NRF2 signaling in cytoprotection and metabolism

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Abstract

The KEAP1-NRF2 system plays a central role in cytoprotection and defense mechanisms against oxidative stress. Because KEAP1 serves as a biosensor for electrophiles by using its reactive thiols and because NRF2 is a transcriptional factor regulating genes involved in the sulfur-mediated redox reactions, the KEAP1-NRF2 system has been regarded as a sulfur-utilizing cytoprotective mechanism. NRF2 is a key regulator of cytoprotective genes, such as antioxidant and detoxification genes, and also to possess potent anti-inflammatory activity. NRF2 has been recently focused as a great modifier/regulator for the cellular metabolism and mitochondrial function. Particularly, the NRF2-mediated regulatory mechanisms of metabolites and mitochondria has been considered diverse, but has not been fully-clarified yet. This review article provides an overview of the molecular mechanisms that regulate NRF2 signaling and its cytoprotective roles, and also highlights NRF2 contribution to the cellular metabolism, particularly in the context of mitochondrial function and newly found sulfur metabolism.

Introduction

Oxygen respiration enables highly efficient energy production by utilizing molecular oxygen, but at the same time, it causes oxidative damage to biomolecules, which is referred to as oxidative stress. The defense mechanism against oxidative stress is essential for the survival of aerobic organisms, and the failure of the defense mechanism causes serious damage to the organisms. The KEAP1 (Kelch-like ECH (erythroid cell-derived protein with CNC homology)-associated protein 1)-NRF2 (nuclear factor erythroid 2-related factor 2) system has been discovered as a defense mechanism against oxidative stress and plays a central role in oxidative stress response and protection (Itoh et al., 1995; Itoh et al., 1997; Itoh et al., 1999; Yamamoto et al., 2018). In addition, the KEAP1-NRF2 system has been reported to contribute to various physiological processes such as regulation of cell differentiation and proliferation (Hochmuth et al., 2011; Mitsuishi et al., 2012; Murakami et al., 2014; Murakami et al., 2017), inflammatory responses (Suzuki et al., 2017; Kobayashi et al., 2017), and anti-aging (Wati et al., 2020; Oishi et al., 2020; Zhao et al., 2022).

Being a transcriptional activator responsible for the cellular redox regulation, NRF2 directly enhances the expression of many antioxidant proteins and enzymes that regulate biochemical reactions. NRF2 is regarded as a master regulator of redox metabolism, coordinately inducing genes encoding enzymes of glutathione system and thioredoxin system as well as NADPH production. In addition, contribution of NRF2 to the mitochondrial function has been also suggested (Dinkova-Kostova & Abramov, 2015; Holmström et al., 2016; Kasai et al., 2019). Mitochondrial are essential for aerobic organisms to be better adapt to the oxidative atmosphere in the current earth environment and also have been an attractive target for the development of therapeutic agents for various diseases, especially aging-related diseases. Recetnly, it has been suggested that sulfur metabolism, especially cysteine metabolism, plays an important role in mitochondrial energy production (Akaike et al., 2017). Interestingly, the KEAP1-NRF2 system has also been shown to regulate cellular cysteine uptake and metabolism, suggesting that it is involved in mitochondrial energy metabolism

via regulation of sulfur metabolism (Alam et al., 2023). In this review article, we overview recent advances in the molecular mechanisms regulating NRF2 signaling and its contribution to metabolism and introduce important roles of sulfur metabolism in the regulation of mitochondrial functions dependent on the KEAP1-NRF2 system.

Physiological roles of the KEAP1-NRF2 system

Piles of studies have been published regarding cytoprotective function of NRF2 in various organisms including *C. elegans* ,*Drosophila* , mouse, and human (Figure 1). NRF2-deficient mice are susceptible to various exogenous stresses, while they are healthy and fertile in a well-controlled and protected environment in a breeding facility for experimental animals. The vulnerability and susceptibility of the NRF2-deficient mice underscore the critical contribution of NRF2 to the response and adaptation to the environmental factors. For example, Nrf2-mediated protection from electrophilic toxicants, such as cigarette smoke (Rangasamy et al., 2004; Iizuka et al, 2005), and ultraviolet from the sun (Hirota et al., 2005) has been reported in mouse models. CncC, which is a *Drosophila* orthologue of NRF2, also confers resistance to the lethal effects of the pesticide malathion (Misra et al., 2011). SKN-1, which is a *C. elegans* orthologue of NRF2, plays beneficial roles in the survival in the presence of oxidative stress generated from paraquat (An & Blackwell, 2003).

In addition to the exogenous environmental factors, NRF2 is also important for the protection from endogenously-generated redox perturbations. In mouse models, NRF2 protects renal cells from reactive oxygen species (ROS) during ischemia-reperfusion injury (Liu et al., 2009; Son et al., 2010; Ashrafian et al., 2012; Nezu et al., 2017), pancreatic beta-cells from proteotoxicity under pathological conditions (Lee et al., 2012; Yagishita et al., 2014; Amin et al., 2021) and neuronal cells from neurodegenerative disorders (Pajares et al., 2016; Rojo et al., 2018; Uruno et al., 2020). Furthermore, inflammation is one of the major causes for the redox disturbance of endogenous origin. NRF2 exerts potent anti-inflammatory function by accelerating resolution of acute inflammation (Itoh et al., 2004; Mochizuki et al., 2005) as well as alleviating chronic inflammation (Suzuki et al., 2017).

Consistent with the antioxidant and anti-inflammatory functions, anti-aging effects of NRF2 activation have been observed in mice (Wati et al., 2020; Oishi et al., 2020; Zhao et al., 2022) and*Drosophila* (Sykiotis & Bohmann, 2008). In mouse salivary glands during physiological aging, age-related alterations including fibrosis, immune cells infiltration, cell senescence, DNA damage and lipid peroxide accumulation are all suppressed by NRF2 activation (Wati et al., 2020). Age-related hearing loss is also delayed in*Keap1* -knockdown mice, in which NRF2 is systemically activated (Oishi et al., 2020). In addition to the effects on age-related functional decline, NRF2 activation by KEAP1 inhibition extends lifespan of Klotho mutant mice, which is a progeria model (Zhao et al., 2022), and alleviates age-related renal phenotypes, such as calcification and fibrosis. The similar lifespan extension can be obsereved in*Drosophila* (Sykiotis & Bohmann, 2008; Rahman et al., 2013).

Contribution of NRF2 to the health promotion in human has been also implicated, based on the polymorphism in the promoter region of*NFE2L2* gene, which generates difference in the expression level of NRF2. Smokers homozygous in the low expressor allele of NRF2 show higher risk of lung cancer (Suzuki et al, 2013). People homozygous in the high expressor allele of NRF2 show lower risk of noise-induced hearing loss (Honkura et al., 2016). Physiological range of NRF2 activation is beneficial in principle.

Regulatory mechanisms of NRF2-mediated transcription

NRF2 is a potent transcriptional activator belonging to the CNC transcription factor group (a family of bZip-type transcription factors homologous to the *Drosophila* transcription factor Cap'n'collar) and has been originally identified as a related member of NFE2 p45 (Itoh et al., 1995). NRF2 heterodimerizes with small MAF proteins and binds to a consensus sequence, antioxidant response element (ARE) ($\mathrm{GCnnn^{G}/_{C}TCA^{C}/_{T}}$) (Motohashi et al., 2000; Motohashi et al., 2004; Katsuoka et al., 2005; Yamamoto et al., 2006). Six functional domains, NRF2-ECH homology (Neh) 1 to 6, have been identified in NRF2 (Figure 2). The Neh1 domain contains a basic region required for DNA binding and a bZip structure required for heterodimer formation with small MAF proteins (Kyo et al., 2004; Kimura et al., 2007; Kurokawa et al., 2009; Sengoku et al., 2022). The Neh2 domain mediates NRF2 binding to KEAP1, an inhibitory regulator of NRF2, via two motifs, DLG

and ETGE motifs. The two-site binding of NRF2 to KEAP1 homodimer enables KEAP1-CUL3 ubiquitin E3 ligase complex to ubiquitinate NRF2 for degradation in the proteasome (Figure 3) (Kobayashi et al., 2004; Zhang et al., 2004; Tong et al., 2006). The DLG motif utilizes only hydrogen bonding and forms a relatively weak binding that exhibits rapid binding and dissociation. The ETGE motif, on the other hand, relies on both hydrogen bonding and hydrophobic interactions to bind to KEAP1 and is thought to exhibit two-step binding and dissociation, resulting in strong binding (Fukutomi et al., 2014; Horie et al., 2021). The Neh3 domain was shown to interact with CHD6 for enhancing NRF2-mediated transcriptional activation (Nioi et al., 2005). The Neh4 and Neh5 domains are known as transcriptional activation domains, and the binding of transcriptional coactivators, such as CREB binding protein (CBP) / p300 and chromatin remodeling factor BRG1, promotes transcriptional activation by NRF2 (Katoh, et al., 2001; Zhang et al., 2007). MED16, a subunit of Mediator complex, is another binding partner of NRF2 to the Neh4 and Neh5 domains, conferring transcriptional activation ability by recruiting the Mediator complex and subsequently RNA polymerase II (Sekine et al., 2015). When glucocorticoid receptor binds to the Neh4 and Neh5 domains, NRF2 activity is suppressed (Alam et al., 2017). The Neh6 domain contains serine residues that can be phosphorylated by GSK3β. NRF2 undergoes proteasome-mediated degradation upon the phosphorylation of the Neh6 domain, indicating that the Neh6 domain mediates KEAP1-independent degradation of NRF2 (Rada et al., 2012; Chowdhry et al., 2013).

Cooperativity with other transcription factors provides another layer of regulation for NRF2 transcriptional activity (Figure 4). ATF4 and NRF2 cooperatively enhance expression of xCT, a cystine transporter (Ye et al., 2014), and enzymes regulating *de novo* synthesis serine (DeNicola et al., 2015). Cooperativity of CEBPB and NRF2, which is uniquely observed in cancer cells with persistent activation of NRF2, promotes the enhancer activity of canonical NRF2 target genes and also generates novel enhancers at the loci that are not normally regulated by transiently-activated NRF2 (Okazaki et al., 2020; Okazaki et al., 2022). The NRF2-CEBPB cooperativity is likely to underly the emergence of a novel enhancer in *NOTCH3* locus for promoting cancer stemness, and at the same time, it also activates canonical NRF2-dependent enhancers, such as in *AKR1C1 -AKR1C2* locus, leading to the increased chemo-resistance of cancer cells (Figure 4). Compared to the persistent activation of NRF2, the transient activation only temporarily induces CEBPB expression, and results in a very short duration of the coexistence of the two factors, which hardly open the enhancers that the NRF2-CEBPB cooperativity does. In contract, the persistent activation of NRF2 leads to continuous expression of CEBPB, leading to constitutive coexistence of the two factors to create a new mode of transcriptional regulation.

KEAP1 is a substrate recognition subunit of CUL3-based ubiquitin E3 ligase and mediates NRF2 ubiquitination, serving as a negative regulator of NRF2 (Kobayashi et al., 2004; Kobayashi et al., 2006). The most unique feature of KEAP1 is to possess highly reactive cysteine residues, which enables KEAP1 to serve as a biosensor for electrophiles. KEAP1 consists of three functional domains: a broad complex tramtrack-bric-à-brac (BTB) domain, an intervening region (IVR), a double glycine repeat and a COOH-terminal region (DC) domain (Chauhan et al., 2013) (Figure 2). KEAP1 forms homodimers via the BTB domain and further forms a complex with Cullin3 (CUL3) and RING-box protein 1 (RBX1) to function as a ubiquitin E3 ligase (Figure 3). The DLG and ETGE motifs of NRF2 interact with DC domains of the KEAP1 homodimer, which allows ubiquitination of NRF2, resulting in proteasome-dependent degradation of NRF2. Intriguingly, the homodimer formation of KEAP1 is essential for the ubiquitin E3 ligase activity of KEAP1-CUL3 complex (Suzuki et al., 2011). The enzymatic activity of KEAP1-CUL3 ubiquitin E3 ligase is inhibited when reactive cysteine residues of KEAP1, such as Cys151, Cys273 and Cys288 in murine KEAP1, are directly modified by electrophiles (Figure 2) (Yamamoto et al., 2008; Saito et al., 2015; Suzuki et al., 2019). Different electrophiles target different cysteine residues of KEAP1, which is regarded as a multimodal sensing system, and the electrophilic signals converge on NRF2 to activate genes for antioxidant response and cytoprotection. Thus, under normal conditions, NRF2 is degraded and functionally repressed by KEAP1, and when electrophiles attack KEAP1, NRF2 is de-repressed and activates transcription. KEAP1 is an electrophilic biosensor regulating NRF2 pathway activity for the stress response.

KEAP1-independent regulation of NRF2 activity has been also described. CUL1-βTrCP ubiquitin E3 ligase

ubiquitinates NRF2 when NRF2 is phosphorylated at the Neh6 domain (Figure 3) (Rada et al., 2012; Chowdhry et al., 2013). GSK3 is responsible for the NRF2 phosphorylation at Ser344 and Ser347 in murine NRF2 (Figure 2), which is suppressed under the active proliferation signals mediated by AKT (Taguchi et al., 2014; Shirasaki et al., 2014).

Metabolism regulated by NRF2

As a key regulator of redox metabolism, NRF2 directly regulates many enzymes and antioxidant proteins involved in the redox regulation. Enzymes and transporters supporting glutathione synthesis and utilization are widely regulated by NRF2, which includes catalytic and regulatory subunits of gamma-glutamylcysteine ligase (GCLC and GCLM), glutathione reductase (GSR), glutathione peroxidases (*e.g.* , GPX2), glutathione-S-transferase (*e.g.* , GSTM1 and GSTP1), and a cystine transporter (xCT) (Figure 4) (Malhotra et al., 2010; Chorley et al., 2012). Thioredoxin system is also under the regulation of NRF2. In NRF2-activated cancer cells possessing hyperactivation of NRF2 and consequently exhibiting NRF2 addiction, which is often caused by somatic mutations of *KEAP1* or *NFE2L2* gene, glutathione synthesis is greatly enhanced and thereby, cysteine, glutamate and glycine are highly consumed and required for glutathione. In the NRF2-activated cancer cells, the demand for cysteine is fulfilled by increased expression of xCT (Sasaki et al., 2002), and the requirement of glycine is covered by increased *de novo* synthesis from serine (DeNicola et al., 2015) and increased dependency on the uptake of extracellular serine and glycine (LeBoeuf et al., 2020). In contrast, glutamate is decreased and short due to glutamate export by xCT and glutamate consumption for the glutathione synthesis, which results in the metabolic vulnerability of NRF2-activated cancer cells (Romero et al., 2017; Sayin et al., 2017).

Another metabolic activity regulated by NRF2 is NADPH synthesis (Figure 4). Pentose phosphate pathway contains two enzymes for the NADPH synthesis, glucose-6-phosphate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD), both of which are target genes of NRF2 (Mutsuishi et al., 2012; Ding et al., 2021). Other NADPH synthesis steps are regulated by isocitrate dehydrogenase 1 (IDH1) and malic enzyme 1 (ME1), which are also regulated by NRF2 (Mutsuishi et al., 2012). Folate metabolism-coupled NADPH production mediated by methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1) and MTHFD2 appears to be partly and indirectly regulated by NRF2, especially in NRF2-activated cancer cells where NRF2 cooperates with ATF4 (Mutsuishi et al., 2012; Fan et al., 2014).

As mentioned above, NRF2 enhances NADPH production by re-wiring metabolism pathway, and offer strong reducing condition. The NRF2-mediated reducing condition is beneficial for maintaining the high efficiency of translation because many ribosomal subunits are rather susceptible to oxidation of cysteine residues and subsequent decline of their functionality (Chio et al., 2016). On the other hand, continuous stabilization of NRF2 is considered to cause excessive cellular reducing force, that is, reductive stress. Inheritable missense mutations in small molecular weight heat-shock proteins promotes hypertrophic cardiomyopathy by forming protein aggregate containing KEAP1, which causes persistent activation of NRF2 (Rajasekaran et al., 2007; Rajasekaran et al., 2011). Under this condition, NRF2-induced reductive stress is regarded to further promotes protein aggregation, which exacerbates cardiomyopathy, as NRF2 suppression mitigates protein aggregation and improves the cardiac function (Kannan et al., 2013). Comprehensive analysis of various lung cancer cell lines also showed that NRF2 activation increases NADH vs. NAD^+, leading to reductive stress (Weiss-Sadan et al., 2023). Consistent with these studies, NRF2-activated cancers exhibit dependency on SLC33A1, which is related to unfolded protein response and autophagy, possibly to avoid protein aggregation under reductive cellular environment caused by NRF2 (Romero et al., 2020).

Mitochondrial function and NRF2

Mitochondria are important organelles as sites of energy metabolism in aerobic respiration. Mitochondria contain TCA cycle as a part of glucose catabolism, β-oxidation pathway as a part of fatty acid catabolism, electron transport chain (ETC), oxidative phosphorylation system and sulfur oxidation pathway as a part of cysteine catabolism. One of the important functions of mitochondria is to produce ATP via oxidative

phosphorylation. TCA cycle and β-oxidation pathway provide NADH and $FADH_2$ as substrates to the ETC that generates proton gradient across the mitochondrial inner membrane leading to the ATP production. Because mitochondrial dysfunction causes increased electron leakage from the ETC and generates ROS, the KEAP1-NRF2 system has been considered to protect cells from mitochondria-derived oxidative stress (Dinkova-Kostova & Abramov, 2015; Kasai et al., 2020; Esteras & Abranov, 2022). However, recent studies have demonstrated that the KEAP1-NRF2 system contributes not only to mitochondrial redox regulation but also to the regulation of energy metabolism.

NRF2 activation contributes to enhance mitochondrial function by regulating mitochondrial biosynthesis and energy production (Dinkova-Kostova & Abramov, 2015; Esteras & Abranov, 2022). For example, NRF2 activation in skeletal muscles by KEAP1 disruption in mice increases oxygen consumption (Uruno et al., 2016), increases myosin heavy chain (MHC) I-positive slow fibers and thereby improves endurance capacity during exercise (Onoki et al., 2021). Regarding mitochondria biogenesis, NRF2 directly promotes transcription of *nuclear respiratory factor-1* (*NRF-1*), a transcription factor required for mitochondrial biogenesis (Piantadosi et al., 2008), and NRF2 inducers have been shown to activate transcription of *περοξισομε προλιφερατορ-αςτιατεδ ρεςεπτορ γαμμα ςοαςτιατορ 1-α* (*ΠΓ-1α*) gene, which serves as a cofactor for NRF-1 (Brose et al., 2012). As to energy production, mitochondrial membrane potential and ATP production are lower in *Nrf2* -deficient mouse embryonic fibroblasts and primary cultured neural cells than wild-type cells (Holmström et al., 2013). Conversely, NRF2 activation by KEAP1 suppression increases mitochondrial membrane potential and ATP production, suggesting that NRF2 promotes energy production in mitochondria. In this regard, recent transcriptome and proteome analyses revealed that factors involved in oxidative phosphorylation and the ETC are regulated downstream of NRF2 directly or indirectly (Cho et al., 2019; Gao et al., 2020; Zhang et al., 2021; Ryan et al., 2022). NRF2 also promotes the production of NADH and $FADH_2$, which are substrates of the ETC, and indeed, the supply of these substrates is reduced in NRF2-deficient cells (Esteras & Abramov, 2022). Consistently, the transcriptome and proteome analyses mentioned above demonstrated that NRF2 activation increases the expression of enzymes related to glucose and fatty acid metabolism and citric acid cycle enzymes involved in the production of NADH and $FADH_2$ (Cho et al., 2019; Gao et al., 2020; Zhang et al., 2021; Ryan et al., 2022).

Fatty acid metabolism and NRF2-mediated mitochondrial activation

In mammals, fatty acids are stored in adipocytes as triglycerides, broken down into fatty acids and glycerol as needed, and released into the blood as free fatty acids. Fatty acids taken into the cells from the blood undergo β-oxidation in the mitochondria and are finally converted to acetyl CoA, which enters the TCA cycle. In the process of β-oxidation, fatty acids produce more $FADH_2$ than NADH compared with glucose, implying that energy production using fatty acids is more dependent on Complex II of the ETC than that using glucose.

As mentioned above, disruption of *Keap1* gene in mouse skeletal muscles increases MHC I-positive slow fibers and improves exercise endurance capacity (Onoki et al., 2021). Intriguingly, the NRF2 activation in the skeletal muscle promotes the fatty acid mobilization and elevates succinate dehydrogenase (SDH) activity, implicating that preferred utilization of fatty acids as energy source enhances the NRF2-mediated endurance capacity. Consistent with the results, it was reported that $FADH_2$ production is reduced in the hearts of NRF2-deficient mice and that fatty acid-stimulated oxygen consumption is increased in the mitochondria of KEAP1-knockdown mice (Ludtmann ert al., 2014), suggesting that NRF2 activation enhances β-oxidation. Carnitine palmitoyl-tansferase 1 (CPT1) and CPT2 are required for the uptake of fatty acids into the mitochondria and are rate-limiting enzymes for fatty acid oxidation and both enzymes were also shown to be decreased in cultured cells and livers from NRF2-deficient mice (Pang et al., 2014; Meakin et al., 2014). The transcriptome and proteome analyses suggest that NRF2 activation increases the expression of CPT1 and CPT2, and in particular, CPT2 was reported to have an ARE sequence to which NRF2 can bind, which implying that CPT2 is a direct NRF2 target gene (Cho et al., 2019; Gao et al., 2020; Ryan et al., 2022). Furthermore, it has been reported that NRF2 also directly promotes gene expression of CD36, which is present in cellular and mitochondrial membranes and involved in fatty acid transport (Maruyama

et al., 2008). These observations suggest NRF2 activation is involved in mitochondrial membrane potential formation by promoting fatty acid uptake and fatty acid oxidation.

Supersulfide and NRF2

Recently, "supersulfides" have been recognized as a new entity of biomolecules (Zhang et al., 2023). Supersulfides are defined as molecules possessing catenated sulfur, and they are present in the form of low-molecular-weight metabolites and in the cysteine residue side chains of proteins. Typical examples are cysteine persulfide (CysSSH) and glutathione persulfide (GSSH) as reduced forms, and cystine trisulfide (CysSSSCys) and glutathione trisulfide (GSSSG) as oxidized forms. A unique chemical property of supersulfides is dual redox reactivity to both electrophiles and nucleophiles, which enables supersulfides to get involved in various biochemical reactions. Because pKa value of the hydropersulfide moiety (-SSH) is lower than that of simple thiol moiety (hydrosulfide moiety; -SH), CysSSH and GSSH are more reactive to electrophiles such as oxidative stress than cysteine (CysSH) and glutathione (GSH) (Ida et al., 2014). Physiological roles of supersulfides include antioxidant functions (Ida et al., 2014; Millikin et al., 2016), anti-inflammatory functions (Zhang et al., 2019; Matsunaga et al., unpublished observation), and signal transduction (Nishida et al., 2012; Nishimura et al., 2019). Supersulfides also contribute to energy metabolism (Akaike et al., 2017; Marutani et al., 2021; Alam et al., 2023), protein quality control (Dóka et al., 2020) and enzymatic activity regulation (Kasamatsu et al., unpublished observation).

Several enzymes have been identified to synthesize supersulfides. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) catalyze transsulfuration, which acts as a conversion pathway of methionine into cysteine, and also reportedly produce hydrogen sulfide/hydropersulfide (H_2S/HS^-). In addition to these activities, CBS and CSE have been shown to possess activities to generate CysSSH from cystine (Ida et al., 2014), which is a very unique reaction because a disulfide bond in cystine is converted to HS^- via C-S lyase-like reaction without consuming reducing equivalent (Figure 5). 3-Mercaptopyruvate sulfertransferase (3-MST) was also reported as the third enzyme generating H_2S and supersulfides (Kimura et al., 2017). However, simultaneous disruption of the three enzymes, CBS, CSE and 3-MST, in mice does not eliminate supersulfide production *in vivo* (Zainol Abidin et al., 2023), strongly suggesting the presence of alternative compensatory mechanisms for supersulfide production. Indeed, cysteinyltRNA synthetase (CARS) has been found to possess cysteine persulfide synthesizing activity as a moonlighting function (Akaike et al., 2017). CARS1 and CARS2 are cytoplasmic and mitochondrial isoforms, respectively, and both isoforms possess four motifs well-conserved among species: two of them are for zinc coordination and the other two are for binding of pyridoxal phosphate (PLP). The former are essential for the cysteinyl-tRNA synthesizing activity and thereby related to protein translation, while the latter are essential for cysteine persulfide synthesizing activity. CARS1 and CARS2 are considered to generate low-molecular-weight supersulfides as well as to conjugate cysteine persulfide with tRNA to generate persulfidated cysteinyl-tRNA, allowing cysteine persulfide to be incorporated into a nascent polypeptide in the ribosome. Although the functional significance of the protein supersulfidation at the translation stage remains to be elucidated, supersulfide production in mitochondria by CARS2 has been shown essential for the mitochondrial function.

Impairment of CARS2-mediated supersulfide production depolarizes mitochondrial membrane potential and reduces oxygen consumption (Akaike et al., 2017; Alam et al., 2023), suggesting an essential role of supersulfides in the mitochondrial energy metabolism. Although a recombinant CARS protein synthesizes CysSSH, accumulation of H_2S/HS^-, rather than CysSSH, was observed in cells. However, when mitochondria are partly depleted in the cells by ethidium bromide treatment, H_2S/HS^- was decreased, but instead CysSSH was increased (Akaike et al., 2017). These results unequivocally indicate that CysSSH is reduced to H_2S/HS^- in the ETC function-dependent manner, implying that supersulfides generated in mitochondria serve as electron acceptors (Figure 5). The consequently-generated H_2S/HS^- is oxidized to supersulfides by sulfide:quionone oxidoreductase (SQOR), which is considered to prevent accumulation of H_2S/HS^- and avoid mitochondrial inhibition by sulfide toxicity (Marutani et al., 2021). Sulfur oxidation enzymes residing in mitochondria, ETHE1 and SUOX, also oxidize supersulfides to generate thiosulfate ($HS_2O_3^-$), sulfite (HSO_3^-) and sulfate (HSO_4^-) using molecular oxygen (Figure 5) (Luna-Sánchez et al., 2017; Ziosi et al., 2017). If

the supersulfide synthesis in mitochondria is impaired, mitochondrial electrons that should be accepted by supersulfides are expected to be transferred to oxygen, leading to the generation of ROS. Thanks to the presence of supersulfides, electrons leaked from the ETC are not accepted by oxygen but by supersulfides and return to the ETC via SQOR. The supersulfide-mediated electron flow is considered as a rescue circuit for leaked electrons, that is, a mechanism avoiding excessive generation of ROS and ensuring the efficiency of the ETC. Therefore, the mitochondrial supersulfide production and the subsequent sulfur oxidation pathway play a critical role in the mitochondrial energy metabolism.

Consistent with the observation that sulfur metabolism makes a substantial contribution to the mitochondrial respiration, cysteine supply is critical for the mitochondrial activity (Alam et al., 2023). One of the supply routes of cysteine is to uptake extracellular cystine via a cystine transporter xCT. Another route is cysteine intracellularly-synthesized from methionine via transsulfuration pathway. As mentioned above, NRF2 directly activates *Slc7a11* gene, which encodes xCT (Sasaki et al., 2002), and thereby increases cellular pool of cysteine, ultimately resulting in the increased production of supersulfides. Importantly, NRF2-mediated mitochondrial activation is canceled either by inhibition of xCT, suppression of CARS2-mediated supersulfide production, or inhibition of the mitochondrial sulfur oxidation pathway, supporting the idea that NRF2 activates mitochondria though promoting the mitochondrial sulfur metabolism (Figure 5). From a different point of view, the role of NRF2 in the mitochondria can be interpreted as another mode of antioxidant function of NRF2: avoiding excessive production of ROS and protecting cells from the oxidative stress derived from mitochondria during oxygen respiration.

NRF2 and supersulfides for understanding various pathogenesis

The experimental results described above suggest that the KEAP1-NRF2 system regulates mitochondrial functions by promoting mitochondrial biosynthesis, electron transport and fatty acid oxidation, in addition to the previously known function of scavenging oxidative stress. NRF2 also enhances mitochondrial activity by promoting cystine uptake, leading to the increase in the cysteine availability for the supersulfide synthesis. This multimodal contribution of NRF2 to the mitochondrial function underscores the requirement of the KEAP1-NRF2 system for the aerobic organisms, particularly, terrestrial life exposed to higher concentrations of oxygen (Yumimoto et al., 2023).

In recent years, the NRF2-mediated regulation of mitochondrial function has attracted much attention from the perspective of disease treatment. For example, in the mouse model, NRF2 activation inhibits the progression and exacerbation of aging-related diseases, such as Alzheimer's disease and Parkinson's disease (Esteras et al., 2016; Uruno et al., 2020). Mitochondrial dysfunction is one of the major causes for these aging-related diseases, which are thought to result from a combination of factors, such as accumulation of oxidative stress, smoldering inflammation and impaired energy production. Therefore, therapeutic agents that ameliorate all of these phenomena are desirable for the treatment of the aging-related diseases, and NRF2 inducers are expected to be ideal because various aging-related phenotypes are more or less alleviated in the genetic model of *Keap1* -knockdown mouse in which NRF2 is activated in the whole body (Wati et al., 2020; Oishi et al., 2020; Zhao et al., 2022). Importantly, it has been suggested that the supersulfides are also associated with the aging-related diseases. The supersulfides have been shown to be decreased during aging in rodents and worms (Zivanovic et al., 2019). Particularly in the brain, decreased protein supersulfidation is closely associated with the progression of the pathological changes (Petrovic et al., 2021). Supersulfides are mostly likely to serve as one of the important downstream effectors of NRF2 for its anti-aging activity as well as the antioxidant and anti-inflammatory functions. Further investigation will clarify how supersulfides contribute to the human health and longevity.

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Figure legends

Figure 1. Physiological roles of NRF2.

NRF2 activates transcription of various genes involved in diverse cytoprotective function under a physiological condition.

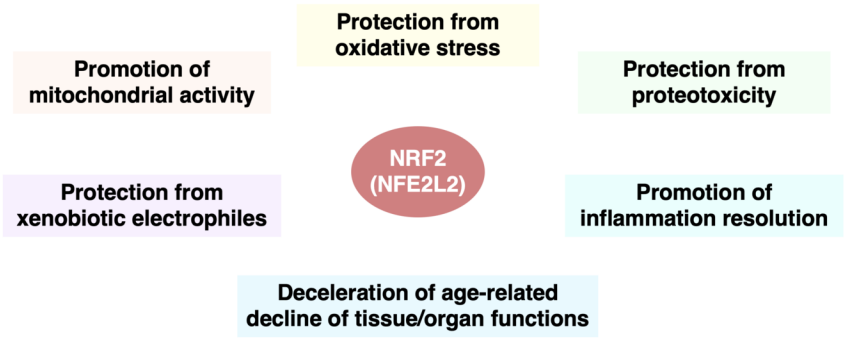

Figure 2. Domain structures of NRF2 and KEAP1.

NRF2 consists of 6 functional domains, Neh1 to Neh6. The function of each Neh domain is shown on the top of the NRF2 structure and some representative factors binding to each domain are also shown. Two serine residues (Ser344 and Ser347) of murine NRF2 are phosphorylation target sites of GSK3. KEAP1 has three domains. Their function is shown on the top of the KEAP1 structure. Three cysteine residues (Cys151, Cys273 and Cys288) of murine KEAP1 are major functional target sites of electrophiles.

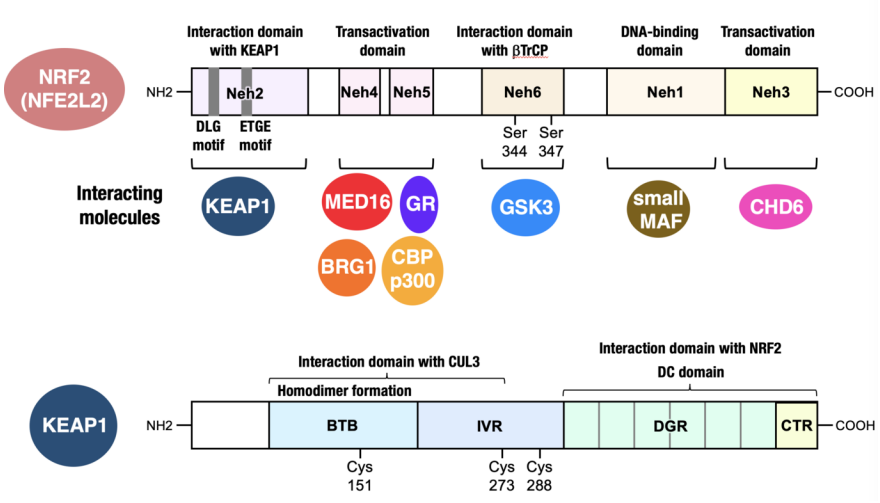

Figure 3. KEAP1-dependent and KEAP1-indepdnednt degradation of NRF2.

KEAP1 serves as an NRF2-recognizing subunit of CUL3-based ubiquitin E3 ligase and leads NRF2 to proteasome-dependent degradation. KEAP1 is inactivated by thiol modification with electrophiles, which allows NRF2 to be translocated to nucleus and activate its target gene transcription. When NRF2 is phosphorylated by GSK3, βTrCP serves as an NRF2-recognizing subunit of CUL1-based ubiquitin E3 ligase.

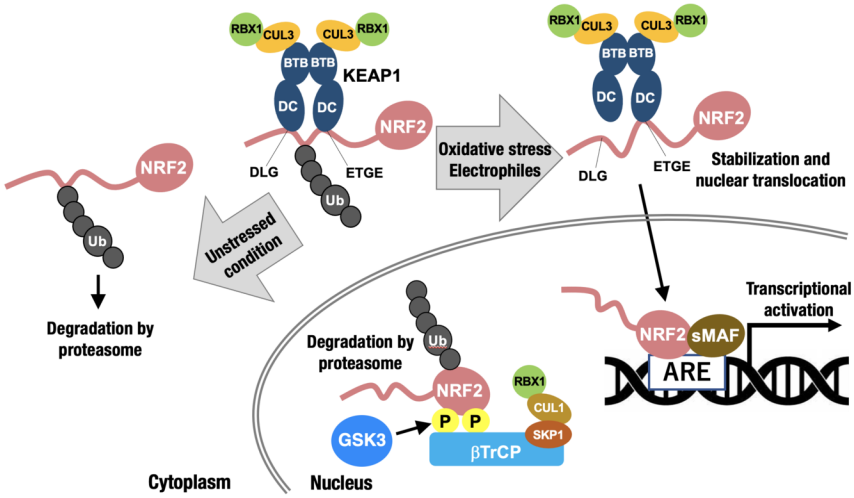

Figure 4. Cooperativity of NRF2 with ATF4 and CEBPB.

Functional cooperativity of NRF2 with ATF4 and CEBPB occurs in cancer cells with constitutive activation of NRF2. Gene categories shown in red are canonical NRF2 target genes. Gene categories shown in green and blue are regulated under the regulation by NRF2-ATF4 and NRF2-CEBPB cooperativity, respectively.

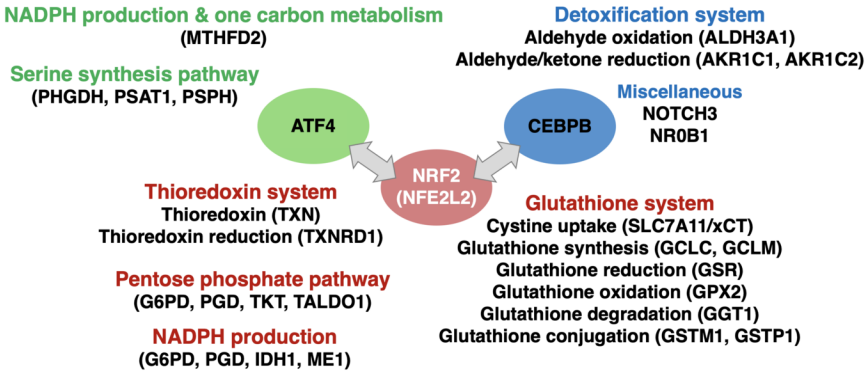

Figure 5. Sulfur metabolism in mitochondria.

Flow of sulfur metabolites in cells and mitochondria is shown with responsible enzymes for each step. Enzymes involved in the supersulfide production and sulfur oxidation pathway are shown in blue and green, respectively. I, II, III and IV indicate complexes in electron transport chain. Q and QH_2 indicate ubiquinone and ubiquinol, respectively.