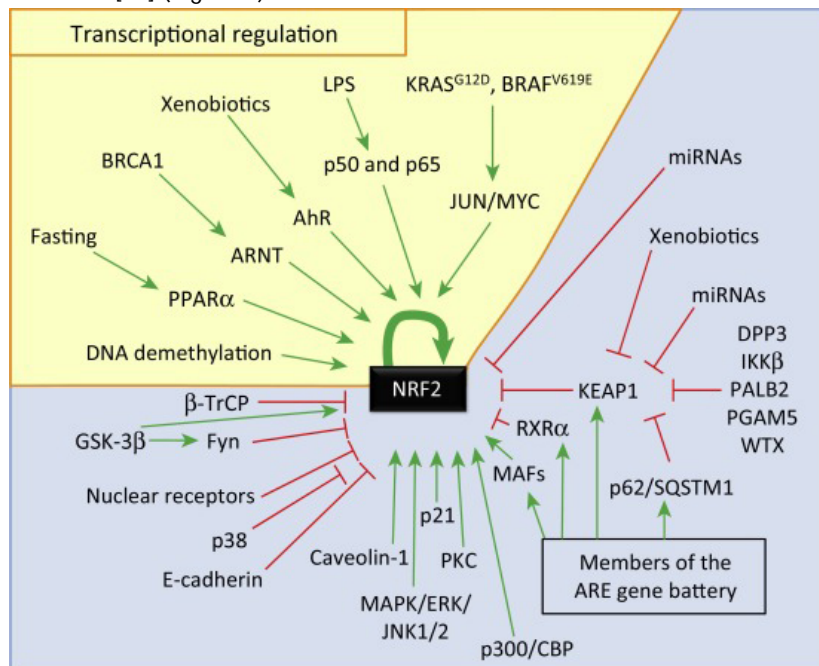
**Figure 3.** NRF2 Controls Various Biological Functions. NRF2 downstream genes identified so far in humans and mice can be grouped into several categories, including genes encoding (i) intracellular redox-balancing proteins that maintain cellular glutathione and thioredoxin levels and reduce ROS levels: glutamate cysteine ligases (GCLC, GCLM), glutathione peroxidase (GPx), thioredoxin 1 (TXN1), thioredoxin reductase (TXNR), peroxiredoxin 1 (PRDX1), sulfiredoxin 1 (SRXN1); (ii) phase I/II/III detoxifying enzymes that metabolize xenobiotics into less-toxic forms or catalyze conjugation reactions to increase the solubility of xenobiotics, thereby facilitating their elimination: glutathione S-transferases (GSTA1, GSTM1), NAD(P)H quinone oxidoreductase-1 (NQO1), UDP-glucuronosyltransferase (UGT), transporters (ABCB6, ABCC2), aldo-keto reductases (AKR1B10, AKR1C1, AKR1C3); (iii) enzymes involved in heme metabolism: heme oxygenase-1 (HMOX-1), ferrochelatase (FECH); (iv) enzymes involved in lipid metabolism: acyl-CoA wax alcohol acyltransferase 1 (AWAT1), fatty acid-binding protein 1 (FABP1), lipase H (LIPH), peroxisome proliferator-activated receptors (PPARA, PPARG), acyl-CoA thioesterase 7 (ACOT7), acyl-CoA oxidase 2 (ACOX2); (v) enzymes involved in lipid and sugar metabolism: ATP citrate lyase (ACLY), fatty acid synthase N (FASN), stearoyl-CoA desaturase (SCD1), sterol regulatory element binding transcription factor 1 (SREBTF1), fibroblast growth factor 21 (FGF21), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1); (vi) enzymes involved in NADPH generation and pentose synthesis: glucose-6-phosphate dehydrogenase (G6PD), isocitrate dehydrogenase 1 (IDH1), aldolase 1 (ALDO1), transketolase (TKT); (vii) proteins involved in autophagy and apoptosis: p62 sequestosome 1 protein (p62), B cell lymphoma 2 (BCL-2), B cell lymphoma-extra large (BCL-xL); and (viii) proteins involved in regulation of xenobiotic response and metabolism: aryl hydrocarbon receptor (AHR). Downregulated genes are noted in red.

Recently, new evidence has indicated that NRF2 plays a role in controlling metabolism[18]. Indeed, in human tumor samples and cell lines, constitutive NRF2 activation increases the expression of genes involved in drug metabolism, thereby sustaining resistance to chemotherapeutic drugs and radiotherapy. Overactive NRF2 also promotes cell



proliferation by inducing metabolic reprogramming towards anabolic pathways, augmenting purine synthesis, and influencing the pentose phosphate pathway (PPP), a metabolic pathway parallel to glycolysis [19] (Figure 3). Indeed, NRF2 redirects glucose and glutamine into anabolic pathways, especially under sustained activation of phosphatidylinositol-3 kinase (PI3K)–AKT signaling; moreover, gain of NRF2 signaling promotes tumorigenesis through an autoregulatory feedback loop involving miRNA-dependent regulation of PPP and histone deacetylase 4 (HDAC4)[20]. All these NRF2-regulated metabolic pathways are likely to be essential contributors to the maintenance of cellular redox and the promotion of tumor growth, suggesting that their targeting may improve the therapeutic outcome in patients with cancer. Finally, a direct role for NRF2 in controlling apoptosis has come from the identification of Bcl-2 and Bcl-xL as ARE-controlled target genes of NRF2 in mammalian cells. Through Bcl-2 and Bcl-xL upregulation, NRF2 can confer apoptosis protection to tumor cells, thus increasing its oncogenic potential 21 and 22.

Experiments performed in several models and species have shown that NRF2 activity is negatively regulated by KEAP1, a substrate adapter protein for the CUL3/RBX1 E3 ubiquitin ligase complex [23]. Under basal conditions, KEAP1 constantly targets NRF2 for ubiquitin-dependent degradation to maintain low NRF2 levels (Figure 2B) 24, 25, 26, 27 and 28. The two-site substrate recognition ‘hinge and latch’ model of NRF2/KEAP1 interaction hypothesizes that each Kelch domain of a KEAP1 homodimer binds to one NRF2 protein through a weak-binding <sup>29</sup>DLG motif (latch) and a strong-binding <sup>79</sup>ETGE motif (hinge), located in the N-terminal Neh2 domain of NRF2 29 and 30. The binding affinity of Kelch 31, 32, 33 and 34 for the <sup>79</sup>ETGE motif is approximately 100-fold higher than for the <sup>29</sup>DLG motif [30]. Through this binding, the seven ubiquitin-accepting lysine residues between these motifs are aligned into a conformation suitable for ubiquitin conjugation 28, 29 and 30. It has been proposed that KEAP1 is able to ‘sense’ a disturbance in redox homeostasis, turning the NRF2-mediated response on or off 7 and 35; indeed, upon oxidative stress, modification of three major KEAP1 cysteine residues, Cys151, Cys273, and Cys288 that individually and/or redundantly act as sensors, imposes a conformational change that disrupts the weak Kelch–<sup>29</sup>DLG binding, resulting in diminished NRF2 ubiquitination without dissociation of NRF2 from KEAP1 29, 30 and 36. As a consequence, NRF2 protein levels are increased, and the NRF2 signaling pathway is activated 24, 26, 27, 28 and 37 (Figure 2C). Although KEAP1 is the major regulator of NRF2 activation, there is further evidence indicating multiple mechanisms of NRF2 regulation, such as phosphorylation by several kinases, including protein kinase C (PKC), MAPK/ERK/JNK, JUN/MYC, and PI3K [38] (Figure 4).



**Figure 4.** Genes and Pathways Regulating NRF2. The yellow section of the figure denotes genes identified in mice and humans which control NRF2 expression at the transcriptional level. The grey section of the figure includes genes and pathways regulating NRF2 at post-transcriptional and post-translational (signaling) levels. Green arrows, increased NRF2 expression/activity; red bars, decreased NRF2 expression/activity.

## NRF2, A Janus Molecule in Cancer

Experimental evidence suggests that NRF2 can have opposite 'Janus' effects in biology (Janus was a Roman god with two faces, one looking to the future and one to the past). Under physiological conditions, NRF2 signaling is turned on by the presence of stressors in the cellular microenvironment and is rapidly deactivated when the insult is blocked. However, under pathological conditions, the tight regulation of NRF2 can be altered. This can result in loss of responsiveness to cell stressors and in subsequent vulnerability of the cell to various insults. Many studies have shown that Nrf2-null mice are highly sensitive to chemical insults {such as benzo[a]pyrene, diesel exhaust, cigarette smoke, N-nitrosobutyl(4-hydroxybutyl)amine, pentachlorophenol, and acetaminophen}, creating a microenvironment which is more prone to tumor development and metastasis 3, 4, 15 and 39. In addition, NRF2 constitutive activation confers a survival advantage to mammalian cells under adverse conditions. As an example, NRF2 pathway activation in A549 cells enhances cell proliferation and resistance to cisplatin [40]. Altogether, several studies have highlighted that aberrant activation of the NRF2 signaling pathway is linked to the detrimental effect of cancer cells because NRF2 target genes can perform crucial functions in cell survival and proliferation, and can promote oncogenesis in many mammalian cancer types 19, 21, 41 and 42. Distinct mechanisms, discussed below, can result in NRF2/KEAP1 pathway activation.

### Somatic Mutations in NRF2 or KEAP1

Gain-of-function mutations of NRF2 (Table 1) and loss-of-function mutations of KEAP1 (Table 2) have been found in several human cancers: these mutations, mainly somatic, lead to overexpression or constitutive activation of the pathway, increasing the antioxidant defense in cancer cells and conferring them survival.

**Table 1.**

*NRF2 Somatic Mutations Identified in Different Human Cancers <sup>a</sup> and <sup>b</sup>*

AA Substitution	CDS Substitution	Primary Tissue
p.L23R	c.68T > G	HCC
p.W24R	c.70T > C	PRCC, ESCC
p.W24C	c.72G > C	Lung SCC, SCLC, ESCC
p.Q26E	c.76C > G	ESCC
p.Q26L	c.77A > T	Lung SCC
p.Q26P	c.77A > C	Lung SCC
p.Q26H	c.78A > C	HCC
p.D27H	c.79G > C	Lung SCC
p.D27Y	c.79G > T	ESCC
p.I28T	c.83T > C	Lung SCC, ESCC
p.D29H	c.85G > C	HCC, lung AC, SCC-UADT
p.D29N	c.85G > A	Lung SCC
p.D29Y	c.85G > T	Cervix SCC, PRCC, lung AC, lung SCC
p.D29G	c.86A > G	HCC, lung SCC, ESCC, SCC-UADT
p.D29V	c.86A > T	CCRCC
p.L30F	c.88C > T	Liver, lung SCC, ESCC, skin, SCC-UADT
p.L30R	c.89T > G	Endometrioid carcinoma
p.G31R	c.91G > A	Central nervous system
p.G31A	c.92G > C	CCRCC, lung SCC, ESCC, skin SCC
p.G31E	c.92G > A	ESCC
p.V32E	c.95T > A	HCC
p.V32G	c.95T > G	Lung SCC



AA Substitution	CDS Substitution	Primary Tissue
p.R34G	c.100C > G	Endometrioid carcinoma, lung SCC
p.R34P	c.101G > C	Lung SCC, ESCC
p.R34Q	c.101G > A	Cervix SCC, lung SCC, LCLC, ESCC, SCC-UADT
p.F71S	c.212T > C	NS
p.Q75H	c.225A > C	ESCC, UADT
p.D77N	c.229G > A	SCC-UADT
p.D77Y	c.229G > T	Liver
p.D77A	c.230A > C	Lung SCC
p.D77G	c.230A > G	HCC, lung SCC, ESCC
p.D77V	c.230A > T	ESCC
p.E78K	c.232G > A	ESCC
p.E79K	c.235G > A	Lung SCC, ESCC
p.E79Q	c.235G > C	Cervix SCC, lung AC, lung SCC, SCC-UADT
p.E79G	c.236A > G	HCC, SCC-UADT
p.T80P	c.238A > C	HCC, ESCC
p.T80I	c.239C > T	HCC, UADT
p.T80K	c.239C > A	PRCC, lung SCC, ESCC
p.T80R	c.239C > G	Lung SCC
p.G81S	c.241G > A	HCC, lung SCC
p.G81D	c.242G > A	Breast, lung AC, lung SCC, ESCC
p.G81V	c.242G > T	HCC, lung SCC, ESCC
p.E82Q	c.244G > C	HCC, lung AC, lung SCC, ESCC
p.E82G	c.245A > G	PRCC, HCC, lung SCC, UADT
p.E82V	c.245A > T	ESCC
p.E82D	c.246A > C	HCC, lung AC, ESCC
p.A124G	c.371C > G	Breast
p.A124V	c.371C > T	Large intestine AC
p.L143R	c.428T > G	Lung AC
p.S164*	c.491C > G	Astrocytoma grade III
p.E185D	c.555G > C	SCC-UADT
p.E380*	c.1138G > T	Large intestine AC
p.P467L	c.1400C > T	CLL
p.R502C	c.1504C > T	Large intestine AC
p.A511V	c.1532C > T	Large intestine AC

a Abbreviations: AA, amino acid; AC, adenocarcinoma; CCRCC, clear cell renal cell carcinoma; CDS, coding sequence; CLL, chronic lymphocytic leukemia; ESCC, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma; PRCC, papillary renal cell carcinoma; SCC, squamous cell carcinoma; UADT, upper aerodigestive tract.

b All the reported mutations are listed in the Cosmic (Catalogue of Somatic Mutations in Cancer) database with the appropriate reference.

Table 2.

*KEAP1 Somatic Mutations Identified in Different Human Cancers<sup>a</sup>*

AA Substitution	CDS Substitution	Primary Tissue	AA Substitution	CDS Substitution	Primary Tissue
p.Q46*	c.136C > T	Lung AC	p.P318L	c.953C > T	Lung SCC
p.Y54D	c.160T > G	CCRCC	p.R320Q	c.959G > A	Lung SCC
p.Y54C	c.161A > G	Lung SCC	p.R320W	c.958C > T	HCC, lung AC, SCC-UADT
p.Q75*	c.223C > T	Lung SCC	p.G333C	c.997G > T	Lung AC
p.K97N	c.291G > T	Lung AC	p.G333S	c.997G > A	Lung AC
p.S102L	c.305C > T	Lung AC	p.Y334H	c.1000T > C	Lung
p.M110V	c.328A > G	Lung AC	p.R336*	c.1006C > T	Lung AC
p.G114W	c.340G > T	HCC	p.R362Q	c.1085G > A	Lung SCC
p.E117K	c.349G > A	Lung AC	p.G364S	c.1090G > A	Lung AC
p.V123L	c.367G > T	Lung AC	p.V369L	c.1105G > C	Lung SCC
p.E138A	c.413A > C	ESCC	p.A407V	c.1220C > T	Prostate AC
p.Y141C	c.422A > G	Lung SCC	p.M409T	c.1226T > C	CCRCC
p.T142M	c.425C > T	Large intestine AC, lung AC	p.R415C	c.1243C > T	Lung AC
p.S144F	c.431C > T	Lung AC	p.G417R	c.1249G > A	Lung AC
p.A159P	c.475G > C	Lung AC	p.G417E	c.1250G > A	Lung AC
p.V155F	c.463G > T	Lung AC, lung SCC	p.V418L	c.1252G > T	Lung SCC
p.V155A	c.464T > C	Lung AC	p.V418M	c.1252G > A	Lung AC
p.I185N	c.554T > A	Lung AC	p.D422N	c.1264G > A	Lung SCC, ESCC
p.G186S	c.556G > A	SCC-UADT	p.G423V	c.1268G > T	Lung AC, lung SCC
p.N189K	c.567C > G	Ovary SC	p.E449*	c.1345G > T	Lung AC
p.A191D	c.572C > A	Large intestine AC	p.R460G	c.1378A > G	Lung AC
p.E218Q	c.652G > C	SCC-UADT	p.R470C	c.1408C > T	Lung AC, SCC, ESCC, UADT
p.E219Q	c.655G > C	Lung AC	p.R470S	c.1408C > A	ESCC
p.S224Y	c.671C > A	Lung SCC	p.R470H	c.1409G > A	Liver
p.L231V	c.691C > G	Lung SCC	p.D479G	c.1436A > G	Lung AC
p.D236N	c.706G > A	HCC, lung AC	p.G480W	c.1438G > T	Lung SCC
p.S243C	c.728C > G	Lung SCC	p.E493D	c.1479G > C	Lung SCC
p.F246L	c.738C > G	Lung AC	p.W497L	c.1490G > T	Lung AC
p.W252C	c.756G > T	Lung AC	p.M503K	c.1508T > A	Lung AC
p.R260Q	c.779G > A	Lung SCC	p.I506V	c.1516A > G	Lung SCC
p.R261P	c.782G > C	Lung AC	p.G509W	c.1525G > T	Lung AC
p.R269W	c.805C > T	HCC, esophagus AC	p.G524C	c.1570G > T	Lung AC

AA Substitution	CDS Substitution	Primary Tissue	AA Substitution	CDS Substitution	Primary Tissue
p.V271M	c.811G > A	Lung AC, ESCC	p.W544R	c.1630T > C	CCRCC
p.P278S	c.832C > T	Lung AC	p.W544C	c.1632G > T	Lung SCC
p.F280Y	c.839T > A	Lung AC	p.S555C	c.1663A > T	Lung AC
p.Q284L	c.851A > T	Lung AC	p.Q563E	c.1687C > G	Lung SCC
p.K287*	c.859A > T	Lung AC	p.E593*	c.1777G > T	HCC
p.L310P	c.929T > C	Lung SCC			

a Abbreviations: see Table 1.

### *NRF2 and KEAP1 Mutations in Lung Cancer*

The majority of mutations involving NRF2 and KEAP1 have been described in lung cancer. The first somatic mutations in the NRF2 pathway were identified in the Kelch-like repeat domain of KEAP1 in two human lung adenocarcinoma cell lines [43]. In this study, KEAP1 mutants showed significantly diminished binding to NRF2, resulting in decreased repressive function and increased NRF2 target gene expression. Later on, many others have widened the list of KEAP1 mutations in lung cancer. Of note, these mutations have been described all along the gene, and not only in the NRF2-interacting region [44], proving that KEAP1 mutations that do not affect the NRF2-binding activity can result in increased NRF2 half-life. As demonstrated by Singh's group, mutations in the KEAP1 gene are present in 25% of lung cancer patients and, importantly, loss of heterozygosity (LOH) for this gene was observed in 41% of the cases [44]. Similar results were found in different cohorts of patients 45, 46 and 47. Interestingly, almost all homozygous mutants presented LOH 44 and 45, meaning that most patients carry a biallelic deactivation of the gene. However, the loss of two copies of the KEAP1 gene is not crucial because KEAP1 proteins harboring mutations on the Kelch-like domain function as dominant-negatives, forming heterodimers with wild-type forms that become unable to associate with NRF2[45]. This hypothesis would explain why heterozygous mutations can also induce NRF2 hyperactivation.

Mutations in the NRF2 coding region, clustered in the KEAP1-binding domain Neh2, were also first described in lung cancer [48]. Of note, NRF2 and KEAP1 mutations are mutually exclusive [48]. Why is lung tissue especially prone to NRF2 hyperactivation? A possible explanation is that respiratory tissue is constantly exposed to oxygen and chemicals present in the air [49]. Indeed, normal lung cells presenting NRF2/KEAP1 mutations could initially take advantage of the stronger activation of the NRF2–KEAP1 pathway to fight overexposure to oxidative stress; however, upon malignant transformation, mutated cells could benefit from this hyperactivated pathway to escape endogenous tumor suppression. Accordingly, these mechanisms have also been described in a variety of squamous cell carcinomas in external body tissues and the upper aerodigestive tract, that are exposed to oxygen and environmental toxicants [50].

### *NRF2 and KEAP1 Mutations in Liver Cancer*

Concerning hepatocellular carcinoma (HCC), two wide studies identified NRF2 mutations in 6.4% and 3.7% of HCC patients 51 and 52. A third study, by contrast, found mutations only in KEAP1 but not in NRF2 [53]. The reason for this discrepancy is not easily understandable.

Dysregulation of the Nrf2/Keap1 pathway has also been described in HCCs developed in two mouse models of hepatocarcinogenesis, namely the c-Myc/TGF- $\alpha$  transgenic mouse model and a phenobarbital-induced model of chemical carcinogenesis 54 and 55. In both models, as well as in humans, mutation and/or dysregulation of the Nrf2/Keap1 pathway have been investigated only at the final biological endpoint, namely HCC. This makes it difficult to understand whether alterations of this pathway are crucial for HCC development. Recently, we have shown that the Nrf2/Keap1 pathway is already dysregulated during the very early steps of the carcinogenic process in the liver of rats using the resistant hepatocyte model [56]. Indeed, in 38 analyzed preneoplastic lesions, we found 25 mutations in the Nrf2 gene and two in Keap1; interestingly, Keap1 mutations were found in samples lacking Nrf2 mutations [57]. All Nrf2 mutations were missense, most frequently at codons 32 (8/25, V32E) and 77 (4/25, D77G). Bioinformatic analysis demonstrated that all the mutations profoundly impaired Nrf2–Keap1 binding and thus should be considered as

activating mutations. Moreover, targeting Nrf2 mutations impaired the tumorigenic ability of HCC cells. Experimental data obtained in the same model indicated that only preneoplastic nodules displaying activation of the Nrf2–Keap1 pathway exhibited metabolic reprogramming, suggesting that Nrf2 contributes to the onset of the metabolic Warburg effect during the very early phases of hepatocarcinogenesis [58]. Notably, inhibition of oxidative phosphorylation, which paralleled increased glucose utilization and PPP activation, occurred only in the most aggressive nodules characterized by activation of the Nrf2–Keap1 pathway, and not in slow-growing lesions [58]. These results, together with the finding of a high frequency of Nrf2 mutations in early steps of hepatocarcinogenesis, suggest that Nrf2 may represent a driver of HCC development. The recent generation of Nrf2 knockout rats has provided an exciting tool to investigate the role of this transcription factor at all stages of the hepatocarcinogenic process [59 and 60].

#### *NRF2 and KEAP1 Mutations in Other Cancers*

In addition to lung and liver cancers, NRF2/KEAP1 mutations have been characterized in several other solid tumors. Most such mutations affect the NRF2/KEAP1 interacting regions and have been defined as activating mutations. A gene profiling study in breast cancer cells identified a point mutation in the N-terminal domain of KEAP1, rendering the protein unable to ubiquitinate NRF2 [61 and 62]. Other somatic KEAP1 mutations have been detected in human gastric (11.1%), colorectal (7.8%), prostate (1.3%) [63], gallbladder (30.7%) [64], and ovarian carcinomas (37%) [65]. These mutations were either heterozygous or associated with LOH, as in lung cancer [48 and 63]. In addition, NRF2 mutations have been found in human head and neck cancer [48], larynx, esophagus, and skin SCC [50], and all of them cluster near or within the Neh2 domain [48 and 50].

#### **Epigenetic Modifications in the KEAP1 Promoter**

Epigenetic modifications in KEAP1 have been shown to decrease the amount of KEAP1 protein, thus promoting NRF2 accumulation in humans [66]. For instance, the promoter region of the KEAP1 gene has been reported to be hypermethylated in human lung [67 and 68], prostate [10], colorectal cancers [69], and gliomas [68]. Moreover, in malignant gliomas, specific CpG island methylation within the KEAP1 promoter region has resulted in a decrease in KEAP1 protein expression by inducing local chromatin remodeling and restricting the ability of the transcriptional machinery to bind to DNA [68]. This hypermethylation appears to inhibit KEAP1 expression, which subsequently results in NRF2 accumulation. These epigenetic modifications have been associated with poor clinical prognosis in patients and may contribute to prediction of disease progression in glioma patients treated with current chemo/radiotherapy regimens [68]. Because deregulation of the NRF2/KEAP1 system has been linked to chemoresistance in several tumor types, the reversal of KEAP1 methylation could also represent a novel strategy to increase the sensitivity of glioma cells to a variety of anticancer drugs.

#### **Negative Regulation of NRF2/KEAP1 by miRNAs**

miRNAs are short, single-stranded, noncoding RNAs that regulate gene expression by sequence-specific binding to mRNA to either inhibit translation or promote mRNA degradation [70]. miR-144 was the first miRNA identified as a NRF2 negative regulator in reticulocytes of patients affected by severe sickle cell anemia, targeting two distinct sites in the NRF2 untranslated region, and decreasing the expression of NRF2 and its target genes such as superoxide dismutase 1, catalase, and glutamate-cysteine ligase subunits [71]. Similarly, miR-28 ectopic expression has been shown to decrease NRF2 mRNA and protein levels [72]. In human breast and liver cancer cells, KEAP1 has been reported to be negatively regulated by miR-200a which, in turn, indirectly induces increases in NRF2 levels [56 and 73] (Figure 4). Similar results have also been obtained in a mouse model of carcinogen-induced mammary hyperplasia in vivo where epigenetic restoration of miR-200a decreased KEAP1 expression, thereby reactivating the Nrf2-dependent antioxidant pathway [73]. All these studies demonstrate the existence of an additional level of complexity in the regulation of the NRF2/KEAP1 pathway.

#### **Aberrant Accumulation of Proteins Disrupting NRF2/KEAP1 Interactions**

From another angle, several studies have revealed that a wide variety of proteins can activate the NRF2 pathway by altering NRF2/KEAP1 binding [74 and 75] (Figure 4).

### *The NRF2 Pathway and p21*

p21 (or CIP1/WAF1) regulates different cellular processes such as cell-cycle arrest, DNA replication and repair, cell differentiation, senescence, and apoptosis [76, 77, 78 and 79]. In response to oxidative stress, p21 is upregulated and directly competes with KEAP1 for NRF2 binding; in fact, the increase in ROS has been found to lead to changes in KEAP1 conformation, loosening the 'latch' (DLG), and this allows p21 to compete with KEAP1 for binding to the DLG motif. Once the latch is open, KEAP1-dependent NRF2 ubiquitination is compromised and NRF2 is stabilized, as demonstrated in colon cancer cells *in vitro* [80]. Moreover, the role of p21 in regulating the NRF2 pathway has also been confirmed *in vivo* in p21-deficient mice which display reduced Nrf2 pathway activation (expression of downstream genes) and antioxidant responses compared to wild-type mice [80]. The same study revealed that the Nrf2-interacting region was localized to the C-terminal KRR motif of p21 [80]. Because this motif is also present in many other p21-interacting proteins [76, 77 and 81], it is conceivable that NRF2 might compete with them for p21 binding, complicating the interactions and regulation of the NRF2 pathway even further.

### *NRF2 Pathway and p62*

Several studies have demonstrated that sequestosome 1 protein (p62/SQSTM1) can modulate NRF2 activity [7, 82, 83 and 84]. p62 is a scaffold protein that binds to polyubiquitinated proteins and targets protein aggregates and damaged organelles for degradation via the autophagy pathway [85]. p62 contains a STGE-binding motif (similar to the NRF2 ETGE motif) [83] that directly interacts with the KEAP1 Kelch domain, thereby disrupting the NRF2-KEAP1 complex, as shown in several human cell lines [7, 82, 83 and 84]. *In vitro* experiments in these cell lines have shown that p62 can also bind to autophagy protein LC3, at the autophagosome membrane, thereby providing a link between NRF2-KEAP1 and the autophagy pathway [83 and 84]. Indeed, ectopic overexpression of p62 or blockade of the autophagosomal flux has been reported to sequester KEAP1 in autophagosomes via a direct interaction between KEAP1 and p62 [83 and 84]. This, in turn, results in decreased NRF2 ubiquitination, which increases its stability and activation, as shown in human hepatocytes and in autophagy-deficient mice [84]. Interestingly, liver-specific autophagy-deficient mice develop adenomas containing p62- and Keap1-positive cellular aggregates, and show increased expression of Nrf2 targets such as Nqo1 and Ho-1 [82, 86, 87 and 88]. Similar aggregates have been identified in more than 25% of human HCCs, together with high expression of NRF2 target genes [89]. Furthermore, p62 accumulation in rat pre-neoplastic liver lesions displaying Nrf2 activation has been recently observed [90]. Of note, ectopic expression of a null p62 in HCC cell lines has resulted in inhibited anchorage-independent cell growth, whereas p62-forced expression has resulted in rescued growth inhibition *in vitro* [89]. Collectively, these findings suggest that the accumulation of p62, and consequently the sustained activation of NRF2, can contribute to tumor development.

### **NRF2 Interaction with Other 'Cancer Master Players'**

The activity of NRF2 is mainly regulated at the protein level through ubiquitination and degradation. However, NRF2 transcription is increased by activated oncogenes, such as KRAS (G12D), BRAF (V619E), and c-MYC (ERT2) [42] (Figure 4). NRF2 augmented expression lowers intracellular ROS, resulting in a more reduced intracellular redox environment. Importantly, Nrf2 genetic targeting in mice can impair K-Ras(G12D)-induced tumorigenesis [42]. In 2014, Tao and colleagues demonstrated that KRAS increased NRF2 transcription via MEK-ERK signaling in several human cell lines and that NRF2 activation promoted drug resistance to cisplatin [91]. Moreover, in a mouse model of mutant KRAS(G12D)-induced lung cancer, brusatol-mediated Nrf2 inhibition enhanced the antitumor efficacy of cisplatin, thus reducing tumor burden and improving survival [91]. Collectively, these data provide strong evidence that NRF2 represents a novel mediator of oncogenesis, acting downstream of many well-known oncogenes.

### **Metabolism-Induced KEAP1 Modifications**

So far, only one example of metabolic-induced KEAP1 modification has been reported [92]. Fumarate is a Krebs cycle metabolite that is normally metabolized to malate by the enzyme fumarate hydratase (FH). Homozygous FH loss-of-function mutations cause the accumulation of high levels of fumarate in hereditary leiomyomatosis and type 2 papillary renal-cell carcinoma [93]. Analyzing the expression profiles of the human hereditary form of type 2 papillary renal-cell carcinoma, fumarate was shown to form adducts with KEAP1 and to activate the NRF2 pathway [94]. Mechanistic

analysis revealed that fumarate modifies cysteine residues within KEAP1, thus abrogating its ability to ubiquitinate NRF2 and causing prolonged NRF2 activation [92 and 94].

## Drugs Targeting NRF2

The activation of NRF2 by dietary compounds has been traditionally considered to prevent cancer development. For example, natural phytochemicals (including sulforaphane, curcumin, resveratrol, lycopene, and carnosol) have been reported to induce NRF2-mediated immune responses as well as acting as chemoprevention agents in different human and animal models [7 and 95].

With the aim of promoting beneficial NRF2 activity against cancer, several synthetic NRF2 activators have been developed. For instance, Oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione) has been shown to inhibit benzo[a]pyrene-induced carcinogenesis in a variety of organs in rat and mouse models [2, 96 and 97]. Given these promising preclinical results, Oltipraz has entered Phase I and II clinical trials in humans. In fact, in a Phase II clinical trial in China, Oltipraz reduced the levels of AFM1 (aflatoxin M1), a toxic hydroxylated metabolite of aflatoxin [98]. In another Phase II trial, Oltipraz did not result in significant decreases in the amount of polyaromatic hydrocarbon DNA adduct levels in lung epithelial cells or blood in tobacco smokers [99]. Because the available results are contradictory, further studies will be necessary to clearly define the effectiveness of NRF2 induction as a chemoprevention strategy.

Dimethyl fumarate (DMF, BG-12, or Tecfidera®) is another synthetic NRF2 activator that alkylates crucial cysteine residues on KEAP1, preventing NRF2 ubiquitination and promoting its stabilization in human cells in vitro and in mouse models of demyelination and neurodegeneration [100]. As a result of a Phase III clinical trial, an oral preparation of DMF has been approved by the FDA for the treatment of multiple sclerosis ([www.fda.gov](http://www.fda.gov)). Recent studies performed in human melanoma and glioblastoma cells have also shown that DMF may harbor potential anticancer properties [101].

Because a pro-tumorigenic role of NRF2 in cancer cells has been clearly shown, pharmacological inhibition of the NRF2 pathway would be of great interest. Unfortunately, no such inhibitors are available at present (Box 1). Several researchers have identified different NRF2 inhibitors, including phytochemicals such as luteolin [102], brusatol [103], procyanidin [104], apigenin [105], and chrysin [106], but currently none has yielded strong and indisputable results. For example, brusatol, a component of *Brucea javanica* seeds, seemed an attractive NRF2 inhibitor because it depletes NRF2 protein in A549 human lung adenocarcinoma cells, sensitizing them to chemotherapy in vitro and in vivo [103]. However, a recent mass-spectrometry study performed in the same cell line reported that brusatol was not a specific NRF2 inhibitor because it rapidly and strongly decreased the expression of a great number of proteins, especially those displaying a short half-life, suggesting that it may play a role as an inhibitor of the protein translation machinery [107]. However, the doses of brusatol used in this study [107] were much higher than those used in the study [103] reporting the specific inhibitory effect of brusatol on NRF2, coupled to an anti-tumorigenic effect. All-trans-retinoic acid (ATRA) has also been proposed as a specific NRF2 inhibitor [108]. In the presence of ATRA, NRF2 forms a complex with RAR $\alpha$ , and can no longer bind to ARE sequences, blocking activation of the pathway. For both brusatol and ATRA, further studies will be necessary to evaluate in depth whether the observed effects are strictly NRF2-dependent or if other mechanisms are involved.

### Box 1.

#### Clinician's Corner

Antioxidant treatments can produce opposite effects on tumor promotion and tumor progression.

NRF2 activation plays a chemopreventive role.

NRF2 inhibition can be advantageous in already established tumors and during chemo- and radiotherapy.

The border between NRF2 anti- and pro-tumorigenic activities is still not well defined. Thus, so far, there are no reliable preclinical or clinical data indicating effective therapeutic strategies.

Currently, no specific NRF2 inhibitors are available for therapeutic purposes. Few, poorly-specific NRF2 activators are being tested in clinical trials.



The identification of the co-crystal structure of the NRF2–KEAP1 complex [29] may provide an opportunity to study new molecules capable of interfering with the binding and activation of this pathway. Moreover, ChIP-seq data [109] have shown that different miRNAs can modulate NRF2 and KEAP1 expression [20, 56, 71, 72 and 73], providing additional putative targets to manipulate this pathway. Recently, the publication of the NRF2 interactome and regulome has allowed the identification of numerous proteins, including different kinases, that could be potentially involved in the regulation of this signaling cascade [110]. The interplay between converging pathways may also offer the opportunity of using pre-existing drugs, such as kinase inhibitors, to modulate the NRF2 pathway and, while striking a balance in NRF2 functions, potentially target cancer development and/or progression in various tissues.

## Concluding Remarks

It has become increasingly apparent that the transcription factor NRF2 is capable of functioning not only as a tumor suppressor but also as an oncogene (Box 1), and that components of its signaling pathway may be dysregulated in various types of cancer via several mechanisms, contexts, and levels. NRF2 pathway hyperactivation can help transformed/malignant cells to escape oxidative stress through the expression of antioxidant target genes or by directly promoting cell survival and proliferation. Moreover, NRF2 can play an important role in chemoresistance, preventing the intracellular accumulation of drugs in cancer cells and subsequently protecting the cells from apoptosis.

Based on the high frequency of tumors displaying aberrant NRF2 activation, including lung, hepatocellular, gall bladder, and ovarian carcinomas, NRF2 should be regarded as an important pharmacological target. Unfortunately, so far specific and effective NRF2 inhibitors are unavailable (see Outstanding Questions). It is evident that the therapeutic utility of inhibiting NRF2 depends on the molecular and clinical context, the type of cancer, disease stage, and on other factors that can contribute to NRF2 activation. Only the achievement of an ideal balance between the disease-preventing and the disease-promoting effects of NRF2 may provide a real benefit for cancer patients in the future. This task is highly ambitious but, nonetheless, is a task which researchers in the NRF2 cancer field should prioritize. Several recent advances in the understanding of NRF2 signaling in cancer have emerged, but it is clear that a great deal of work remains to be carried out to determine the specific mechanistic and functional underpinnings of the dual role of NRF2 in health and disease. Indeed, the elucidation of the specific antioxidant effects of NRF2 in different cancer types will be something to watch for.

How can we define the boundary between health benefits and side-effects of a diet rich in antioxidants? What might be the effect of a chemopreventive diet in a patient already displaying pre-neoplastic lesions?

Because at present there are no specific and effective inhibitors of NRF2, would it still be possible to pharmacologically target NRF2 crosstalk pathways to treat cancer? How can we exploit the role played by NRF2 in the modulation of genes involved in the antioxidant response and ROS detoxification, cell growth induction, regulation of apoptosis, promotion of cell survival and metastasis, autophagy, metabolism control, and chemoresistance? In turn, are these pathways important in the regulation of the NRF2 pathway?

Should we concentrate on designing inhibitors of NRF2 downstream effectors?—or, instead, on specific inhibitors that can selectively interfere with binding between NRF2 and its modulator KEAP1? Or on microRNAs that modulate the expression of NRF2 and KEAP1?

Why have some liver cancer studies reported mutations in NRF2 only, while others have reported mutations only in KEAP1? Could this be due in part to different etiologic factors of the examined cohorts (such as geographic origin)? How do these different mutations affect the NRF2/KEAP1 signaling pathway and are they necessarily redundant?

## Acknowledgments

This work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC), grants IG-15464 to S.G. and IG-15279 to A.C.

## References

1. H. Motohashi, M. Yamamoto. Nrf2–Keap1 defines a physiologically important stress response mechanism *Trends Mol. Med.*, 10 (2004), pp. 549–557
2. M. Ramos-Gomez, et al. Interactive effects of Nrf2 genotype and oltipraz on benzo[a]pyrene-DNA adducts and tumor yield in mice. *Carcinogenesis*, 24 (2003), pp. 461–467
3. K. Iida, et al. Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res.*, 64 (2004), pp. 6424–6431
4. H. Satoh, et al. Nrf2-deficiency creates a responsive microenvironment for metastasis to the lung. *Carcinogenesis*, 31 (2010), pp. 1833–1843
5. G. Rachakonda, et al. Increased cell migration and plasticity in Nrf2-deficient cancer cell lines. *Oncogene*, 29 (2010), pp. 3703–3714
6. J.D. Hayes, et al. Cancer chemoprevention mechanisms mediated through the Keap1–Nrf2 pathway. *Antioxid Redox Signal.*, 13 (2010), pp. 1713–1748
7. A. Lau, et al. Dual roles of Nrf2 in cancer. *Pharmacol. Res.*, 58 (2008), pp. 262–270
8. X.J. Wang, et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis*, 29 (2008), pp. 1235–1243
9. M.B. Sporn, K.T. Liby. NRF2 and cancer: the good, the bad and the importance of context. *Nat. Rev. Cancer*, 12 (2012), pp. 564–571
10. P. Zhang, et al. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Mol. Cancer Ther.*, 9 (2010), pp. 336–346
11. P. Moi, et al. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. U.S.A.*, 91 (1994), pp. 9926–9930
12. K. Chan, et al. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. U.S.A.*, 93 (1996), pp. 13943–13948
13. D.D. Zhang. Mechanistic studies of the Nrf2–Keap1 signaling pathway *Drug Metab. Rev.*, 38 (2006), pp. 769–789
14. K. Itoh, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.*, 236 (1997), pp. 313–322
15. M. Ramos-Gomez, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in Nrf2 transcription factor-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.*, 98 (2001), pp. 3410–3415
16. T. Iizuka, et al. Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes Cells*, 10 (2005), pp. 1113–1125

17. S.A. Reisman, et al. Altered disposition of acetaminophen in Nrf2-null and Keap1-knockdown mice. *Toxicol. Sci.*, 109 (2009), pp. 31–40
18. D.V. Chartoumpekis, et al. Keap1/Nrf2 pathway in the frontiers of cancer and non-cancer cell metabolism. *Biochem. Soc. Trans.*, 43 (2015), pp. 639–644
19. Y. Mitsuishi, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell*, 22 (2012), pp. 66–79
20. A. Singh, et al. Transcription factor NRF2 regulates miR-1 and miR-206 to drive tumorigenesis. *J. Clin. Invest.*, 123 (2013), pp. 2921–2934
21. S.K. Niture, A.K. Jaiswal. Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J. Biol. Chem.*, 287 (2012), pp. 9873–9886
22. S.K. Niture, A.K. Jaiswal. Nrf2-induced antiapoptotic Bcl-xL protein enhances cell survival and drug resistance. *Free Radic. Biol. Med.*, 57 (2013), pp. 119–131
23. A. Kobayashi, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell Biol.*, 24 (2004), pp. 7130–7139
24. K. Itoh, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.*, 13 (1999), pp. 76–86
25. A. Kobayashi, et al. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Mol. Cell Biol.*, 26 (2006), pp. 221–229
26. M. McMahon, et al. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J. Biol. Chem.*, 278 (2003), pp. 21592–21600
27. D.D. Zhang, M. Hannink. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell Biol.*, 23 (2003), pp. 8137–8151
28. D.D. Zhang, et al. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell Biol.*, 24 (2004), pp. 10941–10953
29. M. McMahon, et al. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a ‘tethering’ mechanism: a two-site interaction model for the Nrf2–Keap1 complex. *J. Biol. Chem.*, 281 (2006), pp. 24756–24768
30. K.I. Tong, et al. Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol. Cell Biol.*, 27 (2007), pp. 7511–7521
31. Y. Katoh, et al. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells*, 6 (2001), pp. 857–868
32. N. Wakabayashi, et al. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. U.S.A.*, 101 (2004), pp. 2040–2045

33. T. Yamamoto, et al. Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. *Mol. Cell Biol.*, 28 (2008), pp. 2758–2770
34. L.M. Zipper, R.T. Mulcahy. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J. Biol. Chem.*, 277 (2002), pp. 36544–36552
35. L. Baird, A.T. Dinkova-Kostova. The cytoprotective role of the Keap1–Nrf2 pathway. *Arch. Toxicol.*, 85 (2011), pp. 241–272
36. R. Saito, et al. Characterizations of three major cysteine sensors of Keap1 in stress response. *Mol. Cell Biol.*, 36 (2015), pp. 271–284
37. M. Kobayashi, M. Yamamoto. Nrf2–Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv. Enzyme Regul.*, 46 (2006), pp. 113–140
38. H.K. Bryan, et al. The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem. Pharmacol.*, 85 (2013), pp. 705–717
39. C. Xu, et al. Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer Res.*, 66 (2006), pp. 8293–8296
40. S. Homma, et al. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin. Cancer Res.*, 15 (2009), pp. 3423–3432
41. I. Gañán-Gómez, et al. Oncogenic functions of the transcription factor Nrf2. *Free Radic. Biol. Med.*, 65 (2013), pp. 750–764
42. G.M. DeNicola, et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature*, 475 (2011), pp. 106–109
43. B. Padmanabhan, et al. Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol. Cell*, 21 (2006), pp. 689–700
44. A. Singh, et al. Dysfunctional KEAP1–NRF2 interaction in non-small-cell lung cancer. *PLoS Med.*, 3 (2006), p. e420
45. T. Ohta, et al.. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res.*, 68 (2008), pp. 1303–1309
46. L.M. Solis, et al. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. *Clin. Cancer Res.*, 16 (2010), pp. 3743–3753
47. T. Takahashi, et al. Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. *J. Surg. Oncol.*, 101 (2010), pp. 500–506
48. T. Shibata, et al. Cancer related mutations in NRF2 impair its recognition by Keap1–Cul3 E3 ligase and promote malignancy. *Proc. Natl. Acad. Sci. U.S.A.*, 105 (2008), pp. 13568–13573
49. D. Siegel, et al. Immunohistochemical detection of NAD(P)H:quinone oxidoreductase in human lung and lung tumors. *Clin. Cancer Res.*, 4 (1998), pp. 2065–2070

50. Y.R. Kim, et al. Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J. Pathol.*, 220 (2010), pp. 446–451
51. C. Guichard, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat. Genet.*, 44 (2012), pp. 694–698
52. A. Fujimoto, et al. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat. Genet.*, 48 (2016), pp. 500–509
53. S.P. Cleary, et al. Identification of driver genes in hepatocellular carcinoma by exome sequencing. *Hepatology*, 58 (2013), pp. 1693–1702
54. C. Coulouarn, et al. Genomic modeling of tumor onset and progression in a mouse model of aggressive human liver cancer. *Carcinogenesis*, 32 (2011), pp. 1434–1440
55. R. Luisier, et al. Phenobarbital induces cell cycle transcriptional responses in mouse liver humanized for constitutive androstane and pregnane X receptors. *Toxicol. Sci.*, 139 (2014), pp. 501–511
56. A. Petrelli, et al. MicroRNA/gene profiling unveils early molecular changes and nuclear factor erythroid related factor 2 (NRF2) activation in a rat model recapitulating human hepatocellular carcinoma (HCC). *Hepatology*, 59 (2014), pp. 228–241
57. P. Zavattari, et al. Nrf2, but not  $\beta$ -catenin, mutation represents an early event in rat hepatocarcinogenesis. *Hepatology*, 62 (2015), pp. 851–862
58. M. Kowalik, et al. Metabolic reprogramming identifies the most aggressive lesions at early phases of hepatic carcinogenesis. *Oncotarget* (2016) Published online April 7, 2016. <http://dx.doi.org/10.18632/oncotarget.8632>
59. K. Taguchi, et al. Generation of a new model rat: Nrf2 knockout rats are sensitive to aflatoxin B1 toxicity. *Toxicol. Sci.* (2016) Published online April 12, 2016 <http://dx.doi.org/10.1093/toxsci/kfw065>
60. J.R. Priestley, et al. The NRF2 knockout rat: a new animal model to study endothelial dysfunction, oxidant stress, and microvascular rarefaction. *Am. J. Physiol. Heart Circ. Physiol.*, 310 (2016), pp. H478–H487
61. T. Sjöblom, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*, 314 (2006), pp. 268–274
62. P. Nioi, T. Nguyen. A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. *Biochem. Biophys. Res. Commun.*, 362 (2007), pp. 816–821
63. N.J. Yoo, et al. Somatic mutations of the KEAP1 gene in common solid cancers. *Histopathology*, 60 (2012), pp. 943–952
64. T. Shibata, et al. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology*, 135 (2008), pp. 1358–1368 [1368.e1351-1354](https://doi.org/10.1053/j.gastro.2008.03.054)
65. P.A. Konstantinopoulos, et al. Keap1 mutations and Nrf2 pathway activation in epithelial ovarian cancer. *Cancer Res.*, 71 (2011), pp. 5081–5089

66. D. Wang, et al. Hypermethylation of the Keap1 gene inactivates its function, promotes Nrf2 nuclear accumulation, and is involved in arsenite-induced human keratinocyte transformation. *Free Radic. Biol. Med.*, 89 (2015), pp. 209–219
67. R. Wang, et al. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem. Biophys. Res. Commun.*, 373 (2008), pp. 151–154
68. L.A. Muscarella, et al. Frequent epigenetics inactivation of KEAP1 gene in non-small cell lung cancer. *Epigenetics*, 6 (2011), pp. 710–719
69. N. Hanada, et al. Methylation of the KEAP1 gene promoter region in human colorectal cancer. *BMC Cancer*, 12 (2012), p. 66
70. D.P. Bartel. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116 (2004), pp. 281–297
71. C. Sangokoya, et al. microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood*, 116 (2010), pp. 4338–4348
72. M. Yang, et al. MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. *Breast Cancer Res. Treat.*, 129 (2011), pp. 983–991
73. G. Eades, et al. miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *J. Biol. Chem.*, 286 (2011), pp. 40725–40733
74. M.S. Yates, et al. Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid-treated mice. *Carcinogenesis*, 30 (2009), pp. 1024–1031
75. N. Wakabayashi, et al. When NRF2 talks, who's listening? *Antioxid Redox Signal.*, 13 (2010), pp. 1649–1663
76. G.P. Dotto. p21(WAF1/Cip1): more than a break to the cell cycle? *Biochim. Biophys. Acta*, 1471 (2000), pp. M43–M56
77. A.L. Gartel, A.L. Tyner. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol. Cancer Ther.*, 1 (2002), pp. 639–649
78. R. Li, et al. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature*, 371 (1994), pp. 534–537
79. M.A. O'Reilly. Redox activation of p21Cip1/WAF1/Sdi1: a multifunctional regulator of cell survival and death. *Antioxid Redox Signal.*, 7 (2005), pp. 108–118
80. W. Chen, et al. Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Mol. Cell*, 34 (2009), pp. 663–673
81. E.S. Child, D.J. Mann. The intricacies of p21 phosphorylation: protein/protein interactions, subcellular localization and stability. *Cell Cycle*, 5 (2006), pp. 1313–1319
82. I.M. Copple. The Keap1–Nrf2 cell defense pathway—a promising therapeutic target? *Adv. Pharmacol.*, 63 (2012), pp. 43–79



83. A. Jain, et al. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.*, 285 (2010), pp. 22576–22591
84. M. Komatsu, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell Biol.*, 12 (2010), pp. 213–223
85. Y. Katsuragi, et al. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J.*, 282 (2015), pp. 4672–4678
86. K. Taguchi, et al. Keap1 degradation by autophagy for the maintenance of redox homeostasis. *Proc. Natl. Acad. Sci. U.S.A.*, 109 (2012), pp. 13561–13566
87. A. Lau, et al. A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol. Cell Biol.*, 30 (2010), pp. 3275–3285
88. A. Lau, et al. Arsenic inhibits autophagic flux, activating the Nrf2–Keap1 pathway in a p62-dependent manner. *Mol. Cell Biol.*, 33 (2013), pp. 2436–2446
89. Y. Inami, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J. Cell Biol.*, 193 (2011), pp. 275–284
90. M.A. Kowalik, et al. Induction of autophagy promotes the growth of early preneoplastic rat liver nodules. *Oncotarget*, 7 (2016), pp. 5788–5799
91. S. Tao, et al. Oncogenic KRAS confers chemoresistance by upregulating NRF2. *Cancer Res.*, 74 (2014), pp. 7430–7441
92. J. Adam, et al. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell*, 20 (2011), pp. 524–537
93. C. Eng, et al. A role for mitochondrial enzymes in inherited neoplasia and beyond. *Nat. Rev. Cancer*, 3 (2003), pp. 193–202
94. A. Ooi, et al. An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. *Cancer Cell*, 20 (2011), pp. 511–523
95. T.W. Kensler, N. Wakabayashi. Nrf2: friend or foe for chemoprevention? *Carcinogenesis*, 31 (2010), pp. 90–99
96. C.V. Rao, et al. Chemopreventive effect of oltipraz during different stages of experimental colon carcinogenesis induced by azoxymethane in male F344 rats. *Cancer Res.*, 53 (1993), pp. 2502–2506
97. S. Sharma, et al. The chemopreventive efficacy of inhaled oltipraz particulates in the B[a]P-induced A/J mouse lung adenoma model. *Carcinogenesis*, 27 (2006), pp. 1721–1727
98. L.P. Jacobson, et al. Oltipraz chemoprevention trial in Qidong, People's Republic of China: study design and clinical outcomes. *Cancer Epidemiol. Biomarkers Prev.*, 6 (1997), pp. 257–265
99. M.J. Kelley, et al. Safety and efficacy of weekly oral oltipraz in chronic smokers. *Cancer Epidemiol. Biomarkers Prev.*, 14 (2005), pp. 892–899

100. J.T. Phillips, R.J. Fox. BG-12 in multiple sclerosis. *Semin. Neurol.*, 33 (2013), pp. 56–65
101. A.F. Chen, R.S. Kirsner. Mechanisms of drug action: the potential of dimethylfumarate for the treatment of neoplasms. *J. Invest. Dermatol.*, 131 (2011), p. 1181
102. X. Tang, et al. Luteolin inhibits Nrf2 leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs. *Free Radic. Biol. Med.*, 50 (2011), pp. 1599–1609
103. D. Ren, et al. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc. Natl. Acad. Sci. U.S.A.*, 108 (2011), pp. 1433–1438
104. T. Ohnuma, et al. Enhanced sensitivity of A549 cells to the cytotoxic action of anticancer drugs via suppression of Nrf2 by procyanidins from Cinnamomi cortex extract. *Biochem. Biophys. Res. Commun.*, 413 (2011), pp. 623–629
105. A.M. Gao, et al. Apigenin sensitizes doxorubicin-resistant hepatocellular carcinoma BEL-7402/ADM cells to doxorubicin via inhibiting PI3K/Akt/Nrf2 pathway. *Carcinogenesis*, 34 (2013), pp. 1806–1814
106. A.M. Gao, et al. Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway. *Chem. Biol. Interact.*, 206 (2013), pp. 100–108
107. S. Vartanian, et al. Application of mass spectrometry profiling to establish brusatol as an inhibitor of global protein synthesis. *Mol. Cell Proteomics*, 15 (2016), pp. 1220–1231
108. S. Magesh, et al. Small molecule modulators of Keap1–Nrf2–ARE pathway as potential preventive and therapeutic agents. *Med. Res. Rev.*, 32 (2012), pp. 687–726
109. B.N. Chorley, et al. Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. *Nucleic. Acids Res.*, 40 (2012), pp. 7416–7429
110. D. Papp, et al. The NRF2-related interactome and regulome contain multifunctional proteins and fine-tuned autoregulatory loops. *FEBS Lett*, 586 (2012), pp. 1795–1802

## Glossary

### *Aflatoxin*

a carcinogenic mycotoxin produced by fungi (mainly *Aspergillus*) that causes mutations through binding of its metabolites to DNA.

### *All-trans retinoic acid (ATRA)*

nutrient generated from vitamin A that helps cells to grow and develop, especially in the embryo. ATRA binds to and activates retinoic acid receptors (RARs), thereby inducing changes in gene expression that lead to cell differentiation and decreased cell proliferation.

### *Autophagy*

natural, destructive mechanism that disassembles, through a regulated process, unnecessary or dysfunctional cellular components. Autophagy allows the orderly degradation and recycling of cellular components.

### *BRAF*

B-Raf encodes a serine/threonine protein kinase that is part of the RAS–MAPK signaling cascade.

### *Cancer chemoprevention*

the use of natural, synthetic, or biological substances, food supplements, or other agents in the diet to inhibit the development or progression of malignant changes in cells.

### *CpG islands*

short stretches of DNA in which the frequency of the CG sequence is higher than in other regions. The 'p' in CpG indicates that 'C' and 'G' are connected by a phosphodiester bond.

### *Histone deacetylase 4 (HDAC4)*

represses transcription when tethered to a promoter and plays a key role in transcriptional regulation, cell cycle progression, and developmental events.

### *JUN/MYC*

c-JUN, in combination with c-FOS, forms the AP-1 early response transcription factor. MYC is a transcription factor that activates expression of many genes through binding enhancer box sequences (E-boxes) and recruiting histone acetyltransferases (HATs).

### *KRAS*

a GTPase that recruits and activates proteins, such as c-RAF and PI3K, that are necessary for the propagation of receptor signals.

### *Oxidative stress*

an imbalance between the generation of reactive oxygen metabolites (free radicals) and antioxidant defense mechanisms.

### *Pentose phosphate pathway*

a metabolic pathway parallel to glycolysis that generates NADPH and pentoses as well as ribose 5-phosphate, a precursor of nucleotide synthesis.

### *Phase I/II/III detoxifying enzymes*

enzymes engaged in biotransformation (through conjugating reactions of endogenous compounds and xenobiotics to more easily excretable forms), as well as in the metabolic inactivation of pharmacologically active substances.

### *Phosphatidylinositol-3 kinase (PI3K)–AKT*

the signaling network defined by PI3K, AKT, and the mechanistic target of rapamycin (mTOR) which controls most hallmarks of cancer. The pathway also contributes to cancer-promoting aspects of the tumor environment such as angiogenesis and inflammatory cell recruitment.

### *Protein kinase C (PKC)*

a family of protein kinase enzymes involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on those proteins.

### *Reticulocytes*

immature red blood cells (typically 1% of red blood cells in humans) that still harbor some RNAs and mitochondria.

### *Sickle cell anemia*

a blood disorder due to a genetic alteration of hemoglobin. This leads to rigid, sickle-like shaped dysfunctional erythrocytes.

### *Xenobiotics*

foreign chemicals or natural compounds that include carcinogens, drugs, drug metabolites, and environmental compounds such as pollutants, synthetic pesticides, and herbicides.

### *Warburg effect*

the phenomenon by which most cancer cells predominantly produce energy by a high rate of glycolysis instead of by mitochondrial pyruvate oxidation as in most normal cells.