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# NRF2 signaling cascade in amyotrophic lateral sclerosis: bridging the gap between promise and reality

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## Abstract

Amyotrophic lateral sclerosis is a very disabling disease due to the degeneration of motor neurons. Symptoms include muscle weakness and atrophy, spasticity, and progressive paralysis. Currently, there is no treatment to reverse damage to motor neurons and cure amyotrophic lateral sclerosis. The only two treatments actually approved, riluzole and edaravone, have shown mitigated beneficial effects. The difficulty to find a cure lies in the complexity and multifaceted pattern of amyotrophic lateral sclerosis pathogenesis. Among mechanisms, abnormal RNA metabolism, nucleocytoplasmic transport defects, accumulation of unfolded protein, and mitochondrial dysfunction would in fine induce oxidative damage and *vice versa*. A potent therapeutic strategy will be to find molecules that break this vicious circle. Sharpening the nuclear factor erythroid-2 related factor 2 signaling may fulfill this objective since nuclear factor erythroid-2 related factor 2 has a multitarget profile controlling antioxidant defense, mitochondrial functioning, and inflammation. We here discuss the interest of developing nuclear factor erythroid-2 related factor 2-based therapy in regard to the pathophysiological mechanisms and we provide a general overview of the attempted clinical assays in amyotrophic lateral sclerosis.

**Key Words:** amyotrophic lateral sclerosis; C9orf72; NRF2; oxidative defense; oxidative stress; sulforaphane; superoxide dismutase 1; TDP43

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## Introduction

Amyotrophic lateral sclerosis (ALS) is a lethal disorder characterized in the first place by movement difficulties. The ALS incidence varies between 0.6 and 3.8 per 100,000 person-year and the prevalence between 4.1 and 8.4 per 100,000 people (Longinetti and Fang, 2019). The disparity in rates may be due to variations in age and geographical differences, associated genetic and environmental factors. Males seem slightly more susceptible to developing ALS with an overall male-to-female sex ratio of 1.28 (Longinetti and Fang, 2019; Fontana et al., 2021).

The ALS disease is due to a degeneration of both upper and lower motor neurons in the spinal cord, brain stem, and motor cerebral cortex. Depending on which motor neurons are first affected, the first clinical signs are different. Indeed, regarding the upper motor neurons, associated symptoms include slowed movements of legs or arms, spasticity, or hyperreflexia while for the lower motor neurons, patients develop muscle atrophy and weakness. Half of the patients also show cognitive impairments like perseveration, disinhibition, apathy, and defects in language fluency and working memory (Marchi et al., 2021). Up to 15% of ALS cases fulfill the diagnostic criteria for frontotemporal dementia (Ringholz et al., 2005). Clinical complications appear with eating and respiratory difficulties. In most cases, unfortunately, ALS leads to death within 2–5 years after the first diagnosis of motor deficits but approximately 20% of patients survive longer. The clinical assays for ALS were mostly based on the repurposing of drugs for other neurodegenerative disorders. So far, only two drugs were approved, riluzole and edaravone, although having limited benefits to patients (Oskarsson et al., 2018). Currently, there is no sufficiently efficient treatment to counteract the progression of the disorder. The main

purpose of this review is to highlight the nuclear factor erythroid-2 related factor 2 (NRF2) involvements in ALS pathology and its therapeutic potential for a new clinical strategy.

## Search Strategy

To realize this review, we gather relevant publications from the PubMed database from 1990 to 2023 by associating the following keywords: NRF2 pathway, ALS, C9orf72, oxidative stress, therapeutic strategy, SOD1, TDP43, inflammation, and mitochondria.

## Amyotrophic Lateral Sclerosis Etiology

Only 10% of ALS cases are familial inherited forms while the remaining 90% are sporadic forms, indicating that both genetic and environmental factors contribute to the ALS etiology (Masrori and Van Damme, 2020). Because mutations in the superoxide dismutase 1 (*SOD1*) gene were the first identified as a cause of ALS (Mejzini et al., 2019), a significant understanding of ALS pathogenesis came first from models expressing mutant forms of *SOD1*. Mutations in *SOD1* account for 15% to 20% of familial ALS cases, and approximately 3% of sporadic ALS patients (Renton et al., 2014). In the last 30 years, an important breakthrough was achieved with the identification of new ALS-linked genes (Figure 1; Mejzini et al., 2019; Wang et al., 2023). They are not restricted to familial cases as rare and potentially pathogenic mutations in those genes have been found in almost 25% of sporadic patients (Cady et al., 2015). Only the most extensively characterized genes will be described hereinafter. The identification of two DNA/RNA binding proteins: fused in sarcoma (*FUS*) and TAR DNA-binding protein of 43 kDa (*TDP43*) have shed light on the importance of RNA metabolism in ALS. While mutations in *TDP43* are only present in 5% of familial cases, mislocalization and aggregation of non-mutated *TDP43* are detected in the majority (up to 97%) of ALS patients, suggesting that deregulation of wild-type *TDP43* mediates both sporadic and familial ALS. Other genes involved in RNA processing have been segregated with ALS such as angiogenin (*ANG*), ataxin-2 (*ATXN2*), matrin 3 (*MATR3*), senataxin (*SETX*), TATA-box binding protein associated factor 15 (*TAF15*), Ewing's sarcoma breakpoint region 1 (*EWSR1*), heterogeneous nuclear ribonucleoprotein A1 (*hnRNPA1*), heterogeneous nuclear ribonucleoprotein A2/B1 (*hnRNPA2/B1*), and T-cell-restricted intracellular antigen-1 (*TIA1*). Another ALS pathogenic mechanism that came to light is the impairment of the two major cellular degradation machinery: the ubiquitin-proteasome system and the autophagy-lysosome pathway. Among genes, we can cite p62/sequestosome-1 (*p62/SQSTM1*), ubiquilin-2 (*UBQLN2*), TANK-binding kinase 1 (*TBK1*), optineurin (*OPTN*), valosin-containing protein (*VCP*), charged multivesicular body protein 2B (*CHMP2B*), and cyclin F gene (*CCNF*). Nevertheless, the chromosome 9 open reading frame 72 gene (*C9orf72*) is by far the most common cause of ALS with 40–50% of familial and 5–10% of

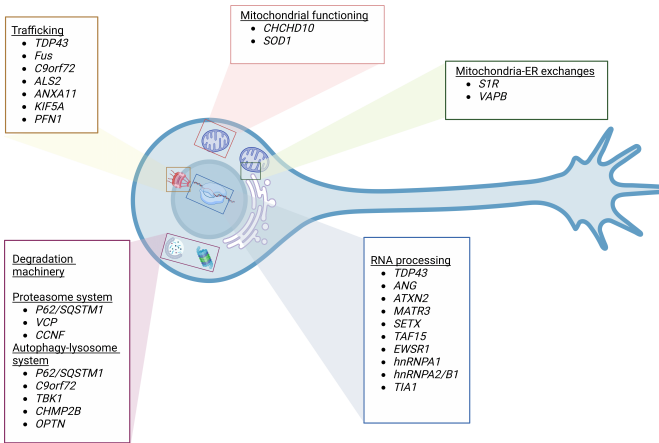
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**Figure 1** | Not exhaustive list of genes linked to ALS pathology and their cellular impacts.

Illustration of genes described as mutated in some ALS cases and their role in cellular machinery such as trafficking, mitochondrial functioning, mitochondria-ER exchanges, RNA processing, and degradation machinery. Created with BioRender.com. ALS: Amyotrophic lateral sclerosis; *ALS2*: alsin; *ANG*: angiogenin; *ANXA11*: annexin A11; *ATXN2*: ataxin-2; *C9orf72*: chromosome 9 open reading frame 72 gene; *CCNF*: F gene; *CHCHD10*: coiled-coil-helix-coiled-coil-helix domain containing protein 10; *CHMP2B*: charged multivesicular body protein 2B; ER: endoplasmic reticulum; *EWSR1*: Ewing's sarcoma breakpoint region 1; *FUS*: fused in sarcoma; *hnRNPA1*: heterogeneous nuclear ribonucleoprotein A1; *hnRNPA2/B1*: heterogeneous nuclear ribonucleoprotein A2/B1; *KIF5A*: kinesin family member 5A; *MATR3*: matrin 3; *OPTN*: optineurin; *P62/SQSTM1*: p62/sequestosome-1; *PFN1*: profilin 1; *S1R*: sigma-1 receptor; *SETX*: senataxin; *SOD1*: copper/zinc superoxide dismutase 1; *TAF15*: TATA-box binding protein associated factor 15; *TBK1*: TANK-binding kinase 1; *TDP43*: TAR DNA-binding protein of 43 kDa; *TIA1*: T-cell-restricted intracellular antigen-1; *VAPB*: membrane-protein-associated protein B; *VCP*: valosin-containing protein.

sporadic ALS cases in Europe and North America. Mutation in *C9orf72* consists in the presence of an abnormally long repeat of a GGGGCC hexanucleotide (G4C2) repeat in the first intron. Expanded G4C2 repeats are well-known to compromise the autophagy pathway (Gao et al., 2017). It is worth mentioning that mitochondrial dysfunction is also a key hallmark of ALS. Indeed several ALS genes are directly implicated in mitochondrial functioning like the coiled-coil-helix-coiled-coil-helix domain containing protein 10 (*CHCHD10*) and *SOD1* that are both present in the intermembrane space of mitochondria (Burstein et al., 2018; Higgins et al., 2002; Ruan et al., 2022). Two other ALS genes, vesicle-associated membrane-protein-associated protein B (*VAPB*) and sigma-1 receptor (*S1R*), regulate crosstalk between endoplasmic reticulum and mitochondria (Liévens and Maurice, 2021; Hartopp et al., 2022). Finally, to complete the none exhaustive list of mechanisms, several ALS genes perturb the trafficking between the nucleus and the cytoplasm, for instance, *C9orf72*, *fus*, and *TDP43* (Zhang et al., 2015; Chou et al., 2018; Lee et al., 2020; Lin et al., 2021) or within the cytosol with alsin (*ALS2*), annexin A11 (*ANXA11*); kinesin family member 5A (*KIF5A*) and profilin 1 (*PFN1*). Thus, all this diversity of genes and mechanisms highlights the complexity and multifaceted pattern of ALS pathogenesis. This raises the question of whether and how a common downstream mechanism leads to the selective degeneration of motor neurons observed in ALS. As a possibility, abnormal RNA metabolism, nucleocytoplasmic transport defects, accumulation of unfolded protein, and mitochondrial dysfunction would in fine induce oxidative damage and *vice versa*. A challenging task is to find a therapeutic strategy to break the vicious circle.

## Oxidative Stress in Amyotrophic Lateral Sclerosis

Oxidative phosphorylation in mitochondria is the main source of free radicals like reactive oxygen species (ROS):  $O_2^-$ ,  $HO^+$ , and  $H_2O_2$ . ROS can, to a lesser extent, be produced in the endoplasmic reticulum (ER), notably during ER stress due to the accumulation of unfolded proteins. Increased ROS amount leads to the generation of other reactive species such as reactive sulfur species and reactive nitrogen species. Reactive species are usually found in cells at limited levels due to scavengers and antioxidant enzymes. The non-enzymatic scavengers include vitamins C and E, glutathione (GSH), lipoic acids, and carotenoids. The antioxidant enzymes comprise SOD, catalase, glutathione peroxidase, peroxiredoxins, and glucose-6-phosphate dehydrogenase. An excessive production of ROS, reactive sulfur species, and reactive nitrogen species together with a defective antioxidant defense may represent a common downstream mechanism in ALS pathogenesis (Singh et al., 2019; Obrador et al., 2021). They progressively damage proteins, lipids, and DNA, targeting primarily the mitochondria and then the whole cell. This begets a deleterious spiral of redox dysregulation, ER stress, inflammatory process, and finally cell death (Mittal et al., 2014; Wang and Kaufman, 2016). Numerous studies have reported increased oxidative stress, including protein oxidation, lipid peroxidation, and DNA damage in biofluids of ALS patients (Park and Yang, 2021). Increased ROS production was also reported in lymphoblasts

from familial ALS patients with *SOD1* mutations (Said Ahmed et al., 2000) or fibroblasts derived from patients with expanded G4C2 repeats in the *C9orf72* gene (Onesto et al., 2016). However, in some other studies, no evidence of oxidative stress was reported in cultured fibroblasts from sporadic ALS cases (Said Ahmed et al., 2000; Codron et al., 2018) or patients bearing a mutation in *TDP43* (Onesto et al., 2016). Cell lines derived from patients may not be the most appropriate model to study ROS levels. Several possible mechanisms leading to oxidative stress in ALS have been already well described in the literature and synergy between genes and environmental factors such as pesticides, solvents, and heavy metals may be of importance (Obrador et al., 2021; Motaiianu et al., 2022).

One important question is whether oxidative stress appears early as a primary cause or just a secondary event in the disease. Interestingly, Babu et al. (2008) reported an increase in lipid peroxidation and reduced activities of antioxidant enzymes, G6PDH, and catalase, as well as reduced levels of GSH in the erythrocytes of 20 sporadic ALS patients with respect to controls. Interestingly, those changes progressed from 6 to 24 months, suggesting a correlation between oxidative stress and ALS duration. In contrast, no significant correlation was found between increased lipid peroxidation and the extent of the disease (Simpson et al., 2004). Further works are still needed to fully determine if oxidative stress could be used as a biomarker in ALS.

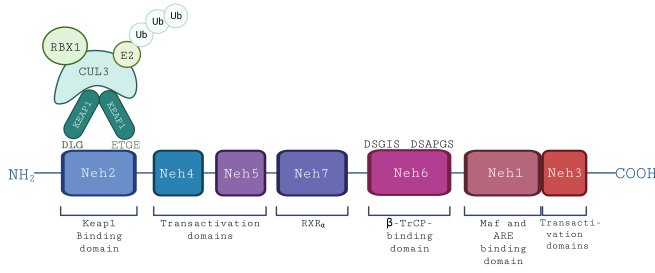
Strategies to prevent mitochondrial dysfunction and ROS accumulation may have a high therapeutic potential. Vitamin E was first evaluated on preclinical models expressing mutant *SOD1* mutation with a Gly93Ala substitution, G93A (*SOD1*<sup>G93A</sup>) (Gurney et al., 1996). Prospective analyses have reported that individuals who regularly used supplements of the vitamin E ( $\alpha$ -tocopherol), which is known to protect membranes from lipid peroxidation, presented almost half less risk of having or dying of ALS than nonusers (Ascherio et al., 2005; Veldink et al., 2007). In fact, the results of a large longitudinal analysis including over 1 million persons suggested that the ALS risk is inversely correlated to the duration of vitamin E supplements in patients (Wang et al., 2011). However, vitamin E failed to reveal an amelioration of motor function and survival in two double-blind, placebo-controlled clinical trials (Desnuelle et al., 2001; Graf et al., 2005). It remains likely that vitamin E intake may delay the risk of ALS in healthy persons but is not able to counteract the disease progression and outcome. Coenzyme Q10 presents a double interest by boosting the mitochondrial energy supply and preventing ROS damage (Beal, 2002). Like coenzyme Q10, MitoQ is an ubiquinone but attached to a positively charged, lipophilic molecule, which allows it to selectively accumulate in mitochondria (Kelso et al., 2001). Despite the beneficial effects of treatments with coenzyme Q10 or MitoQ in the *SOD1*<sup>G93A</sup> mouse model (Matthews et al., 1998), no clear evidence was found in a phase II trial for coenzyme Q10 (Kaufmann et al., 2009) and there is no trial of MitoQ on patients with ALS. Among other properties, dexamipexole presents mitochondria-targeted antioxidant effects (Danzeisen et al., 2006). Treatment with this compound was efficient to prolong survival time and preserve motor function in contrast to Pramipexole in *SOD1*<sup>G93A</sup> mice (Danzeisen et al., 2006). Unfortunately, the beneficial impact of dexamipexole was not replicated in the same transgenic mice (Vieira et al., 2014). Finally, dexamipexole was evaluated in a phase III trial where it was well tolerated, although it failed to improve the ALS pathology in patients (Cudkovic et al., 2013). So far, the only compound with antioxidant properties (Yoshino and Kimura, 2006) that has been approved for use as a treatment for ALS is edaravone in Japan (June 2015), South Korea (December 2015), US (May 2017), Canada (October 2018), Switzerland (January 2019), China (July 2019), Indonesia (July 2020) and Thailand (April 2021). Edaravone was found to ameliorate the quality of life but in a small proportion of ALS patients with well-defined inclusion criteria (Oskarsson et al., 2018). More recently, a retrospective study using health claim data from an US-based administrative database between August 2017 to December 2021 revealed that intravenous edaravone treatment prolonged the survival rate of ALS patients (Brooks et al., 2022).

Overall, while antioxidant molecules may provide a unique opportunity to slow ALS progression, they offered so far mitigated efficacy in clinical trials. As just described, ALS pathology is caused by various gene variants and likely influenced by multiple environmental risk factors. This calls into question the concept of ALS as a single disease and further challenges the identification of a medication. Most antioxidant drug candidates were in the first place validated on preclinical models expressing *SOD1*<sup>G93A</sup>, although mutations in *SOD1* only represent a minority (3%) of total ALS cases. It is now important to validate therapeutic strategies also on other ALS contexts. Several interconnected pathophysiological mechanisms are involved in ALS and this may represent further difficulty to find a cure. Addressing several of them together may be more successful in breaking the vicious circle. Future therapeutic attempts must tackle those issues.

## Nuclear Factor Erythroid-2 Related Factor 2 Signaling Pathways

### NRF2 and oxidative defense

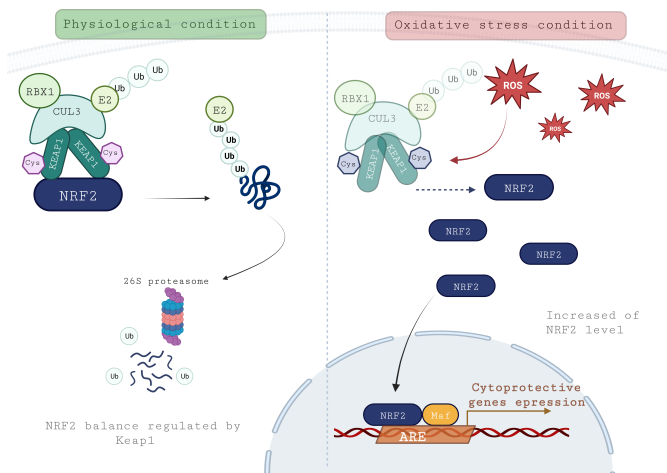
A remarkable therapeutic strategy refers to molecules that will sharpen the antioxidant response to a large extent. Glutathione-based and thioredoxin-based systems with nicotinamide adenine dinucleotide phosphate (NADPH)-generating enzymes are the major mechanisms with an important role in the catalytic removal of ROS. NRF2 is a master regulator of antioxidant response. This transcription factor consists of 605 amino acids and is divided into 7 domains named Neh1-7 (NRF2-ECH Homology) with specific roles (Figure 2).



**Figure 2 | NRF2 peptide with its seven Neh domains and their roles.**

The Neh2 domain contains two motifs (DLG and ETGE sequences) regulating the NRF2 stability through its binding site to KEAP1 protein and CUL3 ubiquitin ligase complex or to the Synoviolin E3 ubiquitin ligase (HDR1). The Neh3, Neh4, and Neh5 domains are necessary for the NRF2 interaction with other coactivators. The Neh7 domain allows NRF2 to bind the RXR $\alpha$  resulting in an inhibition of the NRF2-ARE signaling pathway. The Neh6 domain serves as a negative regulator by binding  $\beta$ -TrCP leading to NRF2 degradation. Finally, the Neh1 domain regulates DNA binding. Created with BioRender.com.  $\beta$ -TrCP:  $\beta$ -transducin repeat-containing protein; CUL3: Cullin3; KEAP1: Kelch-like ECH-associated protein 1; Neh: NRF2-ECH Homology; NRF2: nuclear factor erythroid-2 related factor 2; RBX1: recruiting ubiquitin enzyme E2; RXR $\alpha$ : retinoic X receptor alpha; Ub: ubiquitin.

In homeostasis condition, the NRF2 balance and stability are controlled by several ubiquitin ligase adaptors. Among them, Kelch-like ECH-associated protein 1 (KEAP1)-NRF2 is considered the canonical pathway and well described (Figure 3). KEAP1 is a cysteine-rich protein (27 cysteines) consisting of five domains, including two protein-protein interaction motifs: Bric-à-Brac and Kelch domains. In physiological conditions, KEAP1 forms a dimer with NRF2 through its Kelch domains and the Neh2 domain of NRF2. Bric-à-Brac domain allows recruiting Cullin3 ubiquitin ligase with recruiting ubiquitin enzyme E2 (RBX1), leading to the ubiquitination of NRF2 and its proteasomal degradation by the 26S proteasome. In oxidative stress conditions, KEAP1 configuration is altered by adduction or oxidation of its cysteine residues (Zhang and Hannink, 2003), leading to conformation changes that will alter its ability to address NRF2 to proteasomal degradation. As a consequence, the KEAP1-NRF2 dimer will dissociate and result in the accumulation of NRF2 and its translocation into the nucleus.

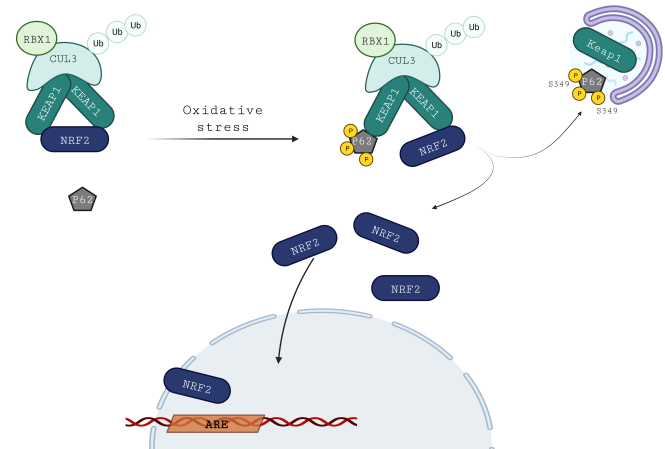


**Figure 3 | KEAP1-NRF2-ARE pathway.**

In physiological conditions (left), KEAP1 binds NRF2 and leads to its proteasomal degradation after ubiquitination by CUL3 and RBX1. In oxidative stress condition (right), reactive oxygen species will modify cysteines residues of KEAP1. This configuration change allows the dissociation of the KEAP1-NRF2 dimer and the NRF2 translocation into the nucleus. Then NRF2 binds the ARE DNA domain upstream of cytoprotective genes to enhance their expression. Created with BioRender.com. ARE: Antioxidant response element; CUL3: Cullin-3; Cys: cysteine; KEAP1: Kelch-like ECH-associated protein 1; Maf: musculo aponeurotic fibrosarcoma proteins; NRF2: nuclear factor erythroid related factor 2; RBX1: recruiting ubiquitin enzyme E2; ROS: reactive oxygen species.

Several non-canonical pathways also regulate NRF2 degradation. Among them, synoviolin E3 ubiquitin ligase (HDR1) is identified as a downstream effector of the inositol-requiring enzyme-1 branch of unfolded protein response to ER stress. A negative correlation was reported between HDR1 and NRF2 expression, since overexpression of HDR1 is associated with a decrease in NRF2 protein level (Wu et al., 2014). More precisely HDR1 enhances the ubiquitylation of NRF2 by a direct interaction on the Neh4 and Neh5 domains of NRF2 (Wu et al., 2014). Another non-canonical pathway is mediated by  $\beta$ -transducin repeat containing E3 ubiquitin-protein ligase (Chowdhry et al., 2013). This ubiquitin ligase interacts with NRF2 on its Neh6 domains (Chowdhry et al., 2013).

It appears clear that oxidative stress defense and autophagic pathway are closely linked, notably through the protein adaptor P62/sequestosome 1 (Figure 4). P62 is an autophagy carrier protein; it serves as a receptor to target the ubiquitinated cargo to autophagic degradation through the recruitment of autophagy-related protein, LC3/ATG8. Then LC3 activity leads to the formation of an autophagosome surrounding the cargo to be degraded by lysosomes. Interestingly, P62 phosphorylation occurs following the oxidative stress signal (Komatsu et al., 2007). As a consequence, the affinity of P62 for KEAP1 is increased leading to its direct binding to KEAP1 via a DPSTGE motif that is similar to the ETGE sequence used by NRF2 to bind KEAP1. This direct interaction results in the inhibition of NRF2-KEAP association (Komatsu et al., 2007). In some conditions like tumors, the prolonged activation of NRF2 through this non-canonical mechanism could be detrimental, contributing to a better resistance of tumoral cells, the "dark side" of NRF2.



**Figure 4 | Regulation of KEAP1-NRF2 by P62 non-canonical pathway.**

Oxidative stress is one of the signals able to activate P62 phosphorylation. As a consequence, P62 will bind KEAP1 leading to the inhibition of KEAP1-NRF2 dimer and the degradation of KEAP1 by autophagy. Then, NRF2 translocates into the nucleus and enhances the expression of antioxidative genes. Created with BioRender.com. ARE: Antioxidant response element; KEAP1: Kelch-like ECH-associated protein 1; Maf: musculo aponeurotic fibrosarcoma proteins; NRF2: nuclear factor erythroid related factor 2; ROS: reactive oxygen species; P: phosphorylation; p62: p62/sequestosome-1; S349: serine 349; Ub: ubiquitin.

After translocation in the nucleus, NRF2 will bind to the antioxidant response element (ARE) DNA regulator sequence. This binding requires its heterodimerization with other transcriptional cofactors on the Neh1 domain of NRF2. The most known and described cofactors are the small musculo aponeurotic fibrosarcoma proteins (MafF, MafG, and MafK) (Andrews et al., 1993; Wild et al., 1999). Nevertheless, it is proposed that NRF2 and activating transcription factor 4 (ATF4) may also form a heterodimer and cooperatively stimulates genes controlling antioxidative defense (Kasai et al., 2020). Moreover, the *ATF4* gene is a direct target of NRF2 as the gene contains an ARE sequence (Miyamoto et al., 2011). This further illustrates the crosstalk between ER stress and oxidative defense.

NRF2 will enhance the expression of detoxifying and antioxidant enzymes. In particular, genes involved in the phase II detoxification such as NADPH-quinone dehydrogenase 1 (*NQO1*), heme oxygenase 1 (*HO-1*), those acting on glutathione production like glutamate-cysteine ligase catalytic subunit (*GCLC*) or glutamate-cysteine ligase modifier subunit (*GCLM*), and detoxification enzymes using glutathione or thioredoxin-system: glutathione S-transferase p11 (*GSTP1*) or peroxiredoxin (*PRDX*). NRF2 also enhances enzymes of NADPH regeneration such as glucose-6-phosphate 1-dehydrogenase or phosphogluconate dehydrogenase. Finally, it is important to notice that NRF2 increases the expression of *KEAP1* as a negative feedback loop.

Besides its role in oxidative stress regulation, NRF2 is also involved in other cellular mechanism defenses. Indeed, previous work has shown its role in inflammatory response and mitochondrial metabolism.

#### NRF2 and mitochondria

NRF2 has an impact on mitochondrial homeostasis regulation that makes it a good strategy to counteract mitochondrial dysfunction existing in neurodegenerative diseases such as ALS. Recently, NRF2 appears to play a role notably in bioenergetics as well as mitochondrial dynamics and integrity.

Indeed, regarding mitophagy, several pieces of evidence showed the key involvement of NRF2. First, p62-mediated mitophagy inducer was shown to induce mitophagy through the upregulation of p62 in an NRF2-dependant manner (East et al., 2014). Moreover, NRF2 induces the expression of several autophagic mediators involved in mitophagy. In fact, NRF2 regulates the expression of PTEN-induced kinase 1 (*PINK1*), containing ARE sequences on its promoter (Murata et al., 2015). *PINK1* kinase activates the ubiquitylation of damaged mitochondria by the ubiquitin ligase Parkin and thereby the



formation of an autophagosome (Khalil and Liévens, 2017). However, the overexpression of NRF2 leads to mitophagy in the *Drosophila* model deficient for PINK1/Parkin system, suggesting alternative mitophagy pathways (Gumeni et al., 2021).

NRF2 influences mitochondrial dynamics by promoting either mitochondrial fusion or fragmentation depending on the tissue type (O'Mealey et al., 2017; Ryan et al., 2022). NRF2 led to the degradation of the pro-fission key protein, dynamin-related protein 1 in fibroblasts and cultured neuronal cells, resulting in a hyperfused mitochondria network (Sabouny et al., 2017). Alternatively, NRF2 activation in alveolar epithelial cells prevented mitochondrial recruitment of dynamin-related protein 1 but also up-regulated mitochondrial dynamin-like GTPase OPA1, a pro-fusion mitochondrial protein (Hou et al., 2021). Conversely, in skeletal muscle and during exercise, NRF2 activation plays a pro-fission role through the stabilization of dynamin-related protein 1 to ameliorate muscle functioning (Huang et al., 2019).

Finally, in microglia, NRF2 was found to be closely linked to the main mitochondrial biogenesis regulator, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Navarro et al., 2017). In addition, NRF2 activation was linked to an upregulation of another mitochondrial biogenesis factor, mitochondrial transcription factor A, in skeletal muscle during exercise (Weiss and Littleton, 2016).

Taken together, these observations highlight that NRF2 is a relevant therapeutic target to also alleviate mitochondrial dysfunctions.

### NRF2 and inflammation

The role of nuclear factor- $\kappa$ B (NF- $\kappa$ B) has been well described in inflammatory, immune response, cellular growth, and apoptosis. NF- $\kappa$ B is sensitive to redox balance changes (Morgan and Liu, 2011). In physiological conditions, NF- $\kappa$ B is kept inactive by its inhibitor I $\kappa$ B $\alpha$  protein. Oxidative stress activates I $\kappa$ B kinase, which phosphorylates I $\kappa$ B $\alpha$ , then leading to its dissociation from NF- $\kappa$ B and its proteasomal degradation. Thus, NF- $\kappa$ B is released and can modulate the expression of proinflammatory genes, including tumor necrosis factor alpha-like (*TNF- $\alpha$* ), interleukin 1 beta (*IL-1 $\beta$* ), and interleukin 6 (*IL-6*), among others. NRF2 signaling plays a protective role in this vicious loop to limit NF- $\kappa$ B effects before it becomes harmful. Indeed, NRF2 has an indirect effect to negatively modulate NF- $\kappa$ B through its downstream genes such as *HO-1* (Soares et al., 2004). Moreover, the activity of NF- $\kappa$ B and expression of proinflammatory cytokines due to scratch injury of cultured astrocytes were further enhanced by the absence of NRF2 (Pan et al., 2012). Interestingly, the treatment with a potent inducer of the NRF2 signaling pathway diminished the inappropriate innate response to septic shock in cells exposed to lipopolysaccharide but not in NRF2 knockout cells (Liby et al., 2005).

Altogether these observations highlight NRF2 as a novel modifier gene that determines survival by modulating the innate immune response.

## Nuclear Factor Erythroid-2 Related Factor 2 Signaling and Amyotrophic Lateral Sclerosis

The role of NRF2 signaling has been explored in the ALS context. Ample evidence has arisen from different studies on ALS patients and models of the disease whatever the gene involved in the pathology. One of the first study on postmortem tissue samples compared *NRF2* and *KEAP1* expression in the primary motor cortex and the spinal cord from ALS patients and age-matched controls (Sarlette et al., 2008). A reduction of *NRF2* mRNA and NRF2 protein levels was observed in both structures of ALS patients while *KEAP1* mRNA expression was increased only in the motor cortex (Sarlette et al., 2008). In another study, authors observed instead an increase in NRF2 protein expression in the motor cortex but not in the spinal cord (Lastres-Becker et al., 2022). However, no correlation was found with levels of *NRF2* transcripts, which remained unchanged in the motor cortex but increased in the spinal cord. The limitation of postmortem studies is that analysis is done at the late stage of the pathology.

We can assume that the mechanisms involved in the initiation of neurodegeneration and those implicated in the progression are different between sporadic and familial cases. To explore earlier stages of ALS, Lastres-Becker et al. (2021) have generated a lymphoblastoid cell line from sporadic patients or cases carrying SOD1 mutations. Lymphoblasts from SOD1 patients displayed a decreased level of NRF2 protein while sporadic patients showed an opposite effect. Thus, the effect on the NRF2 pathway seems to be dependent on the causative mutation, age, sex, and clinical characteristics in patients (Lastres-Becker et al., 2021).

To better identify specific or common mechanisms of NRF2 between the different causative mutations, several studies have been done using transgenic ALS models. A reduction of NRF2 levels occurred in cultured primary motoneurons of SOD1<sup>G93A</sup> mice (Pehar et al., 2007) and SOD1<sup>G93A</sup>-transfected NSC34 motoneuron-like cells (Kirby et al., 2005). *In vivo*, the expression of NRF2 and its target GCLC was significantly reduced in the spinal cord of SOD1<sup>G93A</sup> mouse (Wang et al., 2022). Moreover, a comparative study revealed that mice with rapid progression of pathology (129Sv-SOD1<sup>G93A</sup>) expressed NRF2 more weakly compared to mice with slow progression of pathology (C57-SOD1<sup>G93A</sup>) (Nardo et al., 2013). This study demonstrates the key role of NRF2 in the SOD1 ALS pathogenesis. In a cell model, NSC-34 transfected with TDP43<sup>M337V</sup>, a reduced expression of total NRF2 and its target NQO1 were observed (Tian, 2017). In fact, it was reported that the presence

of TDP43<sup>M337V</sup> led to an increase in *NRF2* transcript levels but a reduced protein expression. This contradictory observation finds an explanation in that, TDP43<sup>M337V</sup> perturbed the interaction between the DNA-binding protein, heterogeneous nuclear ribonucleoprotein K, and *NRF2* mRNAs, then altering the translation of *NRF2* transcripts (Moujalled et al., 2017). Although the impact of C9orf72 mutation on NRF2 signaling has been poorly explored, NSC34 cells expressing expanded dipeptide repeats altered the activation of NRF2 expression consecutively to a treatment with the NRF2 inducer, dimethyl fumarate (Jiménez-Villegas et al., 2022). Again, it was associated with a translational shunt down of NRF2 by dipeptide repeats (Jiménez-Villegas et al., 2022).

The effect on NRF2 signaling seems to depend on the cell type. In a time-course analysis on SOD1<sup>G93A</sup> mice, increased NRF2/ARE signaling was reported early in distal muscles before the onset of the disease (Kraft et al., 2007). This supports the implication of oxidative stress in the initial phase of the disease and the “dying back” hypothesis, proposing that the disease first occurs at the neuromuscular junction. In rat and mouse SOD1<sup>G93A</sup> models, *NRF2* mRNA and protein levels were reported to increase in reactive astrocytes of the spinal cord (Vargas et al., 2005; Kraft et al., 2007). Similarly, an accumulation of NRF2 was observed in glial-like cells of SOD1<sup>G93A</sup> mice (Mimoto et al., 2012). The increase in NRF2 in the glia or muscles may represent an attempt of those cells to counteract oxidative stress.

## Nuclear Factor Erythroid-2 Related Factor 2 Cascade as a Therapeutic Target in Amyotrophic Lateral Sclerosis

The “one target, one weapon” idea has proven to be a challenging task for therapeutic strategies to cure multifactorial neuropathologies like ALS. Oxidative stress, mitochondrial dysfunction, ER stress, and inflammation are interconnected key actors in ALS. Future medication must be designed on targets with multifaceted cellular impact. In that respect, NRF2 appears as a valuable target for therapeutic prospects.

Crossbreeding SOD1<sup>G93A</sup> mice with NRF2-null mice had little effect on the pathology of ALS mice (Guo et al., 2013; Vargas et al., 2013). The neuronal loss in the spinal cord appeared earlier and the astrocytes proliferated more in SOD1<sup>G93A</sup> mice when NRF2 was lacking (Guo et al., 2013). Surprisingly, this translated into a very slight decrease in disease onset and lifespan (Guo et al., 2013) or no detrimental effect at all on the onset or median survival in SOD1<sup>G93A</sup> or SOD1<sup>G85R</sup> mice (Vargas et al., 2013). These data suggest that NRF2 has little implication for the disease progression in those mice. Interestingly, Guo et al. (2013) found that some NRF2 target genes (*HO-1*, *GCLM*, *GCLC*) are still upregulated and at a similar extent in SOD1<sup>G93A</sup> mice null for NRF2 as compared to SOD1<sup>G93A</sup> mice. GSH levels also remained unchanged at the onset when NRF2 was lacking. NRF2-independent pathways may compensate for the loss of NRF2 and this may explain the modest impact. However, overexpressing NRF2 under the control of the astrocytic promoter glial fibrillary acidic protein delayed muscle denervation and extended the lifespan of SOD1<sup>G93A</sup> mice (Vargas et al., 2008). This was accompanied by increased expression levels of ARE-driven genes including *NQO1*, *HO-1* as well as *GCLM* and *GCLC*, resulting in a twofold gain of GSH content. Increased NRF2 activation in astrocytes also extended median survival in another mouse model overexpressing *SOD1* with a disrupted copper-binding site, SOD1<sup>H46R/H46Q</sup> (Vargas et al., 2008). This can be compared to the benefits of pre-treating astrocytes derived from SOD1<sup>G93A</sup> mice with extracellular vesicles (EV) isolated from interferon- $\gamma$  stimulated mesenchymal stem cells (Provenzano et al., 2022). Whereas SOD1<sup>G93A</sup> astrocytes exacerbated the degeneration of SOD1<sup>G93A</sup> motor neurons, their pre-treatment with EV significantly reduced their toxicity. Similarly induced astrocytes reprogrammed from fibroblasts from two patients carrying the SOD1<sup>AAV</sup> mutation were toxic to wild-type motoneuron (Provenzano et al., 2022). Nevertheless, in contrast pre-treating those astrocytes with EV led to an increased survival of motoneurons (Provenzano et al., 2022). A key neuroprotective mechanism that emerged from this study concerns NRF2. While the nuclear localization of NRF2 was found decreased in SOD1<sup>G93A</sup> mouse astrocytes or SOD1<sup>AAV</sup> reprogrammed astrocytes, the pre-treatment with EV restored the NRF2 levels in the nuclear compartment. In the same way, overexpressing Sirtuin 6 (*SIRT6*) or increasing nicotinamide adenine dinucleotide in astrocytes prepared from mutant SOD1<sup>G93A</sup> mice abrogates their toxicity toward cocultured motoneuron (Harlan et al., 2019). In both cases, neuroprotection depends on NRF2 signaling and antioxidant protein upregulation in astrocytes (Harlan et al., 2019). Taken together, these observations provide strong evidence that astrocytes may be a key lever to prevent ALS pathogenesis and, notably through NRF2 signaling. Several studies have also reported a more direct role of NRF2 in neurons. Vargas et al. (2013) observed a slight delay in disease onset when NRF2 was overexpressed selectively in neurons or type II skeletal muscle fibers in SOD1<sup>G93A</sup> mice. NSC34 motoneuron-like cells expressing SOD1<sup>G93A</sup> were transduced by lentiviral vectors to overexpress *NRF2* (Nanou et al., 2013). Interestingly, this led to a decrease in oxidative stress and an improvement in cell survival. However, the same group showed that the AAV6 viral delivery of NRF2 in the neuronal population of the spinal motor cord failed to ameliorate the phenotype of the SOD1<sup>G93A</sup> mouse (Nanou et al., 2013). Treatment of TDP43<sup>M337V</sup>-expressing NSC34 cells with a conditioned medium of umbilical cord mesenchymal stem cells increased NRF2 nuclear translocation and the survival of these cells (Lan et al., 2023).

Natural compounds and synthetic agents that are supposed to enhance NRF2 signaling have been evaluated for their neuroprotective potential in the ALS context. Among them, curcumin known to boost NRF2 signaling was found to ameliorate abnormal electrophysiological features and mitochondrial dysfunction of NSC34 cells expressing mutant TDP43 (Lu et al., 2012; Dong et al., 2014). Treatment of mutant SOD1 mice with a curcumin derivative GT863 significantly slowed the progression of motor dysfunction (Kato et al., 2022). In two preclinical studies, curcumin was shown to improve the disease progression of ALS patients and to reduce oxidative damage (Ahmadi et al., 2018; Chico et al., 2018). Despite these encouraging results, the main obstacles to the use of curcumin as medication are its chemical instability, poor tissue distribution, and insolubility in water. The screening of 3 260 000 commercially available compounds resulted in the identification of N-(4-(2-pyridyl)(1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide (CPN-9), which showed selective neuroprotection against oxidative-stress induced-cell death by increasing ARE-dependent NRF2 targets (Kanno et al., 2012). CPN-9 not only alleviated motor disease progression and motor neuron loss but also extended the survival interval after onset in SOD1<sup>H46R</sup> mice (Kanno et al., 2012). However, CPN-9 could not result in medication due to its poor water solubility and blood-brain barrier permeability. Thus, novel small molecules were designed *in silico* using CPN-9 as a primary mother compound and among them, a top molecule, 2-[mesityl(methyl)amino]-N-[4-(pyridin-2-yl)-1H-imidazol-2-yl] acetamide trihydrochloride (WN1316), emerged with efficacy at doses remarkably inferior to CPN-9 (Tanaka et al., 2014). WN1316 rescued motor function and survival of SOD1<sup>H46R</sup> and SOD1<sup>G93A</sup> mice (Tanaka et al., 2014). A phase 1 clinical trial with this compound was then disclosed in 2014 to assess the safety and pharmacokinetics in healthy adults but data are still not known. Two synthetic triterpenoid analogs, 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid ethylamide (CDDO-EA) and 2-cyano-3, 12-dioxooleana-1,9-dien-28-oic acid trifluoroethylamide (CDDO-TFEA) that potentially activated Nrf2/ARE pathway were tested on SOD1<sup>G93A</sup> mice. Both of these compounds significantly enhanced motor performance and slowed disease progression when the treatment started at the presymptomatic or symptomatic age (Neymotin et al., 2011). Edaravone currently used as medication in ALS also promotes NRF2 signaling besides its anti-inflammatory efficacy (Zhang et al., 2019). While NRF2 reporter imaging increased in SOD1<sup>G93A</sup> mice accordingly to the progression of the disease, edaravone treatment reduced it with clinical improvement (Zhang et al., 2019). Using RNA sequencing on rat cells overexpressing TDP43, edaravone appeared to significantly affect NRF2 signaling among other enriched pathways (Soejima-Kusunoki et al., 2022).

Electrophilic compounds that modify cysteine residue at position 151 of KEAP1 disrupt the interaction between KEAP1 with Cul3 and thereby prevent the degradation of NRF2. One of them, 1-isothiocyanato-4-(methanesulfinyl) butane or sulforaphane is naturally abundant in its inactive form in seeds and sprouts of cruciferous plants and is known to activate NRF2 signaling interaction between KEAP1 and Cul3 (Tarozzi et al., 2013). Sulforaphane was beneficial in reducing cell damage and lipid peroxidation on NSC34 cells transfected with wild-type TDP43 or mutant (TDP43<sup>G331K</sup> and TDP43<sup>M337Y</sup>) (Duan et al., 2010). Levels of NRF2 itself and two of its targets were found to increase after sulforaphane exposure on wild-type TDP43-expressing cells but surprisingly not on mutant TDP43. Our group showed *in vivo* that sulforaphane treatment was beneficial on zebrafish larvae expressing mutant TDP43<sup>G348C</sup> (Lasbleiz et al., 2022). While TDP43<sup>G348C</sup>-expressing larvae displayed a decline in their locomotor response to tactile stimulation of the tail fin, a pre-treatment with sulforaphane was efficient to rescue the locomotor performances (Lasbleiz et al., 2022). In our condition, sulforaphane increased levels of NRF2 and several targets such as *GCLC*, *GCLM*, or *KEAP1* on physiological or mutant TDP43 conditions. Contrary to data on NSC34 (Duan et al., 2010), the presence of mutant TDP43 in zebrafish larvae did not modify the NRF2 expression (Lasbleiz et al., 2022). Dimethyl fumarate exerts an anti-inflammatory as well as an antioxidant effect through NRF2 and is used to treat multiple sclerosis. A phase 2 study with dimethyl fumarate was conducted at six medical centers on randomized patients with a diagnosis of possible or definite sporadic ALS (Vucic et al., 2021). Despite a good tolerance and safety of dimethyl fumarate, no significant amelioration was observed at 36 weeks between placebo and treated groups. Further works are needed to analyze the impact of sulforaphane or dimethyl fumarate and their derivatives.

In conclusion, the multifaceted profile of NRF2 is an asset that places NRF2 as a key therapeutic target. It makes clear that future studies must endeavor to demonstrate the efficacy of the NRF2 pathway to face ALS pathology. Given the growing interest in NRF2-therapy from pharmaceutical companies, the future challenge will be to define limitations between the efficacy and safety of NRF2-related compounds.

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