

# Cellular Signalling

## Targeted Therapy of NRF2high Esophageal Squamous Cell Carcinoma

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<b>Abstract:</b>	Esophageal squamous cell carcinoma (ESCC) is a deadly disease and one of the most aggressive cancers of the gastrointestinal tract. As a master transcription factor regulating the stress response, NRF2 is often mutated and becomes hyperactive, and thus causes chemo-radioresistance and poor survival in human ESCC. There is a great need to develop NRF2 inhibitors for targeted therapy of NRF2 high ESCC. In this review, we mainly focus on three aspects, NRF2 inhibitors and their mechanisms of action, screening novel drug targets, and evaluation of NRF2 activity in the esophagus. A research strategy has been proposed to develop NRF2 inhibitors using human ESCC cells and mouse models.
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Re: Invited Review

Dear Dr. Hao,

Thank you for inviting us to submit a review article entitled *Targeted Therapy of NRF2<sup>high</sup> Esophageal Squamous Cell Carcinoma* to *Cellular Signalling*. We have submitted all the required files online, which include Abstract, Manuscript, Figure, Supplementary Material, Declaration of Interest Statement.

Since NRF2 signaling has been extensively reviewed in the literature especially in the recent years, we only described the signaling pathway in brief. We mainly focus on three aspects, NRF2 inhibitors and their mechanisms of action, screening novel drug targets, and evaluation of NRF2 activity in the esophagus. Our long-term goal is to translate some of the inhibitors into clinical trials.

We look forward to working with your team to get this manuscript published.

Sincerely yours,

Xiaoxin Luke Chen

## **Abstract**

Esophageal squamous cell carcinoma (ESCC) is a deadly disease and one of the most aggressive cancers of the gastrointestinal tract. As a master transcription factor regulating the stress response, NRF2 is often mutated and becomes hyperactive, and thus causes chemo-radioresistance and poor survival in human ESCC. There is a great need to develop NRF2 inhibitors for targeted therapy of NRF2<sup>high</sup> ESCC. In this review, we mainly focus on three aspects, NRF2 inhibitors and their mechanisms of action, screening novel drug targets, and evaluation of NRF2 activity in the esophagus. A research strategy has been proposed to develop NRF2 inhibitors using human ESCC cells and mouse models.

# Targeted Therapy of NRF2<sup>high</sup> Esophageal Squamous Cell Carcinoma

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**Abbreviations:** ARE, antioxidant response element; ESCC, esophageal squamous cell carcinoma; KEAP1, Kelch-like ECH associated protein 1; NRF2/NFE2L2, nuclear factor erythroid 2-related factor 2;

**Keywords:** Esophageal squamous cell carcinoma; NRF2

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## Abstract

Esophageal squamous cell carcinoma (ESCC) is a deadly disease and one of the most aggressive cancers of the gastrointestinal tract. As a master transcription factor regulating the stress response, NRF2 is often mutated and becomes hyperactive, and thus causes chemo-radioresistance and poor survival in human ESCC. There is a great need to develop NRF2 inhibitors for targeted therapy of NRF2<sup>high</sup> ESCC. In this review, we mainly focus on three aspects, NRF2 inhibitors and their mechanisms of action, screening novel drug targets, and evaluation of NRF2 activity in the esophagus. A research strategy has been proposed to develop NRF2 inhibitors using human ESCC cells and mouse models.

## Introduction

Esophageal cancer is expected to affect 19,260 adults and cause 15,530 deaths in the US in 2021 (Cancer Facts & Figures 2021). In the world, it is the seventh most prevalent cancer and the sixth leading cause of cancer-related death, with more than 604,100 new cases and 544,076 deaths in 2020<sup>1</sup>. Two main histological types of esophageal cancer exist, squamous cell carcinoma (ESCC) and adenocarcinoma. Human ESCC develops from precancerous lesions, and its histopathology follows a step-wise pattern of hyperplasia, dysplasia, and SCC. The 5-year survival rate for ESCC is ~18%, a number that reflects late diagnosis, the aggressiveness of the disease, and a lack of effective treatment strategies<sup>2, 3</sup>. Thus, there is a great need to further elucidate the molecular mechanisms and develop more effective treatment strategies for ESCC.

Nuclear factor (erythroid-derived 2)-like 2 (*NRF2* or *NFE2L2*) mutations are commonly seen in ESCC, with frequencies between 5% and 30%. As a major cellular defense mechanism, the NRF2 signaling pathway regulates the expression of enzymes involved in detoxification and anti-oxidative stress response. NRF2 forms heterodimers with small MAF proteins and binds to the antioxidant response elements (ARE) of target genes when cells are exposed to oxidative stress or xenobiotics. Kelch-like ECH associated protein 1 (KEAP1) inhibits the function of NRF2 by retaining NRF2 in the cytoplasm and facilitating its ubiquitination-dependent degradation under normal physiological conditions. Oxidative stress and electrophilic modification of KEAP1 results in NRF2 nuclear translocation and ARE-dependent gene transcription (Figure 1). In addition to the KEAP1-CUL3 E3 ubiquitin ligase complex, NRF2 can also be ubiquitinated by  $\beta$ -TrCP-CUL1 or HRD1 for proteasomal degradation<sup>4, 5</sup>.

The NRF2 signaling pathway is a double-edged sword in the context of carcinogenesis. Chemical or genetic activation of NRF2 induces cytoprotective enzymes conferring protection against chemical carcinogenesis in multiple models. *Nrf2*<sup>-/-</sup> mice are more susceptible to chemical carcinogenesis than wild-type counterparts<sup>6, 7</sup>. On the other hand, cancer cells can hijack the NRF2 signaling pathway for their survival through mechanisms that lead to constitutive activation of NRF2 signalings, such as somatic mutations of *KEAP1/NRF2/CUL3*, accumulation of disruptor proteins, skipping of *NRF2* exon 2, KEAP1 succinylation, *KEAP1* hypermethylation, increased NRF2 expression, and electrophilic attack of KEAP1 by oncometabolites. NRF2 hyperactivation promotes cell proliferation and metabolic reprogramming, accelerates distant metastases, and confers chemo- and radio-resistance<sup>8, 9</sup>. *NRF2* and *KEAP1* are classified as high-confidence cancer driver genes<sup>10</sup>. Although not yet realized, the NRF2 signaling pathway is regarded as a tractable molecular target for cancer therapy<sup>11</sup>.

Using gene microarray data of human ESCC (GEO23400; n=53)<sup>12</sup>, we performed clustering analysis with a list of esophageal NRF2 target genes<sup>13</sup> and identified two subtypes, NRF2<sup>high</sup> cases (n=17) and NRF2<sup>low</sup> cases (n=36)<sup>14</sup>. Similarly, human ESCC can be clustered into NRF2<sup>Mut</sup> and NRF2<sup>WT</sup> cases according to DNA mutations. NRF2<sup>Mut</sup> ESCC was associated with a significantly worse prognosis than NRF2<sup>WT</sup> ESCC<sup>15</sup>. These data suggest

subtyping human ESCC according to the NRF2 status is essential for targeted therapy, and potent NRF2 inhibitors are highly desirable.

The esophagus is a unique organ site for studies on NRF2 hyperactivation in cancer. Genetic activation of NRF2 in *Keap1*<sup>-/-</sup> mice results in robust phenotypes in the esophagus, esophageal hyperplasia and hyperkeratosis<sup>16</sup>. To date, all transcriptionally impacted genes downstream of *Keap1* knockout are NRF2 responsive, although it remains possible that other KEAP1 substrates could alter transcription by an NRF2-independent mechanism. Overexpression of KEAP1 substrates (e.g., WTX, PALB2, SQSTM1, DPP3, CDK20) containing competitive binding motifs (e.g., ETGE, DLG) results in NRF2 displacement and subsequent NRF2 activation<sup>17-24</sup>. However, in the esophagus, NRF2 is the most important KEAP1 substrate as the esophageal phenotype of *Keap1*<sup>-/-</sup> mice can be completely rescued in *Nrf2*<sup>-/-</sup>;*Keap1*<sup>-/-</sup> and *K5Cre*;*Nrf2*<sup>fl/fl</sup>;*Keap1*<sup>-/-</sup> mice<sup>16, 25</sup>.

### NRF2 inhibitors and their mechanisms of action

At least 5 strategies have been proposed to target the NRF2 signaling pathway for cancer therapy: (1) transcriptional downregulation of *NRF2*; (2) increased degradation of *NRF2* mRNA or decreased translation; (3) enhancement of NRF2 degradation through up-regulation/activation of E3 ubiquitin ligase complexes for NRF2; (4) blocking the dimerization of NRF2 with small MAF proteins; and (5) blocking the NRF2-sMAF DNA-binding domain<sup>26</sup>. Many small-molecule compounds with NRF2-inhibitory activities have been reported in the literature (Table 1). Some NRF2-inhibitory compounds, *i.e.*, 4-methoxychalcone, apigenin, ascorbic acid, BET bromodomain inhibitor JQ1, chrysin, cryptotanshinone, epigallocatechin 3-gallate, luteolin, metformin, N-N-dimethylformamide, trichostatin A, triptolide, valproic acid, and wogonin, may in fact activate NRF2 expression and/or activity in some other experimental settings. They have been well-reviewed in the literature and may have limited potential for further drug development<sup>11, 27, 28</sup>.

High-throughput screening of chemical compound libraries containing natural products, synthetic compounds, and FDA-approved drugs is a popular approach to identify candidate compounds or compound classes with NRF2-inhibitory activities<sup>29-33</sup>. Apart from small molecule inhibitors, siRNA is also promising for targeting NRF2<sup>34, 35</sup>. However, siRNA formulation and delivery into esophageal epithelial cells *in vivo* are major hurdles in drug development. Following the screen, compounds are subject to validation of their NRF2-inhibitory activities in cultured cells and animal models.

While the NRF2-inhibitory activity of many compounds can be validated *in vitro*, they have rarely been validated in animal models. In order to develop NRF2 inhibitors for NRF2<sup>high</sup> ESCC, CRISPR-Cas9 was used to create a novel mouse carrying the *Nrf2*<sup>E79Q</sup> mutation within the endogenous *Nfe2l2* locus, which is the most commonly observed activating mutation in human cancer and is known to activate NRF2<sup>36</sup>. This mouse line allows conditional esophagus-specific activation of NRF2 when crossed with *K14Cre* line or *Sox2CreER* line<sup>37</sup>. Four weeks after tamoxifen exposure, *Sox2CreER*;*LSL-Nrf2*<sup>E79Q/+</sup> mice developed strong NRF2<sup>high</sup>-driven esophageal phenotype in the esophagus, similar to *Keap1*<sup>-/-</sup> mice (data not shown).

## Drug targets in NRF2<sup>high</sup> ESCC

In addition to NRF2 itself, some other genes may represent viable therapeutic targets if they prove functionally critical for NRF2<sup>high</sup> cancer. For example, NR0B1 was identified as a selectively expressed protein in NRF2<sup>high</sup> lung cancer and small molecules that disrupted NR0B1 protein complexes inhibited NRF2-dependent lung cancer growth<sup>38</sup>. To find additional drug targets for NRF2<sup>high</sup> ESCC, we downloaded the publicly available omics data of human cancer cell lines among which 22 human ESCC cell lines were included (Table 2; Supplementary Material). Using the RNAseq data and a list of differentially expressed genes in NRF2<sup>high</sup> ESCC<sup>14</sup>, we performed clustering analysis and principal component analysis to subtype ESCC cell lines into two clusters, NRF2<sup>high</sup> and NRF2<sup>low</sup> (Figure 2A, B). Among the NRF2<sup>high</sup> ESCC cell lines, KYSE70 (*NFE2L2*<sup>W24C</sup>), KYSE180 (*NFE2L2*<sup>D77V</sup>; *KEAP1*<sup>P278Q</sup>), KYSE520 (*NFE2L2*<sup>T80I</sup>), OE21 (*NFE2L2*<sup>G81S/D318H</sup>), TE6 (*NFE2L2*<sup>F71\_D77del</sup>), and TE11 (*NFE2L2*<sup>D29G</sup>) are NRF2<sup>Mut</sup> cells, and KYSE510, TE9, and TT are NRF2<sup>WT</sup> cells. KYSE510 has an NRF2<sup>high</sup> status probably due to *PIK3CA*<sup>E545K</sup> mutation. TE9 has been reported to express a high level of NRF2<sup>39</sup>.

To validate the clustering analysis, we compared the metabolomics data of NRF2<sup>high</sup> ESCC cells and NRF2<sup>low</sup> ESCC cells. Several metabolites (e.g., glutathione oxidized, glutathione reduced, NADP) are significantly higher, and several others (e.g., 6-phosphogluconate, glycine, aspartate, glutamate) are significantly lower in NRF2<sup>high</sup> ESCC cells than NRF2<sup>low</sup> ESCC cells. These observations are in agreement with several studies on the functional role of hyperactive NRF2 in cellular metabolomics<sup>14, 39, 40</sup>. Using the combined RNAi screen data of 20 ESCC cell lines (9 NRF2<sup>high</sup> and 11 NRF2<sup>low</sup>), we also found that NRF2<sup>high</sup> ESCC cells were more dependent on the *NFE2L2* gene than NRF2<sup>low</sup> ESCC cells (Figure 2C). These data support the clustering result of NRF2<sup>high</sup> and NRF2<sup>low</sup> ESCC cells.

We then compared gene dependency scores of NRF2<sup>high</sup> ESCC cells with those of NRF2<sup>low</sup> ESCC cells using data from two screening techniques, RNAi and CRISPR. It is generally believed that these two techniques are complementary especially when an improved bioinformatics tool is used to eliminate the off-target effect of RNAi<sup>41</sup>. The precision of the two libraries in detecting essential genes is similar and combining data from both screens improves performance, even though results from these two screens show little correlation, which can be partially explained by the identification of distinct essential biological processes with each technology<sup>42, 43</sup>. In total, 9 genes (*ABL1*, *ALDH3A1*, *C1QTNF9B*, *CASC3*, *DDX4*, *EXOSC3*, *GPAA1*, *SNAP47*, *SYT2*) were found to have higher dependency (*i.e.*, lower dependency scores) in NRF2<sup>high</sup> ESCC cells by both screens. For example, *ABL1*, as a non-receptor tyrosine kinase, is known to be essential and actionable for lung cancer cell survival<sup>44</sup>. *ABL1* promotes NRF2 nuclear localization in kidney cancer cells<sup>45</sup>. *ALDH3A1* oxidizes various aldehydes to the corresponding acids. NRF2 is known to regulate *ALDH3A1* expression in pancreatic cancer cells<sup>46</sup>. *ALDH3A1* is strongly expressed in human ESCC tissue, but barely detectable in the non-malignant esophageal epithelium. Knockdown of *ALDH3A1* in ESCC cells suppresses cell viability and clonogenic capacity



as well as tumor growth *in vivo*<sup>47</sup>. These data suggest that ABL1 and ALDH3A1 are potential drug targets for NRF2<sup>high</sup> ESCC.

There is a strong rationale to target kinases in combination with NRF2 inhibition for NRF2<sup>high</sup> ESCC. Multiple signaling pathways, for example, PI3K/AKT/mTOR can activate NRF2<sup>48</sup>. AKT can also increase the stability of NRF2 by activating p21 which disrupts the NRF2-KEAP1 interaction, and by inhibiting GSK3 $\beta$  that leads to reduced NRF2 phosphorylation, prevents its nuclear translocation, and promotes its ubiquitination and degradation<sup>18, 49</sup>. On the other hand, NRF2 overexpression is known to activate PI3K/AKT signaling in melanocytes and hepatocytes<sup>50, 51</sup>. NRF2 directly regulates mTOR transcription when the PI3K pathway is intact, whereas this function is lost when PI3K is activated<sup>52</sup>. It should be noted that keratinocyte-specific deficiency of *Pten* resulted in AKT activation and esophageal hyperplasia and hyperkeratosis<sup>53</sup>, similar to the NRF2<sup>high</sup>-driven esophageal phenotype. Recently, the NRF2 and PI3K pathways have been shown to synergize in driving non-small-cell lung cancer which was associated with metabolic reprogramming and altered immune microenvironment<sup>54</sup>. These data suggest that the NRF2 and PI3K pathways play a synergistic role in promoting carcinogenesis through mutually reciprocal positive reinforcement. In fact, in a pan-cancer analysis of TCGA datasets, the strongest co-occurrence of affected pathways was between genomic alterations of the NRF2 and PI3K pathways. These co-occurring alterations appeared most frequently in lung cancer, ESCC, head and neck SCC, and uterine carcinoma<sup>55</sup>.

Synthetic lethality is another potential approach to identify additional drug targets for NRF2<sup>high</sup> ESCC<sup>56, 57</sup>. Using isogenic ESCC cells with varying NRF2 status and high-throughput screening technologies, this approach may open up novel therapeutic opportunities.

### Drug candidates for NRF2<sup>high</sup> ESCC

Using drug sensitivity data from three databases (PRISM, CTRP V2, and GDSC), we compared drug sensitivity scores of NRF2<sup>high</sup> ESCC cells in comparison to NRF2<sup>low</sup> ESCC cells (Supplementary Material). From the PRISM database, we found NRF2<sup>high</sup> ESCC cells are significantly more sensitive to 62 drugs than NRF2<sup>low</sup> ESCC cells. These 62 drugs mainly fall into two classes, microtubule inhibitors and kinase inhibitors (especially Aurora A kinase inhibitors). For example, alisertib (an Aurora A kinase inhibitor) is known to induce oxidative stress and inhibit the expression of NRF2 in osteosarcoma cell lines<sup>58</sup>. Crizotinib (an inhibitor of ALK and c-Met) induces hepatotoxicity to reduce cancer cell viability, by activating oxidative stress responses, stimulating mitochondrial apoptosis and necrosis, accumulating reactive oxygen species, and inhibiting NRF2 signaling<sup>59</sup>. Apatinib (a VEGFR2 tyrosine kinase inhibitor) promotes oxidative stress-dependent apoptosis, by suppressing glutathione and NRF2 signaling in ovarian cancer cells<sup>60</sup>.

From the CTRP V2 database, we found 4 drugs are more sensitive for NRF2<sup>high</sup> ESCC cells than NRF2<sup>low</sup> ESCC cells: fluorouracil, leptomycin B, BRD-K71781559, tanespimycin. From GDSC1 and GDSC2 databases, we found motesanib and navitoclax, respectively. Motesanib (sorafenib) is a multi-kinase inhibitor that selectively

inhibits EGFR1, VEGFR2, and VEGFR3, and inhibits tumor growth. A study of sorafenib in the treatment of advanced gastric and gastroesophageal cancers showed that when combined with docetaxel and cisplatin, the regimen was effective in reducing tumor size and increase progression-free survival and overall survival<sup>61</sup>. Lung cancer cells that were sensitive to trametinib plus navitoclax (a BCL-xL/BCL-2 inhibitor) expressed higher levels of NRF2 than did those that were resistant<sup>62</sup>. Navitoclax also induces apoptosis and synergizes with chemotherapy by targeting stemness pathways in esophageal cancer<sup>63, 64</sup>.

Furthermore, in a recent study on the pharmacogenomics landscape of protein-protein interaction (PPI)-perturbing mutations, NRF2-KEAP1 PPI was identified as one of the top among 470 putative PPIs in a pan-cancer analysis of 33 cancer types. Sensitivity to 8 drugs was found higher in cancer cells with mutant NRF2-KEAP1 PPI as compared with those with wild-type NRF2-KEAP1 PPI: 17-AAG, docetaxel, temsirolimus, JNJ-2684165, midostaurin, mitomycin C, ZG-10, and embelin<sup>65</sup>. Interestingly, both 17-AAG and mitomycin C have been reported in the literature as NRF2 inhibitors which are more potent for NRF2<sup>high</sup> cancer cells and act through synthetic lethality<sup>66, 67</sup>.

### Monitoring NRF2 activity in ESCC

When NRF2 inhibitors are used for clinical trials on NRF2<sup>high</sup> ESCC, there will be a need to assess the NRF2 activity in the cancer tissue to serve two clinical needs: (1) Diagnosis of NRF2<sup>high</sup> ESCC: We currently depend on exome sequencing to detect gene mutations, RNAseq to elucidate mRNA expression profiles, or quantitative multiplex immunohistochemistry to evaluate protein expression, for selecting cancer patients for targeted therapy. Although this tissue-based invasive approach has been widely used and is reasonably successful in clinical practice, they all require invasive biopsy. In addition, more or less they downplay tumor heterogeneity by using one piece of tumor or biopsy tissue to represent the whole tumor. (2) Evaluation of the efficacy of NRF2 inhibitors: The efficacy of cancer-targeted therapy is assessed in the same way as traditional cancer therapy, and clinicians depend on symptoms, biomarkers, and radiology to guide treatment planning. More efficient and non-invasive tools that can reveal molecular changes as well as functional abnormalities are highly needed (Table 3).

We have generated an NRF2<sup>high</sup> mRNA signature which is responsive to NRF2 inhibition and an NRF2<sup>high</sup> protein signature. Algorithms will be generated for the assessment of NRF2 activity in ESCC tissue samples. In addition, hyperactive NRF2 is known to cause metabolic reprogramming in the esophagus through transcriptional regulation of metabolic genes, for example, glucose transporter/enzymes (GLUT1, HK1, HK2) and acetate enzyme (ACSS2)<sup>14, 68</sup>. NRF2 also positively regulates the expression of a monocarboxylate transporter (MCT1) in mouse skeletal muscle, colonic epithelial cells, and mouse liver<sup>69 70</sup>. It has become feasible to use PET/CT imaging tools with radionuclides to evaluate whether an individual's cancer is NRF2<sup>high</sup> before treatment and whether an NRF2 inhibitor successfully hits NRF2 after treatment. For example, <sup>18</sup>F-FDG, is widely used for clinical staging and follow-up of human cancer including ESCC. A multicenter prospective trial showed that <sup>18</sup>F-

FDG PET/CT had a sensitivity of 79% and a specificity of 95% for stage IV human ESCC, while early-stage T1 and T2 tumors tended to have minimal or no FDG uptake<sup>71</sup>. <sup>18</sup>F-FDG is transported into the cells via glucose transporters on the membrane (e.g., GLUT1) and metabolized in the cytosol into <sup>18</sup>F-FDG-6P by hexokinases (e.g., HK1, HK2), which are overexpressed in NRF2 hyperactive tissues<sup>72</sup>. In patients with lung SCC, the maximum standardized uptake value (SUVmax) was significantly higher in NQO1<sup>high</sup> tumors than NQO1<sup>low</sup> tumors (NQO1 is a *bona fide* NRF2 target)<sup>73</sup>. GLUT1 expression is significantly correlated with SUVmax of <sup>18</sup>F-FDG in human ESCC tissues<sup>74</sup>. <sup>11</sup>C-acetate is transported into the cells via monocarboxylate transporters on the membrane (e.g., MCT1), and converted into <sup>11</sup>C-acetyl-CoA by acyl-CoA synthetase short-chain family members (e.g., ACSS2). <sup>11</sup>C-acetyl-CoA will then be used for *de novo* lipogenesis, histone acetylation, and the tricarboxylic acid cycle. <sup>11</sup>C-acetate PET/CT is primarily used for detecting prostate cancer due to the poor uptake of <sup>18</sup>F-FDG and the proximity of the prostate to the bladder<sup>75</sup>.

We have conducted a preliminary imaging study on wild-type and *Sox2CreER;LSL-Nrf2<sup>E79Q</sup>* mice using <sup>18</sup>F-FDG/<sup>11</sup>C-acetate PET imaging in combination with contrast-enhanced CT. Both <sup>18</sup>F-FDG uptake and <sup>11</sup>C-acetate uptake were significantly elevated in the NRF2<sup>high</sup> esophagus as compared to the control. *Ex vivo* autoradiography of <sup>11</sup>C-acetate showed dramatic accumulation in the NRF2<sup>high</sup> esophagus (data not shown). Further studies are ongoing to develop dual <sup>18</sup>F-FDG/<sup>11</sup>C-acetate PET for assessment of NRF2 activity in the esophagus.

## Conclusion

There is a pressing need for NRF2 inhibitors for targeted therapy of NRF2<sup>high</sup> ESCC and other NRF2<sup>high</sup> cancers. Promising inhibitors and novel drug targets have been identified and await further validation, in particular, in animal models. NRF2 activity assays with tissue-based methods and imaging tools are highly needed. Further research is expected to develop the inhibitors and elucidate their mechanisms of action for NRF2<sup>high</sup> ESCC.

**Table 1. NRF2 inhibitors**

Compound	Mechanisms of Action	Note
(E)-3-(3,5-dimethoxyphenyl)-1-(2-methoxyphenyl)prop-2-en-1-one <sup>76</sup>	Unknown	
1-(4-(tert-Butyl)benzyl)-3-(4-chlorophenyl)-N-hydroxy-1H pyrazole-5-carboxamide <sup>77</sup>	Unknown	
3',4',5',5,7-pentamethoxy flavone <sup>78</sup>	Upregulates KEAP1 expression and inhibits ERK	
Ailanthone <sup>79, 80</sup>	Post-translational mechanisms, i.e., increase of KEAP1 and decrease of UCHL1 deubiquitinase	
<i>All-trans-retinoic acid</i> <sup>81-83</sup>	Inhibits NRF2-ARE binding through a direct interaction between NRF2 and RAR $\alpha$	
AEM1 <sup>29</sup>	Unknown	High-throughput screen
Bexarotene <sup>82, 84</sup>	RXR $\alpha$ activation which interacts with the Neh7 domain of NRF2 and antagonizes ARE-dependent mRNA expression	RXR $\alpha$ -specific ligand
Brusato <sup>85-87</sup>	Global translation inhibitor	
Camptothecin <sup>88</sup>	May inhibit NRF2 transcription, translation and/or promoting mRNA degradation	DNA topoisomerase I inhibitor
Clobetasol propionate <sup>30 89</sup>	Binds to glucocorticoid receptor and recruits a corepressor to suppress ARE-dependent transcription; Prevents NRF2 nuclear translocation; Promotes NRF2 degradation by promoting $\beta$ -TrCP-dependent degradation in a glucocorticoid receptor and GSK3-dependent manner	High-throughput screen
Corvallatoxin (digoxigenin, cymarin) <sup>90</sup>	Activates GSK3 $\beta$ and thus promotes NRF2 degradation	Na <sup>+</sup> /K <sup>+</sup> -ATPase inhibitor
Costunolide <sup>91</sup>	Inhibits telomerase	
Halofuginone <sup>92</sup>	Inhibits prolyl-tRNA synthetase and thus global translation	
HER2 antibodies (Trastuzumab, Pertuzumab) <sup>93</sup>	<i>NRF2</i> promoter hypermethylation	
Homoharringtonine <sup>94</sup>	Stabilizes GC-rich sequence in 5'-UTR of <i>NRF2</i> DNA	
HSP90 inhibitors (17-AAG, 17-DMAG, IPI-504) <sup>66</sup>	Metabolism by NRF2 target genes (NQO1 and TXNRD1) into more potent HSP90 inhibitors	
IM3829 (4-(2-Cyclohexylethoxy)aniline) <sup>95</sup>	Inhibits NRF2 nuclear translocation	
Isoniazid <sup>96, 97</sup> , Ethionamide <sup>98</sup>	Activates SIRT1; Inhibits NRF2 nuclear translocation by inhibiting ERK1 phosphorylation	Anti-tubercular drug
K67 and its derivatives <sup>99, 100</sup>	Inhibits KEAP1-phospho-p62 protein-protein interaction and thus promotes NRF2 degradation	
Malabaricone-A <sup>101</sup>	Unknown	
Mitomycin C <sup>67</sup>	Bioactivation by NRF2 target genes (cytochrome P450 reductase, NQO1 and PPP enzymes) causes DNA damage	
ML385 <sup>31</sup>	Binds to the Neh1 domain of NRF2 and thus interferes with the DNA binding activity of NRF2-MAFG complex	High-throughput screen
Ochratoxin A <sup>102-104</sup>	Inhibits NRF2 nuclear translocation, NRF2-ARE binding, and histone acetylation, and increases miR-132	

Sorafenib <sup>105</sup>	Unknown	Inhibitor of multiple kinases (VEGFR, PDGFR and RAF)
Tetrahydrocarbazoles <sup>106</sup>	May stabilize KEAP1-NRF2 interaction	
Trigonelline <sup>107, 108</sup>	Inhibits NRF2 nuclear translocation	
Vorinostat <sup>109</sup>	Downregulates c-Myc, increases KEAP1 expression, and inhibits NRF2 nuclear translocation	
<ul style="list-style-type: none"> <li>• PHA-767491</li> <li>• AZ-628</li> <li>• SL-327<sup>33</sup></li> </ul>	Inhibits NRF2 nuclear translocation	<ul style="list-style-type: none"> <li>• Cdc7/CDK9 inhibitor</li> <li>• RAF inhibitor</li> <li>• MEK inhibitor</li> </ul>
<ul style="list-style-type: none"> <li>• Stattic</li> <li>• Grassypeptolide A</li> <li>• Cardiac glycosides (lantoside C, strophanthidin, peruvoside, proscillaridin, ouabain)</li> <li>• Emetin and anisomycin</li> <li>• Actin-disrupting agents (lyngbyabellin A, dolastatin 12)<sup>30, 32</sup></li> </ul>	<ul style="list-style-type: none"> <li>• STAT3 inhibitor</li> <li>• May inhibit DPP8 and DPP4 and thus promote NRF2 degradation</li> <li>• May inhibit Na<sup>+</sup>,K<sup>+</sup>-ATPase pump and thus decrease KEAP1 phosphorylation<sup>110</sup>, or through HIF pathway</li> <li>• Protein synthesis inhibitor</li> <li>• Possibly interferes actin polymerization and NRF2 nuclear translocation</li> </ul>	High-throughput screen
• Antimetabolites (e.g., methotrexate) <sup>30</sup>		High-throughput screen

**Table 2. Multi-omics data of human ESCC cell lines**

Data	Spreadsheet in Supplementary Material	Weblink	Reference
RNAseq	CCLE_RNAseq-genes-rpkm_ESCC	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	111
Metabolomics	Metabolomics data	<a href="https://portals.broadinstitute.org/ccle/data">https://portals.broadinstitute.org/ccle/data</a>	112
CRISPR gene dependency screen	Achilles CRISPR Dependency	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	113
RNAi gene dependency screen	Combined RNAi Dependency	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	41
PRISM drug sensitivity screen	PRISM Drug Sensitivity	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	114
CTRP V2 drug sensitivity screen	CTRP V2	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	115
GDSC1 drug sensitivity screen	GDSC1	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	116 117
GDSC2 drug sensitivity screen	GDSC2	<a href="https://depmap.org/portal/download/">https://depmap.org/portal/download/</a>	116 117
Protein-Protein Interaction and drug sensitivity	Original data not available	<a href="https://mutanome.lerner.ccf.org">https://mutanome.lerner.ccf.org</a>	65

**Table 3. Approaches for the evaluation of NRF2 activity in the esophagus**

Sample	Assay	Measurement	Pros	Cons
Surgical/biopsy specimens	Whole exome sequencing	<i>NRF2</i> , <i>KEAP1</i> , <i>CUL3</i> mutations	Accurately reflect the NRF2 status in cancer tissues	Invasive procedure to harvest tissue samples; Potential variations due to tumor heterogeneity; High quality of tissue samples;
	RNAseq or qPCR	NRF2 <sup>high</sup> signature		
	Quantitative multiplex IHC	Nuclear localization of NRF2 and overexpression of NRF2 and its target genes		
	NanoString	A combination of DNA, mRNA, proteins		
Live animal or human patients	PET/CT	Radionuclide avidity	Reflect the functional status of NRF2 in the whole cancer tissues; Non-invasive and convenient for clinical follow-up	Possible lack of specificity for NRF2 status

## Figure legends

**Figure 1. NRF2 signaling pathway.** Under basal conditions, NRF2 is bound to KEAP1, which is a CUL3-based E3 ubiquitin ligase adapter that regulates NRF2 ubiquitination and proteasomal degradation. When cells are exposed to oxidative or electrophilic stress, KEAP1 changes its conformation and releases NRF2. Then NRF2 translocates into the nucleus, forms a heterodimer with its obligatory partner (small MAF proteins), binds to the ARE, and activates the transcription of downstream genes, such as antioxidative genes and phase II detoxification enzymes.

**Figure 2. Subtyping 22 ESCC cells into two subtypes, NRF2<sup>high</sup> and NRF2<sup>low</sup>, using RNAseq data.** (A) Clustering analysis; (B) Principal component analysis; (C) Significant dependency on the *NFE2L2* gene by NRF2<sup>high</sup> ESCC cells as compared to NRF2<sup>low</sup> ESCC cells. Student's *t* test is used for statistical evaluation of *NFE2L2* gene dependency score using the original data from the combined RNAi screen.

## Supplementary Material:

**Comparison of NRF2<sup>high</sup> and NRF2<sup>low</sup> ESCC cells.** This file contains the RNAseq data (22 cell lines), metabolomics data (22 cell lines), combined RNAi gene dependency screen data (cell viability as the readout, 20 cell lines), Achilles CRISPR gene dependency screen data (cell viability as the readout, 16 cell lines), PRISM drug sensitivity data (viability as the readout, 20 cell lines), CTRP V2 drug sensitivity data (cell viability as the readout, 20 cell lines), GDSC1 drug sensitivity data (cell viability IC<sub>50</sub> and AUC as readouts, 22 cell lines), and GDSC2 drug sensitivity data (cell viability IC<sub>50</sub> and AUC as readouts, 20 cell lines).



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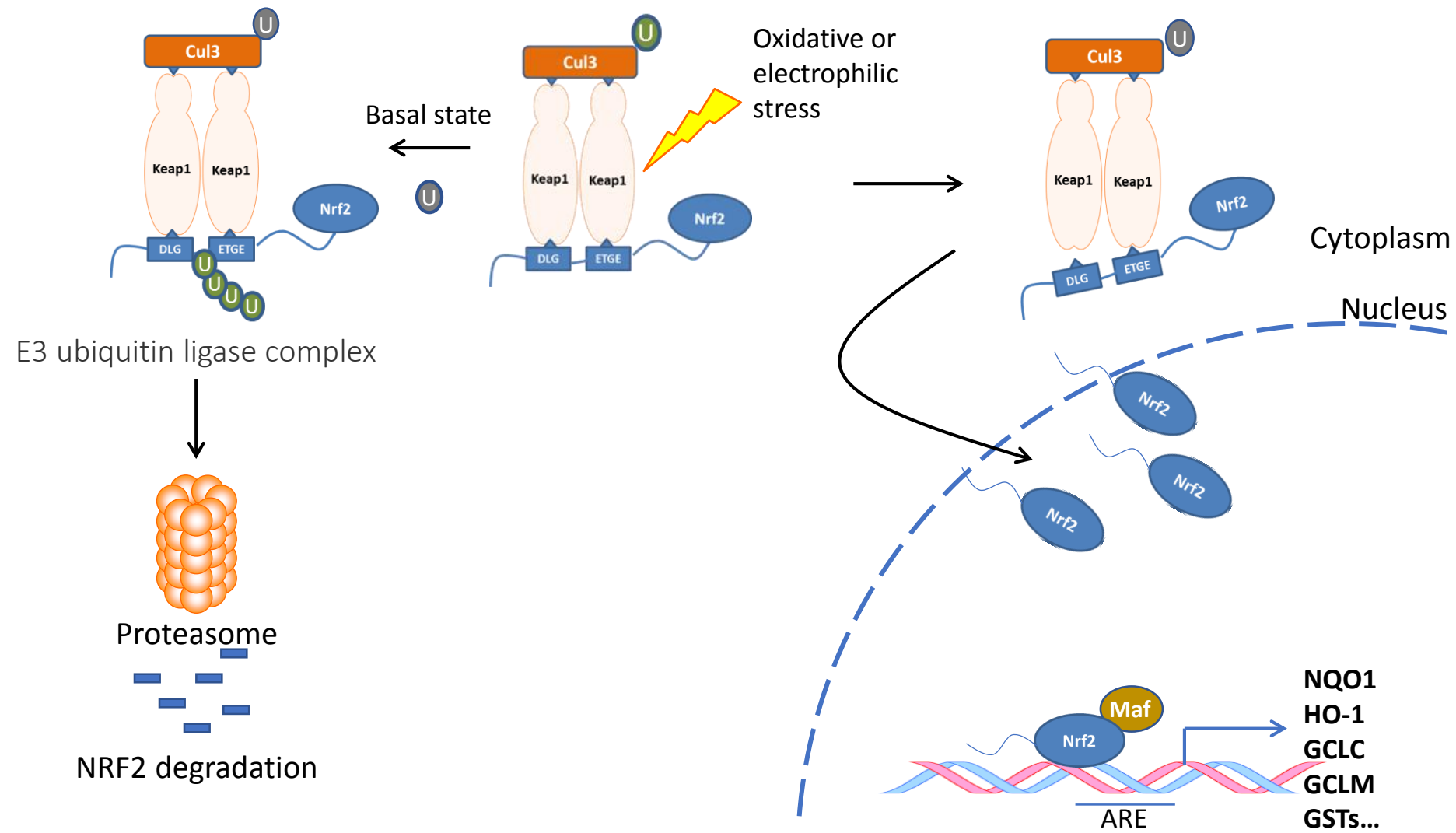
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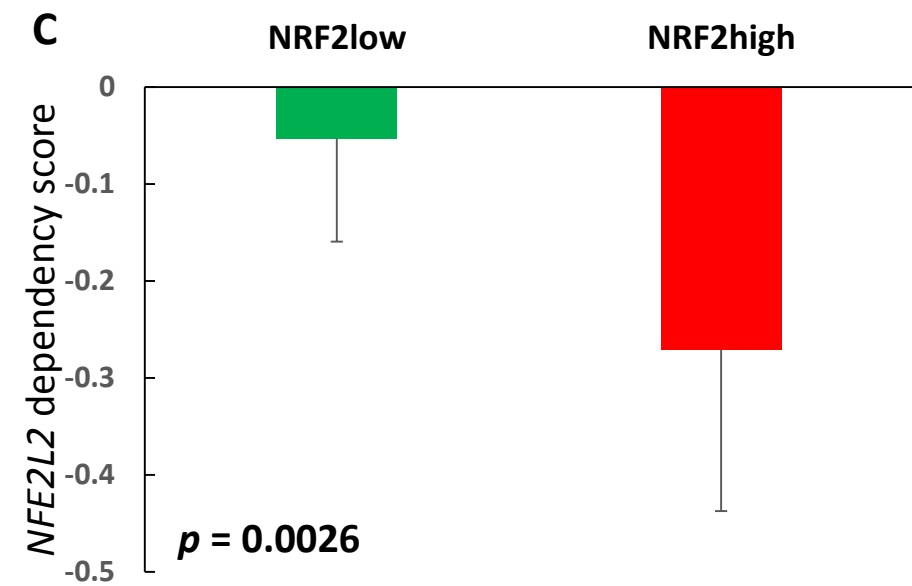
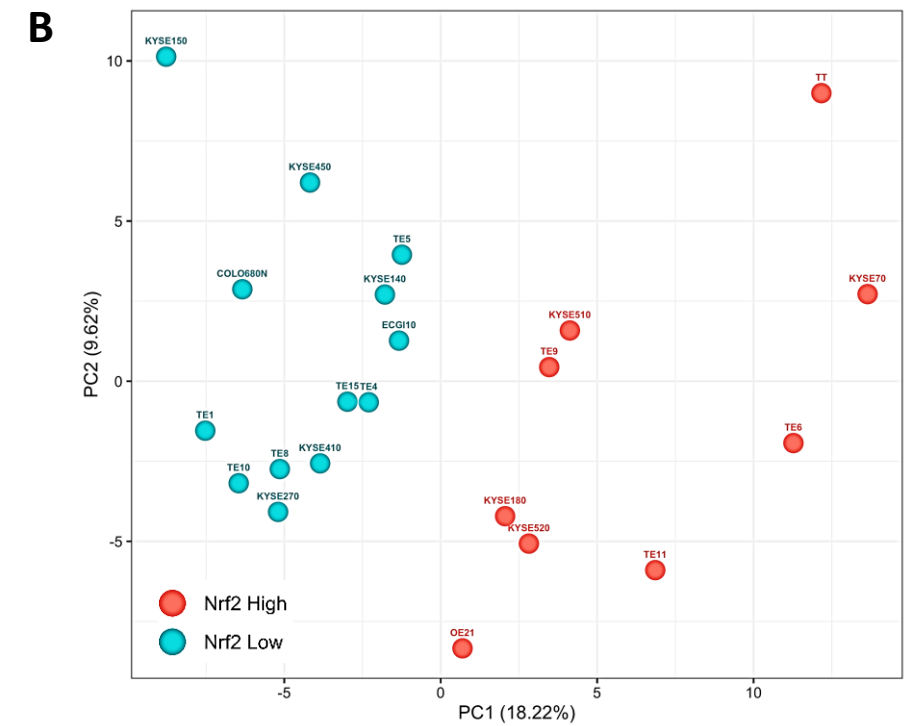
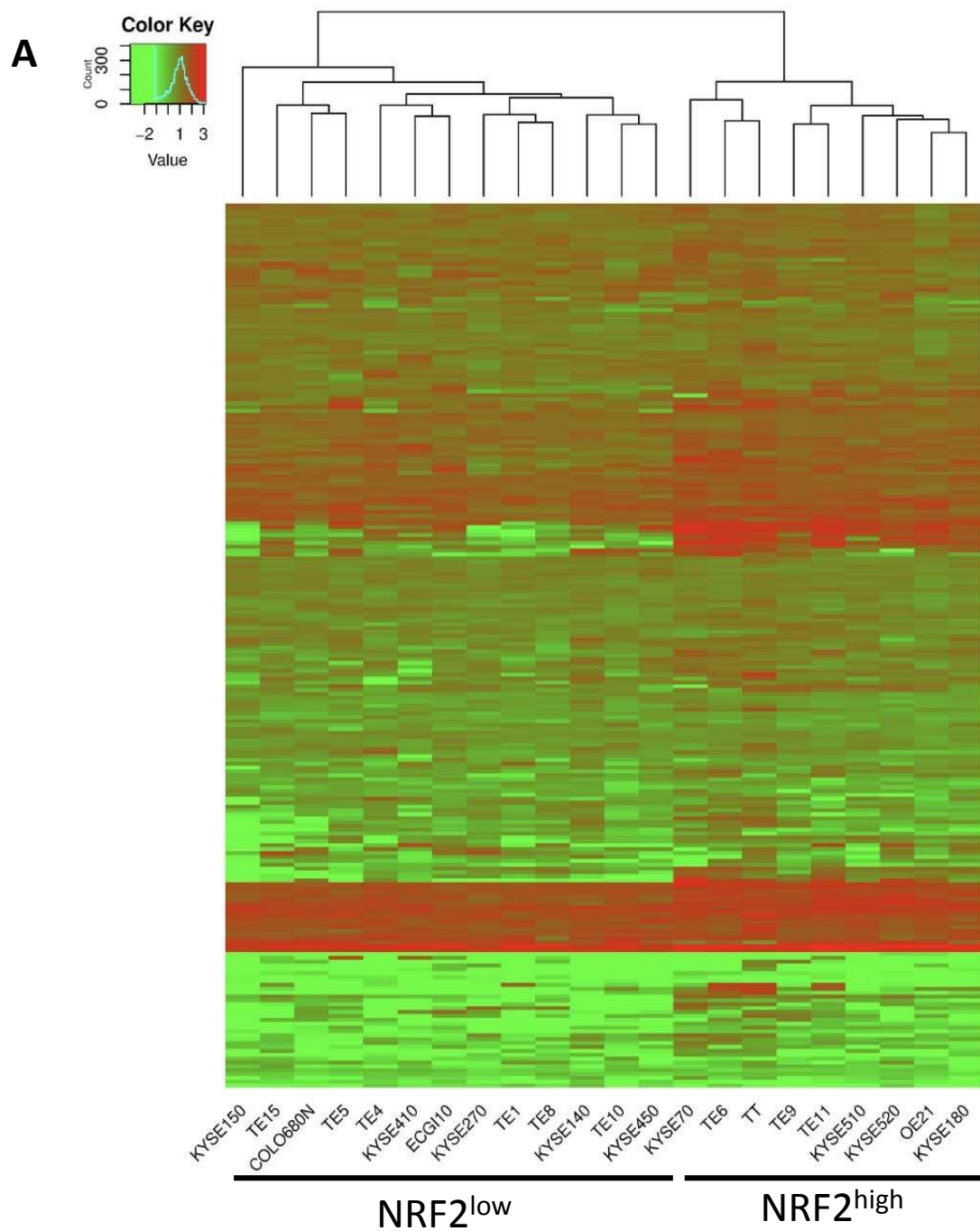
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**Conflict of Interest Statement:** The authors have no competing interests to declare.