NRF2: KEAPing Tumors Protected

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ABSTRACT The Kelch-like ECH-associated protein 1 (KEAP1)/nuclear factor erythroid 2–related factor 2 (NRF2) pathway plays a physiologic protective role against xenobiotics and reactive oxygen species. However, activation of NRF2 provides a powerful selective advantage for tumors by rewiring metabolism to enhance proliferation, suppress various forms of stress, and promote immune evasion. Genetic, epigenetic, and posttranslational alterations that activate the KEAP1/NRF2 pathway are found in multiple solid tumors. Emerging clinical data highlight that alterations in this pathway result in resistance to multiple therapies. Here, we provide an overview of how dysregulation of the KEAP1/NRF2 pathway in cancer contributes to several hallmarks of cancer that promote tumorigenesis and lead to treatment resistance.

Significance: Alterations in the KEAP1/NRF2 pathway are found in multiple cancer types. Activation of NRF2 leads to metabolic rewiring of tumors that promote tumor initiation and progression. Here we present the known alterations that lead to NRF2 activation in cancer, the mechanisms in which NRF2 activation promotes tumors, and the therapeutic implications of NRF2 activation.

INTRODUCTION

The transcription factor nuclear factor erythroid 2–related factor 2 (*NFE2L2*, NRF2) plays a pivotal role in cellular physiology and tumorigenesis. The canonical role of NRF2 is to shift cellular metabolism to maintain redox balance. NRF2 protein levels are negatively regulated by the ubiquitin ligase scaffold protein Kelch-like ECH-associated protein 1 (KEAP1), which binds to NRF2 and facilitates its ubiquitination and proteasomal degradation (1, 2). In the presence of cytotoxic oxidative stress, NRF2 accumulates and translocates to the nucleus, where it regulates the transcription of a plethora of genes that promote antioxidant defenses (Fig. 1; refs. 2–7). In addition to its role in maintaining cellular redox homeostasis, NRF2 plays crucial roles in regulation of immune responses (8) and drug detoxification (2, 9, 10). The KEAP1/NRF2 pathway is genetically, epigenetically, and post-transcriptionally altered in multiple cancers, including lung, breast, liver, esophageal, and pancreatic cancers (6, 11–17). Mutually exclusive loss-of-function (LOF) mutations in *KEAP1* or gain-of-function (GOF) mutations in *NRF2*, both of which result in stabilization of NRF2, have been identified to promote both tumorigenesis and resistance to multiple therapies (13, 17–20). Alterations in this pathway also lead to metabolic vulnerabilities that can be exploited therapeutically. This review provides an overview of the KEAP1/NRF2 pathway in normal physiology and its importance in tumor development, cancer metabolism, mechanisms of treatment resistance, and novel therapeutic strategies to target tumors with NRF2 activation.

PHYSIOLOGIC IMPORTANCE OF THE KEAP1/NRF2 PATHWAY

Yamamoto and colleagues were the first to describe the NRF2 transcriptional factor and its cytoprotective role through the regulation of a battery of genes that protect cells from toxins, drugs, and other toxic xenobiotics (4, 21). Since then, multiple groups have evaluated the physiologic role of the KEAP1/NRF2 pathway using a series of genetically engineered mouse models (GEMM). Although *NRF2* loss has no gross impact on normal mouse development (22), the importance of NRF2 becomes apparent under various forms of environmental stress. Iizuka and colleagues (23) used *NRF2*-null mice to demonstrate increased susceptibility to cigarette smoke–induced emphysema. Beyond cigarette exposure, *NRF2*-null mice and rats have increased susceptibility to multiple insults and the development of diverse pathologies (24–30).

To assess the impact of constitutive NRF2 activation, Wakabayashi and colleagues (31) developed *Keap1* knockout mice. At birth, *Keap1*-null animals are normal in size

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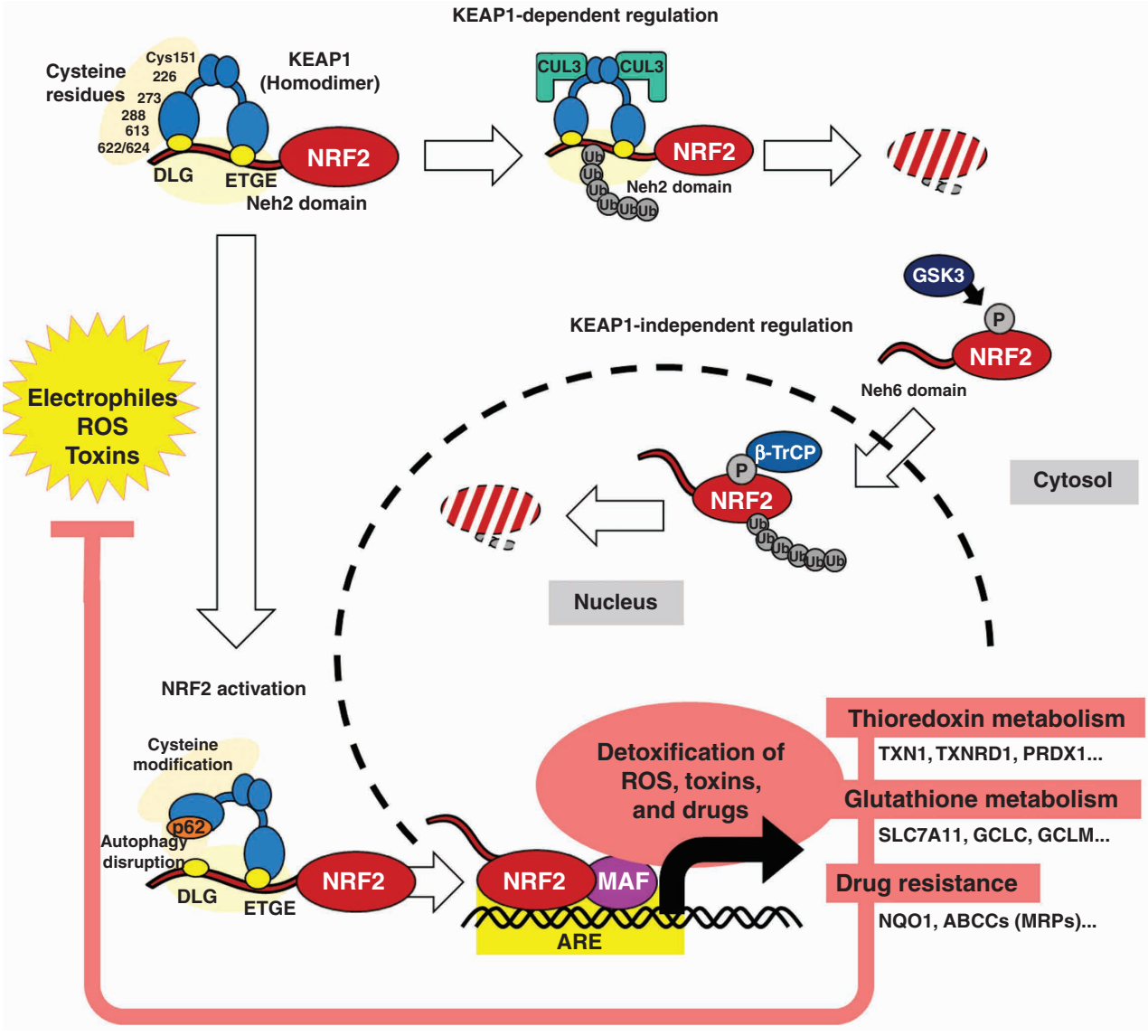

Figure 1. Physiologic activation and regulation of NRF2. Under basal conditions, NRF2 is bound by KEAP1 via the DLG and ETGE motifs in the Neh2 domain of NRF2 in cytosol and leads to binding of CUL3, polyubiquitination, and proteasomal degradation. NRF2 is also regulated by KEAP1-independent mechanisms via phosphorylation of the Neh6 domain by GSK3 and proteasomal degradation by β-TrCP. ROS, drugs, and toxins react with cysteine residues on KEAP1, resulting in structural changes and the accumulation of NRF2 to translocate to the nucleus and function as a transcriptional factor. In the nucleus, NRF2 heterodimerizes with small MAF proteins and binds to antioxidant response elements to induce a series of target genes for detoxification of ROS, toxins, and drugs. ABC, ATP-binding cassette; MAF, musculoaponeurotic fibrosarcoma; PRDX1, peroxiredoxin 1; TXN, thioredoxin; TXNRD1, thioredoxin reductase 1; Ub, ubiquitin.

but fail to survive past day 21 due to hyperkeratosis of the esophagus and forestomach that causes gastric obstruction and death (31). Critically, loss of *Nrf2* in the context of *Keap1* loss prevents the development of hyperkeratosis and death, demonstrating the epistatic relationship of KEAP1 and NRF2. To dissect the physiologic importance of NRF2 across tissues, multiple studies have performed tissue-specific deletion of *Nrf2* or achieved NRF2 activation through *Keap1* deletion. Okawa and colleagues (32) showed that hepatocyte-specific *Keap1* loss was associated with increased tolerance to acetaminophen toxicity due to NRF2 activation and drug detoxification. NRF2 can suppress immune responses in multiple mouse models. In M1 macrophages, NRF2 suppresses proinflammatory cytokines by blocking transcription of *Il6* and *Il1β* (8, 33). The significance of tissue-specific deletion of *Keap1* has been shown to regulate cell differentiation (34). Moreover, tissue-specific activation of NRF2 reduces tissue damage in sickle cell disease mouse models (35) and the development of type 1 diabetes in a nonobese diabetic mouse model (36). Although global loss of *Nrf2* has minimal effects under homeostasis, in the context of cytotoxic stress, tissue-specific NRF2 activity and downstream biological consequences become evident.

PHYSIOLOGIC ACTIVATION OF NRF2 AND DYSREGULATION IN CANCER

Structural Features of KEAP1 and NRF2

Given the frequency of NRF2/KEAP1 pathway aberration in cancers (Fig. 2A), the structural features of these proteins are important to comprehend how mutations disrupt interactions that lead to hyperactive NRF2 responses. Herein, we provide an overview of the structures of NRF2 and KEAP1, major domains, and the functions of these domains (Fig. 2B and C). NRF2 is part of the cap 'n' collar (CNC) basic leucine zipper family of transcription factors. The CNC domain is a 43–amino acid sequence located at the N-terminus of the DNA binding domain of this family, which includes the transcription factors NRF1, NRF2, NRF3, BACH1, and BACH2 (37). The structure of NRF2 is broken down into seven NRF2–ECH homology domains (Neh1–Neh7; Fig. 2C). The Neh1 domain mediates heterodimerization with the protein small musculoaponeurotic fibrosarcoma (sMAF), which together bind to DNA at designated antioxidant response elements (ARE) with the sequence 5′-GTGAC NNNGC-3′ (also called a CNC sMAF binding element; refs. 38, 39; Fig. 1). Neh1 also contains a nuclear localization signal while Neh5 has a redox-sensitive nuclear export signal. The Neh2, 6, and 7 domains are involved in regulating NRF2 activity. Collectively, the Neh domains mediate specific protein–protein and protein–DNA interactions, resulting in the fine-tuning of the oxidative/xenobiotic stress signals governed by NRF2. The primary role of KEAP1 is to act as a substrate adaptor for the Cullin 3 (CUL3)–RING E3 ubiquitin ligase complex. Each of the major domains of KEAP1 has an important role in this degradation process. KEAP1 contains five main domains (Fig. 2B): the N-terminal region, Broad complex, Tramtrack and bric-à-brac (BTB) domain (40), intervening region (IVR), KELCH domain, and C-terminal region (Fig. 2B). The KELCH domain contains six KELCH motif repeats that form six β sheets (41). It is these KELCH repeats that bind to the Neh2 domain of NRF2 (42). Specifically, KEAP1 binds to two conserved degron motifs located in the Neh2 domain (43), ETGE and DLG (Fig. 2C), with KEAP1 binding more strongly to the ETGE motif (44). The BTB domain facilitates the homodimerization of KEAP1 and binding to the CUL3–RING E3 ubiquitin ligase complex (45–48). This complex is formed by three components: CUL3, RING-box protein 1 (RBX1), and E2. CUL3 binds a range of BTB-containing proteins, including KEAP1 (Fig. 1). These BTB proteins serve as substrate adaptors for the CUL3–RING E3 ligase complex. RBX1 serves to bind E2, which has been conjugated to ubiquitin. Once the substrate is bound to this complex, it is ubiquitinated and degraded. The IVR of KEAP1 plays an important role in redox homeostasis as this region contains key cysteine residues, including C226, C273, and C288. These cysteine residues along with those in other domains seen in Fig. 2B (C151, C613, and C622/624) are oxidized in the presence of electrophiles (49), reactive oxygen species (ROS; ref. 50), and toxins (51). Modification of these cysteine residues results in a conformational change in KEAP1, impairing the degradation of NRF2 and enabling the accumulation of *de novo* NRF2 (52).

Posttranslational Regulation of NRF2

The sensory role of KEAP1 is enabled through cysteine residues that react with ROS (50) or electrophiles (49). Work by Wakabayashi and colleagues (53) identified that reactive cysteine groups C273 and C278 respond to ROS and induce conformational changes in KEAP1 to promote NRF2 stabilization. In addition, there are a series of known electrophilic molecules capable of activating NRF2. Dinkova-Kostova and colleagues (54) demonstrate that electrophiles such as sulforaphane react with cysteine to form disulfide links mainly at C257, C273, C288, and C297. Other NRF2 inducers, such as dexamethasone mesylate and tertiary butylhydroquinone, are known to react with cysteine residues on KEAP1, resulting in NRF2 stabilization (55, 56). Similarly, McMahon and colleagues (57) show that KEAP1 has three distinct sensor regions containing cysteines that react with Zn^{2+}, nitrous oxide, or alkenals.

Multiple electrophilic metabolites, which can be aberrantly regulated in cancer, modify KEAP1 cysteine residues to alter KEAP1 conformation. This has been described especially in the context of the tricarboxylic acid (TCA) cycle–derived metabolites fumarate (58–61) and itaconate (62) and glycolysis-derived metabolites such as methylglyoxal (55). Other metabolites such as polyunsaturated fatty acid alkenals can posttranslationally stabilize NRF2. These findings suggest that KEAP1/NRF2 signaling responds not only to xenobiotic stressors and ROS but also to alteration in metabolites due to disruption of endogenous metabolism in cancer. The important role of metabolites in NRF2 activation and cancer risk is demonstrated by germline mutations of fumarate hydratase (FH), which results in the development of hereditary leiomyomatosis and renal cell carcinoma cancer syndrome (63). Inactivation of FH results in accumulation of the TCA cycle intermediate fumarate, which promotes NRF2 activation, likely through succination of KEAP1 (59–61).

In addition to degradation through the KEAP1–CUL3 ubiquitin ligase complex, NRF2 can also be degraded by the SKP1–CUL1 ubiquitin ligase complex. Chowdhry and colleagues (64) show that NRF2 has two β-transducin repeat containing protein (β-TrCP) binding motifs—DSGIS and DSAPGS—in the Neh6 domain. Like KEAP1, β-TrCP acts as a substrate adaptor for CUL1-mediated degradation. Furthermore, binding at the DSGIS motif is enhanced by phosphorylation through glycogen synthase kinase 3 (GSK3; Fig. 1). AKT inhibition results in an increase in GSK3 activity, increased degradation of NRF2, and sensitivity to cisplatin in *KEAP1*-mutant A549 lung cancer cells (64). Activation of the PI3K/AKT mitogenic pathway mediates phosphorylation and inhibition of GSK3, resulting in activation of NRF2. In breast cancer, in which PI3K/AKT signaling is frequently dysregulated, NRF2 is posttranslationally activated by PI3K/AKT to increase antioxidant capacity via the above mechanism (65).

Competitive Binding

The interaction between KEAP1 and NRF2 can be disrupted by proteins that compete with NRF2 from binding to KEAP1. For example, Ma and colleagues (66) demonstrated that PALB2, which is known to bind and regulate

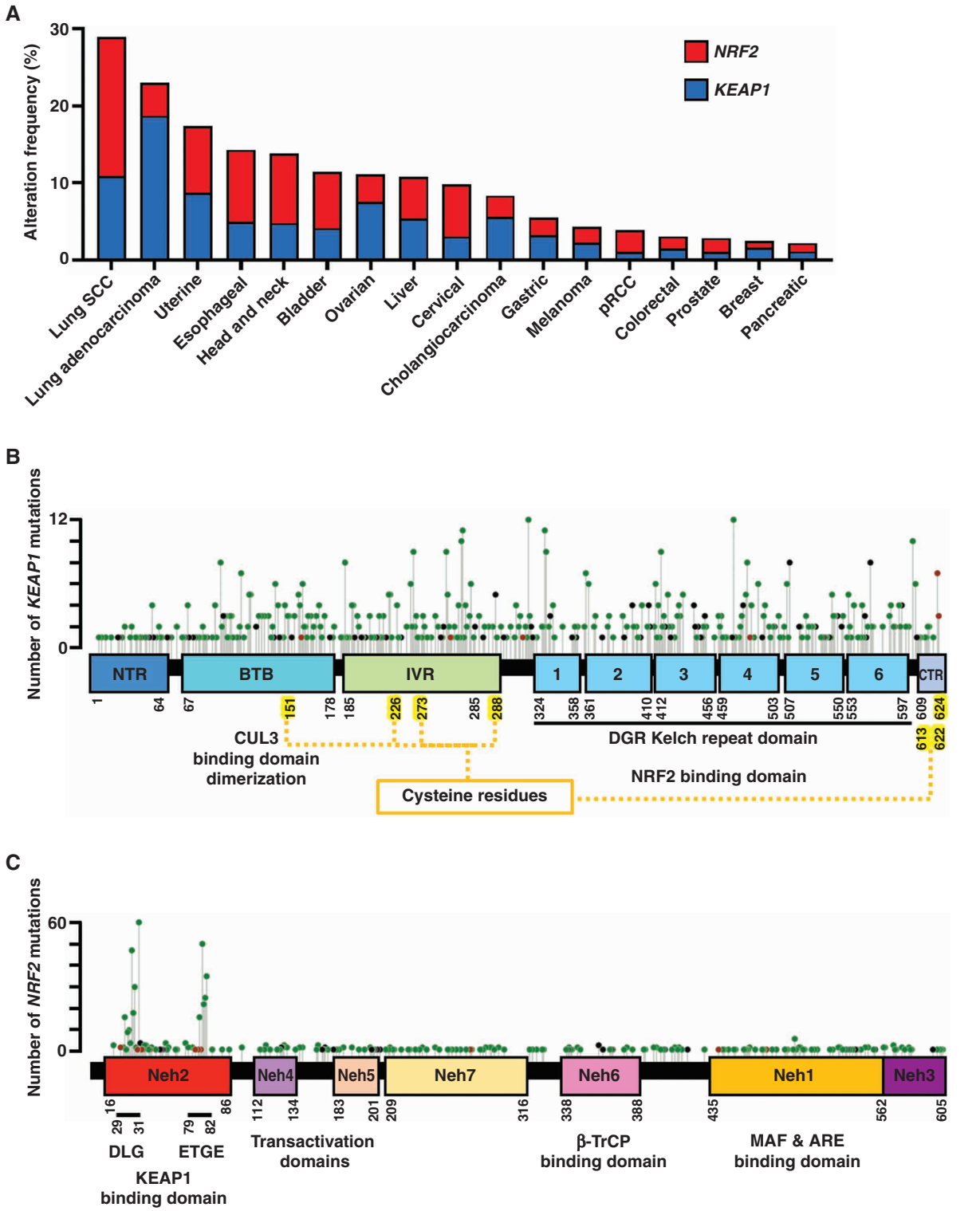

Figure 2. Mutation spectrum in the KEAP1/NRF2 pathway. **A,** Frequency of mutations in *NRF2* and *KEAP1* in solid tumors generating using cBioPortal data from The Cancer Genome Atlas (TCGA). **B,** Map of KEAP1 with mutations based on cBioPortal TCGA data sets. KEAP1 is divided up into the following domains: NTR, BTB, IVR, 6 Kelch domains, and CTR. Key cysteine residues for sensing ROS and toxins are indicated. **C,** Map of NRF2 with seven Neh domains and mutations labeled. The KEAP1-binding motifs, DLG and ETGE, are indicated in the Neh2 domain, whereas the loci of phosphorylation by β-TrCP are located in the Neh6 domain. Somatic mutations in *NRF2* are highly concentrated in DLG and ETGE motifs. CTR, C-terminal region; DGR, double glycine repeat; IVR, intervening region; MAF, musculoaponeurotic fibrosarcoma; NTR, N-terminal region; pRCC, papillary renal cell carcinoma; SCC, squamous cell carcinoma.

the intranuclear localization and stability of BRCA2, has an ETGE motif identical to NRF2 and competes for binding with KEAP1. Increased protein levels of PALB2 result in decreased KEAP1-dependent degradation of NRF2 (66). In a similar manner, p62 (SQSTM1), an important component of autophagy and an NRF2 target, binds to KEAP1 through a KEAP1-interacting region (KIR) that competes with the ETGE motif of NRF2, resulting in inhibition of NRF2 degradation (67–69). Several other proteins are known to compete with NRF2 for binding to KEAP1, including BRCA1, p21, and DPP3 (70, 71). These competitive KEAP1 client proteins represent alternative nonoxidative stress–related mechanisms by which NRF2 can be stabilized and thus feed multiple signaling inputs toward direct regulation of NRF2 and the downstream antioxidant response network.

Genetic Alterations in the KEAP1/NRF2 Pathway

Hyperactivation of NRF2 plays a critical role in multiple tumor types, including lung, liver, gastric, ovarian, breast, and colorectal cancers (Fig. 2A). Among them, activation of NRF2 is most extensively characterized in non–small cell lung cancer (NSCLC). Inactivating mutations in *KEAP1* are located throughout the gene (Fig. 2B). Hast and colleagues (72) identified multiple classes of *KEAP1* mutations that have differing mechanisms and degree of NRF2 activation. These somatic mutations result in *KEAP1* LOF, leading to stabilization and accumulation of NRF2 (18, 73–77). Using an ARE-luciferase reporter, Hast and colleagues (72) demonstrated that these mutations enhance NRF2 activation. Interestingly, none of the mutations were in domains required for NRF2 binding. However, five of these mutations, including the more frequent G333C mutation, impaired NRF2 binding. Some of these mutations enhanced binding to NRF2, including R470C. Despite demonstrating increased NRF2 affinity, expression of *KEAP1* super-binding mutants resulted in increased stability and nuclear localization of NRF2 with a concomitant increase in NRF2 transcriptional target genes. Furthermore, many *KEAP1* mutations found in human lung tumors appear to be dominant negative mutations which establish varying degrees of NRF2 activation (43, 78).

Kerins and Ooi (79) provide a thorough review of key NRF2 mutations in cancer. Unlike *KEAP1*, activating GOF mutations in *NRF2* occur in specific hotspot regions corresponding to the ETGE and DLG domains (ref. 80; Fig. 2C). Mutations in *NRF2* are more prevalent in lung squamous cell carcinoma, whereas *KEAP1* mutations are more frequent in lung adenocarcinoma (81–83). LOF mutations in *KEAP1* and GOF mutations in *NRF2* are generally mutually exclusive, suggesting that NRF2 activation is the main driver for selection of these mutations rather than other KEAP1-mediated effects. Work by Jamal-Hanjani and colleagues (84) evaluated intratumoral heterogeneity and genomic evolution by analyzing multiple regions of lung tumors. They found that *KEAP1* mutations are an early clonal driver mutation, emphasizing that mutations in *KEAP1* are likely selected for during tumor initiation or progression.

Mutations in *KEAP1* frequently co-occur with other mutations, suggestive of cooperative events leading to selection. Notably, mutation in *KRAS* frequently co-occurs with *KEAP1* mutation in lung adenocarcinoma (20). Furthermore, multiple patient cohorts have demonstrated that mutations in *KEAP1* frequently co-occur with mutations in liver kinase B1 (*LKB1/STK11*; refs. 20, 85). Mutations in either *KEAP1* or *LKB1* are independently associated with unfavorable clinical outcomes (86). Interestingly, both genes are located on chromosome arm 19p along with *SMARCA4*, another frequently comutated gene with *KEAP1* and *LKB1* (86). Although these mutations confer a survival advantage to tumors, they may provide the opportunity to therapeutically target genotype-specific vulnerabilities in these aggressive tumor subtypes.

Epigenetic Modification

KEAP1 can be epigenetically regulated, reflecting another mechanism to increase NRF2 levels. *In vitro*, Muscarella and colleagues (12) found that 50% of NSCLC and 42% of small-cell lung cancer cell lines had methylation in the promoter of *KEAP1* and decreased expression. In their analysis of 47 patient NSCLC samples, methylation of *KEAP1* was seen in 47% of cases (12). NRF2 activation as a result of *KEAP1* promoter methylation has been associated with poor patient outcomes in glioma, renal cell carcinoma, and colorectal cancer (12, 87, 88). An analysis of breast tumors found that *KEAP1* promoter methylation was frequently observed and associated with estrogen receptor (ER)–positive, HER2-negative tumors. In addition, patients with triple-negative breast cancer with *KEAP1* promoter hypermethylation demonstrated a higher mortality (89).

Transcriptional Regulation

NRF2 can be regulated transcriptionally through multiple mechanisms. Interestingly, the promoter of *Nrf2* contains an ARE sequence, and therefore NRF2 can reinforce its own transcription (90). The aryl hydrocarbon receptor has been shown to regulate *Nrf2* by directly binding to xenobiotic response elements found within the NRF2 promoter (91). Wakabayashi and colleagues (92) showed that Notch transcriptionally upregulates *Nrf2*. Notch-specific NRF2 regulation was shown to be important for liver development, as liver-specific deletion of *Nrf2* through albumin-Cre results in reversal of both Notch-induced hepatomegaly and an increase in intrahepatic bile ducts. Using mouse embryonic fibroblasts (MEF), DeNicola and colleagues (6) demonstrated that multiple oncogenes, including $Kras^{G12D}$, $Braf^{V619E}$, and *Myc*, transcriptionally upregulate *Nrf2* to mitigate ROS. In breast cancer, BRCA1 is reported to regulate *NRF2* through direct promoter binding (70, 93) or via posttranslational methods that lead to increased NRF2 expression (70).

Posttranscriptional Modifications

NRF2 levels can be regulated by various posttranscriptional mechanisms. Goldstein and colleagues (94) identified a subset of tumors that had high expression of NRF2 and its transcriptional signatures without mutations in *KEAP1* or *NRF2*. They identified an *NRF2* splice variant that skipped exon 2, which corresponds to the Neh2 domain regulated by KEAP1 (94). In a series of *in vitro* experiments, they demonstrated that skipping of exon 2 resulted in NRF2 activation. This activity was not enhanced with knockdown of *KEAP1* or impaired with *KEAP1* overexpression, validating that the interaction between KEAP1 and NRF2 was lost through exon skipping.

Multiple miRNAs have also been found to regulate NRF2. Yang and colleagues (95) identified that miR-28 negatively regulates *Nrf2* transcripts. This same group also demonstrated that miR-200a negatively regulates *Keap1* and thus stabilizes NRF2 in breast cancer cells (96, 97). The miRNA miR-421 has been shown to reduce KEAP1 levels and is associated with resistance to paclitaxel in A549 lung cancer cells (98). Other miRNAs, such as miR-155, miR-27a, miR142-5p, and miR144, have also been found to reduce NRF2 levels (99).

ROLE OF NRF2 IN TUMORIGENESIS

NRF2 has been shown to play a multifaceted role at different stages of tumor development across several types of cancer. ROS accumulation promotes DNA damage, leading to activation of oncogenes and inactivation of tumor suppressor genes, resulting in cellular transformation and tumor initiation. Many carcinogens used to initiate tumors in animals promote mutagenesis partly through buildup of ROS and DNA damage (100, 101). Given the important role of NRF2 in suppressing ROS and drug detoxification, several studies have shown that NRF2 activation can suppress the formation of carcinogen-induced tumors. However, both cancer genomics and preclinical GEMM data suggest that activation of NRF2 through LOF mutations in *KEAP1* or GOF mutations in *NRF2* is associated with poor prognosis and more aggressive disease (18, 102). Furthermore, there is evidence for tissue-specific protective roles of the KEAP1/NRF2 pathway in solid tumors driven by the same oncogenes. These observations suggest that NRF2 likely has dual stage-specific protumorigenic and antitumorigenic roles depending on the context. Here, we highlight the studies implicating the KEAP1/NRF2 pathway in tumorigenesis and discuss future work needed to clearly define the role of NRF2 in tumor initiation and progression across different malignancies.

To study carcinogenesis using mouse models, both chemical carcinogen-induced models or GEMMs have been used. In some carcinogen-induced tumor models, NRF2 activation has been shown to suppress tumor initiation (30, 103–105). Pharmacologic activation of NRF2 by CDDO-Im, an NRF2 inducer currently in clinical trials for the treatment of diabetic kidney disease (106), suppressed tumorigenesis in a carcinogen-induced lung cancer model (107). In addition, germline *Nrf2* knockout (KO) mice accelerated tumor formation in a vinyl carbamate–induced lung cancer model (108). Similarly, *Nrf2* KO mice formed more lung tumors compared with *Nrf2* wild-type mice upon exposure to urethane (101). Both the vinyl carbamate– and urethane-induced models acquire recurrent somatic mutations in known oncogenes, such as *Hras* and *Kras*, and NRF2 activation in these models can suppress the initiation of carcinogen-induced tumors. *Nrf2* KO mice also fail to develop tumors in carcinogen-induced models of hepatocellular carcinoma and bladder cancer (109, 110).

On the basis of genomic and clinical data of patients with lung cancer, it is clear that alterations in the KEAP1/NRF2 pathway are recurrent (20, 73) and enriched in smokers and nearly absent in young never-smokers (111, 112). This correlation and the above preclinical findings have led to the hypothesis that smoking may induce NRF2 activation as a protective mechanism against damage induced by carcinogens to suppress tumor initiation. However, once an oncogenic driver mutation (e.g., *KRAS*) has occurred, high NRF2 expression may be selected for by protecting tumors from insults, supporting proliferative processes, and potentially suppressing antitumor immune responses.

DeNicola and colleagues (6) were the first to demonstrate that *Nrf2* is required for *Kras*-driven lung and pancreas tumorigenesis. They demonstrated that both *Kras* and *Myc* oncogenes constitutively increase *Nrf2* transcription to elevate the basal activity of the antioxidant and cellular detoxification program required for tumorigenesis. Subsequent studies in *Kras*-driven pancreatic cancer models showed that *Nrf2* KO repressed tumor formation (113). Using patient-derived organoid models, Chio and colleagues (114) demonstrated that *Nrf2* knockdown suppressed tumor initiation and maintenance. These studies support the idea that NRF2 is necessary for tumorigenesis in tumors with known oncogenic drivers such as *Kras*. Future studies using inducible approaches to suppress *Nrf2* at initiation or progression will more precisely clarify the stage-specific requirement for *Nrf2*.

Genomic studies suggest that activation of the NRF2 pathway by genetic alterations in KEAP1/NRF2 plays an important role in cancer development (13, 18, 75, 76, 102, 115). The KEAP1/NRF2 axis in tumorigenesis has been most extensively studied in the setting of lung cancer. Romero and colleagues (116) were the first to demonstrate the tumor-suppressive role of KEAP1 in *Kras*-driven GEMMs of lung adenocarcinoma. Targeted somatic CRISPR/Cas9-based LOF of *Keap1* resulted in increased tumor growth and histologic grade (116). In addition, multiple studies have used conditional deletion of LOF point mutant (85, 117–119) *Keap1* alleles to demonstrate the tumor-suppressive role of KEAP1 in *Kras*-driven lung adenocarcinoma. Furthermore, conditional *Keap1* and *Pten* loss in the lung generated papillary lung adenomas, suggesting that PI3K/AKT pathway activation may cooperate with NRF2 activation in the absence of *Kras* alterations (120). Loss of *Keap1* in basal cells also promotes the development of squamous cell carcinomas in the context of *Trp53* loss (121).

A major caveat with some of the studies discussed above is that both the pharmacologic and genetic modulation of *Nrf2* is systemic, which would also affect cells in the tumor microenvironment that play a role in tumor initiation and maintenance, including myeloid-derived cells whose differentiation and function are known to be regulated by NRF2 (8, 27). Satoh and colleagues (122) systematically tested the dual role of NRF2 activation in cancer development. Using a urethane-induced lung cancer model, they observed that *Keap1* knockdown mice, with higher levels of NRF2, developed smaller tumors compared with wild-type mice. However, when tumors from these animals were transplanted into immunodeficient mice, *Keap1* knockdown tumors grew faster than *Keap1* wild-type tumors (122).

The role of NRF2 activation during tumorigenesis may also be tissue-specific. Hamada and colleagues demonstrated that conditional loss of *Keap1* in a *Kras*-driven pancreatic cancer GEMM leads to pancreatic atrophy and decreased tumor growth (123), whereas conditional loss of *Keap1* in

a *Kras*-driven lung cancer GEMM leads to more aggressive tumors (116). However, the dosage of NRF2 activation by *Keap1* LOF likely also has a role in tumorigenesis (120). This is suggested given that human and mouse pancreatic tumors upregulate *NRF2* in the setting of *KRAS* mutation but likely not to the extent seen in the setting of *KEAP1* mutations (6).

Li and colleagues (124) demonstrated that NRF2 activation not only leads to more aggressive tumors but may also play a role in driving histologic subtypes of NSCLC. Using a GEMM with conditional mutation of *Kras* and loss of *Lkb1* that forms both adenocarcinoma and squamous lung carcinoma, they demonstrated that modulation of oxidative stress by overexpression of *Nrf2* or treatment with N-acetyl cysteine (NAC) reduced the frequency of squamous tumors, suggestive of ROS as a major driver of differentiation of tumors between lung adenocarcinomas versus squamous cell carcinomas (124). Interestingly, NRF2 is significantly upregulated in type II endometrial cancer (serous and clear cell carcinomas) compared with type I, suggesting that NRF2 is involved in driving histologic subtypes of endometrial cancer (125).

In addition to the importance of the KEAP1/NRF2 pathway in tumor initiation and maintenance, NRF2 has been shown to also promote metastases through its interaction with BACH1 (126). Accumulation of NRF2 in lung cancer causes the stabilization of BACH1 by induction of heme oxygenase 1 (HO1), the enzyme that breaks down heme (127). Using a *Kras*-driven model, they demonstrate that loss of *Keap1* or *Fbxo22* induces metastasis in a BACH1-dependent manner. Furthermore, human metastatic lung cancers display higher levels of HO1 and BACH1, which correlate with poor prognosis and increased incidence of metastasis in patients with lung cancer. These findings were further supported by a study that demonstrated that dietary antioxidants lead to BACH1 stabilization and increased metastasis in the same *Kras*-driven adenocarcinoma model (128). Finally, the dipeptidyl peptidase 4 inhibitors saxagliptin and sitagliptin can stabilize NRF2, resulting in an increased metastatic potential in xenograft models (129). This study also observed that increased NRF2 expression also correlated with increased metastasis in liver cancer. Overall, NRF2 activation by any number of mechanisms, including somatic mutations, transcriptional regulation, or metabolic reprogramming–triggered modifications of KEAP1, promotes tumorigenesis.

NRF2 METABOLIC REWIRING

Redox Metabolism

NRF2 is traditionally known for its role in redox homeostasis through the regulation of glutathione and thioredoxin, which are responsible for scavenging ROS, among other functions. Two genes critical for glutathione synthesis are transcriptionally regulated by NRF2: glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM; ref. 130). GCLC catalyzes the reaction ligating glutamate and cysteine to form y-glutamyl cysteine, whereas GCLM increases the affinity of GCLC for its substrates (Fig. 3). Glutathione is then produced via glutathione synthetase using y-glutamyl cysteine and glycine as substrate and consuming ATP in the process. In addition, NRF2 transcriptionally regulates glutathione reductase (131) and thioredoxin reductase 1 (132), both of which require NADPH generated by the pentose phosphate pathway (PPP), to reduce oxidized glutathione and thioredoxin (Fig. 3).

NADPH has two key roles in metabolism. First, it is used in the synthesis of multiple macromolecules, including nucleotides (133), cholesterol (134), and fatty acids (135). Second, it is used to regenerate the reduced form of glutathione and thioredoxin described above. In addition to these well-established roles, it also acts as a cofactor for NAD(P)H quinone dehydrogenase 1 (NQO1), a target of NRF2 (136). NADPH can principally be generated from metabolism of TCA cycle intermediates, from glycine/tetrahydrofolate metabolism, and by the PPP. The TCA cycle intermediates isocitrate and malate can be metabolized by isocitrate dehydrogenase and malic enzyme, respectively, to produce NADPH, with both enzymes being regulated by NRF2 (7). The PPP enzymes involved in NADPH metabolism, including G6PD, PGD, TKT, and TALDO1, seem to be directly or indirectly regulated by NRF2 and are shown in Fig. 3 (7). Overall, NRF2 activation serves to increase the intracellular pool of NADPH by regulating enzymatic activity of key NADPH synthesis reactions.

Redox balance plays a critical role in cancer initiation and progression. Generation of ROS can induce tumor-promoting mutations through DNA damage. However, contrary to common belief, use of antioxidants has been linked to cancer progression and poor outcomes in clinical trials. In a randomized clinical trial, Omenn and colleagues treated patients with high-risk exposure to smoking or asbestos with either a placebo or a combination of the antioxidants β-carotene and vitamin A (137). The treatment arm had a significant increase in incidence of lung cancer and death. The study was stopped early because of these findings. In a more recent clinical trial, use of vitamin A, vitamin C, vitamin E, coenzyme Q10, and carotenoids was associated with increased risk of recurrence (138). Preclinical evidence also supports that supplementation with antioxidants promotes tumor progression. Using $Kras^{G12D}$- or $Braf^{V600E}$-driven GEMMs of lung cancer, Sayin and colleagues (139) showed that the addition of dietary antioxidants (NAC or vitamin E) resulted in increased tumor burden and higher tumor grade. In a melanoma mouse model, treatment with NAC had no impact on primary tumor growth but increased circulating melanoma cells and metastases (34). Inhibition of NRF2 function results in reduced proliferation *in vivo*, highlighting the importance of NRF2 activation in tumor progression (6, 116). However, in addition to redox homeostasis, NRF2 regulates multiple other cellular pathways that also play a role in tumor progression.

Amino Acid Metabolism

Although NRF2 is mainly known for regulating the cellular redox state, NRF2 activation has a key role in multiple pathways involving amino acid transport and metabolism (Fig. 3). The synthesis of glutathione requires three amino acids: glutamate, cysteine, and glycine. NRF2 regulates not only the synthesis of glutathione but also the intracellular abundance of these amino acids. *SLC7A11* is an NRF2 target that encodes for a protein that dimerizes with SLC3A2 to form the x_c^- antiporter system (xCT). xCT functions as a concentration-dependent antiporter, which exports

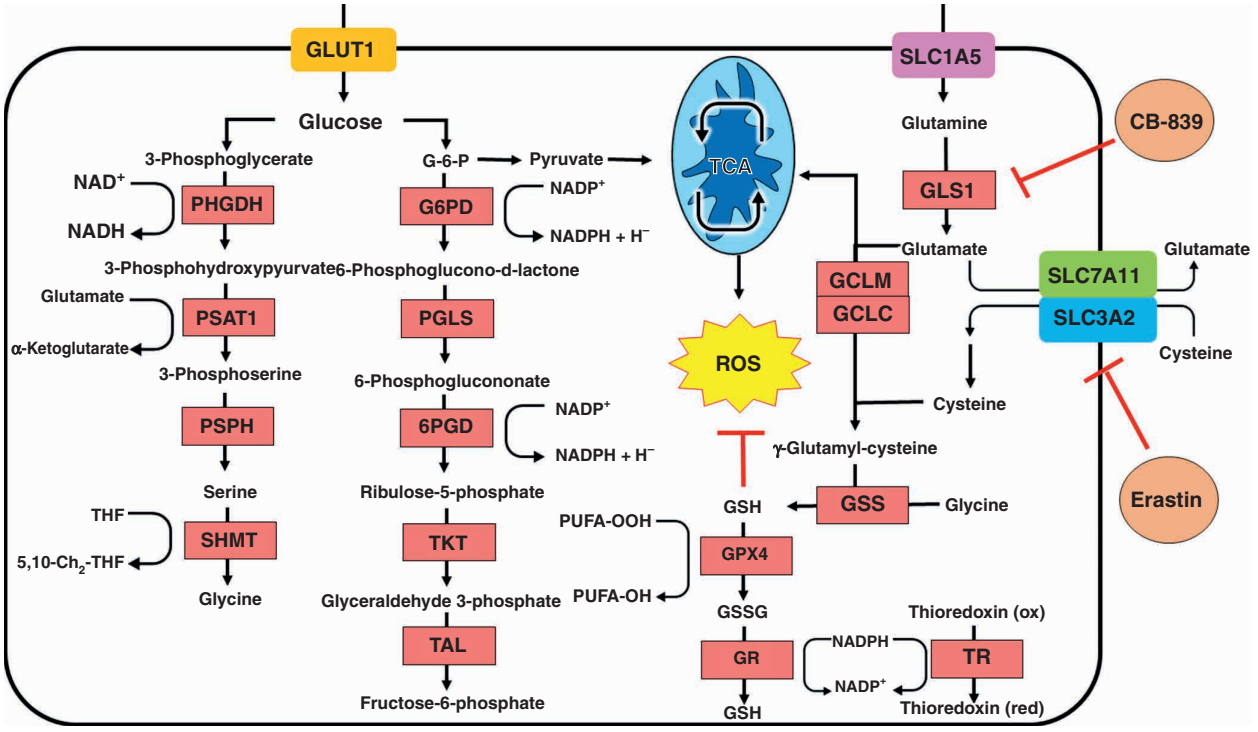

Figure 3. Metabolic rewiring by NRF2. Activation of NRF2 dramatically enhances generation of glutathione by increasing synthesis of glutathione from intracellular glutamate, cysteine, and glycine. Intracellular glutamate is derived from glutamine through GLS1. Cystine is imported by the NRF2 target SLC7A11. Serine and glycine are synthesized via NRF2-dependent processes. NADPH is synthesized to support redox metabolism by the pentose phosphate pathway. GLS1 and SLC7A11 function can be impaired by CB-839 and erastin, respectively. 6PGD, 6-phosphogluctonate dehydrogenase; G6PD, glucose-6-phopshate dehydrogenase; GLS1, glutaminase 1; GPX4, glutathione peroxidase 4; GR, glutathione reductase; GSS, glutathione synthetase; NAD(P)H, nicotinic adenine dinucleotide (phosphate); PGLS, 6-phosphogluconolactonase; PHGD, phosphoglycerate dehydrogenase; PSAT1, phosphoserine aminotransferase; PSPH, phosphoserine phosphatase; PUFA, polyunsaturated fatty acid; SHMT, serine hydroxymethyltransferase; TAL, transaldolase; THF, tetrahydrofolate; TKT, transketolase; TR, thioredoxin.

glutamate in exchange for cystine, the dimerized form of cysteine. This transporter serves to maintain intracellular stores of cysteine for glutathione synthesis (7, 116, 140). Sayin and colleagues (141) show that NRF2-mediated depletion of intracellular glutamate stores through export (xCT) and consumption [glutathione (GSH) synthesis] results in cancers that are dependent on extracellular glutamine. These tumors can be therapeutically targeted by inhibiting glutaminase, which ultimately catalyzes the conversion of glutamine to glutamate (Fig. 3), and therefore depleting intracellular glutamate (55, 62, 141). Not only is glutamate a critical carbon source for many biosynthetic reactions, but glutamate also serves as a nitrogen donor for the synthesis of nonessential amino acids, including serine and glycine.

Under normal physiologic conditions, depletion of intracellular glutamate due to NRF2 activation likely has no impact on serine-dependent reactions, as serine and other nonessential amino acids can be imported from the microenvironment. However, LeBoeuf and colleagues (142) have demonstrated that in serine-deprived conditions, hyperactive NRF2 creates a metabolic vulnerability by depleting glutamate needed for serine synthesis. One central feature of NRF2 activation is the dependency on extracellular glutamine to replenish intracellular glutamate for numerous downstream pathways. Glutamine enters the cell through multiple transporters, including SLC1A5 (143), whereas GLS1 catalyzes the rate-limiting step in glutaminolysis to produce glutamate (144, 145). This dependency on external glutamine can be exploited therapeutically.

Using *KRAS*-driven mouse and patient-derived xenograft models, loss of *KEAP1* sensitizes tumors to CB-839 (116, 141). Several studies have demonstrated that inhibiting GLS1 with CB-839 or BPTES has been successful in impairing the growth of NRF2-addicted cancer cells (116, 141, 146, 147). This has been further validated by Galan-Cobo and colleagues (148), who show that activation of NRF2 sensitizes human cell lines to glutaminase inhibition. Glutaminase inhibition sensitivity has been attributed to depletion of intracellular pools of glutamate, which is exported by xCT in NRF2-active tumors as above. Sayin and colleagues (141) showed that blocking xCT using the small-molecule erastin, and therefore blocking the export of glutamate, rescues sensitivity of *Keap1* LOF- or *Nrf2* GOF–mutant cell lines to CB-839. This sensitivity is not specific to lung malignancies. *Kras*-mutant pancreatic cancers upregulate NRF2 activity in response to chemotherapy (149) or eIF4A inhibitors (150) and become sensitized to CB-839. In a HER2-driven breast cancer model, HER2 downregulation leads to ROS-mediated apoptosis. Resistant dormant cells escape apoptosis and lead to tumor recurrence by NRF2-driven activation of antioxidant pathways, which sensitizes them to glutaminase inhibition (151).

Another potential liability in NRF2-addicted tumors targets cysteine metabolism. Kang and colleagues (119) identified that *KEAP1*-mutant tumors epigenetically silence the expression of cysteine dioxygenase 1 (*CDO1*), which metabolizes the entry of cysteine toward taurine biosynthesis. Overexpression of CDO1 in NRF2 hyperactive cells resulted in impaired proliferation, suggesting a potential therapeutic vulnerability.

Autophagy

The KEAP1/NRF2 pathway interacts with the autophagy pathway through the adaptor p62. Normally, p62 binds proteins (such as those misfolded under ER stress) and shuttles the protein to the autophagolysosome by interacting with LC3. As discussed above, p62 can bind KEAP1 but with lower affinity compared with NRF2. However, after phosphorylation at serine 349 by mTORC1, p62 binds KEAP1 with much higher affinity than NRF2 through competitive binding with the DLG motif (68, 152). This interaction then leads to degradation of the p62–KEAP1 complex. Interestingly, NRF2 transcriptionally regulates p62 through an ARE and therefore forms a positive feedback loop (153).

The pathologic role of the p62–KEAP1 interaction can be seen in a subset of patients with bladder cancer with increased p62 expression. Overexpression of p62 in bladder cancer cell lines increased NRF2 levels and *in vitro* proliferation, whereas knockout of p62 impaired tumor growth in a xenograft model (154). Umemura and colleagues (155) demonstrated, using multiple hepatocellular carcinoma models (carcinogen-induced, constitutive mTORC1 activation, and a nonalcoholic steatohepatitis model), that hepatocyte-specific loss of p62 reduced tumor initiation. They further showed that hepatocyte overexpression of p62 resulted in tumor formation, and deletion of the KIR region of p62 (which interacts with KEAP1 and stabilizes NRF2) reversed the development of tumors. NRF2 stabilization by p62–KEAP1 binding has also been shown specifically in patients with hepatitis C who have hepatocellular carcinoma (156).

Using a CRISPR/Cas9-based screens, Romero and colleagues (118) found that *KEAP1*-mutant cells are vulnerable to loss of *Slc33a1*, an endoplasmic reticulum–associated protein implicated in ER homeostasis. Loss of this transporter results in an induction of autophagy markers and transcriptional signatures enriched in the unfolded protein response (UPR) pathway. The sensitivity of *Keap1*-mutant cells to loss of *Slc33a1* was thought to be mediated by added UPR in the setting of increased glutathione synthesis. Targeting SLC33A1/UPR induction may be a promising therapeutic target in *KEAP1*-mutant lung adenocarcinoma.

Iron and Heme Metabolism

NRF2 transcriptionally induces a large transcriptional program that regulates heme and iron metabolism, which need to be tightly regulated and play a role in multiple physiologic processes. Although heme is mostly known for its role in coordinating oxygen in hemoglobin, heme and its derivatives are important prosthetic groups for at least 100 different enzymes that catalyze multiple redox reactions, including complex II, III, and IV of the electron transport chain (157, 158) and lipid desaturation (159–161). Heme synthesis is a complex process that involves multiple reactions to synthesize the protoporphyrin IX (PPIX) to which ferrous iron (Fe^{2+}) is bound in the mitochondria to generate heme. NRF2 induces the expression of the mitochondrial porphyrin transporter ATP-binding cassette subfamily B member 6 (ABCB6), which imports porphyrin into the mitochondria, and ferrochelatase (FECH), which loads ferrous Fe^{2+} iron onto the PPIX ring (162). NRF2 also regulates heme degradation by inducing the expression of *HO1*, which degrades heme into ferrous iron and biliverdin (3). Regulation of heme plays a pivotal role in lung cancer metastasis. As discussed above, Lignitto and colleagues (126) demonstrated that loss of *KEAP1* leads to activation of NRF2, leading to an HO1-dependent heme degradation that then stabilizes the prometastatic transcription factor BACH1.

The iron atom in heme is a potential catalyst for Fenton reaction–mediated lipid oxidation through the production of superoxide that is scavenged by antioxidants (163). As a result, free heme levels must be tightly regulated in order to maintain redox homeostasis (127, 163, 164). The iron liberated from heme is recycled for heme synthesis, storage, or export from the cells. Therefore, NRF2 plays a critical role in regulating free iron to prevent Fenton reaction–mediated lipid peroxidation, which triggers a form of cell death called ferroptosis. These lipid peroxide radicals can be reduced to nontoxic lipids by glutathione in a reaction catalyzed by glutathione peroxidase 4 (Fig. 3). Lipid radicals can accumulate in conjunction with free iron and in the absence of sufficient glutathione to trigger ferroptotic cell death. The KEAP1/NRF2 pathway likely plays a critical role in blunting ferroptotic cell death as several components of ferroptosis are regulated by NRF2. NRF2 prevents iron-dependent generation of hydroxyl radicals by promoting free iron sequestration through the transcriptional regulation of ferritin light (FTL) and heavy (FTH1) chains that together make up the 24 subunits that form ferritin (5). Export of free iron is solely facilitated by ferroportin 1, which has also been shown to be transcriptionally regulated by NRF2 in macrophages (165). In addition, NRF2 regulates glutathione synthesis at multiple levels, including through regulation of xCT expression that facilitates cysteine availability for GSH synthesis. Reduction of lipid peroxides requires NADPH produced by NRF2-regulated enzymes in the PPP. There is a growing interest in exploiting ferroptosis to therapeutically target cancer cells. Despite this, there are limited data showing the importance of NRF2 activation suppressing ferroptosis *in vivo*. *In vitro* work has shown that NRF2 activation results in resistance to ferroptosis inducers, suggesting that these classes of drugs may have limited efficacy in NRF2-activated tumors (166). Recently, Takahashi and colleagues (167) used three-dimensional spheroid cultures that better mimic *in vivo* growth conditions to demonstrate that the inner cells of tumor spheroids are exposed to higher ROS and lipid peroxidation and are susceptible to cell death after *NRF2* knockdown. Furthermore, suppressing ferroptosis may be a key mechanism by which NRF2-active tumors resist radiotherapy. Lang and colleagues (168) have shown in preclinical models that radiation therapy induced ferroptosis through downregulation of xCT. Therefore, upregulation of xCT may allow tumors to evade radiation-induced ferroptosis. Kang and colleagues

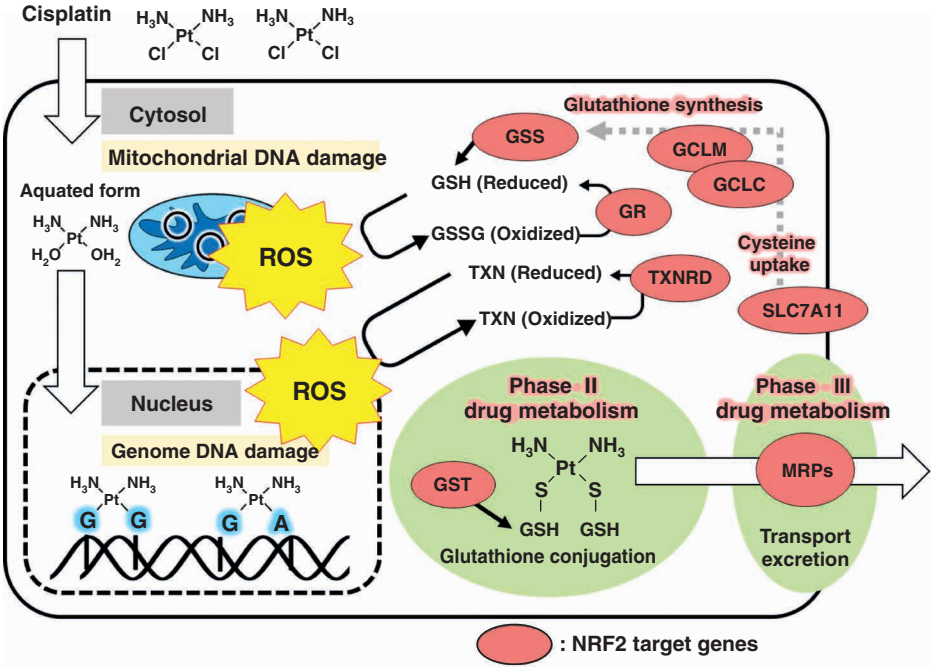

Figure 4. NRF2-dependent drug detoxification. Aquated form of cisplatin targets both mitochondrial and genomic DNA by anchoring to nucleotides at guanine–guanine or guanine–adenine bonds, which cause severe DNA damage and generate ROS. Hyperactivated NRF2 maintains high glutathione and thioredoxin levels, which scavenge ROS. Toxic aquated forms of cisplatin are conjugated with glutathione by a NRF2-induced transferase, GST, and then excreted by multidrug-resistant pumps, which are also transcriptional targets of NRF2. GSSG, glutathione disulfide; MRP, multidrug resistance protein.

(169) demonstrated that the NRF2 target GCLC suppresses ferroptosis by a GSH-independent mechanism. They show that GCLC activity serves as a glutamate sink through synthesis of γ-glutamyl dipeptides. This depletion of intracellular glutamate inhibited cystine starvation–dependent ferroptosis. Further work needs to be done to validate the role of NRF2 in ferroptosis in both humans and mouse models.

THE ROLE OF KEAP1/NRF2 IN THERAPY RESISTANCE

Chemotherapy and Radiotherapy Resistance

Disease recurrence and development of resistance to therapy pose a challenge to the clinical treatment of cancer. Hyperactivation of NRF2 in cancer is associated with chemotherapy and radiotherapy resistance and, as a result, poor prognosis (115, 170, 171). For example, NRF2-addicted cancer cell lines are less sensitive to the typical anticancer drugs such as cisplatin and doxorubicin (172). Platinum-based chemotherapy induces tumor cell death through two key mechanisms: the formation of DNA adducts that impair DNA replication and the induction of mitochondrial ROS (173, 174). As previously described, in normal physiology, NRF2 functions in a cytoprotective manner but also enables a mechanism for NRF2-mediated cisplatin resistance. Cancer cells with hyperactivation of NRF2 by somatic mutations in *KEAP1* or *NRF2* show increased expression of phase II detoxification enzymes such as NQO1 and GST to conjugate reactive molecules such as cisplatin with the reduced form of glutathione, GSH. Once conjugated to GSH, cisplatin is excreted by phase III detoxification pumps MRP4 and MRP5 (ref. 175; Fig. 4). Other typical anticancer drugs, such as 5-fluorouracil, 6-TG, gemcitabine, and cytarabine, are also pumped out by this activation of ATP-binding cassette family transporters induced by NRF2. In addition, cisplatin is known to induce cell death through increased mitochondrial ROS, which can be mitigated by the antioxidant pathways activated by NRF2. From a screening comparison of tumor suppressor versus chemotherapy and targeted therapy, *KEAP1* was detected as a marker of resistance across different organ backgrounds against arsenic trioxide (176). NRF2 hyperactivation can also sensitize to chemotherapy as NQO1 can activate mitomycin C, increasing its cytotoxicity (177, 178). For similar reasons, hyperactive NRF2 tumors have been known to be resistant to radiotherapy likely mediated through regulation of radiation-induced ROS (124). However, recent work has demonstrated that addition of the glutaminase inhibitor CB-839 can sensitize *KEAP1*-mutant cell lines to radiation therapy (18).

Targeted Therapy Resistance

Therapeutic targeting of mutations in *EGFR* and *ALK* is a key component of treatment for patients with NSCLC with these specific driver mutations. Multiple lines of evidence suggest that LOF mutations in *KEAP1* can reduce responses to targeted therapy. Using an *in vitro* CRISPR screen in human lung cancer cell lines, Krall and colleagues (179) identified that *KEAP1* loss results in resistance to the MEK inhibitor trametinib. Using a similar approach, it was demonstrated that *KEAP1* mutations drive resistance to sorafenib in hepatocellular carcinoma cell lines (180). In addition, loss of *KEAP1* in the

BRAF^{V600E}-mutant lung cancer cell line HCC364 desensitizes cells to the BRAF inhibitor vemurafenib. The authors further verified targeted therapy resistance in *EGFR*- and *ALK*-mutant cells with *KEAP1* mutations. Hellyer and colleagues (181) validated these experimental findings in a cohort of 228 patients with *EGFR* mutations with or without *KEAP1*/*NRF2*/*CUL3* mutations. Activation of NRF2 in these *EGFR*-mutant tumors was associated with treatment failure. Other drugs including axitinib, a VEGF inhibitor, also appear to have reduced effectiveness *in vitro* in the context of elevated NRF2 activity in renal cell carcinoma (182). Recently, in a phase II clinical trial using the *KRAS*^{G12C} inhibitor sotorasib, there was a trend for reduced response rates in *KEAP1*-mutant tumors (183). The mechanism in which activation of NRF2 induces drug resistance in the context of targeted therapy is not clearly delineated but is likely related to already-described mechanisms, including drug detoxification and regulation of ROS.

Modulation of Antitumor Immune Responses

Extensive clinical work has demonstrated that NRF2 activation in lung adenocarcinoma impairs antitumor immune response. In addition to reducing sensitivity to chemotherapy, both LOF mutations in *KEAP1* and activating mutations in *NRF2* confer worse overall survival for patients treated with checkpoint blockade (20, 184). These findings are not specific to lung, as a pan-cancer analysis has shown similar findings across multiple tumor types (44, 80). Surprisingly, mutations in *KEAP1* were associated with high tumor mutational burden as well as increased PD-L1 expression, which is strongly correlated with favorable responses to immune checkpoint blockade (185).

The mechanism of this resistance remains unclear, and little work has been done to characterize the immune microenvironment of *KEAP1*-mutant tumors. Kadara and colleagues (186) showed that in early stages, *KEAP1*-mutant tumors had higher peritumoral CD57$^+$ and granzyme B$^+$ cells suggestive of natural killer (NK) cells. Using a *Keap1*$^{flox/flox}$; *Pten*$^{flox/flox}$ (K1P) mouse model, Best and colleagues (85) analyzed immune infiltration in tumor-bearing and healthy mice. They found that lungs from tumor-bearing mice had a reduced number of NK, B, and T cells. In addition, these studies showed that loss of *Keap1* impairs the expansion of CD11c$^+$ immune populations observed in the K1P mouse model.

The activation of NRF2 in specific immune populations can alter their function. Kobayashi and colleagues (8) showed that NRF2 can inhibit lipopolysaccharide (LPS)-induced expression of *Il6* and *Il1β* in M1 macrophages. In a similar fashion, Thimmulappa and colleagues (187) demonstrated that *Nrf2*$^{-/-}$ peritoneal neutrophils produce less IL6 and TNFα in response to LPS stimulation. NRF2 can also alter cytokine expression in a tumor-intrinsic context. The alarmin/cytokine IL33 has been implicated in promoting tumor progression, but the mechanism of IL33 is not entirely clear. Using a skin squamous cell carcinoma, it has been demonstrated that NRF2 promotes IL33 release, resulting in accumulation of protumor macrophages (188). NRF2 also has been shown to regulate *Il11*. Using a mouse colorectal cell line, Nishina and colleagues (189) induced *Il11* transcription by treatment with the electrophile 1,2 naphthoquinone and further demonstrated that this upregulation was NRF2-dependent. Kitamura and colleagues (190) demonstrated that in human breast cancer samples, IL11 protein levels correlated with NRF2 levels. They further showed that *Keap1*-null MEFs upregulated *Il11* in three-dimensional culture and that tumor engraftment was impaired by knockout of *Il11*. These studies demonstrate the importance of NRF2 activity in regulating cytokine production, which may have an impact on tumorigenesis.

Hayashi and colleagues (191) reported that *Keap1*-null and *Kras*^{G12D}-mutant lung tumors display infiltrating CD8$^+$ T cells with higher expression of PD-L1 compared with *Keap1* wild-type and *Kras*^{G12D}-mutant tumors. In addition, they observed that NRF2 activation in the extratumoral area (described as an NRF2-charged microenvironment) resulted in reduction in area of *Keap1*-mutant *Kras*^{G12D}-driven tumors. The NRF2-charged microenvironment also regulated the histopathology of tumors that developed. Tumors with an NRF2-charged microenvironment favored a more lepidic phenotype while in the noncharged microenvironment displayed a more aggressive papillary phenotype. Interestingly, *Tnfα* expression in CD8$^+$ T cells was induced in the NRF2-charged microenvironment (191). Multiple other studies have shown that global NRF2 activation or NRF2 inactivation in immune populations can regulate tumor growth (122, 192). These reports demonstrate the potential role for NRF2 modulation to suppress NRF2 hyperactivated cancers through enhancement of anticancer immunity. Extensive additional work is needed to characterize the alterations in the tumor immune microenvironment to determine the mechanism through which *KEAP1*-mutant tumors develop resistance to checkpoint blockade.

It is attractive to hypothesize that tumor cell metabolic rewiring driven by hyperactivation of NRF2 can alter the metabolic milieu of the tumor microenvironment and regulate immune cell effector functions (Fig. 5). Nutrient competition is characterized by increased production of immunosuppressive metabolites such as lactate (193, 194) and kynurenine (195) by the tumor cells but also increased consumption of metabolites necessary for immune cell activation such as glucose (194, 196), glutamine (197–199), serine (200), and tryptophan (195, 201). It is likely that the increased utilization of nutrients such as glucose, glutamine, alanine, glycine, and cystine (116, 143) in *Keap1*-mutant tumors used to support anabolic metabolism may dampen immune responses via restricting effector immune cells of essential nutrients. *Keap1* mutation could also be affecting immune responses via increased antioxidant production. This is an exciting line of investigation considering that immune populations have differential redox sensitivity. Lipid peroxidation products can inhibit dendritic cell function and subsequent T-cell activation (202). Oxidative stress can increase the immunosuppressive ability of T regulatory cells (Treg; ref. 203). Interestingly, Wang and colleagues (204) showed that IFNγ released by CD8$^+$ T cells downregulates the two subunits of the glutamate-cystine antiporter xCT promoting ferroptosis (SLC7A11, *SLC3A2*). Increased SLC7A11 expression in the setting of *KEAP1* LOF or *NRF2* GOF may enable tumors to evade IFNγ-induced ferroptosis.

We previously discussed that NRF2 plays a major role in regulating heme and iron metabolism. Lignitto and

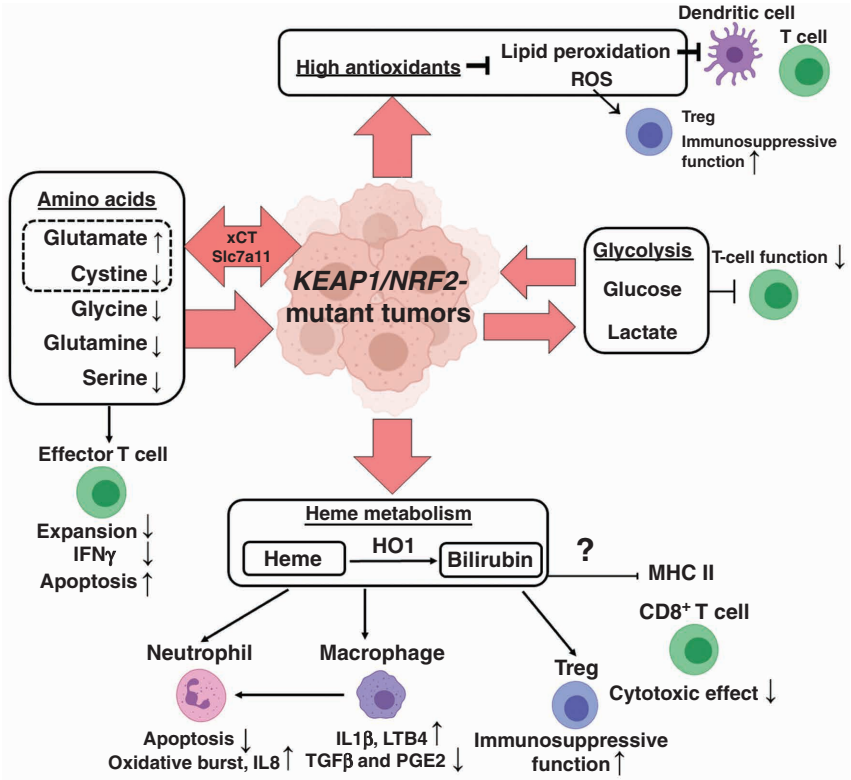

Figure 5. Impact of *Keap1/Nrf2* mutation on tumor microenvironment and antitumor immune responses. *Keap1* LOF/*Nrf2* GOF mutation–harboring tumors display increased uptake of nonessential amino acids such as glycine, serine, and glutamine. Cystine is imported through NRF2-regulated transporter xCT. Overall, in the microenvironment of *Keap1/Nrf2*-mutant tumors, glutamate is increased, whereas cystine, glycine, glutamine, and serine are depleted. These metabolic changes can inhibit effector T-cell function (expansion, production of IFNγ) and induce apoptosis. Besides amino acids, *Keap1/Nrf2* mutations result in increased glycolysis and thus increased glucose consumption and lactate secretion, which can be deleterious for T-cell function. NRF2 is a master regulator of antioxidants that decrease ROS and lipid peroxides, which can affect dendritic cells, T-cell effector cells, and Treg cells. Hyperactivation of NRF2 results in altered heme metabolism, and the by-products of these pathways can affect neutrophils, macrophages, T regulatory cells, and cytotoxic T lymphocytes.

colleagues (126) showed heme to be important for tumor growth and metastasis. NRF2 can induce multiple genes (*ABCB6*, *FECH*, *HO1*, *BLVRB*) central to heme regulation. Heme metabolism by-products as well as heme itself have been suggested to have immunomodulatory functions. In neutrophils, heme decreases apoptosis and promotes oxidative burst and IL8 production (205). In macrophages, it promotes IL1β production via inflammasome (NLRP3) activation and LTB4 production that drives neutrophil recruitment, and it also impairs production of anti-inflammatory molecules such as TGFβ and PGE_2 (206). Bilirubin, which is produced by biliverdin reductase B, the last step of heme degradation, has been proposed to have potent immunosuppressive activity, including the ability to downregulate MHC II expression (207, 208), induce $CD8^+$ T-cell apoptosis (207), and promote Treg cell infiltration (209). Interestingly, carbon monoxide, a by-product of HO1-catalyzed reactions, has been shown to suppress allograft rejection, pointing to its immunosuppressive activity (210).

FUTURE THERAPEUTIC PERSPECTIVES

LOF *KEAP1* mutations and GOF *NRF2* mutations lead to more aggressive tumors through the mechanisms discussed above and serve as a prognostic marker associated with poor survival and poor response to multiple types of therapy (20, 211, 212). Furthermore, *KEAP1/NRF2* mutations also lead to metabolic vulnerabilities in preclinical models, making these mutations predictive biomarkers of responsiveness to metabolic therapies (116). Currently, there are four clinical trials specifically targeting *KEAP1* mutations (summarized in Table 1). The most promising trial is the KEAPSAKE trial, which combines the glutaminase inhibitor CB-839 (telaglenastat) with standard first-line therapy (pembrolizumab and chemotherapy) in patients with tumors that carry LOF mutations in *KEAP1* or activating mutations in *NRF2*. Although inhibiting glutaminase may improve outcomes in patients with *KEAP1* mutations by targeting tumor-intrinsic vulnerabilities, CB-839 may have the added benefit of augmenting antitumor T-cell responses. Leone and colleagues (213) demonstrate that glutaminase inhibition can augment immune responses in preclinical mouse tumor models without hyperactivated NRF2. They show that glutaminase inhibition has unique effects on T-cell subsets, including improved cytokine production, activation, and proliferation. The prodrug DRP-104 (sirpiglenastat) is currently in phase I trials (Table 1; trial ID: NCT04471415). The active moiety of DRP-104,

Table 1. Clinical trials targeting *KEAP1/NRF2*-mutant tumors

Trial	Drug	Study type	Inclusion	Intervention	Primary outcomes
KEAPSAKE NCT04265534	CB-839 (telaglenastat)	Phase II Randomized placebo controlled	Metastatic NSCLC with mutation in KEAP1, NRF2, or LKB1	Pembrolizumab + carboplatin + pemetrexed ± CB-839	Progression-free survival Safety and tolerability Dosing
BeGIN NCT03872427	CB-839	Phase II Open-label single arm	Advanced tumor with mutation in NF1, KEAP1, or LKB1	CB-839	Best overall response rate
NCT04471415	DRP-104 (sirpiglenastat)	Phases I and IIa Dose escalation Dose expansion	Advanced NSCLC with mutation in KEAP1, NRF2, or LKB1 and already received first-line therapy	DRP-104 + atezolizumab	Maximum tolerated dose Area under plasma concentration C_{max} of DRP-104
NCT02417701	Sapanisertib	Phase II Randomized open label	Stage IV or recurrent squamous cell carcinoma with KEAP1 or NRF2 mutation	Docetaxel + sapanisertib	Progression-free survival

6-dizao-5-oxo-L-norluecine (DON), is a potent glutamine antagonist. Although DON has previously been shown to reduce tumor growth, its high toxicity has prevented its therapeutic development (214). However, DRP-104 itself is inactive and was designed to limit systemic exposure to DON while targeting tumor cells.

Although current clinical trials focus on targeting metabolic vulnerabilities in NRF2-addicted tumors, future trials are likely to involve targeting NRF2 signaling in itself. Several promising *in vitro* and *in vivo* studies have demonstrated the feasibility of inhibiting NRF2 to suppress tumor growth as a single therapy or as a sensitizing agent. Tang and colleagues (215) demonstrate that luteolin impairs the binding of NRF2 to AREs and promotes NRF2 degradation in A549 cells. They go on to show that luteolin sensitizes A549-derived cells to oxaliplatin, bleomycin, and doxorubicin. In subsequent work, the authors showed that combination luteolin and cisplatin treatment significantly impaired tumor growth compared with monotherapy in A549 xenograft models (216). Of note, luteolin has NRF2-independent effects, including impairing EGFR signaling, which may contribute to these results (217). In a similar set of experiments, Ren and colleagues (218) demonstrated that brusatol, a drug that inhibits NRF2 activity among other effects, sensitizes tumors to platinum chemotherapy in an A549 xenograft model. Inhibiting NRF2 activity has potential clinical implications, yet no clinical trials have used this approach to target tumors. The off-target effects of these drugs and the possibility of impairing physiologic antioxidant and immune responses may limit their use clinically.

CONCLUDING REMARKS

The KEAP1/NRF2 pathway has a key role in tumorigenesis. Although NRF2 activation increases resistance to chemotherapy and radiotherapy, activation of these pathways leads to metabolic liabilities. Glutaminase inhibition has been extensively explored in preclinical models and has rapidly moved to clinical trials. But therapeutic vulnerabilities in other NRF2-dependent pathways, including the PPP and heme synthesis, need to be explored. In addition, although activation of NRF2 is clearly driving tumor-intrinsic protumor changes, there are NRF2-independent effects of *KEAP1* mutations that should be explored and potentially exploited therapeutically. And finally, understanding additional co-occurring mutations in the context of *KEAP1* mutation may lead to novel therapies in targeting not only *KEAP1*-mutant tumors but also other aggressive co-occurring mutations such as *LKB1*.

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