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NRF2 and cancer: the good, the bad and the importance of context

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Abstract

Many studies of chemopreventive drugs have suggested that their beneficial effects on suppression of carcinogenesis and many other chronic diseases are mediated through activation of the transcription factor NFE2- related factor 2 (NRF2). More recently, genetic analyses of human tumours have indicated that NRF2 may conversely be oncogenic and cause resistance to chemotherapy. It is therefore controversial whether the activation, or alternatively the inhibition, of NRF2 is a useful strategy for the prevention or treatment of cancer. This Opinion article aims to rationalize these conflicting perspectives by critiquing the context dependence of NRF2 functions and the experimental methods behind these conflicting data.

NFE2-related factor 2 (NRF2) is a transcription factor that integrates cellular stress signals and responds by directing various transcriptional programmes. NRF2 was a somewhat esoteric protein little more than 10 years ago, when only a limited number of investigators were studying its protective roles in suppressing oxidative or electrophilic stress and inhibiting carcinogenesis (1–4), but more recently NRF2 has become the subject of widespread interest and investigation. This regulatory protein and its own negative regulator, Kelch-like ECH-associated protein 1 (KEAP1), have stimulated many publications and have become the topic of an important controversy. The controversy is centred on whether NRF2 is tumour suppressive or, conversely, oncogenic, leading to the question of whether NRF2 should be targeted for anticancer therapeutic approaches (5).

There are strong opinions that further pharmacological development of drugs that enhance NRF2 activity should be pursued for preventing not only cancer but also many other diseases in which oxidative and inflammatory stress are crucial for pathogenesis (6–8). Indeed, many new drugs that activate NRF2 (in addition to other targets) are now in clinical trials for numerous indications. These drugs include sulphoraphane (9) and curcumin (10) (for the prevention of cancer), dimethyl fumarate (11,12) (for the treatment of multiple sclerosis), bardoxolone methyl13 (for the treatment of diabetic nephropathy) and resveratrol (14) (for multiple indications). However, recent genetic analyses have shown that mutations in *NRF2* or *KEAP1* are found in some cancers; these mutations enhance NRF2 activity and are associated with resistance to standard chemotherapy and poor survival from cancer (15–17).

NRF2 cellular functions

Under basal conditions NRF2 is kept transcriptionally inactive through binding to its inhibitor, KEAP1, which targets NRF2 for proteasomal degradation. A third protein in this complex is the cullin 3 (CUL3) ubiquitin ligase, which directs this degradation. The fine structure of this complex and its molecular and physiological regulation have been studied in

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great detail (16,18) and will not be discussed at length here. Instead, in the following sections we focus on the functions of NRF2 that are most pertinent for its roles in cancer.

Stress sensing through modifications of NRF2–KEAP1

The NRF2–KEAP1 module is of primary importance in maintaining the homeostatic milieu because cells need to respond adaptively to many types of stress. Cells have incorporated highly toxic molecules into physiological signalling systems. These molecules include reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), and reactive nitrogen species (RNS), such as nitric oxide (NO). Low concentrations of these potentially toxic molecules are used for adaptive intracellular signalling, and higher concentrations are used for self-defence against microorganisms (22). However, physiological concentrations of molecules such as H2O2 and NO need to be tightly regulated, and NRF2 plays a crucial part in this process.

KEAP1 has more than 20 free sulphhydryl (-SH) groups in its constituent cysteine residues. These highly reactive functional groups act as stress sensors. Various oxidative or electrophilic cellular stresses, including ROS and RNS, modify KEAP1 cysteine residues (16,18,23,24). These modifications (which include adduct formation) result in a conformational change of KEAP1, thereby reversing the proteasomal degradation of NRF2, which then becomes transcriptionally active [FIG. 1]. The NRF2–KEAP1 module is part of an entire network of proteins (the thiol proteome) (25) whose activity is regulated through modifications of cysteine residues in response to the cellular redox state. The reactivity of these cysteine residues can be modulated not only by redox reactions (26) but also by NO (*S*-nitrosylation) (27) or guanine (*S*-guanylation). Specific cysteine residues are thus thiol-based cellular switches that regulate the activity of their respective proteins (25–28) to provide a link between the cellular redox state and important cell fate decisions. Classic examples are the multiple protein tyrosine phosphatases, all of which contain reactive cysteine residues in their active sites (29) and are crucial determinants of many aspects of cell physiology, differentiation and proliferation.

Different stressors may react differentially with various cysteine residues in KEAP1, suggesting that specific cysteine residues, individually or in combination, contribute to the overall activity of KEAP1 in a unique manner (23,24). This fine-tuning, called the ‘cysteine code’, indicates that the NRF2–KEAP1 module is not a simple ‘on’ or ‘off’ switch but can instead respond differentially to distinct patterns of adduct formation by various stressors (16,23,24). Other modifications also regulate NRF2 and its interaction with KEAP1. These proteins can be phosphorylated (30,31) or acetylated (32,33), and KEAP1 can be modified by succination under pathological conditions as discussed below (34); thus, NRF2–KEAP1 interacts with multiple cellular networks. How-ever, the *in vivo* importance of all of these additional modifications is not yet totally clear.

NRF2 effector functions

There are more than 100 genes that are regulated by NRF2. NRF2 binds to response elements on DNA, known as antioxidant response elements (AREs) or electrophile response elements (EpREs) (16,18), and regulates the expression of genes involved in the response to cellular stress. For example, NRF2 can reduce RNS and ROS levels by directly controlling the enzymatic formation of such molecules (in the case of NO by suppressing the expression of inducible nitric oxide synthase (iNOS; also known as NOS2) (35), or by its ability to induce the expression of enzymes, such as catalase, that destroy H2O2 (REF. 18). Furthermore, cells need protection from toxic xenobiotic molecules, and NRF2 again has a major role here, especially through its potent induction of glutathione, the primary cellular scavenger of electrophiles, as well as by its induction of enzymes of glucuronidation, which

conjugate xenobiotics for excretion18. Indeed, there are more than 20 such cytoprotective 'phase 2' enzymes that are upregulated by NRF2. NRF2 can also influence drug transport through the induction of the multidrug resistance-associated gene family (18). Thus, NRF2 can transduce aspects of cellular stress into adaptive, cytoprotective responses.

Moreover, it appears that NRF2 may also regulate many genes other than classic cyto-protective ones, particularly with respect to cell differentiation and proliferation. Thus, newborn *Keap1*-knockout mice (in which *Nrf2* is constitutively overexpressed) die from hyperplastic keratinization of the oesophagus and forestomach, which obstructively limits intake of food; this lethality can be reversed by simultaneous knockout of *Nrf2* (REF. 36). Recently there have been reports of the effects of NRF2 on differentiation or proliferation of stem cells in the bone marrow (37) or intestine (38). Indeed, NRF2 enhances signalling by the multifunctional transcription factor NOTCH1, which is a known regulator of differentiation (39); there are ARE sites on the *NOTCH1* promoter. Thus, NRF2 is a multifunctional transcription factor that induces a broad range of biological responses upon its activation.

Beyond this immediate homeostatic response, NRF2 activity has longer-term consequences, which have been described in a recent review (40). Accordingly, there is now an extensive literature describing functional connections between NRF2 and signalling pathways involving nuclear factor-κB (NF-κB) (41,42), p53 (REFS 43,44), aryl hydrocarbon hydroxylase receptor (AhR) (45), mTOR (46), heat shock proteins (47), activator protein 1 (AP1) (48) and NOTCH1 (REF. 39). Thus, NRF2 is able to modulate many cellular activities beyond its immediate homeostatic, cytoprotective role and to influence processes as diverse as inflammation, proliferation, apoptosis, cell differentiation, tissue regeneration and even metabolism.

The diverse network of potential activities of NRF2 provides a helpful background to the present controversy surrounding the protein, and this should be borne in mind when the 'good' and 'bad' functions of NRF2 are discussed in the ensuing sections. The capacity of cells to deal with stress is fundamental to life, and KEAP1 and NRF2 are at the core of this process, especially as they stabilize the thiol proteome of the cell.

Tumour suppressor functions of NRF2

There is abundant evidence that activation of NRF2 can suppress carcinogenesis, especially in its earliest stages. This topic has been extensively reviewed (5,18,19); thus we only provide brief details here. Suppression of carcinogenesis has been demonstrated in several experimental designs, either by showing that many drugs (as well as genetic alterations) that enhance the activity of NRF2 inhibit carcinogenesis, or by showing that genetic deletion of *Nrf2* enhances susceptibility to development of cancer. In particular, the anti-carcinogenic activity of chemopreventive drugs (many of which activate NRF2) has been shown to be either abolished or markedly decreased in *Nrf2*-null mice.

Chemopreventives signal through NRF2 and other proteins

Chemically diverse chemopreventive drugs have been used in studies of NRF2, including sulphoraphane, phenethyl isothiocyanate, oltipraz, curcumin, resveratrol, fumaric acid and its esters, and synthetic oleanane triterpenoids. Because many of these molecules are natural products that occur in food, it has been relatively easy to obtain acceptance by patients for their use in clinical trials of cancer prevention, although approval by regulatory agencies may still be required. As would be expected of NRF2 activators, all have been shown to induce multiple cytoprotective and antioxidative enzymes (phase 2 response), and many are

anti-inflammatory as well; why such responses might be tumour preventive in these settings is discussed later.

A common property of these compounds is their ability to react with cysteine residues on target proteins, most notably KEAP1, and this thiol reactivity has been correlated with their ability to induce activity of NRF2. However, these drugs can target cysteines in multiple proteins: for example, sulphoraphane targets NF-κB (49) as well as both JUN and FOS of the AP1 complex (50), and proteomic and other analyses have shown that synthetic oleanane triterpenoids target a range of proteins containing reactive cysteines (51). An additional layer of complexity is that the reactivity of cysteine thiols is highly variable within proteins and among proteins because it depends on the immediate context of neighbouring amino acid residues; adjacent lysine and arginine residues enhance the formation of the reactive thiolate (S−) anion in cysteine. Thus, different concentrations of any chemopreventive drug are expected to react with different subsets of cysteines in KEAP1 and other proteins to produce distinct biological responses (16,23,24).

Chemopreventive activity of NRF2 activators

As just two examples of NRF2 activators with chemopreventive activity, sulphoraphane (a natural product) and synthetic oleanane triterpenoids have been used extensively in mouse models of cancer. Sulphoraphane inhibits carcinogenesis at multiple organ sites, including skin, lung, bladder, breast, colon and stomach (5,18,19,52–57). In many studies, the beneficial effects were obtained by administering sulphoraphane during the promotion or progression phase of carcinogenesis; in the case of suppression of carcinogenesis by sulphoraphane in the *Apc*Min mouse (which expresses a truncated, non-functional form of the adenomatous polyposis coli protein), the benefit is clearly that of altering the further consequences of the expression of a genetic lesion (53,56). Human cancer chemoprevention studies with sulphoraphane-rich extracts of broccoli are currently being pursued in China (9,58).

Similarly, synthetic oleanane triterpenoids suppress the promotion and/or progression of cancers of the lung, breast, skin and pancreas (59,60). In these studies, drugs were not acting by minimizing DNA damage associated with initiation of carcinogenesis because they were not given until after mutation of DNA by a chemical carcinogen had occurred, or they were used in transgenic mouse models (59–61). In prevention studies using genetic models of carcinogenesis, synthetic oleanane triterpenoids have delayed the onset of tumorigenesis driven by oncogenes as diverse as *Kras*, *Trp53*, *Brca1* and *Erbb2* (also known as *Her2* or *Neu*) in organs such as the pancreas or breast (61,62). There has been particularly strong suppression of lung carcinogenesis by synthetic oleanane triterpenoids in a model in which *Kras* activity or mutations are induced by the carcinogen vinyl carbamate (59,60).

Tumour suppression and *Nrf2* mouse models

Because chemopreventive drugs have multiple cellular targets, a key tool for determining whether their tumour-suppressive effects are NRF2-dependent is the use of *Nrf2*-knockout mice (18,19), which were originally developed by Yamamoto and colleagues (1). Thus, chemoprevention by oltipraz was greatly diminished in *Nrf2*-knockout mice (4,63), and many similar studies in *Nrf2*-knockout mice have been carried out with agents that protect against skin carcinogenesis induced by ultraviolet light or chemicals (18,19,64). Likewise, the ability of the triterpenoid CDDO-imidazolide to protect against aflatoxin-induced liver carcinogenesis appears to depend on its ability to induce NRF2-dependent cytoprotective enzymes, as such enzymes cannot be induced in *Nrf2*-knockout mice (65).

Knockout mice have also shown a tumour-suppressive activity of NRF2 outside the setting of chemoprevention. The susceptibility to carcinogenesis — induced by polycyclic hydrocarbons in the forestomach and skin, by inflammation in the colon or by a nitrosamine carcinogen in the bladder — is markedly increased in *Nrf2*-knockout mice (4,63,64,66). Additionally, mice with *Nrf2* overexpression resulting from *Keap1* knockout have been shown to have increased resistance to cancer cell metastasis to their lungs (67).

The importance of NRF2 activation for the action of chemopreventive agents has been reinforced by multiple studies using many of the same agents for protection from diseases other than cancer [BOX 1]. Such widespread protective effects have created strong incentives to develop new potent enhancers of NRF2 activity for the prevention and treatment of many diseases (several of which are presently incurable) in which both inflammatory and oxidative stress have a key pathogenic role. However, within the past 5 years there have been many new reports of the oncogenic activity of NRF2. These reports have led to increasing concerns about the safety of a long-term increase in NRF2 activity.

Oncogenic functions of NRF2

Cancer-associated activation of NRF2

As NRF2 promotes cell survival under stress, it is logical to argue that increased NRF2 activity could be tumour promoting by being protective for cancer cells. Indeed, the Yamamoto laboratory and others have identified cancer-associated mutations that activate NRF2 [TABLE 1], although they occur in a much smaller percentage of cancers than common mutations such as *KRAS* or *TP53*.

Gain-of-function mutations in *NRF2* are found mostly in squamous cell carcinomas of the oesophagus, skin, lung and larynx (68). The mutant proteins typically retain their transcriptional activity but have lost their KEAP1-binding capacity, meaning that they overcome KEAP1-mediated inhibition and KEAP1-triggered ubiquitylation. Using immunochemistry, an increase in NRF2 levels in tumour nuclei has been shown in tumour cells with *NRF2* mutations, together with an increase in the expression of cyto-protective enzymes (69). Loss-of-function mutations in human *KEAP1* have been found in carcinomas of the lung (69–73), gallbladder (74), ovary (75), breast (76,77), liver (73) and stomach (73); these mutations result in constitutive NRF2 activity. Additionally, *KEAP1* mutations may have oncogenic roles beyond NRF2 activation, such as dysfunctional binding of KEAP1 to other proteins that regulate proliferation and apoptosis. As an example, under basal conditions, wild-type KEAP1 binds to inhibitor of NF-κB kinase (IKK), which is the kinase that leads to the activation of NF-κB. Binding to KEAP1 enhances proteasomal degradation of IKK, which in turn diminishes the pro-inflammatory and pro-survival activity of the NF-κB pathway. This inhibition of the pro-tumorigenic transcriptional activity of NF-κB is lost when *KEAP1* is mutated (41,42).

NRF2 activation, clinical outcomes and chemotherapy resistance

Although the frequencies of *NRF2* and *KEAP1* mutations in tumours are often low, other contributing mechanisms — such as epigenetic hypermethylation of the *KEAP1* or *NRF2* promoters (78,79) — have been found, and disruptions of *KEAP1* and *NRF2* expression levels are frequently observed in cancer. Clinically, it has been shown that decreased expression of KEAP1 and increased expression of NRF2 may be associated with poor prognosis. In an extensive study of 304 lung cancer specimens (69), immunohistochemical analysis of non-small-cell lung cancers showed abnormally high expression of NRF2 in the entire range of carcinomas in many patients, whereas relatively low expression of KEAP1 was only seen in patients with adenocarcinomas. Both abnormalities could be correlated with poor prognosis, measured either as recurrence-free or overall 5-year survival (69).

Furthermore, this lung cancer study suggested that nuclear expression of NRF2 may play a part in resistance to platinum-based chemotherapy of squamous cell carcinoma.

Similar findings have been made in a recent study of 30 patients with epithelial ovarian carcinoma (75), which showed that NRF2 staining in tumour cell nuclei was found in more than half of the patients (by contrast, no staining was seen in control normal ovarian epithelium), with associated upregulation of NRF2-dependent genes. Furthermore, mutation of *KEAP1* or absence of *KEAP1* mRNA expression was found in almost half of the tumour specimens that were positive for NRF2 staining. Most importantly, this study suggested that high levels of NRF2 expression could be correlated with a relative resistance to platinum-based chemotherapy and with an inferior survival rate (75).

Thus, there are serious clinical concerns about the adverse effects of enhanced NRF2 activity with respect to drugs used in cancer chemotherapy. Beyond the studies on clinical material, there have been extensive studies in cancer cell lines and in experimental animals, which have documented the ability of NRF2 to enhance drug resistance. These studies have included a diverse range of drugs, such as cisplatin, carboplatin, 5-fluorouracil, paclitaxel, bleomycin, doxorubicin and etoposide (74,80,81). It is clearly a serious problem, and many articles on this topic have concluded with the suggestion that the development of new inhibitors of NRF2 should be considered as a novel approach to the treatment of carcinomas (71,80–83).

The effector functions of NRF2 might cause chemotherapy resistance by several mechanisms: through suppression of the oxidative stress that can play an important part in the cytotoxic effects of chemotherapy; through drug detoxification by glutathione and other conjugating mechanisms; and through stimulation of ATP-dependent drug efflux pumps such as the multidrug resistance system, which again would lower effective drug levels.

NRF2 may promote tumorigenesis through stress protection

An important new concern is the finding that common oncogenes, such as *KRAS*, *BRAF* and *MYC*, all increase the transcription and activity of NRF2, resulting in an increase in cytoprotective activity in the cell and, most notably, a decrease in ROS levels (84). Thus, oncogenes may promote tumorigenesis in part through an NRF2-dependent creation of a more favourable intracellular environment for the survival of tumour cells. Support for this contention was obtained in mouse models of pancreatic cancer, as well as in human pre-invasive pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDA) (84). Elevated protein expression of the NRF2 target gene NAD(P)H:quinone oxidoreductase 1 (*Nqo1*) and decreased immunoreactivity of 8-oxo-deoxyguanosine (a marker for DNA mutation) were found in KRAS-mutant murine and human PanIN and PDA.

One question still remains in interpreting these data: does an increase in NRF2 levels directly promote tumorigenesis, or are increased NRF2 levels a response to other, more fundamental stressful changes induced in cells by oncogenes? The finding that KRAS and BRAF stimulate transcription of *Nrf2* by elevating levels of JUN, which in turn binds to known start sites for transcription of *Nrf2*, indicates that some of these effects are direct (84). These studies again raise important issues on the complex roles of ROS in cancer: mutagenic ROS may be involved during carcinogenesis in promoting and maintaining the oncogenic phenotype, suggesting that ROS levels should be suppressed for the prevention of cancer (5,18). Conversely, drugs that increase ROS production and activity might be useful chemotherapy agents by further increasing oxidative stress and thus killing cancer cells (85,86). Understanding the context of action and the dose–response to ROS would appear to be critical for practical drug development in this area.

Can the paradox be resolved?

How can we resolve the paradox that although high expression of NRF2 in cancer cells has poor prognostic implications for a patient, drugs and herbal agents that enhance activation of NRF2 are safely used worldwide to improve human health and even to try to delay the process of ageing? There is little, if any, evidence that these chemopreventive drugs themselves are carcinogenic; in fact, many are potent and safe agents for the suppression of carcinogenesis in mouse models of cancer.

The simplest answer is that it is all a matter of context, particularly the different experimental contexts in which genetic or pharmacological data are obtained, and how these data are interpreted. Thus, genetic studies leave little doubt that *NRF2* or *KEAP1* may be mutated in human cancers and that the resultant increase in NRF2 activity can lead to resistance to classic cytotoxic chemotherapeutic agents. Likewise, genetic approaches have been used to demonstrate that KRAS, BRAF and MYC increase transcription and activity of *Nrf2* and its cytoprotective action. If enhancement of oxidative stress represents an important therapeutic approach to cancer, then there is good reason to suggest that we should consider blocking NRF2 activity in fully malignant cells and thereby increase oxidative stress (85,86), contrary to the NRF2-activating effects of the chemopreventive agents discussed above.

Roles of NRF2 may depend on the stage of tumorigenesis

Fully malignant cells, which are characterized by their autonomy, are very different from dysplastic (but not yet fully neoplastic) cells in a premalignant lesion. Premalignant cells are under much greater control from inflammatory cells and other stromal cells in their microenvironment and, moreover, they have not yet reached a level of DNA damage that makes them autonomous. Therefore, enhancement of NRF2 activity, which would lessen both inflammatory and further oxidative or mutagenic stress, appears to be beneficial during premalignant states, and thus for the suppression of carcinogenesis. As such, the biological time context is important: NRF2 activity is desirable (for the host organism) in early stages of tumorigenesis, when the host is seeking to control premalignant carcinogenesis, but is undesirable in later stages of tumorigenesis, when it could make fully malignant cancer cells become resistant to treatment [FIG. 2]. An analogous situation exists with transforming growth factor-β (TGFβ) (87), which can suppress early stages of carcinogenesis but enhance tumour growth and metastasis in later stages, or be involved in, at different times, the initiation or termination of the inflammatory process (88).

Genetic and pharmacological approaches differ

Furthermore, as noted in an excellent recent review on NRF2 (REF. 5), there are major contextual differences between the use of pharmacological agents in animals and the use of animals with either gene knockout or knock-in [TABLE 2]. Essentially, there is no dose–response (or even time-dependent response) in classic genetic studies. In such studies, a given gene is either expressed or silenced maximally, although new methods for conditionally regulating gene expression now add some fine-tuning to gene-based studies. However, genetic studies cannot provide the same fine-tuning and evaluation of dose-dependency and dosage scheduling that is possible with pharmacological agents. Thus, a biological response is transient with a pharmacological agent but is usually constitutive following a genetic mutation, and the amplitude of the response is not as high with a pharmacological agent as is possible with genetic approaches. Indeed, as others have noted, the dose–response curve for drugs may be U-shaped, with low doses of a drug causing a specific effect, and higher doses of the same drug causing an opposite effect (89). This has noticeably been the case for synthetic oleanane triterpenoids that enhance NRF2 activity:

these agents can be antioxidative and cytoprotective at low doses, but be pro-oxidative and cause DNA damage and apoptosis at higher doses (59,60). Because NRF2 effector functions are contextually integrated in a complex network and because NRF2 activators are able to titrate the overall activity of NRF2, it is plausible that different amounts of NRF2 activity result in qualitatively (rather than just quantitatively) different outputs.

Another major difference between genetic and pharmacological studies of NRF2–KEAP1 is the 'purity' of the approach. When genes are deleted with a proper technique, a single, unique molecular lesion is created. By contrast, essentially all of the drugs that have been used to enhance NRF2 activity in carcinogenesis studies also have other molecular targets; we are not aware of any drug that has KEAP1 or NRF2 as its sole target. Almost all of these drugs react with -SH groups, and this offers the possibility of binding to many targets other than KEAP1, as mentioned earlier. Thus it is entirely conceivable that the overall health benefits that have been achieved with many 'NRF2 enhancers' are also partially due to additional actions on protein targets other than NRF2. Experimental studies showing loss of drug benefit in *Nrf2*-knockout mice do not prove that NRF2 is the sole target. They only show that *Nrf2* is necessary, but it may not be totally sufficient, for the beneficial effects of the drugs. Thus, in an important study that compared genetic versus pharmacological effects on NRF2 signalling, it was found that CDDO-imidazolide (a strong NRF2 activator) induced the expression of many regulatory genes for cell signalling that were not activated following genetic deletion of *Keap1* (REF. 90), which is consistent with NRF2 activators having targets in multiple cellular networks.

Another distinction between genetics and pharmacology is that whereas drugs are typically administered systemically, genetic lesions — whether they are experimentally engineered or naturally occurring cancer mutations — can be restricted to particular cell or tissue types. As an illustration that the effects of organism-wide NRF2 activation differ from tissue-specific NRF2 activation, germline *Keap1*-null mice die at an early age (36) (see above), whereas liver-specific *Keap1* knockout results in healthy mice that are markedly resistant to the lethal toxicity of *N*-acetyl-*p*-aminophenol (91). Moreover, human tumour-cell-specific *KEAP1* loss-of-function mutations have been observed.

Fumarate as a paradigm for context-dependent effects

It has long been known that the Krebs cycle metabolite fumarate inhibits the development of chemical carcinogenesis in rodent skin, forestomach, lung and liver, which is accompanied by the induction of classic phase 2 cytoprotective enzymes (18,92–94). More recently, fumarate has been found to form adducts with KEAP1 and is thus another NRF2 activator (34,95,96). Fumarate also has pronounced anti-inflammatory and neuroprotective activities (11,12), and dimethyl fumarate has now completed advanced clinical trials for the treatment of multiple sclerosis (11,12). In the Krebs cycle, intracellular fumarate is rapidly metabolized to malate by the enzyme fumarate hydratase (FH). Importantly, however, homozygous *FH* loss-of-function mutations are a known cause of papillary renal carcinoma in humans. The loss of FH enzyme activity causes the accumulation of high levels of fumarate in the kidney, which then forms succinate adducts with KEAP1 by thio-alkylation [FIG. 3]. This leads to an increase in NRF2 activity, which is presumed to be causative for kidney carcinoma (95,96).

Other mechanisms for oncogenesis driven by fumarate, such as the activation of transcriptional pathways regulated by hypoxia-inducible factor 1α (HIF1α), as well as mitochondrial dysfunction, have also been proposed (97–99). However, recent genetic and biochemical studies have shown that renal pathology (cyst formation) caused by *FH* mutation is independent of HIF signalling (96); moreover, these studies provide extensive evidence for the activation of genes regulated by NRF2 (REF. 95). The complex disruptive

effects of FH deficiency on the Krebs cycle and the subsequent impairment of mitochondrial ATP synthesis (both of which in turn lead to 'glycolytic addiction') remain to be explored (100,101). Overall, although there are alternative models offering explanations for how loss of *FH* leads to tumorigenesis, we believe that NRF2 is a key mechanism; however, whether the tumorigenesis is impaired in an *NRF2*−/− background remains to be determined.

In a broader sense, fumarate sheds light on the context dependence of NRF2 in carcinogenesis. At physiological levels, fumarate — an integral metabolite in the Krebs cycle — is essential for life. Like other NRF2 activators, at appropriate pharmacological doses it can prevent cancer in many organs in animals. However, when the levels of intracellular fumarate are chronically increased to very high levels by mutation of *FH*, it becomes a carcinogen; this parallels the effect of NRF2 hyperactivation that is observed in various tumours. "The dose makes the poison", an axiom attributed to Paracelsus, is appropriate for this NRF2 activator.

Conclusions and future perspectives

Overall, the question of whether NRF2 activation is 'good' or 'bad' is inadequately framed. We would suggest that both sides of the argument over this apparent paradox have merit, but the answer of whether to use drugs to stimulate or inhibit the NRF2 pathway depends on context [FIG. 2]. For the prevention of cancer and other chronic diseases in which oxidative and inflammatory stress contribute to the pathogenesis, enhancing NRF2 activity remains an important approach. This is especially true if the drugs interact with other important homeostatic networks in the cell, as is the case for the drugs presently available. At the same time there seems to be a strong rationale for the development of new NRF2-inhibitory agents for use in cancers in which genetic mutations cause constitutive activation of the NRF2 pathway. Again, it should be emphasized that drugs cause only an intermittent and variable effect on NRF2, whereas the activation of NRF2 through mutations is constant and invariable. Hopefully, rational discussion and the importance of context will prevail to allow the optimal development and use of new drugs in this important new area of pharmacology.

In summary, there is abundant evidence that activation of NRF2 can be a safe and effective strategy for the chemoprevention of cancer and many other diseases. People have been safely ingesting NRF2 activators in their diet for millennia. However, the example of fumarate and its ester, dimethyl fumarate, tells us that it is essential for both basic scientists and clinicians to understand the correct dosage of NRF2 activators if they are to be used safely and effectively for the prevention of chronic disease. It will be essential to pay careful attention to any new studies on the genetics of *NRF2* and *KEAP1* to ensure that NRF2 activators are not given to genetically inappropriate subsets of people. Furthermore, the topic of the potential use of NRF2 inhibitors to increase the therapeutic usefulness of chemotherapy in patients with invasive cancer needs further consideration. Context remains quintessential.

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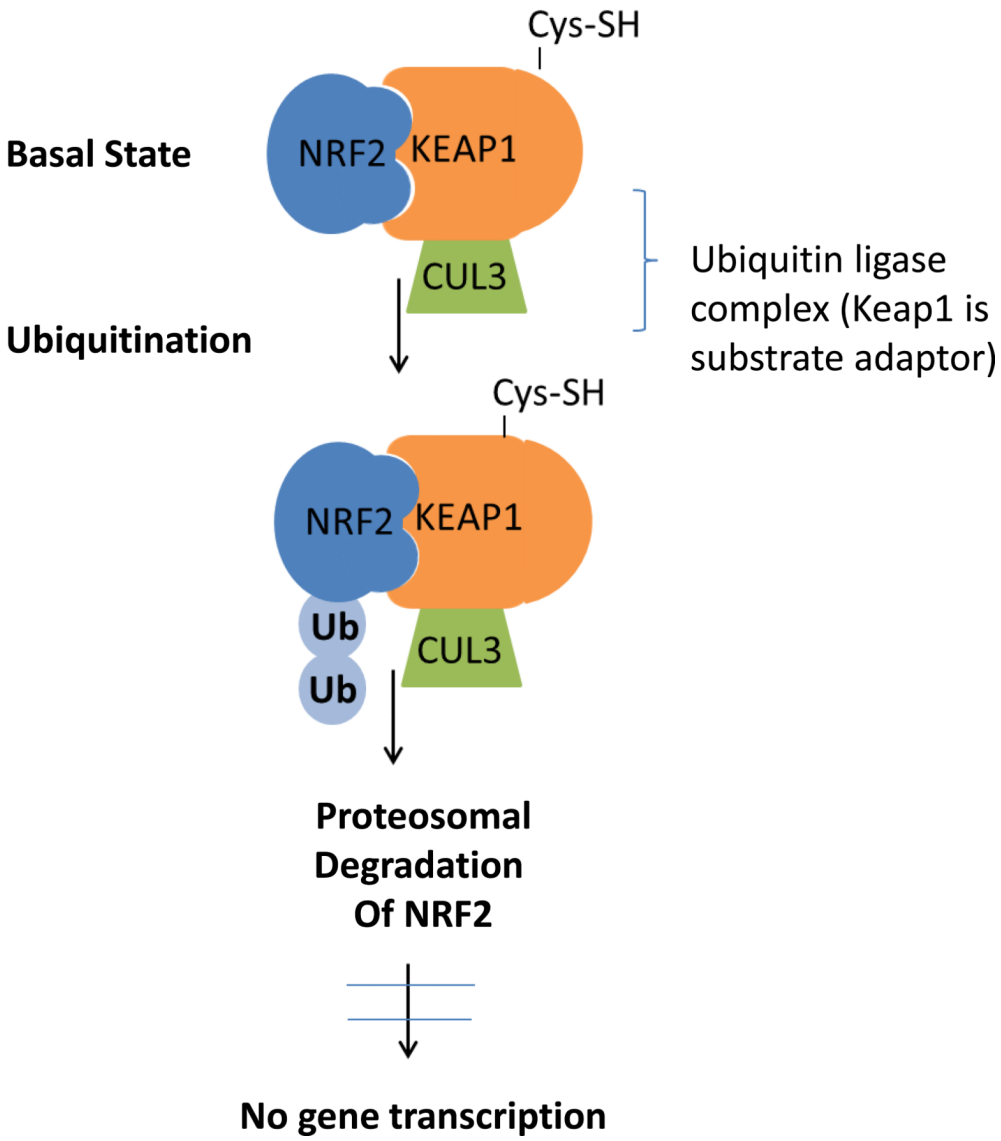
Box 1. NRF2 and chemoprevention of other diseases

In many disease states, in addition to cancer, oxidative and/or inflammatory stress has a crucial role in pathogenesis, and often causes the mutation of DNA. NFE2-related factor 2 (NRF2) is able to suppress such stress (even the mutation of DNA) and thus prevent disease, so the ability of pharmacological agents to activate NRF2 is an essential component of their desirable actions. Selected examples of the beneficial effects of drugs that activate NRF2 include protection from acute insults to the lung 8,102, kidney 103, brain 6,104, liver 105, eye 106 and heart 107 that are caused by diverse factors such as cigarette smoke 107, hyperoxia 108, ischaemia–reperfusion injury 109 and chemical toxins (such as heavy metals 103, streptozotocin 110 and the neurotoxin MPTP 111). Data have also been obtained for NRF2-activating drugs that have a disease-preventive role in chronic diseases such as diabetes 112 and obesity 113, and in genetic models of multiple neurodegenerative diseases 114–116. Importantly, although these drugs can target proteins other than NRF2 and thus have additional NRF2-independent mechanisms of action, their beneficial effects in many studies are NRF2-dependent because the effects are abolished or greatly diminished in *Nrf2*-knockout mice.



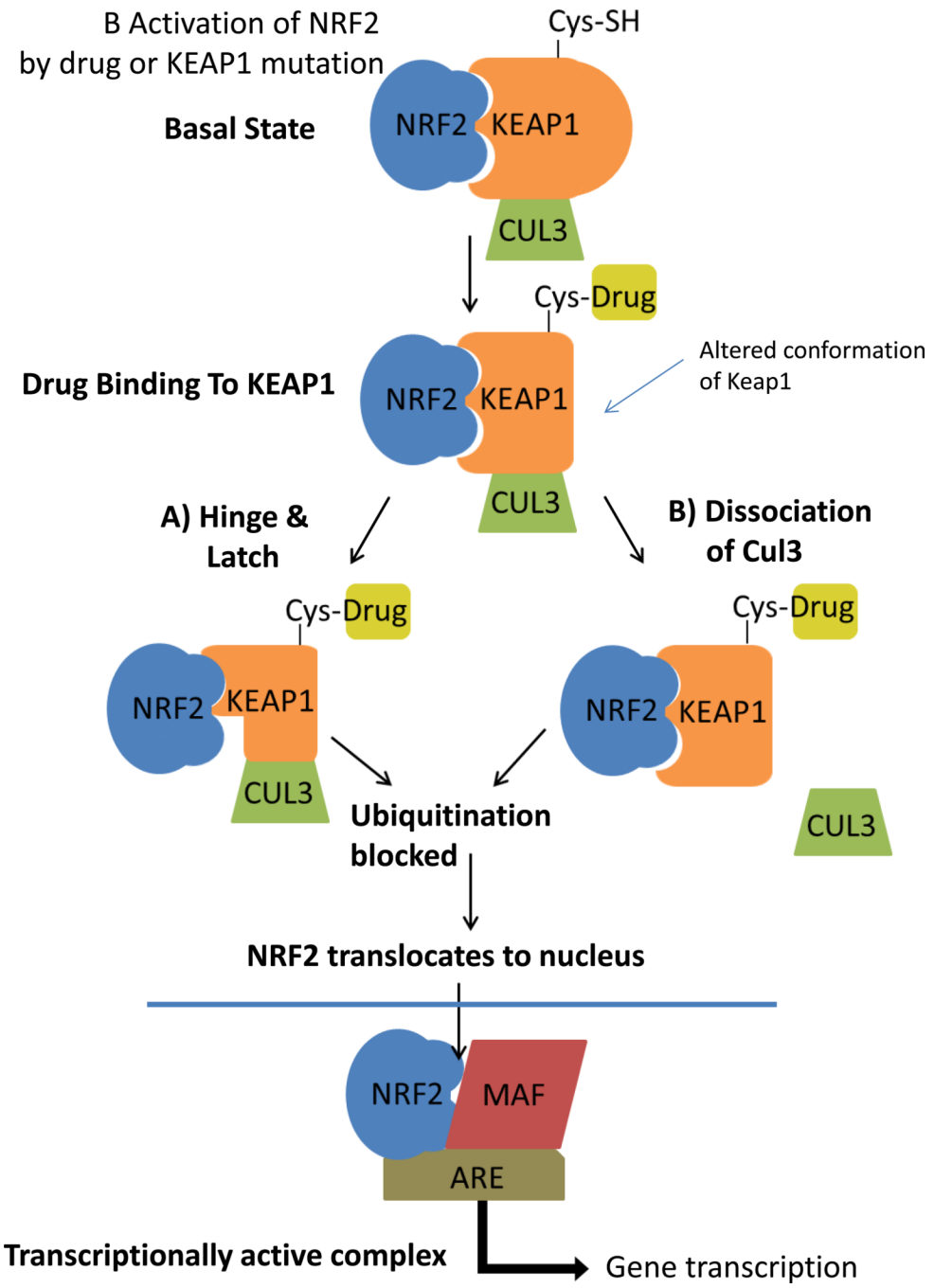


Figure 1. Suppression of NRF2 activity by KEAP1, and disruption by drugs or mutations

Regulation of ubiquitylation of NFE2-related factor 2 (NRF2) is a key process in the cellular response to drugs or oxidative and electrophilic stress. **a** | In a basal state, in the absence of drugs or oxidative or electrophilic stress, NRF2 is polyubiquitylated by the Kelch-like ECH-associated protein 1 (KEAP1)– cullin 3 (CUL3) complex. CUL3 is a ubiquitin ligase and KEAP1 is a substrate adaptor. This polyubiquitylation results in NRF2 being degraded by the proteasome. **b** | The ubiquitylation of NRF2 is blocked when KEAP1 is rendered non-functional by the conformational change resulting from the binding of a drug or another electrophile to one of the reactive cysteine residues of KEAP1, or by the mutation of

KEAP1. Two mechanisms have been suggested for this inactivation, namely: a ‘hinge and latch’ process, which loosens the association of NRF2 with KEAP1; or dissociation of CUL3 from KEAP1. Note that ubiquitylation of NRF2 can also be blocked by mutations in NRF2 (not shown). If NRF2 is not degraded, it can then migrate to the nucleus, where it becomes transcriptionally active after binding with one of the MAF proteins. Recent studies also suggest that interactions between NRF2–KEAP1 and a drug may occur in the nucleus 117, and that KEAP1 may also be regulated by ubiquitylation 118 (not shown). This figure is a greatly simplified representation of a complex process; molecular details are still being elucidated 16. ARE, antioxidant response element.

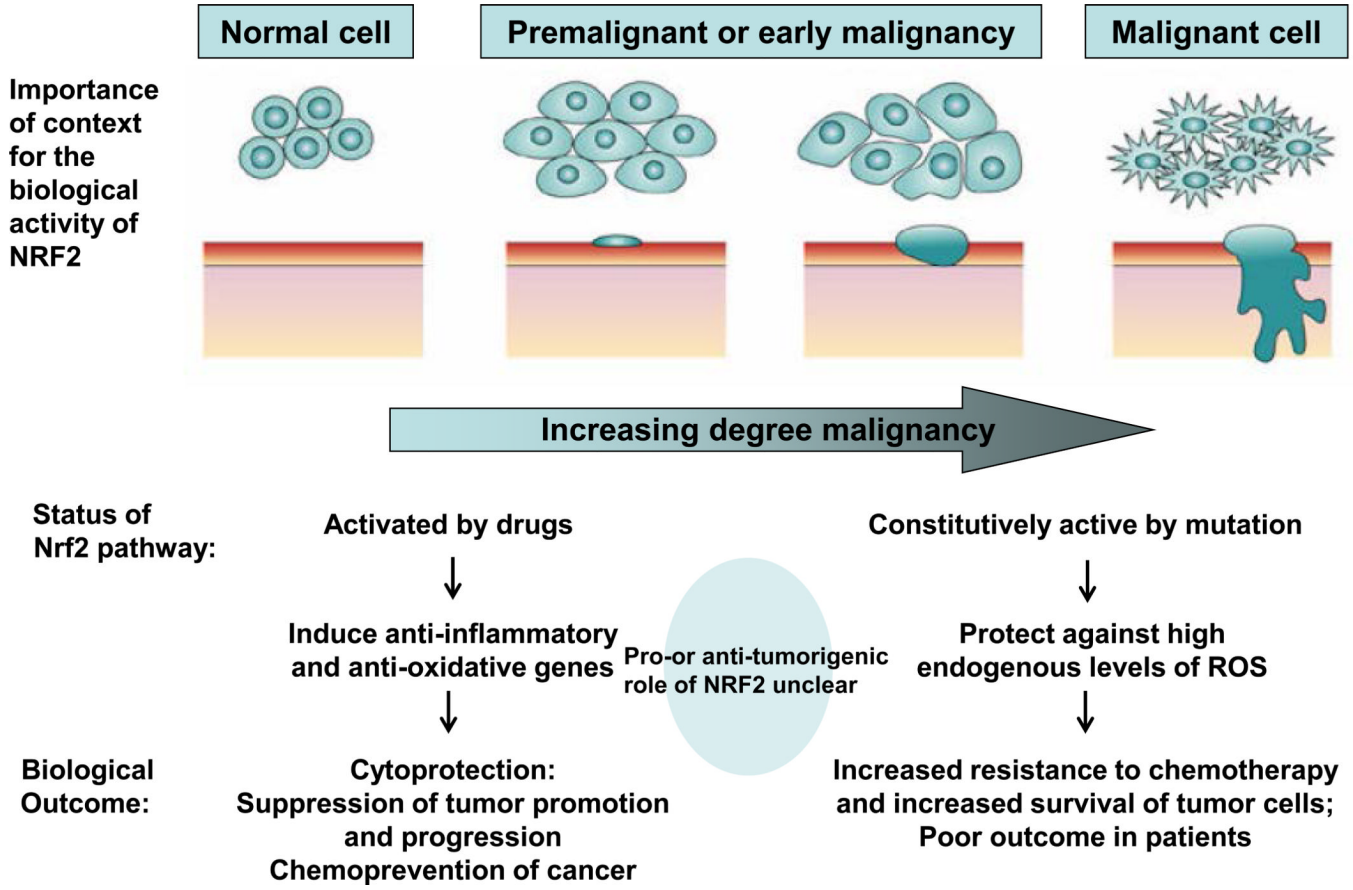


Figure 2. A model for the importance of the context of tumour stage for the biological consequences of NRF2 activation

Enhancing NFE2-related factor 2 (NRF2) activity is important for the prevention of cancer, especially if low doses of drugs are used during the earliest stages of carcinogenesis. However, in fully malignant cells, enhancement of NRF2 activity (caused by mutations) can protect tumours from the cytotoxic effects of reactive oxygen species (ROS) that are induced by chemotherapy or that may be produced endogenously by oncogenic signalling in advanced tumours. The effects of NRF2 inducers on cells at intermediate stages of tumorigenesis are still largely unknown and need further investigation. Carcinogenesis is a continuum, and there may be many different premalignant genotypes and phenotypes within a given susceptible organ *in vivo*.

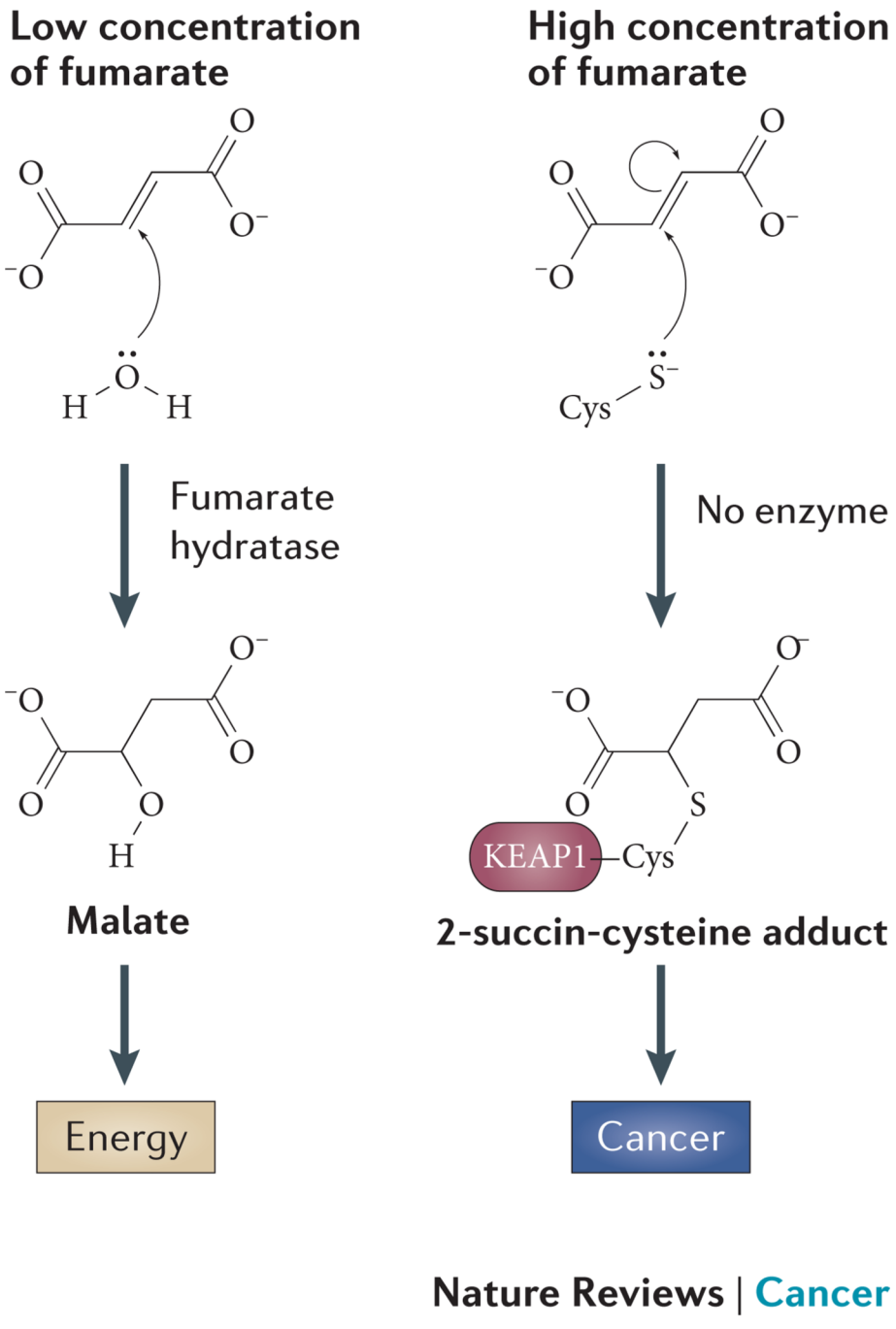


Figure 3. Beneficial or carcinogenic effects of fumarate depend on its dose and the presence or absence of the enzyme fumarate hydratase

In normal tissue, the Krebs cycle enzyme fumarate hydratase catalyses the rapid attack of water on the double bond of fumarate, resulting in the formation of malate. Intracellular levels of fumarate are thus kept low in the energy-producing process of the Krebs cycle. However, when fumarate hydratase is mutated and therefore inactive, the concentration of fumarate increases, and it is then susceptible to non-enzymatic attack by cysteine (as the thiolate anion). Reactive cysteine residues (such as Cys151) on the Kelch-like ECH-associated protein 1 (KEAP1) molecule can thus form covalent succinate adducts. This results in a conformational change in KEAP1 and transcriptional activation of NFE2-related

factor 2 (NRF2), as shown in FIG. 1. The classic-α,β-unsaturated ketone (eneone) structure of the fumarate molecule is paradigmatic for many other drugs that activate NRF2. Figure courtesy of G. Gribble, Dartmouth College, Hanover, New Hampshire, USA.

Table 1

KEAP1 or *NRF2* mutations in human cancers

Tissue	Mutated gene	Zygosity	Frequency	Reference
Breast cancer	*KEAP1* C23Y	N/A	N/A	76,77
Lung cancer	*KEAP1*	Homozygous and heterozygous	80/181 lung cancer cell lines showed LOH (44%) and 6/12 showed point mutations (50%); 10/54 NSCLC samples showed mutations (19%)	70
Lung cancer	*KEAP1*	Heterozygous	5/65 lung cancers (8%)	72
Gallbladder cancer	*KEAP1*	Homozygous	4/13 gallbladder cancers (31%)	74
Lung and head/neck cancer	*NRF2*	Homozygous	11/103 lung cancer (11%); 3/12 head/neck cancer (25%)	71
Lung cancer	*KEAP1*	N/A	4/79 patients (5%)	119
Lung cancer	*KEAP1*	N/A	6/130 tumors (4.6%)	73
Lung cancer	*KEAP1* or *NRF2*	N/A	*KEAP*1, 1/31 tumours (3%); *NRF2* - 2/29 tumours (7%)	69
Oesophagus, skin, lung, and larynx cancers	*NRF2*	Heterozygous	Oesophagus, 8/70 tumours (11%); skin, 1/17 tumours (6%); lung, 10/125 tumours (8%); larynx, 3/23 tumours (13%)	68
Ovarian cancer	*KEAP1*	Heterozygous	4/14 clear cell samples (29%)	75
Liver cancer	*KEAP1*	N/A	4/45 tumors (8.9%)	73
Gastric cancer	*KEAP1*	N/A	6/54 tumors (11%)	73

*
The biological effect of a tumorigenic mutation in Kelch-like ECH-associated protein 1 (*KEAP1*) or NFE2-related factor 2 (*NRF2*) is typically the activation of NRF2 target genes; additional details are summarized in REF. 16. LOH, loss of heterozygosity; NA, not available; NSCLC, non-small-cell lung cancer.

Table 2

Contrasting genetic and pharmacological approaches to cancer

Approach	Genetic Alteration	Pharmacological
Duration	Constitutive	Transient
Amplitude	On or off(no dose-response)	Dose-responsive
Target	Single gene	Networks
Location	Specific cell or organ	Whole organism