Minireview

Molecular Basis of the KEAP1-NRF2 Signaling Pathway

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Transcription factor NRF2 (NF-E2-related factor 2) is a master regulator of cellular responses against environmental stresses. NRF2 induces expression of detoxification and antioxidant enzymes and suppresses inductions of pro-inflammatory cytokine genes. KEAP1 (Kelch-like ECH-associated protein 1) is an adaptor subunit of CULLIN 3 (CUL3)-based E3 ubiquitin ligase. KEAP1 regulates the activity of NRF2 and acts as a sensor for oxidative and electrophilic stresses. NRF2 has been found to be activated in many types of cancers with poor prognosis. Therapeutic strategies to control NRF2-overreactivated cancers have been considered not only by targeting cancer cells with NRF2 inhibitors or NRF2 synthetic lethal chemicals, but also by targeting host defense with NRF2 inducers. Understanding precise molecular mechanisms how the KEAP1-NRF2 system senses and regulates the cellular response is critical to overcome intractable NRF2-activated cancers.

Keywords: cancer therapy, defense system, KEAP1, NRF2, stress sensing

INTRODUCTION

Our body is equipped with defense systems that upregulate expression of cytoprotective enzymes and NRF2 (NF-E2-related factor 2) is the central player in the inducible expression of cellular defense enzymes (Ishii et al., 2000; Itoh et al., 1997). NRF2 belongs to the CNC (cap-n-collar) subfamily of basic region-leucine zipper (bZIP)-type transcription factors (Itoh et al., 1995). NRF2 dimerizes with one of the small MAF (musculoaponeurotic fibrosarcoma) proteins (sMAF). The NRF2-sMAF heterodimer binds to antioxidant or electrophile response element (ARE/EpRE), which is now collectively referred to as the CNC-sMaf binding element (CsMBE), located in the regulatory regions of the cytoprotective enzyme genes (Friling et al., 1990; Otsuki et al., 2016; Rushmore et al., 1991).

Kelch-like ECH-associated protein 1 (KEAP1) was identified as a negative regulator of NRF2 (Itoh et al., 1999). KEAP1 acts as a substrate-recognition subunit of the CULLIN 3 (CUL3)-based E3 ubiquitin ligase complex that specifically targets NRF2 (Itoh et al., 2003; Kobayashi et al., 2004). In the absence of stress stimuli, NRF2 is efficiently ubiquitinated by the KEAP1-CUL3 E3 ligase and degraded rapidly through the proteasome pathway, such that cellular NRF2 activity is constitutively suppressed in unstressed conditions. Upon exposure to oxidative or electrophilic stresses, KEAP1 loses the ability to ubiquitinate NRF2, allowing NRF2 to accumulate in the nucleus and activate transcription of its target genes. It should be noted that recent studies expand extraordinary our knowledge about the KEAP1-NRF2 system and its relation to intractable cancers with massive NRF2 activation.

It has been shown that in many types of cancers NRF2 activity is overactivated with high frequency (Padmanabhan

et al., 2006; Romero et al., 2017; Shibata et al., 2008). These NRF2 overactivations are brought by somatic mutations, epigenomic errors, exon skipping, etc., which destroy protein-protein interactions (PPI) of NRF2 and KEAP1 and make NRF2 stable (Adam et al., 2011; DeNicola et al., 2011; Goldstein et al., 2016; Komatsu et al., 2010; Ooi et al., 2011; 2013; Padmanabhan et al., 2006; Shibata et al., 2010; Wang et al., 2008). The NRF2-overactivated cancers manifest strong resistance to currently standard therapies of cancers and brings about poor prognosis. Therefore, as summarized in Fig. 1, therapeutic strategies to control malignant NRF2-overeactivated cancers have been designed and developed. These approaches include not only targeting cancer cells with NRF2 inhibitors or NRF2 synthetic lethal chemicals, but also by targeting host defense with NRF2 inducers (Hayashi et al., 2020; Hiramoto et al., 2014; Satoh et al., 2010). Of note, these strategies are supported by in-depth understanding the mechanistic basis of the KEAP1-NRF2 system function as well as understanding the system's contribution to the fundamental cellular defense machinery. In this review, we especially introduce molecular mechanisms underpinning how this system senses various environmental stresses and potentiates our defense systems.

EXPANSION OF NRF2 TARGET GENES

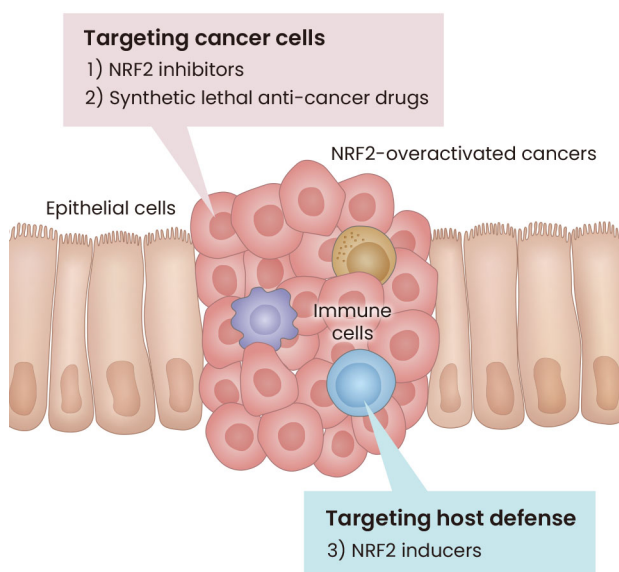Various NRF2 target genes have been identified through

Fig. 1. Therapeutic strategies to control NRF2-overeactivated cancers. In the tumor, NRF2 inhibitors help suppress tumor growth and metastasis by decreasing NRF2 protein levels. In addition, synthetic lethality anti-cancer drugs are transformed into strongly active drugs by NRF2-targeted drug-metabolizing enzymes. In contrast, activation of NRF2 in the microenvironment inhibits the progression of NRF2-overactivated malignant tumors. NRF2, NF-E2-related factor 2.

gene expression profiling analysis and chromatin immunoprecipitation (ChIP) analysis (Chorley et al., 2012; Hirotsu et al., 2012). Of the target genes, major group is the cytoprotective genes encoding anti-oxidative stress enzymes, detoxifying enzymes and enzymes related to metabolic reprogramming in cancer. For instance, *heme oxygenase 1* (*HO-1*) gene is one of the representative NRF2 target genes, which acts for anti-oxidative stress response. Recent analyses of peritoneal macrophages from HO-1-DsRed knock-in mice lacking NRF2 demonstrate that electrophilic NRF2 inducers, such as diethylmaleate (DEM) and 1-[2-cyano-3,12,28-triox-ooleana-1,9(11)-dien-28-yl]-1H-imidazole (CDDO-Im), activate expression of *HO-1* gene in an NRF2-dependent manner, while HO-1 substrate hemin induces expression of *HO-1* gene in an NRF2-independent manner (Zhang et al., 2021). NRF2 also regulates glucose metabolism genes, which becomes important in the metabolic reprogramming in cancer (Mitsuishi et al., 2012). In addition, NRF2 has also been shown to attenuate inflammation by suppressing the induction of pro-inflammatory cytokine gene transcription (Kobayashi et al., 2016; Suzuki et al., 2020a).

These NRF2 target genes are regulated by the heterodimer composed of NRF2 and one of the sMAF proteins, e.g., MAFF, MAFG, or MAFK, through CsMBE. Recently, Katsuoka et al. (2019) provided direct and compelling evidence for the NRF2-MAFG heterodimer-mediated transcription of NRF2-target genes. By introducing a tethered NRF2-MAFG heterodimer using a flexible linker peptide into the cells lacking all three sMaf proteins, which ensured no interference from other endogenous CNC-sMAF heterodimers or sMAF homodimers, they showed that the NRF2-MAFG heterodimer acts as a dominant transcriptional activator of NRF2-dependent gene transcription through CsMBE (Katsuoka et al., 2019). Structural analysis conducted by Sengoku et al. (2022), strongly supports this notion. Their crystal structure analysis revealed that the NRF2-MAFG heterodimer has approximately 200-fold stronger affinity for CsMBE than do the canonical bZIP proteins, such as activator protein 1 (AP-1), and this is realized by utilization of the CNC motif in NRF2 protein that forms extensive contact with the DNA backbone phosphates (Sengoku et al., 2022).

FLOODGATE MODEL FOR THE KEAP1-MEDIATED ACTIVATION OF NRF2

KEAP1 interacts with the Neh2 (NRF2-ECH homology domain 2) degron domain of NRF2 (Itoh et al., 1999). The stoichiometry of KEAP1 and NRF2 within the KEAP1-NRF2 complex is 2:1, which is shown by means of isothermal calorimetry analysis (Tong et al., 2006a), nuclear magnetic resonance (NMR) titration analyses (Horie et al., 2021; Tong et al., 2006a), and an analytical centrifugation analysis (Iso et al., 2016). A single NRF2 protein binds to a KEAP1 homodimer using a high-affinity ETGE motif and a low-affinity DLGex motif. This two-site recognition/binding mechanism between the NRF2 Neh2 domain and KEAP1 homodimer is critical for the efficient ubiquitination of NRF2 (Tong et al., 2006a; McMahon et al., 2010; Suzuki et al., 2015).

KEAP1 mainly localize in the cytoplasm loosely attaching

perinuclear cytoskeleton network (Watai et al., 2007). Oxidative or electrophilic stresses provoke nuclear accumulation of NRF2 without altering the cytoplasmic localization of KEAP1 (Watai et al., 2007). It should be noted that these stresses appear not to trigger dissociation of the KEAP1-NRF2 complex (Kobayashi et al., 2006), but rather repress strongly the KEAP1-based E3 ubiquitin ligase activity. Thus, reduction of the NRF2 ubiquitination leads to the stabilization and nuclear accumulation of *de novo* synthesized NRF2 (Kobayashi et al., 2006).

Quantitative biochemical studies revealed that in the basal state, NRF2 protein is maintained at a level significantly lower than the levels of KEAP1. When challenged with the electrophilic NRF2 inducer DEM, abundance and localization of KEAP1 protein does not change, whereas nuclear NRF2 protein rises to the level considerably higher than that of KEAP1 (Iso et al., 2016). Thus, in the basal uninduced conditions the KEAP1-based E3 ubiquitin ligase acts as a "floodgate" that degrades NRF2 efficiently in collaboration with the proteasome system (Fig. 2) (Iso et al., 2016). On the contrary, in response to oxidative or electrophilic stimuli, i.e., in response to inducers, the KEAP1-CUL3 complex loses the floodgate function, i.e., the ubiquitin ligase activity, and NRF2 accumulates in the nucleus and activates the target gene expression (Iso et al., 2016; Suzuki et al., 2017; Yamamoto et al., 2018).

CYSTEINE CODE FOR THE KEAP1 SENSING OF ELECTROPHILIC NRF2 INDUCERS

A variety of chemical NRF2 inducers are reported, and the majority are electrophiles that react with nucleophilic thiols, including cysteine sulfhydryl groups (Dinkova-Kostova and Ta-

laly, 2008). Specific patterns of KEAP1 cysteine modifications by individual chemical NRF2 inducers have been identified by mass spectrometry analyses (Eggler et al., 2005; Kobayashi et al., 2009) and these modifications affect the KEAP1-based E3 ubiquitin ligase activity.

Functional significance of the cysteine residues in KEAP1 has been examined by means of site-directed mutagenesis (Saito et al., 2016; Suzuki et al., 2019; Takaya et al., 2012; Yamamoto et al., 2008; Zhang and Hannink, 2003). The chemicals triggering NRF2 activation appear to modify KEAP1 cysteine residues in a multiple pattern. This unique use of cysteine residues as sensors has led to the "cysteine code" concept (Suzuki et al., 2013). The concept explains the unique feature of the KEAP1-NRF2 system, which responds to a diverse array of chemical and oxidative insults (Suzuki et al., 2013). Our recent results revealed molecular basis as to how Keap1 employs multiple cysteine residues as sensors enabling activations of various NRF2-mediated cytoprotective responses (Suzuki et al., 2019). Based on the analyses, we classified the Nrf2 inducers into five classes (Fig. 3), which are going to be introduced below.

Class I; KEAP1-Cys151-preferring inducers

The importance of KEAP1-Cys151 as a sensor has been verified by means of knock-in or transgenic mice expressing a KEAP1 mutant in which Cys151 of KEAP1 is substituted with serine, which are referred to as KEAP1^C151S (Eggler et al., 2009; Kobayashi et al., 2009; McMahon et al., 2010; Saito et al., 2016; Takaya et al., 2012; Yamamoto et al., 2008; Zhang and Hannink, 2003). In KEAP1^C151S mouse embryonic fibroblasts (MEFs) and peritoneal macrophages, the Cys151 residue was found to be indispensable for the accumulation of NRF2 in
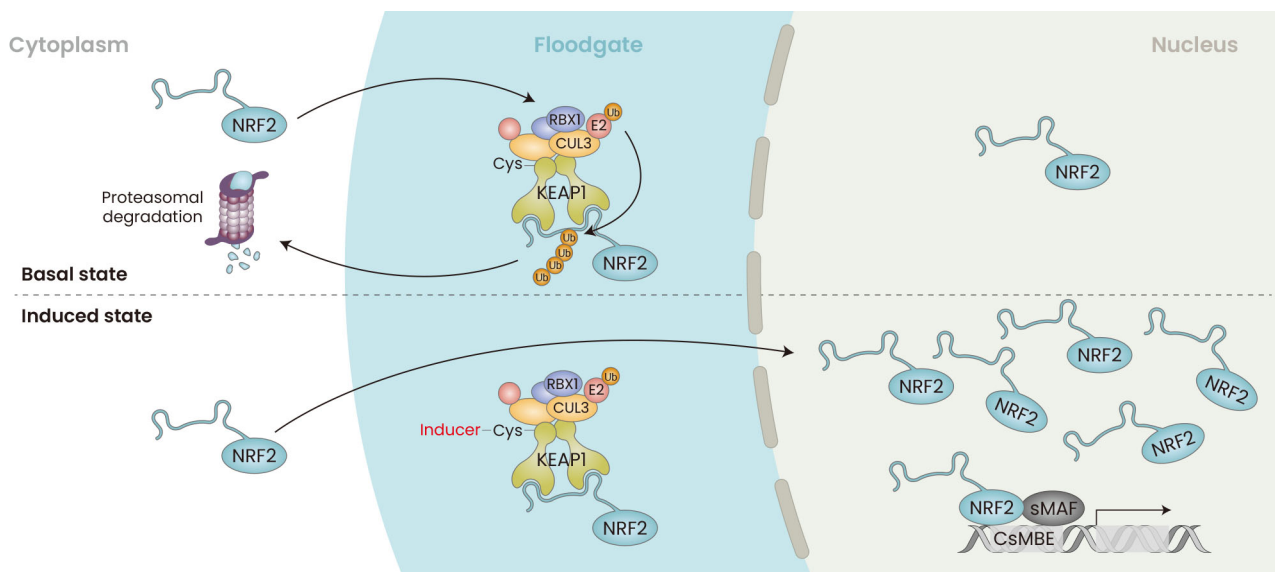

Fig. 2. Floodgate Model for the KEAP1-mediated Activation of NRF2. In the basal state, the ubiquitin ligase KEAP1-CUL3 complex acts as a floodgate and ubiquitinates NRF2. Ubiquitinated NRF2 degrades through the proteasome system. In response to oxidative and electrophilic stimuli, NRF2 accumulates significantly in the nucleus. These stimuli do not affect the abundance and subcellular localization of KEAP1 and CUL3. NRF2 dimerizes with one of the small MAF proteins (sMAF) to form an NRF2-sMAF heterodimer that recognizes the CNC-sMAF binding element (CsMBE) and activates target gene expression. KEAP1, Kelch-like ECH-associated protein 1; NRF2, NF-E2-related factor 2; CUL3, CULLIN 3; CNC, cap-n-collar.

response to an array of electrophilic chemicals. These NRF2 inducers include DEM, tBHQ (*tert*-butyl hydroquinone), DMF (dimethylfumarate), nitric oxide (NO), sulforaphane (SFN), and CDDO-Im (Saito et al., 2016; Takaya et al., 2012). These are categorized into Class I or Cys151-preferring inducers (Fig. 3).

Cys151 is located in the BTB domain of KEAP1, which is responsible for the interaction of KEAP1 with CUL3. Consistent with these observations, several reports have shown that modification of Cys151 inhibits the KEAP1-CUL3 interaction and prevents ubiquitination of NRF2 (Cleasby et al., 2014; Eggler et al., 2009; Zhang et al., 2004). It should be noted that there are a couple of reports that disagree with this model (Baird et al., 2013; Li et al., 2012). In this regard, pull-down and analytical centrifugation analyses show that many of the Cys151-targeting electrophilic inducers do not provoke dissociation of the KEAP1-CUL3 complex, except in the case of CDDO-Im (Iso et al., 2016). Chemical inducers produce conformational changes in KEAP1, as shown by a hydrophobicity probe (Dinkova-Kostova et al., 2005). We surmise that cysteine modification elicits structural alterations in KEAP1 by affecting the complex status of KEAP1 and CUL3 without causing their dissociation. This structural alteration in KEAP1 prevents NRF2 ubiquitination. It is possible that Cys151 modification affects the angle of orientation of the KEAP1-CUL3
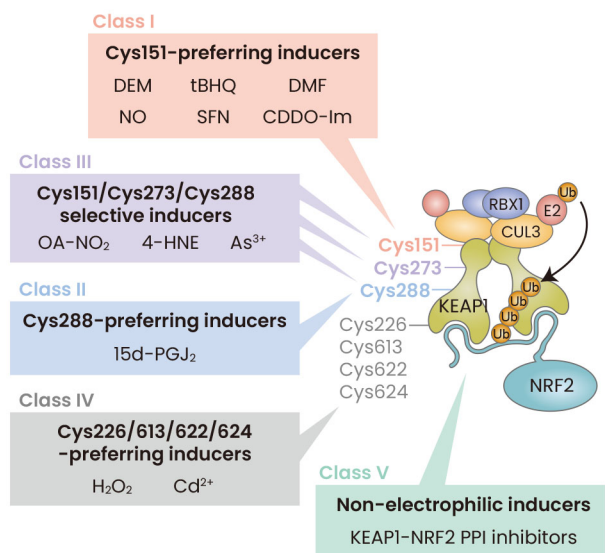

Fig. 3. Model for the multiple stress-sensing mechanisms acting through KEAP1. There are five classes of NRF2 inducers: Class I, Cys151-preferring; Class II, Cys288-preferring; Class III, Cys151/Cys273/Cys288-selective; Class IV, Cys226/613/622/624-preferring; and Class V, Non-electrophilic KEAP1-NRF2 PPI inhibitors. Chemicals representing each class are shown. KEAP1, Kelch-like ECH-associated protein 1; NRF2, NF-E2-related factor 2; DEM, diethylmaleate; tBHQ, tert-butyl hydroquinone; DMF, dimethylfumarate; NO, nitric oxide; SFN, sulforaphane; CDDO-Im, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-imidazol; OA-NO$_2$, nitro-octadec-9-enoic acid; 4-HNE, 4-hydroxy-nonenal; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; H$_2$O$_2$, hydrogen peroxide; CUL3, CULLIN 3; PPI, protein-protein interactions.

association, thereby significantly altering the distance between ubiquitin and the target lysine residues in the Neh2 domain.

Class II & III; NRF2 inducers consisting of Cys273/288 on KEAP1-IVR domain

It has been assumed that other cysteine residues, especially Cys273 and Cys288 in the intervening region (IVR), may also contribute to the stress-sensor function of KEAP1. The Cys273 and Cys288 residues have been suggested to react with 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) (Kobayashi et al., 2009). However, substitution of Cys273 and Cys288 with serine or alanine failed to repress NRF2 activity in both a reporter co-transfection transactivation assay (Wakabayashi et al., 2004; Zhang and Hannink, 2003) and in transgenic complementation rescue mouse experiments (Yamamoto et al., 2008), precluding a simple validation of this notion.

To overcome this problem, we systematically introduced amino acid substitutions into Cys273 and Cys288 and identified amino acids that do not affect the ability of KEAP1 to repress NRF2 accumulation (Saito et al., 2016). Finally, we found that KEAP1 mutants in which Cys273 was replaced with tryptophan and Cys288 was replaced with glutamate (Saito et al., 2016) retained the ability to repress NRF2 accumulation. In unstressed conditions, it seems that Cys273 and Cys288 are kept in hydrophobic and hydrophilic conditions, respectively. These characteristics of Cys273 and Cys288 might be critical for the structural integrity of KEAP1 for maintaining ubiquitin ligase activity. However, conclusive insights into these structural requirements await further elucidation of the structure surrounding the IVR that includes Cys273 and Cys288. It was shown in MEFs from KEAP1-Cys-288Glu (*Keap1*^{C288E}) knock-in mice that 15d-PGJ$_2$ is recognized by KEAP1 Cys288. Therefore, 15d-PGJ$_2$ belongs to Class II chemicals known as Cys288-preferring inducers (Fig. 3).

On the other hand, all three cysteine residues (Cys151, Cys273, and Cys288) are indispensable for the activity of KEAP1 that senses 9-nitro-octadec-9-enoic acid (9-OA-NO$_2$), 4-hydroxy-2-nonenal (4-HNE) and sodium arsenite (NaAsO$_2$). These chemicals therefore belong to Class III and are referred to as Cys151/Cys273/Cys288-selective inducers (Fig. 3).

Class IV; KEAP1-Cys226/613/622/624-selective NRF2 inducers

Oxidative stress is involved in the development and progression of many diseases, including Alzheimer's disease, atherosclerosis, and cancer (Sies et al., 2022). Hydrogen peroxide (H$_2$O$_2$) is classified into the Cys151/Cys273/Cys288-independent inducer (Saito et al., 2016). NRF2 accumulation by H$_2$O$_2$ was not affected in KEAP1$^{C151S\&C273W\&C288E}$-expressing MEFs, indicating that the three cysteine residues are dispensable for NRF2 activation in response to H$_2$O$_2$. This suggests that alternative cysteine residues are critical for stress recognition by KEAP1.

To address this hypothesis, we generated a KEAP1 mutant protein with 11 cysteine residue substitution along with various individual and combination mutations of these cysteine residues. Functional analyses of an additional range of KEAP1 mutants showed that the H$_2$O$_2$ sensor actually resides

in the 11 cysteine residues, and finally revealed that Cys226, Cys613, Cys622, and Cys624 are involved in the sensing of H_2O_2 by KEAP1 (Suzuki et al., 2019). Of note, despite the fact that our results delineate that the four cysteine residues are involved in the H_2O_2 sensor activity, no individual cysteine residue is critical for the sensor function, as the single mutation of any of the four cysteine residues does not inactivate the oxidative stress sensor. These observations support our contention that disulfide bond between Cys226, Cys613, and Cys622/624 is formed in response to oxidative stress, and the sensor for the H_2O_2 contains a robust compensatory mechanism. Thus, these findings indicate that an elaborate fail-safe mechanism consisting of Cys226, Cys613, and Cys622/Cys624 ensures the KEAP1's response to H_2O_2 across a range of conditions.

These mechanistic studies revealed that various electrophiles are sensed by unique multiple cysteine residues, including Cys151, Cys273, and Cys288. Electrophiles make thiol-alkylation covalent bondage with these cystines. On the other hand, H_2O_2 forms a disulfide bond between Cys226, Cys613, Cys622, and Cys624. A pair between these residues is formed between two of these cysteine residues. These results thus indicate that electrophiles and ROS employs distinct cysteine residues of KEAP1. However, precise mechanisms as to how these cysteine modifications alter ubiquitin ligase activity of KEAP1 remain to be elucidated.

Class V; non-electrophilic KEAP1-NRF2 PPI inhibitor or NRF2 inducers

Classic inducers of NRF2 interact with the cysteine residues of KEAP1 by virtue of their electrophilic nature and can therefore inherently react with glutathione or thiol in proteins. Since an over-dose of such thiol-reactive chemicals could cause electrophilic damage in cells, chemicals that directly inhibit the PPI of KEAP1 and NRF2 are emerging as candidates of attractive novel NRF2 inducers. We refer these small chemical inducers to as Class V (Fig. 3). For instance, the isoquinoline PRL-295 (Lazzara et al., 2020) was developed with the safety and physicochemical properties of the earlier naphthalene- and isoquinoline-based KEAP1 inhibitors (Richardson et al., 2018). The potency of PRL-295 in cell-based assays is similar to that of sulforaphane (Dayalan et al., 2022). We have utilized PRL-295 recently to test the "Hinge-Latch" model of NRF2 activation. The results revealed that the PPI inhibitors disrupt the DLGex-KEAP1 interaction preferentially over the ETGE-KEAP1 interaction (Horie et al., 2021). The "Hinge-Latch" model will be discussed more in detail in the next section. Another example is that Scohia Pharma, Inc. claimed a patent of WO2020116660 that described macrocyclic KEAP1-NRF2 inhibitors harboring the tetrahydroisoquinoline scaffold.

UPDATED "HINGE-LATCH" MODEL OF THE KEAP1-NRF2 SYSTEM

Various structure-function analyses of the KEAP1-NRF2 interaction revealed the importance of the two-site binding model between the KEAP1 homodimer (i.e., two DC domains) and the Neh2 domain of NRF2 (via DLGex and ETGE motifs) (Fukutomi et al., 2014; Tong et al., 2006a). The Hinge-

Latch model was proposed as a plausible mechanism for the KEAP1-mediated NRF2 activation (Tong et al., 2006b). This model is based on the fact that the DLGex and ETGE motifs show approximately two-orders of magnitude difference in the binding affinity to the KEAP1-DC domain (Tong et al., 2007). Various lines of evidence including somatic mutation analyses in clinical cancer studies support the presence of this model (Shibata et al., 2008). However, the model was not validated to date due to technical difficulties in examining the DLGex-KEAP1 binding and ETGE-KEAP1 binding simultaneously.

To overcome this problem, Horie et al. (2021), utilized NMR titration, which is capable of simultaneous and sequences-specific assignment of the DLGex and ETGE motifs. By utilizing this method, Horie et al. (2021), found that the Hinge-Latch mechanism indeed operates during the activation of NRF2 by p62 accumulation, which can be induced by autophagy deficiency, as well as pharmacological KEAP1-NRF2 PPI inhibitors such as PRL-295, but not by electrophilic NRF2 inducers such as CDDO-Im, SFN or 15d-PGJ$_2$ (Fig. 4).
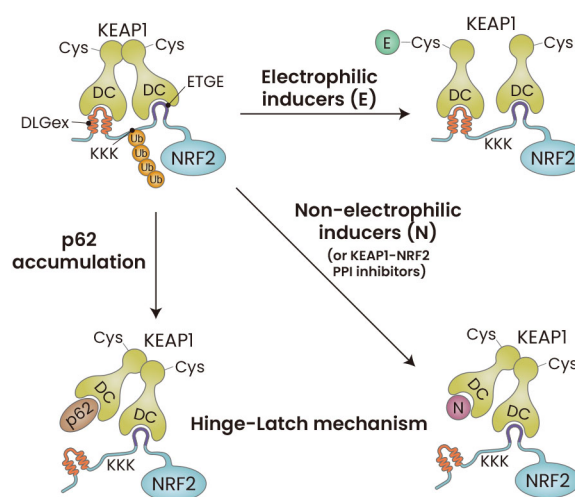

Fig. 4. Updated Hinge-Latch model. Autophagy chaperone p62 accumulation disrupts the DLGex-Keap1 interaction and activates NRF2, and this mechanism conforms to the concept of the Hinge-Latch model. Similarly, pharmacological KEAP1-NRF2 PPI inhibitors disrupt the DLGex-KEAP1 interaction preferentially to the ETGE-KEAP1 interaction, and molecular basis for the efficacy of these chemicals is the utilization of the Hinge-Latch mechanism. In contrast, electrophilic NRF2-activating compounds activate NRF2 without disrupting the Neh2-KEAP1 interaction, implying that these KEAP1 thiol-modifying chemicals utilize mechanisms distinct from the Hinge-Latch mechanism. KEAP1, Kelch-like ECH-associated protein 1; NRF2, NF-E2-related factor 2; PPI, protein-protein interactions.

THERAPEUTIC STRATEGIES TO CONTROL NRF2-OVERACTIVATED CANCERS

NRF2 plays diverse role in the context of cancer. As the KEAP1-NRF2 pathway is an essential cell defense system against environmental stresses, NRF2 provides important anti-cancer ability (Aoki et al., 2001; Ohkoshi et al., 2013; Ramos-Gomez et al., 2003). On the other hand, aberrant NRF2 overactivation has been found in several types of cancer cells, and such NRF2 overactivation in cancer cells correlates strongly with poor clinical outcomes (Padmanabhan et al., 2006; Romero et al., 2017; Shibata et al., 2008). Aberrant NRF2 activation in cancer cells induces expression of cellular defense enzymes and metabolic reprogramming conferring therapeutic resistance and aggressive proliferation properties (Homma et al., 2009; Mitsuishi et al. 2012). Previous studies have shown multiple mechanisms of NRF2 activation in cancer cells, such as somatic mutations (Ooi et al., 2013; Padmanabhan et al., 2006; Shibata et al., 2010), loss of *NRF2* gene exon 2 (Goldstein et al., 2016), enhanced transcription of NRF2 (DeNicola et al., 2011), p62 accumulation (Komatsu et al., 2010), epigenetic modification of *KEAP1* gene (Wang et al., 2008) and production of oncometabolites (Adam et al., 2011; Ooi et al., 2011).

Over the course of analyses for molecular features of the KEAP1-NRF2 signaling pathway, we have shown some promising compounds to treat NRF2-activated cancers (Fig. 1). Initially, halofuginone was identified as an NRF2 inhibitor using a high-throughput screening (Tsuchida et al., 2017). Halofuginone suppresses NRF2 protein accumulation by inhibition of prolyl-tRNA synthetase. Recently, we developed novel compound "halofuginone-micelle" which attacks NRF2-activated cancers with less adverse effect (Panda et al., 2022). Another promising approach is to isolate synthetic lethal drugs including mitomycin C and the geldanamycin-derived HSP90 inhibitor (Baird and Yamamoto, 2021; Baird et al., 2020) (Fig. 1). These compounds are anticancer prodrugs and bioactivated by NRF2-dependent drug metabolizing enzymes. Targeting the NRF2 transcriptome enables to reduce toxicity in normal cells and provide specificity to NRF2 activated cancers.

In addition, NRF2 activation in cancer microenvironment can also be an effective approach against NRF2-activated cancers (Hayashi et al., 2020) (Fig. 1). In a mouse model experiment with systemic and myeloid cell specific NRF2 knocked out as well as transplanted 3 Lewis lung carcinoma (3LL) cells, higher ROS accumulation in the myeloid-derived suppressor cells (MDSCs) was observed (Hiramoto et al., 2014; Satoh et al., 2010). These mice also displayed a higher rate of cancer incidence and spontaneous lung metastasis, demonstrating that high NRF2 levels in the tumor microenvironment suppress tumor cell development by reducing the ROS levels in MDSCs. These observations indicate that NRF2 activation in host myeloid cells can be a therapeutic target in clinical settings. Several NRF2 inducers have been provided and some of them have been already used in clinical setting, so further studies of NRF2 inducer will lead to novel anticancer drug development (Gold et al., 2012; Szczesny-Malysiak et al., 2020; de Zeeuw et al., 2013).

FUTURE PERSPECTIVES

Accumulating lines of evidence have proven that KEAP1 senses a wide range of NRF2-inducing chemicals (Saito et al., 2016; Suzuki et al., 2019). Nonetheless, how KEAP1 senses a variety of chemicals utilizing multiple and distinct sets of cysteine residues remains to be clarified. Additional studies on the sensing mechanisms will advance our understanding of the environmental stress response.

NRF2 activators targeting the cysteine thiols of KEAP1 have been extensively developed as discussed above and NRF2 activators targeting the KEAP1-NRF2 PPI are also entering the stage of extensive development. It is interesting to note that Cuadrado (2015) has been developing β-transduction repeat-containing protein (β-TRCP)-NRF2 PPI inhibitor in patent (WO2022152800) as [β-TRCP-S-phase kinase-associated protein 1-CULLIN1 (β-TRCP-SKP1-CUL1) E3 ligase complex is also responsible for ubiquitination of NRF2 (Cuadrado, 2015; Kuga et al., 2022). Novel NRF2 activators targeting other sites will also be developed.

Of the many interesting recent topics in the NRF2 field, one highly interesting topic is the *Nrf2*-knockout mouse travel of international space station. The Nrf2 knockout mice stayed there for 31 days and all the mice returned safely to the ground (Suzuki et al., 2020b). Analyses of the mice revealed that NRF2 acts to preserve homeostasis of the skeletal muscle, kidney, and epididymal white adipose tissue (Hayashi et al., 2021; Suzuki et al., 2022; Uruno et al., 2021). Next challenge of the space mouse study is to verify that the NRF2 activation indeed acts to maintain homeostasis of whole body against the space stress and the study will continue.

AUTHOR CONTRIBUTIONS
T.S., J.T., and M.Y. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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