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NRF2 as a therapeutic opportunity to impact in the molecular roadmap of ALS

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a devastating heterogeneous disease with still no convincing therapy. To identify the most strategically significant hallmarks for therapeutic intervention, we have performed a comprehensive transcriptomics analysis of dysregulated pathways, comparing datasets from ALS patients and healthy donors. We have identified crucial alterations in RNA metabolism, intracellular transport, vascular system, redox homeostasis, proteostasis and inflammatory responses. Interestingly, the transcription factor NRF2 (nuclear factor (erythroid-derived 2)-like 2) has significant effects in modulating these pathways. NRF2 has been classically considered as the master regulator of the antioxidant cellular response, although it is currently considered as a key component of the transduction machinery to maintain coordinated control of protein quality, inflammation, and redox homeostasis. Herein, we will summarize the data from NRF2 activators in ALS preclinical models as well as those that are being studied in clinical trials. As we will discuss, NRF2 is a promising target to build a coordinated transcriptional response to motor neuron injury, highlighting its therapeutic potential to combat ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease that involves the progressive degeneration of motor neurons in the cerebral cortex, brain stem, and spinal cord [1]. Although ALS is perceived as a rare disease, it is the third most common neurodegenerative disease after Alzheimer's (AD) and Parkinson's (PD) [2], and has a lifetime risk of approximately 1 in 300. Death usually occurs within two to three years from diagnosis, often as a consequence of respiratory failure [1,3]. The pathological hallmarks of this disease and their correlation with clinical manifestations have been studied in detail. Thus, degeneration of upper motor neurons (MN) leads to spasticity in the limbs and bulbar musculature, overall clumsiness, markedly brisk reflexes, and pyramidal distribution weakness. Degeneration of lower MN neurons in brainstem motor nuclei and in the ventral horn of the spinal cord leads to muscle weakness, atrophy and fasciculations. In addition to the MN related symptoms, up to 50 % of cases also show evidence of mild cognitive impairment and approximately 5 % develop overt features of frontotemporal dementia (FTD) [4,5].

In spite of the good clinical knowledge of ALS pathogenesis, no relevant disease-modifying drug is currently available [6], turning this disease into a rapidly growing social and economic burden [7]. Recently approved treatments for ALS only marginally influence survival (riluzole) or disability progression (edaravone) [8,9]. This lack of therapeutic efficacy is probably due to the fact that the genetic and clinical landscape of ALS is very complex and clinical manifestations inherent to MN degeneration most likely reflect a variety of different upstream molecular pathomechanisms [10]. Indeed, mutations in more than 30 genes have been implicated in ALS pathogenesis, therefore indicating that ALS is not a single disease but rather belongs to a disease spectrum. This fact must be considered in searching for disease-modifying treatments, shifting from targeting common clinical manifestations to acting on the underlying molecular pathophysiology. Data from disease model systems, and from human biosamples, provide strong evidence in all

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cases of ALS for defects in RNA metabolism, redox imbalance, inflammation, and altered proteostasis, which exhibit a high degree of molecular connectivity and that are most likely related to the progressive loss of homeostatic functions. However, therapies targeting each of these pathways individually have consistently failed to produce robust evidence of benefit in the clinic. Therefore, a strategic shift in approach is needed to develop therapies which target these multiple pathophysiological pathways together by reinforcing the endogenous homeostatic systems. A very promising strategy is the activation of transcription factor NRF2 (nuclear factor (erythroid-derived 2)-like 2), nowadays recognized as a master regulator of multiple cytoprotective responses. NRF2 is ubiquitously expressed [11], but at different levels depending on the cell type. Glial cells exhibit more robust NRF2 expression than neuronal cells [12]. Therefore, the role of NRF2 in astrocytes and microglia is expected to be especially relevant to the health of MN, combining both direct and indirect mechanisms.

2. Genetic alterations associated with ALS

Most ALS cases are considered sporadic (SALS), but 5–10 % are familial (FALS), usually with autosomal dominant inheritance. More than 30 genes have been identified as causative for ALS and the genetic cause can now be identified in 60–70 % of familial ALS cases [13]. Moreover, large-scale genome-wide association studies (GWAS) of apparently SALS, without a family history, indicates a genetic variability for these patients, based predominantly on rare variants. Thus, one recent study showed clinically reportable causative genetic changes in 21 % of ALS patients, only 7 % of whom had a positive family history, and a further 21 % of cases had variants of unconfirmed significance after removal of benign or non-specific variants [14].

The most common genetic cause of ALS is a hexanucleotide expansion in the first intron of the *C9ORF72* gene, present in approximately 40 % of FALS cases and 7 % of SALS cases [15]. Mutations in *SOD1*, *TARDBP* and *FUS* are the next most frequent, and are present in respectively 20 %, 4 % and 4 % of FALS cases, and in some sporadic cases [16] (Fig. 1A). Other reported ALS genes are less common and participate in the disruption of various cellular pathways, including RNA processing, endosomal trafficking, autophagy/protein homeostasis, mitochondrial integrity, and cytoskeletal function/axonal transport [17]. Beyond *TARDP43* and *FUS*, exome sequencing studies revealed mutations in other genes involved in RNA metabolism, such as *MATR3* [18], *hnRNPA2B1* and *hnRNPA1* [19]. These studies have led to an increased interest in the role of RNA metabolism in ALS.

3. ALS mouse models

The predominant approach to modelling the pathology of ALS has relied on the use of genetic mouse models with transgenic (over) expression of human mutant ALS associated genes that cause profound phenotypes. More recently, knock-in approaches have begun to yield more physiological models, but with milder phenotypes. Transgenic overexpression of the human SOD1 gene with disease-relevant mutations is the first described and to-date most extensively studied model. In 1994, humanized mice carrying a human SOD1 transgene with the G93A mutation under control of the human SOD1 promoter were reported [20]. These mice displayed a motor phenotype with significant loss of spinal MN. This model provided evidence that SOD1 mutations lead to a toxic gain of function [20] and has led to multiple observations related to ALS pathophysiology. These include the concept of non-cell autonomous MN degeneration [21], the role of glial inflammation in driving disease progression [22], the contribution of SOD1 misfolding and aggregation to pathology [23], and validation of Neurofilament Light Chain levels as biomarkers of MN degeneration [24]. Mice expressing the wild-type sequence with a similar expression level lacked a discernible phenotype [20], although a mild phenotype has been seen in subsequent studies [25]. Controversy surrounds the use of this model, particularly in regard to replication of the effects of pharmacological agents in different laboratories. However, these issues seem to be related predominantly to differences in study design [26].

After the identification of TDP-43 proteinopathy as a characteristic feature of ALS [27] and the identification of mutations in its coding gene, *TARDBP*, as causative in a small proportion of cases [28], human TDP-43 transgenic mice with mutant specific phenotype were generated. This was an imperative because essentially all ALS cases carry TDP-43 proteinopathy, except for patients with mutations in *SOD1* and *FUS* [29], suggesting a mechanistic difference in MN degeneration driven by these gene mutations. Transgenic overexpression above a certain threshold of either wild-type or mutant TDP-43 led to significant MN degeneration and severe phenotypes [30,31]. Interestingly, more physiological levels of expression, generated milder phenotypes that included MN degeneration in the absence of toxic protein aggregates, suggesting that proteinopathy may only be prevalent at later stages of

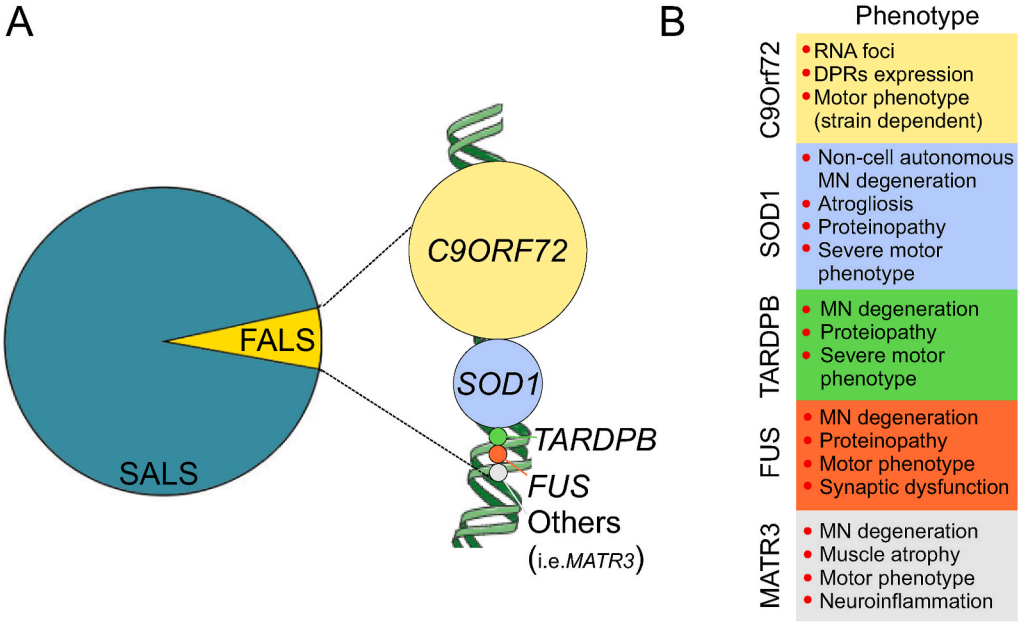

Fig. 1. *Overview of ALS human genetics and mouse models.* A, familiar ALS (FALS) represents the 5–10 % of the total cases, usually with autosomal dominant inheritance. The hexanucleotide expansion of the *C9ORF72* gene is present in approximately 40 % of FALS cases. Mutations in *SOD1*, *TARDBP* and *FUS* represent 20 %, 4 % and 4 % of FALS cases, respectively. Other ALS genes are less common and are implicated in disruption of various cellular pathways. B, the discovery of these genes has allowed the development of enormously useful mouse models in preclinical research. The figure illustrates representative hallmarks of each mouse model.

disease [32]. Similar results are replicated in mouse models carrying knock-in mutations of the murine *Tardbp* gene, which show milder neurodegeneration, no proteinopathy and a mixed ALS/FTD phenotype [33].

To date several groups have generated *FUS* over-expressing mice. Recombinant adeno-associated viruses have been used to express V5-tagged human wild type or mutant FUS^{R521C} or $FUS^{\Delta 14}$ (which lacks the nuclear localization signal) in neurons of the mouse brain. Although these mice do not model ALS, this study showed that the cytoplasmic accumulation of mutant FUS versions was higher than the wild type version. In fact, over-expression of $FUS^{\Delta 14}$, recapitulated many aspects of FUS proteinopathies, including insoluble aggregates of FUS, ubiquitin, p62/SQSTM1, α-internexin, and the poly-adenylate(A)-binding protein 1 (PABP-1). However, TDP-43 did not localize to these inclusions [34]. Regarding *FUS* transgenic mice, it is interesting that whereas the homozygous mice overexpressing the wild type *FUS* under the control of a modified mouse prion promoter exhibit profound neurodegeneration, hemizygous mice are unaffected. Despite that total FUS levels and subcellular location was similar in both genotypes, homozygous mice showed severe motor dysfunction with a 60 % loss of lower MNs. At the cellular level, these mice exhibited FUS-positive, ubiquitin-negative cytoplasmic inclusions in surviving motor neurons of the spinal cord, as well as glial activation [34]. In a recent study, the phenotypes of FUS knock-in mice expressing miss-localized cytoplasmic FUS and complete FUS knockout mice has been compared. Interestingly, FUS knock-in mice exhibited a reduced number of MN at birth, associated with exacerbated MN apoptosis, which was rescued by neuron-specific CRE-mediated expression of wild type FUS. These studies suggest that in addition to FUS nuclear loss of function, cytoplasmic accumulation of FUS leads to MN death through a toxic gain of function [35]. Over-expression of a truncated version of FUS ($FUS^{(1\text{-}359)}$) lacking the nuclear localization signal leads to synaptic and dendritic spine dysfunction in the frontal cortex before neuronal loss, recapitulating some phenotypic features of ALS related to disruption of dendritic homeostasis [36]. The transcriptional profile of $FUS^{(1\text{-}359)}$ transgenic mice was analyzed by RNA-seq. The resulting data show that, prior to clinical symptoms, FUS-mediated miss-localization is asymptomatic until it reaches the terminal stage of disease progression. Massive FUS-positive inclusions were detected in spinal cord after this time, accompanied by a transcriptional shift including acquisition of pro-inflammatory phenotype of microglia and malfunction of cholinergic transmission in MNs [37].

Other murine models replicate *MATR3* mutations. Mice expressing human MATR3 protein in either wild type or mutant $MATR3^{F115C}$ under the control of the mouse prion promoter exhibit robust expression of the transgenic mRNA in muscle but minimal in spinal cord. Whereas the lifespan of mice expressing wild type MATR3 is more than 20 months with no change in motor tests performance, $MATR3^{F115C}$ mice exhibit weak limbs earlier than 1 month of age. Although the muscle fibers of both mice showed an increase in the number of vacuoles in an age-dependent manner, vacuolation was noticeably more severe in $MATR3^{F115C}$ mice [38]. CRISPR/Cas9 technology has allowed the generation of a new $MATR3^{S85C}$ knock-in mouse. Interestingly, these mice recapitulate behavioral and neuropathological features of early-stage ALS such as motor impairment, muscle atrophy, neuromuscular junction defects, Purkinje cell degeneration and neuroinflammation in the cerebellum and spinal cord and emerge as a promising tool for preclinical studies [39].

After the identification of hexanucleotide repeat expansions in *C9ORF72*, a range of BAC transgenic mice have been generated carrying the full-length gene from an ALS patient with up to 500 repeats. Multiple studies show in these mice a molecular phenotype characterized by RNA foci and Dipeptide Repeated Protein (DPR) expression [40–42], but only one model demonstrated a significant motor phenotype [42]. Nevertheless, behavioral findings are under dispute due to variable outcomes between different laboratories [43,44]. Other strategies for replicating DPR toxicity include viral mediated over-expression of DPR producing transgenes [45,46] (Fig. 1B). As we will discuss, the use of these animal models contributed immensely to our understanding of ALS pathology, allowing the identification of therapeutic molecular targets.

4. NRF2 regulation and ARE-gene targets

The transcription factor NRF2 is a basic region-leucine zipper transcription factor that forms heterodimers with small musculoaponeurotic fibrosarcoma proteins (MAFs) in the nucleus. The heterodimer recognizes an enhancer sequence termed Antioxidant Response Element (ARE) that is present in the regulatory regions of approximately 250 genes (ARE-genes). ARE-genes encode a broad network of enzymes involved in phase I, II, and III biotransformation reactions, antioxidant mechanisms encompassing NADPH, glutathione- and thioredoxin-mediated reactions, lipid and iron catabolism, interaction with other transcription factors, and autophagy gene expression. These pleiotropic actions lead to significant neuroprotective effects. Attenuation of the NRF2 response has been observed in ALS [47,48]. In human biosamples, biochemical evidence also indicates that reduced NRF2 activity is a hallmark of ALS [49,50]. Importantly, the observed attenuation of NRF2 responses can be overcome pharmacologically as will be described later.

NRF2 is continuously synthesized and degraded, exhibiting a half-life of just 20–30 min depending on the cell type [51]. This apparently futile cycle allows cells to rapidly respond to insults and, through its transcriptional network, coordinate multifaceted responses for maintaining a stable internal environment. NRF2 stability is controlled at least by three ubiquitin E3 ligase adapters: Kelch-like ECH-associated protein 1 (KEAP1) [52–54], beta-transducin repeat containing protein (β-TrCP) [55,56] and synoviolin E3 ubiquitin ligase (HDR1) [57], which target this protein for proteasomal degradation. The main and pharmacologically most relevant mechanism of regulation of NRF2 stability is KEAP1. KEAP1 is a homodimeric E3 ligase adapter that presents NRF2 to a complex formed by CULLIN3/RBX proteins, leading to its ubiquitination and subsequent proteasomal degradation [58,59]. KEAP1 binds one molecule of NRF2 at two amino acid sequences with low (aspartate, leucine, glycine; DLG) and high (glutamate, threonine, glycine, glutamate; ETGE) affinity [60]. There are two well established mechanisms of KEAP1 regulation. The canonical pathway is based on the fact that KEAP1 is an electrophilic sensor that contains several electrophile- and redox-sensitive cysteine residues. Ectopic or endogenous electrophiles, modify sulfhydryl groups of specific redox-sensitive cysteines of KEAP1, including C151, C273, and C288 [61]. Following these cysteine modifications KEAP1 is no longer capable of presenting NRF2 for ubiquitination [62]. As a result, newly synthetized NRF2 escapes KEAP1-dependent degradation, translocates to the nucleus, and activates ARE genes (Fig. 2). In the non-canonical pathway, KEAP1 is sent to autophagy degradation through interaction with the autophagy carrier protein SQSTM1 [63]. This protein contains an STGE motif that, upon serine phosphorylation by several signaling kinases such as casein kinase 1 (CK1), transforming growth factor beta-activated kinase 1 (TAK1), mTORC1 and PKCσ [64–67] resembles the high affinity ETGE motif of NRF2. Then, by binding to KEAP1, SQSTM1 leads to autophagic degradation of KEAP1, relieving NRF2 from KEAP1-mediated repression. Importantly, mutations affecting *SQSTM1* have been implicated in both ALS and frontotemporal dementia [68] (Fig. 3).

5. Dysregulation of the NRF2 in ALS

Although studies employing NRF2-null mice backcrossed with ALS-mice reported only a modest protection of NRF2 to disease hallmarks, probably due to compensatory mechanisms [69,70], strong evidence indicates that NRF2 activity decreases in ALS models and patients. Primary MN cultures from $SOD1^{G93A}$ transgenic mice showed reduced NRF2 levels compared with those from wild type littermates [71]. Accordingly, motor neuron-like NSC34 cells overexpressing mutant human SOD1 revealed downregulation of the NRF2 transcriptional

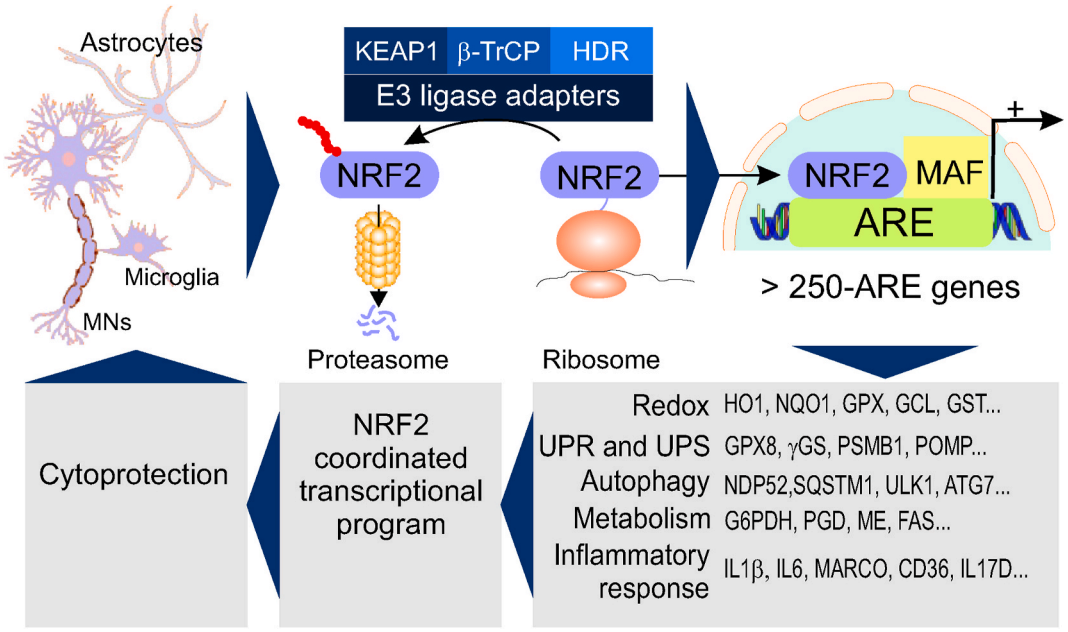

Fig. 2. *Transcriptional program coordinated by NRF2 to underpin a protective response.* Newly synthetized NRF2 in neurons and glial cells is constantly targeted for degradation by the action of the indicated E3-ligase adaptors. Under several stimuli, these E3-ligases could be inhibited and NRF2 escapes degradation. Then, NRF2 enters the nucleus and heterodimerizes with the members of small MAF family through their bZip domain. The heterodimer binds to an enhancer sequence termed Antioxidant Response Element (ARE) that is present in the regulatory regions of over 250 genes (ARE genes). These genes participate in the control of redox, inflammation, metabolism and proteostasis functions, as indicated. Activation of this NRF2-ARE response favors a cytoprotective wave. ATG-7, autophagy related 7; FAS, fatty acid synthase; GCL, glutamyl cysteine ligase; Gpx, glutathione peroxidase; Gpx8, glutathione peroxidase; GST, glutathione S-Transferase; G6PDH, glucose-6-phosphate dehydrogenase; γGS, γ-glutamate cysteine synthetase; HO1, heme oxygenase-1; IL1β, interleukin 1 beta; IL6, interleukin 6; IL17D; interleukin 17D; MARCO, macrophage receptor with collagenous structure; ME, malic enzyme; NQO1, NADPH quinone oxidase 1; NDP52, calcium-binding and coiled-coil domain-containing protein 2; PSMB1, proteasome subunit b type-1; POMP, proteasome maturation protein; PGD, phosphogluconate dehydrogenase; SQSTM1, sequestosome 1/p62; ULK1, unc-51 like autophagy activating kinase 1.

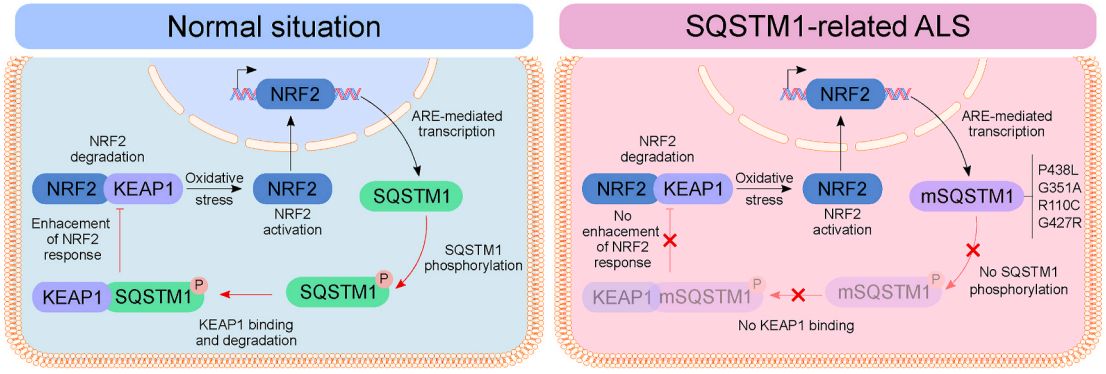

Fig. 3. *ALS-related SQSTM1 mutations impair feedforward loop in the SQSTM1/KEAP1/NRF2 pathway.* In the normal situation (left panel), NRF2 is activated upon oxidative stress and enters the nucleus to drive ARE genes transcription, which gives rise to SQSTM1 synthesis. Phosphorylated SQSTM1 binds KEAP1, targeting it for autophagic degradation and thus enhancing the NRF2 response. In SQSTM1-related ALS (right panel) mutations in SQSTM1 (mSQSTM1) either impede its phosphorylation or binding to KEAP1, hindering its autophagosome degradation, and increasing repression of NRF2 signaling.

signature [47]. A different approach has consisted on developing ALS pathology in mice expressing a reporter cassette of ARE-driven human placental alkaline phosphatase. The authors showed an early and intense NRF2 activation in skeletal muscles, but a lower level of activation at symptoms onset in spinal cord MNs and astrocytes [72]. A MNs transcriptomic study investigating differences between two strains of *SOD1* transgenic mice with fast and slow disease progression showed that the fast-progressing strain (129Sv) had a much weaker NRF2 response compared to the slow-progressing strain (C57bl/6) [73]. Strong evidence for a role of NRF2 in ALS comes from genetic studies performed in 522 sporadic ALS patients compared with 564 asymptomatic controls. One *NFE2L2* haplotype was associated with decreased risk of ALS while one haplotype in *KEAP1* correlated with later ALS onset. Importantly the *NFE2L2* haplotype, which includes three functional promoter SNPs associated with high NRF2 protein expression, correlated with four years later disease onset [50]. Among the new genes associated with ALS, 10 mutations in the *SQSTM1* gene, comprising 9 heterozygous missense mismatches and 1 deletion, have been found in both sporadic and familial forms of ALS. Interestingly, predictive *in silico* analysis classified 8 of the 9 missense variants as pathogenic. This finding links NRF2/KEAP1/autophagy with ALS pathology, suggesting that NRF2-mediated regulation of protein degradation might represent an important therapeutic target to combat ALS [74]. NRF2 and KEAP1 expression levels were analyzed in *postmortem* samples of primary motor

cortex and spinal cord [75]. Compared to controls, NRF2 mRNA and protein levels were reduced in spinal cord, whereas KEAP1 mRNA levels were increased in the motor cortex. Because the *postmortem* studies were performed at the terminal stage of ALS, NRF2 reduction does not exclude the possibility that the NRF2 axis could be activated at onset or at early ALS stages. Nevertheless, these results suggest that alterations in this signaling pathway occur in MNs of ALS patients and that they may contribute to chronic MN degeneration. The NRF2 target gene encoding Peroxiredoxin 3 (*PRX3*), is also downregulated in spinal MNs from sporadic and SOD1-related MN degeneration, suggesting the disruption of mitochondrial antioxidant defense as an alternative mechanism to generate oxidative stress within these cells [48]. In fact, evaluation of temporal and spatial changes of KEAP1, NRF2 and its downstream response proteins Heme oxygenase-1, Thioredoxin, and Heat shock protein 70 (HO-1, TRX, and HSP70, respectively) throughout the MN degeneration in the spinal cord of ALS model mice revealed that, while KEAP1 protein levels progressively decreased from early to late symptomatic stages, in parallel NRF2 dramatically increased in the anterior lumbar cord with accumulation in the MN nucleus and in glial cells. In contrast, downstream stress response proteins showed only a small increase in MNs that correlated with increased number of glial cells after the symptomatic stage [76]. Altogether, these studies support the hypothesis that reinforcement of NRF2 activity could represent a promising therapeutic strategy for neuroprotection in ALS.

It is not clear yet how the NRF2 signature is altered in ALS and this fact might be related to RNA handling. In Neuro2A, SH-SY5Y and HEK293 cell lines as well as in ALS spinal cord samples, the RNA binding protein RBM45 is redistributed from the nucleus to the cytosol after oxidative insult, accompanied by KEAP1 binding. The functional consequences of this interaction were analyzed in HEK293 cells showing that RBM45 stabilized KEAP1 with consequent dampening of the transcription of an ARE-reporter [77]. Moreover, RNA binding immunoprecipitation studies in patient fibroblasts revealed robust enrichment of NFE2L2 and GPX1 (glutathione peroxidase 1) transcripts bound to hnRNP K protein in TDP-43 mutant cells. Despite the elevated expression of several antioxidant transcripts, glutathione levels were reduced in TDP-43^{M337V} patient fibroblasts and astrocyte cultures from TDP-43^{Q331K} mice compared with control cells, indicating elevated oxidative stress and failure of some upregulated antioxidant genes to be properly translated into protein [78].

6. Molecular hallmarks of ALS associated with insufficient NRF2 activation

The variety of biological pathways affected by multiple FALS-related genes reveals the complex etiology of this disease [10,79]. Importantly, the genetic heterogeneity of ALS inferred from genomics is also mirrored by transcriptomics studies. To determine whether NRF2 could be crucial in combating ALS molecular hallmarks, we screened microarray and RNA-seq public datasets generated from spinal cord, brain cortex and MNs differentiated from induced pluripotent stem cells. In total, we downloaded 9 lists of differentially expressed genes in neural tissue or cells implicated in ALS alterations (Table 1) [80–87]. As shown in Fig. 4, analysis of the dysregulated genes by Gene Ontology Biological Process enrichment depicted several altered pathways including cell death/-cycle, signaling, immune response, subcellular localization, neurodevelopment and migration, adhesion, proteostasis, response to oxygen levels alterations, vascular homeostasis, and RNA metabolism. This analysis provides a starting point to discuss the role of these pathways in the pathogenesis of ALS and the possible contribution of dysregulated NRF2 to some of them. Although excitotoxicity was not detected at the transcriptomic level, we will address it because of the involvement of NRF2.

Table 1
Sources of datasets used for network analysis of transcriptomic pathways.

Reference[a]	ALS type	Sample type	Transcriptome technology[b]	DEGs criterio[c]	# of DEGs
Alves et al., 2015 [80]	SALS	MN from hiPSCs	Microarray (8 × 60 K)	p-value < 0.05.	1592
Aronica et al. 2015-I [81]	SALS	Cerebral cortex	Microarray (4 × 44K)	FDR threshold of 0.05	3610
Aronica et al. 2015-II [81]	SALS	Cerebral cortex	Microarray (4 × 44K)	FDR threshold of 0.05	11350
Brohawn et al., 2016 [82]	SALS	LCM neurons from spinal cord	RNA-seq	Adjusted p-value < 0.05	84
Dols-Icardo et al., 2020 [83]	SALS	Motor cortex	RNA-seq	Adjusted p-value < 0.05	124
Harjuhaahto et al., 2020 [84]	FALS	MN from hiPSCs	RNA-seq	Adjusted p-value < 0.05	2968
Krach et al., 2018 [85]	SALS	LCM neurons from spinal cord	RNA-seq	Adjusted p-value < 0.05	1040
Lederer et al., 2007 [86]	SALS	Motor cortex	Microarray	Adjusted p-value < 0.05	59
Wong et al., 2019 [87]	FALS	MN from hiPSCs	RNA-seq	FDR threshold of 0.1	415

[a] The study by Aronica et al. depicted two different subsets of sALS patients based in their different transcriptional signatures. In this analysis we used both subsets as separated entities two construct gene ontology network.
[b] Microarray studies were conducted in the different versions of Whole Human Genome Microarray (Agilent). The version of the microarray is indicated in parentheses.
[c] Statistical stringencies of the original studies were used to filter DEGs genes.

6.1. Redox control

Dysregulation of the antioxidant defenses and increased production of reactive oxygen species (ROS) contribute to progression of neurodegenerative disease. The analysis of cerebrospinal fluid (CSF), blood and tissue samples from ALS patients shows evidence of oxidative stress and provide reliable biomarkers. Most studies have focused on the identification of oxidized macromolecules or antioxidant capacity. Thus, the lipid peroxide 4-hydroxynonenal (HNE) [88], was significantly increased in the CSF of SALS patients compared to patients with other central nervous system diseases [89,90], although this finding was not reproduced in another study [91]. Lipid peroxidation levels were also increased in plasma and serum [92] but were not modified by riluzole, further highlighting the need for combined therapies. In addition, lumbar spinal cord from ALS patients exhibited increased HNE-peroxidated products measured by immunohistochemistry [93,94], as well as malondialdehyde [95,96]. The increase in lipid peroxidated products is also found at late stages in the spinal cord of the SOD1^{G93A} mice, indicating that oxidative stress is a common mechanism for MN degeneration in animal models and patients [97,98].

The levels of 3-nitrotyrosine (3NT), a post-translational protein marker of nitrosative stress, are extensively altered in ALS [99,100]. 3NT is increased in the CSF [101] and in the spinal cord of ALS patients [102], and in one study this was accompanied by a concomitant increase in the expression of the inducible isoform of nitric oxide synthase (iNOS) [103]. Increased nitrosative stress could lead to further oxidative stress in a positive feedforward loop, because nitrosylated SOD2 was significantly enriched in CSF [104] and it is known that nitrosylated forms of SOD2 are inactive [105].

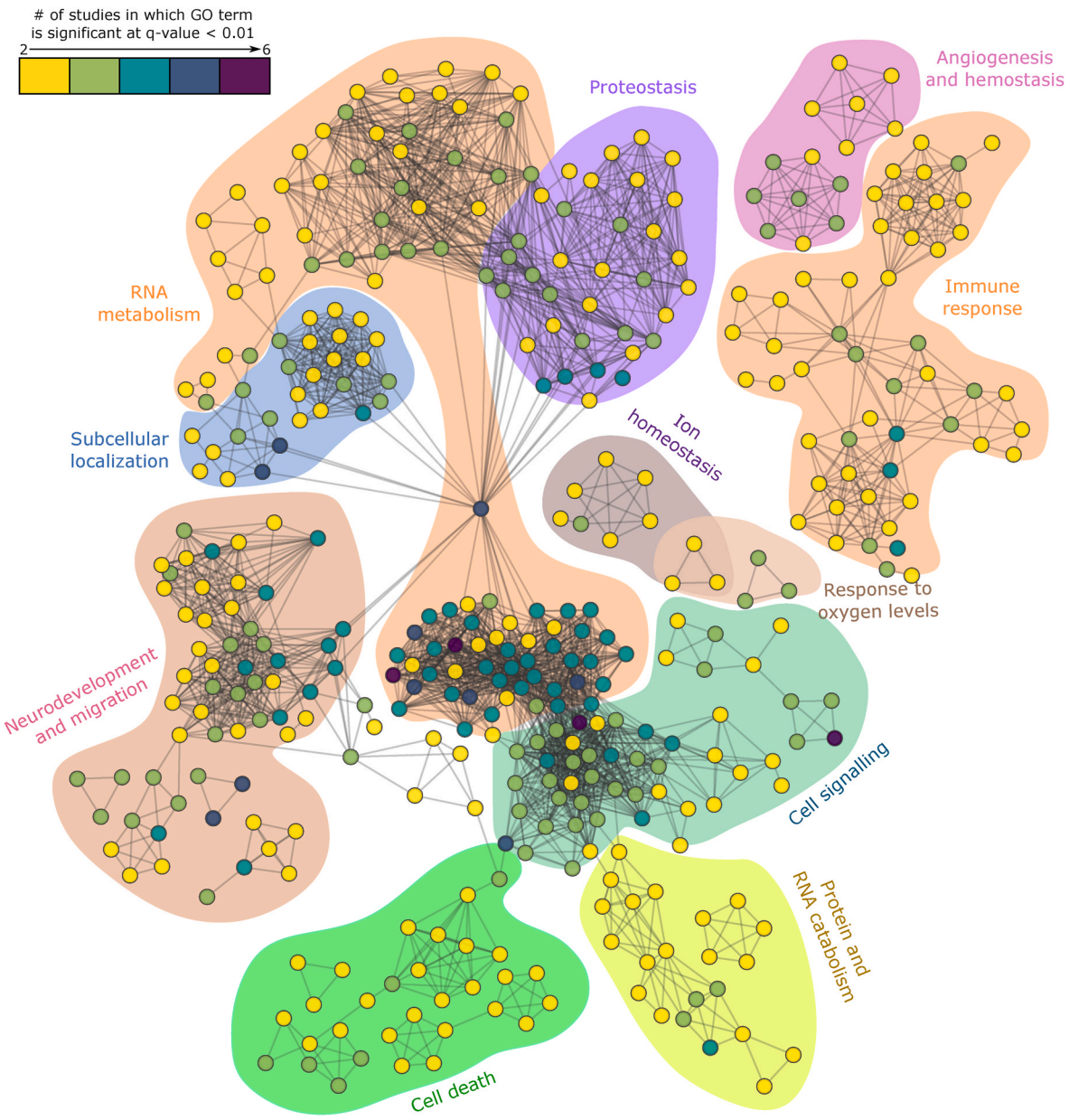

Fig. 4. *Network analysis of dysregulated pathways in ALS.* Lists of dysregulated genes from several ALS transcriptomic studies were subjected to overrepresentation analysis of Gene Ontology (GO) Biological Process Slim (April 2021 release) in Cytoscape using BinGO plugin [262]. Then, EnrichmentMap plugin [263] was used to produce a network of overrepresented terms across all studies. A q-value (FDR-adjusted p-value) < 0.01 was used as an statistically significance threshold for term enrichment. Only terms overrepresented in at least two lists of dysregulated genes were used to construct the GO term network. The color of each node represents the number of dysregulated-gene lists in which the gene ontology term is enriched. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

HNE activates NRF2 in HeLa cells [106] and in rat L2 provokes the NRF2-dependent activation of glutathione synthesis [107]. More in the context of ALS, NRF2 protects from the nitrosative stress produced by spinal cord astrocytes of $SOD1^{G93A}$ mice *in vitro* by inducing glutathione biosynthesis [108]. By contrast, reduction of the glutathione pool in the $SOD1^{G93A}$ mice and in knock-out mice for *Gclm (Glutamate-Cysteine Ligase Modifier Subunit)* worsens the course of the disease. Activation of glutathione synthesis and use with NRF2 activators could help indirectly against other ALS pathological hallmarks that are in part caused by oxidative stress. For example, HNE produces the partial cytoplasmic localization of TDP-43 *in vitro*, resembling what happens in ALS-related MN injury [109] but treatment of $TDP43^{A315T}$-expressing NSC34 cells with glutathione monomethyl ester decreases in TDP-43 nuclear clearance and induces cytoplasmic accumulation [110].

6.2. Resolution of inflammation

Changes in the immune response associated with ALS are not simply a consequence of MN degeneration, but actively contribute to disease progression. In the early stages of disease, resident glial cells and infiltrating T-lymphocytes may display protective programs aimed at mounting a neuroprotective response, while at later disease stages, persistent inflammation contributes to damaging MNs, and accelerates disease progression [111]. Numerous preclinical studies have reported that activation of NRF2 leads to potent anti-inflammatory effects in myeloid leukocytes [112], macrophages [113] and glial cells [114,115]. In observational studies, a polymorphism in *NFE2L2* was associated with reduced transcriptional activity, correlating with increased risk of inflammatory disease [116].

Nuclear factor-kappa B (NF-κB), a master regulator of inflammation, is upregulated in the spinal cord of ALS patients [117] and SOD1^{G93A} mice [118]. NRF2 and NF-kB crosstalk through feedforward and feedback mechanisms. Both transcription factors compete for the same coactivator, CBP/p300, shifting the transcriptional activity in favor of the most abundant factor within the nucleus. Additionally, NF-kB may promote interaction of histone deacetylase-3 with small MAF proteins, therefore preventing their dimerization with NRF2 [119]. NF-kB binds and translocates KEAP1 to the nucleus, thus favoring NRF2 ubiquitination and degradation in this cellular compartment [120]. On the other hand, *NFE2L2* transcription is activated by NF-kB, thus inducing a negative feedback loop [121]. The E3 ligase adapter β-TrCP tags both IkBα [122] and NRF2 [55,56] for proteasomal degradation, and this can lead to increased NF-kB activity. Various pro-inflammatory signals activate the Rho GTPase RAC1, which leads to NF-kB and NRF2 activation. Then, NRF2 inhibits RAC1-mediated activation of NF-kB [123]. NF-κB inhibition in microglia could be considered a molecular mechanism to delay MN injury. Thus, selective NF-κB inhibition in microglia rescued MNs from microglial-mediated death *in vitro* and extended survival in SOD1 mice. In contrast, constitutive activation of NF-κB selectively in myeloid cells of wild type mice produces some of the pathological features of ALS in the spinal cord including gliosis and MN death [124].

Secreted mSOD1 and mTDP-43 proteins, and lysophosphatidylcholine, a lipid related to neurodegeneration, may act as a damage-associated molecular pattern (DAMP), triggering inflammasome activation [125–127]. The NLRP3 inflammasome is a multiprotein complex that activates caspase-1 and promotes the processing of the pro-inflammatory cytokine IL-1β. A growing body of evidence supports the concept that NRF2 activation attenuates NLRP3 inflammasome activation in monocytes, macrophages, and microglia [128,129]. Accordingly, NRF2-null mice exhibit enhanced brain NLRP3 inflammasome activation and neuroinflammation [130]. Cooperating with NLRP3 inhibition, NRF2 can also inhibit the recruitment of RNA pol II to the promoter region of the pro-inflammatory IL6 and IL1β genes, repressing their transcription upon LPS stimulation [131].

The role of TGF-β1 in ALS has been extensively studied. Serum and CSF of ALS patients show elevated TGF-β1 levels that correlate with a more aggressive disease course [132]. Astrocytes from ALS patients and SOD1^{G93A} mice also express high TGF-β1 levels. On the contrary, ablation of SOD1^{G93A} expression in astrocytes decreased TGF-β1 levels and slowed disease progression [133]. Although in the central nervous system the relationship between NRF2 with TGF-β1 is still unknown, in liver NRF2 suppresses the profibrotic TGF-β1/Smad3 pathway [134]. Considering that ALS progression is slowed by pharmacological inhibition of TGF-β1, increasing the survival of SOD1^{G93A} mice [133], NRF2 activation might provide a strategy to attenuate the over-production of TGF-β1.

Upon stimulation microglia acquire a dynamic state ranging from pro-inflammatory (M1-classic) to the wound healing/neuroprotective (M2-alternative) phenotypes, depending on the disease stage and surrounding environmental triggers. Microglial activation can be detected by position emission tomography imaging in ALS patients using a specific radioligand for the peripheral benzodiazepine binding site, [^{11}C](R)-PK11195. This signal is significantly increased in motor cortex, pons, and thalamus in ALS patients, with significant correlation between binding in the motor cortex and the burden of upper MN clinical features [135]. The M1/M2 balance is altered in microglia derived from SOD1^{G93A} mice. At early disease stages, microglial cells exhibit high M2-markers (*Ym1*, *CD163* and *Bdnf)* and low M1-inflammatory markers (Nox2) compared to end-stage disease [136]. NRF2 also induces an anti-inflammatory phenotype modulating the M1/M2 phenotypes [137,138]. The modulation of the glutathione pool exerted by NRF2 is instrumental in determining the microglial phenotype. NRF2 activation reinforces cysteine and glutathione levels through upregulation of the cystine/glutamate transporter and the glutamate-cysteine ligase catalytic and modulatory subunits (GCLC and GCLM, respectively) leading to acquisition of the anti-inflammatory M2-phenotype. On the contrary, glutathione depletion sensitizes macrophages to LPS-induced inflammation [139].

In summary, these studies indicate that NRF2 is a plausible candidate to modulate the microglial fate in ALS. The benefits of the M2-phenotype have been recently described employing induced pluripotent stem cells from ALS patients. M2-induced cells suppressed pro-inflammatory mediators (IL-6 and TNF-α) released by M1-like cells and reduced the proliferation of effector T cells. Moreover, M2-derived cells transformed effector T cells into regulatory T cells promoting beneficial immunomodulatory effects [140]. Overall, these studies point towards a key role of NRF2 as a modulator of the inflammatory response and highlight an important aspect of the therapeutic potential of NRF2 activation in ALS.

6.3. Protein quality control

The KEAP1/NRF2 pathway has been considered for many years a defense mechanism against oxidative stress, but recent evidence has put this pathway at the center of an overall cellular stress-response, which is tightly linked with regulation of proteostasis. Indeed, dysregulation of protein folding, and degradation is common to several neurodegenerative diseases, which display characteristic protein aggregates such as amyloid-β and Tau (AD), α-synuclein (PD) or TDP-43 (FTD/ALS) among others. NRF2 and proteostasis intersect through direct interaction between SQSTM1 and KEAP1. As highlighted above, in the non-canonical pathway, KEAP1 is tagged by SQSTM1 and redirected for autophagic degradation, thus leaving NRF2 free to accumulate and translocate to the nucleus [63]. The link between NRF2 and proteostasis, however, is twofold, as NRF2 also transcriptionally regulates several genes involved in the un-folded protein response (UPR), the ubiquitin proteasome system (UPS) and autophagy [141].

Since mutations in SQSTM1 are associated with ALS, it is likely that also the NRF2 pathway is affected due to impairment of the SQSTM1-KEAP1 interaction [142], leading to downregulation of the antioxidant response. Interestingly, disruption of the SQSTM1/KEAP1 interaction is a common hallmark in SQSTM1-related ALS. Thus, P348L and G351A mutations in SQSTM1, located in the KEAP1-interaction domain, produce reduced binding to KEAP1 and thus a decrease in the activation of NRF2 as measured in ARE-luciferase reporter assays [143]. Similarly, the R110C mutation produces a decrease in the phosphorylation of Ser 403 and Ser 349 [144], which are needed for the SQSTM1/KEAP1 interaction [67], also resulting in impaired NRF2 activity. Neurons with mutated SQSTM1, exhibit an increase in stress granules, cytoplasmic TDP-43 aggregates, and abnormal dendrite morphology [142]. Consistently, NRF2 downregulation exacerbates the impairment in protein degradation, resulting in misfolded protein accumulation and proteotoxicity.

A further link between TDP-43 proteinopathy and dysregulation of the NRF2 pathway was highlighted in NSC34 cells expression mutant TDP-43^{M337V}. Interestingly, these cells showed increased nuclear accumulation of NRF2 and downregulation of its target genes [145]. These opposing effects were caused by reduced expression of the NRF2 *trans*-activators MAFK and Jun dimerization protein (JDP2). Similarly, mutant TDP-43 interacts with hnRNPK, which, in turns, binds NRF2-regulated transcripts, blocking their translation into proteins and leading to downregulation of the antioxidant repose [78].

Interestingly, *C9ORF72*-linked ALS mutations, as well as other variants of ALS, display autophagy impairment and SQSTM1 accumulation. SQSTM1 cytosolic accumulation might lead to constitutive activation of NRF2 via the non-canonical pathway and, consequently, of its downstream gene targets. Studies in *Atg7*-deficient mice, displaying severe autophagy defects, not only feature SQSTM1 accumulation and constitutive NRF2 activation, but also increased levels of polyubiquitylated protein aggregates and inclusion bodies [146]. Surprisingly, these can be completely suppressed by either SQSTM1 or NRF2 downregulation.

This effect has been associated with the ability of NRF2 to transcriptionally regulate genes involved in protein ubiquitination [147] as a stress-response mechanism. These studies indicate that NRF2 activation in the treatment of ALS needs to be aimed at restoring homeostatic balance, as NRF2 deficiency, as well as hyperactivation, can lead to significant proteostasis defects.

6.4. RNA processing

The identification of mutations in RNA binding proteins such as TDP-43 and FUS, involved directly in RNA metabolism, and in C9orf72 which leads to both sequestration of RNA binding proteins and disruption of nucleocytoplasmic transport, suggest a key pathogenic mechanism in ALS [148]. Appropriate processing and control of RNA splicing, transport and stability is essential for neuronal health. As discussed previously, some of these mechanisms mediate the transcriptional dysregulation of NRF2 target genes [47,78]. This finding suggests a straightforward strategy for overcoming this dysregulation by enhancing NRF2 activation [48].

Various RNA binding proteins important in RNA metabolism and processing undergo changes in subcellular localization during stress. This is the case for sodium arsenite-induced translocation of TDP-43 from the nucleus into stress granules as a result of oxidative stress [149]. These stress granules form as a result of liquid-liquid phase separation, and long-term arsenite treatment leads to formation of a separated phase of TDP-43 droplets that lose their liquid-like state and transit to gels or solids likely enabling TDP-43 aggregation [150].

Considering that oxidative stress deeply impacts in microRNAs (miRs) dynamics, it could be expected that miRs modulate in part the NRF2-driven cytoprotective program. In fact, it is a two-direction way in which miRs directly or indirectly target NRF2 but also NRF2 modulate miRs biogenesis. Proper stress granule formation and complete maturation of miRs require the coordinated action of a multiprotein complex which includes the ribonuclease type III, DICER. Conditional inactivation of DICER in MNs is enough to cause spinal neurodegeneration *in vivo* [151]. Accordingly, laser capture microdissection demonstrates that total miRs levels are reduced in MNs isolated from the lumbar spinal cord SALS patients [152]. Very relevant, the 5′-flaking region of human and mouse *DICER* gene contains an ARE site [153]. Since enhancing DICER activity is beneficial for MN function in two independent ALS mouse models [152], we speculate that activation of NRF2 could ameliorate DICER reduction improving MN function.

NRF2 modulates the levels of miR-106b-25 cluster through a harmonized action with ATF4 in response to UPR, repression of the miR-106b-25 cluster increases the levels of the apoptotic protein BIM. Some evidence supports the relevance of this axis in ALS pathology. First, UPR response has been strongly associated with MN degeneration [154]. Second, the downregulation of miR-106b-25 cluster has been reported in the symptomatic stage of $SOD1^{G86R}$ mice together with BIM upregulation [155], while BIM ablation reduced cellular apoptosis in the ventral horn of a transgenic mouse model of FALS, increasing their lifespan [156]. Finally, ALS patients show a misbalance between ATF4/NRF2 expression since they exhibited upregulation of ATF4 [157] and downregulation of NRF2 as discussed above. Altogether, these observations provide a rational for NRF2 activation to reinforce crucial players of RNA metabolism.

6.5. Intracellular transport

Intracellular trafficking of vesicles and organelles is essential to maintain key cellular functions, such as metabolic homeostasis, protein synthesis and recycling, and membrane maintenance [158]. This cellular process is particularly important in neurons, which are highly specialized and compartmentalized cells. Neurons rely on axonal transport to shuttle mitochondria along the axon to provide the energy necessary for maintenance of membrane potentials, signal transduction and to transport vesicles and neurotransmitters [159]. This function is so essential for neurons that perturbation of this system is at the center of several neurodegenerative conditions [159].

Very relevant here, intracellular transport is highly sensitive to oxidative stress, in fact, hydrogen peroxide treatment of neurons leads to inhibition of axonal transport before any sign of neurodegeneration is observed [160]. Strikingly, studies in *C. elegans* have highlighted a strong link between NRF2 activation and synaptic activity [161]. Consistently, several findings have demonstrated that oxidative stress causes microtubule depolymerization, likely due to oxidation of cysteine residues on both α- and β-tubulin [162]. Particularly important in the context of ALS, glutathionylation of tubulin in MNs occurs during oxidative events, thus altering microtubule dynamics and structure [163]. Indeed, dysregulation of intracellular trafficking is a consistent characteristic of ALS, observed in transgenic SOD1 [164], TDP-43 [165] and C9orf72 mouse models [166], which all share dysregulation of the antioxidant response and NRF2 transcription [167,168]. Besides damaging microtubules and, thus, inhibiting axonal transport, oxidative stress has a direct impact on intracellular cargo trafficking, i.e. organelles and vesicles shuttled within the cell. Compelling evidence indicates that oxidative stress causes mitochondrial damage [169], as well as ER stress and impairment of vesicle formation and trafficking [96]. In this context, activation of the antioxidant response through the NRF2 pathway is likely to restore several mechanisms that impair intracellular trafficking. Indeed, activation of NRF2 has beneficial effects in a preclinical model of tauopathy [170,171] and counteracts the adverse effects of hydrogen peroxide on axonal transport [160].

6.6. Angiogenesis and vascular homeostasis

At least two angiogenic molecules, angiogenin (ANG) and vascular-endothelial growth factor (VEGF), are altered in ALS. First, several ANG mutations are causative of sporadic and familial cases of ALS [172]. Second, patients bearing a specific haplotype in the promoter region of the *VEGF* gene are at increased risk for developing ALS [173] at least in male patients [174]. In the same way, VEGF-deficiency in $SOD1^{G93A}$ mice worsens MN degeneration [175]. Together, the contribution of these two genes to the pathophysiology of ALS underline the importance of angiogenesis in ALS.

Both *VEGF* and *ANG* are hypoxia-inducible genes [176]. The master regulator of hypoxic response is Hypoxia inducible factor 1α (HIF1α), a transcription factor that undergoes stabilization in the absence of oxygen, being key in the development of new blood vessels [177]. Interestingly, both ALS patients and mouse models show HIF1α accumulation in spinal cord [178], suggesting an impairment in hypoxia-resolving abilities. This is supported by the low levels of ANG and VEGF proteins in CSF from hypoxemic ALS patients compared to ALS controls [179], and by impairment in HIF1α transcription monocytes from ALS patients [180]. Indeed, recent *in vivo* studies using hypoxia-sensitive probes for positron emission tomography revealed that the spinal cord of ALS patients is under hypoxic-stress, maybe resulting impaired formation of new blood vessels [181].

Although NRF2 also participates in angiogenesis, the direct crosstalk with HIF1α is complex [182]. For instance, fumaric acid esters, acting as activators of both NRF2 and HIF1α, promote the release of VEGF in astrocytes from wild type but not $SOD1^{G93A}$ mice, suggesting a limited response for the NRF2/HIF1α axis in ALS [183]. Much more established is the role of the NRF2 target *HMOX1*, encoding Heme oxygenase-1 (HO-1). HO-1 catalyses the biotransformation of the heme group, with the aid of NADPH and O_2, into bilirubin, carbon monoxide (CO) and free Fe^{2+} [184]. Interestingly, CO augments VEGF expression in vascular smooth muscle cells and endothelial cells [185,186], leading to angiogenesis. HO-1 also functions downstream of VEGF signaling as it is crucial for formation of new vessels upon VEGF stimulation [187]. Similarly proper HO-1 catalytic activity is needed for HIF1α stabilization, since *Hmox1* knockout mice exhibit a decreased revascularization

after limb hypoxia [188].

Regression of the vascular network in spinal cord is a common hallmark of $SOD1^{G93A}$ and $FUS^{(1-359)}$ mice and precedes MN degeneration [189,190]. Nevertheless, the vascular disturbances in these models, although fundamentally similar, display a different responsiveness to ANG treatment. On one hand, $SOD1^{G93A}$ benefit from exposure to ANG, exhibiting improved motor performance, survival and reduced vascular regression [191]. On the other, $FUS^{(1-359)}$ mice do not respond to ANG treatment [191]. This lack of responsiveness is probably due to the low expression of miR-126 in $FUS^{(1-359)}$ mice, which is abundant in endothelial cells and is involved in angiogenesis [192] due sensitization to VEGF signaling. Hence, low miR-126 levels in $FUS^{(1-359)}$ mice would result in decreased angiogenic capacity and would hamper the therapeutic potential of ANG treatment. NRF2 activation with oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine upregulates miR-126 expression in HUVEC cells [193]. NRF2 activation renders endothelial cells more responsive to angiogenic stimuli through miR-126 upregulation, acting as an essential partner in a possible combination therapy. Importantly, miR-126 downregulation is partially responsible for other ALS hallmarks such as muscle denervation and axonal degeneration in the SOD^{G93A} mice, making NRF2-mediated activation of miR-126 a multi-hit strategy [194]. Moreover, miR-126 upregulates NRF2 in HUVEC cells [195], providing a positive feedback loop probably through upregulation of PI3K signaling [192].

6.7. Excitotoxicity

Excitotoxicity is defined as the damage provoked in neurons due to an excessive increase in their excitatory inputs or a lack of the homeostatic mechanisms needed to regulate this activity [196]. The mechanisms involved in the death of neurons by excitotoxicity include a massive influx of Ca^{2+} ions that cannot be readily cleared. These ions accumulate in mitochondria and lead to energy failure and ROS overproduction [197]. Initial studies revealed an imbalance in the levels, metabolism and transport of glutamate, the main excitatory neurotransmitter in the CNS, suggesting a possible pathogenic role [198,199]. Furthermore, evidence from *in vitro* cultures suggested that cerebrospinal fluid from ALS patients was toxic to cultured rat neurons in an AMPA glutamate receptor-dependent manner [200]. This imbalance in the glutamatergic system supported the therapeutic use of the anti-glutamatergic agent riluzole, with modestly improves the survival of ALS patients [201].

NRF2 has been implicated in both basal susceptibility and inducible protection against excitotoxic damage. On one hand, hippocampal slices from NRF2-null mice displayed an increase in cell death upon exposure to kainate accompanied by an increase in oxidative stress [202]. On the other, activation of NRF2 by several compounds has provided protection against glutamate-mediated excitotoxicity, in *in vitro* cell cultures [203,204] and rat spinal cord explants [205]. Moreover, NRF2 activation also protected against the excitotoxic damage mediated by quinolinic acid, agonist of glutamate NMDA receptors [206], in rat cortical slices [207] or striatum [208].

Although some studies have pointed out the possible role of increased glutamate levels in ALS (reviewed in Ref. [209]), the major mechanisms driving excitotoxicity in this condition seems to be related to dysregulation of glutamate clearance attributed to a dysfunction or reduction in the levels of the main high affinity glutamate transporter, EAAT2 [209]. However, $SOD1^{G93A}$ causes a marked decrease in EAAT2 levels at early disease stages in rats [210], but only at later stages of disease in mice [211–213]. In the first case, EAAT2 loss could operate to neurodegeneration, while in second case it may occur as a secondary result of earlier pathogenic events. In ALS patients, initial studies reported a specific selective decrease in glutamate transport in synaptosomes derived from motor and somatosensory cortex, and spinal cord, consistent with reduced EAAT2 levels at the same locations [214–216].

Oxidative stress impairs glutamate clearance in ALS in rat cortical astrocytes and mouse spinal cultures [217]. Mechanistically, EAAT2-mediated glutamate transport decreases in an oxidative environment [218]. Interestingly, EAAT2 is modified by HNE in the lumbar spinal cord of ALS patients [218], suggesting feedforward loop between excitotoxicity and oxidative stress. NRF2 activation might break this injurious cycle, by reducing ROS levels and thus rendering EAAT2 more efficient at clearing extracellular glutamate.

Several studies have highlighted the potential benefits of joined targeting of EAAT2 and NRF2 in ALS. One study using organotypic cultures of rat spinal cord revealed that combined treatment with riluzole, which enhances EAAT2 transport [219] and the NRF2 activator sulforaphane provided superior protection against excitotoxicity compared to riluzole or sulforaphane. Similarly, combined lentiviral delivery of *GDH2*, *EAAT2* and *NFE2L2* genes in $SOD1^{G93A}$ mice delayed disease onset and improved survival better than each of these genes separately [220].

Extracellular glutamate and redox balance crosstalk at the level of the cysteine/glutamate antiporter SLC7A11, whose expression is upregulated by NRF2. In astrocytes, this transporter imports cystine, the oxidized form of cysteine, in concert with the export of glutamate to the extracellular space [221]. Thus, an increase in the levels of EAAT2 could support cystine import that, upon reduction to two cysteines, will be used for glutathione synthesis [222]. Unfortunately, ceftriaxone, an antibiotic that induces both EAAT2 and NRF2 simultaneously, did not show efficacy in an ALS clinical trial [223].

NRF2 also participates in calcium homeostasis. Primary neuronal cultures from NRF2-null mice display decreased levels of calcium buffering proteins, such as CALBINDIN D28K [224], and are prone to raise intracellular calcium levels. This finding correlates with the reduced level of CALBINDIN D28K described in MNs of ALS patients and suggests that NRF2 activation could reinforce calcium buffer capacity of MNs and, therefore, prevent the deleterious activation of some calcium-activated enzymes like calpain protease [225]. NRF2 activation by dimethyl fumarate has demonstrated calcium homeostatic abilities in human astrocytes harbouring SOD1 mutation through a mechanism involving its downstream target Glutathione peroxidase 8 (GPX8) [226]. GPX8 is enriched in mitochondria-associated membranes at the endoplasmic reticulum and activates the sarco-endoplasmic reticulum ATPase 2b (SERCA2b). Mechanistically, GPX8 expression reduced the level of calcium in endoplasmic reticulum and in resting cytosolic calcium levels [226,227].

7. NRF2 as a druggable target to combat ALS hallmarks

The incomplete understanding of pathophysiological mechanisms in ALS, limitations of disease models, the genetic and phenotypic heterogeneity of the disease and the limitations in clinical trial design have all undoubtedly contributed to the lack of predictive early biomarkers and adequate neuroprotective treatments for ALS [228]. Since 1996, riluzole is the only marketed therapy for ALS patients. Riluzole delays the onset of ventilator-dependence or tracheostomy in some patients and increases survival by only approximately 3 months [229]. As discussed above, many pathomechanisms contribute to MN injury [230] and riluzole, as an anti-glutamate agent, only ameliorates one of these mechanisms, i.e. excitotoxicity, and it is therefore not surprising that the effect of this drug is modest. This emphasizes the need for a crucial reassessment of methods used in ALS drug development at the level of molecular target identification and clinical trial design and implementation.

Therapies targeting individual pathways have universally failed to achieve positive effects in ALS clinical trials for more than 50 years. In fact, the promising results obtained in preclinical models submitted to antioxidant therapy have been poorly translated into clinical advances for ALS patients. Placebo-controlled randomized clinical trials aimed to address efficacy of vitamin E, α-tocopherol, β-carotene, lutein and lycopeno did not appear to affect survival and motor function in ALS

[231]. All these studies retrieved contradictory, inconclusive or scarcely statistically significant results. The principal disadvantages seem to be related with the higher antioxidant needed dosages and poor bioavailability. In this review, we discuss an exciting and innovative class of drugs that activate the transcription factor NRF2 and might provide protection against many if not all hallmarks of ALS pathology (Fig. 5A).

7.1. Therapeutic effects of NRF2 activation in preclinical models of ALS

Genetic activation of NRF2 in astrocytes results in beneficial neuroprotective effects. The toxicity of astrocytes expressing ALS-linked mutant SOD1 to co-cultured MNs was reversed by NRF2 overexpression. NRF2 overexpression in astrocytes significantly delayed onset and extended survival in two ALS-mouse models [72,232].

The physiological activation of NRF2 by endogenous antioxidant molecules has been analyzed in ALS models. Nitro-fatty acids (NO2-FA) are electrophilic signaling mediators released during inflammation, which can activate NRF2-responsive genes in astrocytes and prevent the death of MNs in cell culture [233]. ANG is a secretory vertebrate RNase that enters neuronal cells and cleaves a subset of tRNAs, leading to the inhibition of translation initiation and the assembly of stress granules. Mutations in the *ANG* gene are a cause of ALS [234] and administration of human ANG to ALS mouse models extends lifespan and improves motor function [235]. Of note, ANG activates NRF2-target genes in murine astrocytes, including *Nqo1*, *Gclm*, and *Gstα4*, resulting in protection against oxidative stress [236]. Urate (2,6,8-trioxy-purine), the end product of dietary purines, has been proposed as prognostic factor for survival in ALS since its levels are reduced in the serum of ALS patients [237]. Interestingly, urate is an important endogenous antioxidant in humans [238] that activates NRF2 in an AKT/GSK3β-dependent manner [239]. Thus, in mutant SOD1-overexpressed NSC34 motor neuron cells and in *Drosophila* models of ALS, urate exerted neuroprotection against oxidative damage by increasing GSH production through the activation of the NRF2/GCLC axis [239].

NRF2 activation has been driven by small molecule electrophiles that react with KEAP1 and previous studies supported the use of two synthetic triterpenoid compounds (CDDO and CDDO-EA). With treatment at the pre-symptomatic stage, both compounds significantly attenuated weight loss, enhanced motor performance, and extended the survival of the $SOD1^{G93A}$ mice. When administered at the symptomatic stage, NRF2 activation was neuroprotective and slowed disease progression [240]. The small molecule S [+]-apomorphine, a receptor-inactive enantiomer of the clinically approved dopamine-receptor agonist (R [-]-apomorphine), was identified as an NRF2 activator emerging from library screening. Notably, mice treated with S [+]-apomorphine showed induction of NRF2 controlled genes in the brain, and significant attenuation of motor dysfunction in $SOD1^{G93A}$ mice. S [+]-apomorphine also reduced pathological ROS levels and improved survival following oxidant challenge to fibroblasts from ALS patients. This molecule emerges as a promising candidate for evaluation as a potential neuroprotective agent in ALS patients in the clinic [241]. The same ALS mouse model has been used to investigate the efficacy of the powerful free-radical scavenging agent TBN (2-[[(1,1-dimethylethyl) oxidoimino]-methyl]-3,5,6-trimethylpyrazine) which activates the NRF2/HO1 axis. When administered after the onset of motor dysfunction, TBN improved motor performance, reduced spinal motor neuron loss and the associated glial response, decreased denervation at the neuromuscular junction and decreased the expression of human SOD1 [242]. NRF2 activation resulting from SIRT6 overexpression abrogates the MN damage exerted by co-cultured astrocytes isolated from mutant SOD1-overexpressing mice [243]. The new compound WN1316 (2-[mesityl (methyl)amino]-N-[4-(pyridin-2-yl)-1H-imidazole-2-yl] acetamide trihydrochloride) boosts the NRF2 transcriptional signature in SH-SY5Y neuronal cells. Oral administration of WN1316 in $SOD1^{H46R}$ and $SOD1^{G93A}$ mice improved motor function and survival. Immunohistochemical analysis revealed less oxidative DNA damage and reduced pro-inflammatory markers as well as repression of both microgliosis and astrocytosis compared to vehicle treated mice [244]. Recently, the antioxidant γ-oryzanol exhibited neuroprotection in MNs of flies expressing $SOD1^{G85R}$, as demonstrated by prolonged survival, improvement of motor deficits, reduced oxidative damage and regulated redox homeostasis when compared with controls. Remarkably, γ-oryzanol significantly increased the pool of reduced GSH by the activation of the NRF2/GCLC axis [245]. CPN-9 (N-(4-(2-pyridyl) (1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide) upregulates NRF2 and the expression of its target genes such as *HMOX1*, *NQO1* and *GCLM*. Systemic administration of CPN-9 to $SOD1^{H46R}$ mice, administered after disease onset, resulted in improved motor function, and delayed disease progression [246].

Fingolimod phosphate (FTY720), the first approved oral therapy for multiple sclerosis was administered to $SOD1^{G93A}$ mice, starting from the onset of motor symptoms to the end stage of the disease. The drug was able to improve the neurological phenotype and to extend the survival. The beneficial effect of fingolimod administration was associated with a significant modulation of neuroinflammatory and protective genes including *Cd11b*, *Foxp3*, *iNOS*, *Il1β*, *Il10*, Arg1, and *Bdnf* [247]. An additional study demonstrated that fingomilod phosphate induces nuclear accumulation of NRF2, enhancing the transcription of its target genes [248].

The free radical scavenger edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one, Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan) was reported as a therapeutic drug for ALS, modestly slowing the progression of disability measured by the ALS functional rating scale (ALSS-FRS-R) and motor neuron degeneration in SOD1 mice and rats [249,250]. The NRF2 signaling pathway was activated after edaravone treatment in cultured neurons, and its neuroprotective effect was abolished after NRF2 knockdown [251]. *In vivo* imaging showed that edavarone reduced the levels of oxidative stress in the spinal cord and skeletal muscle of in SOD^{G93A} mice [252].

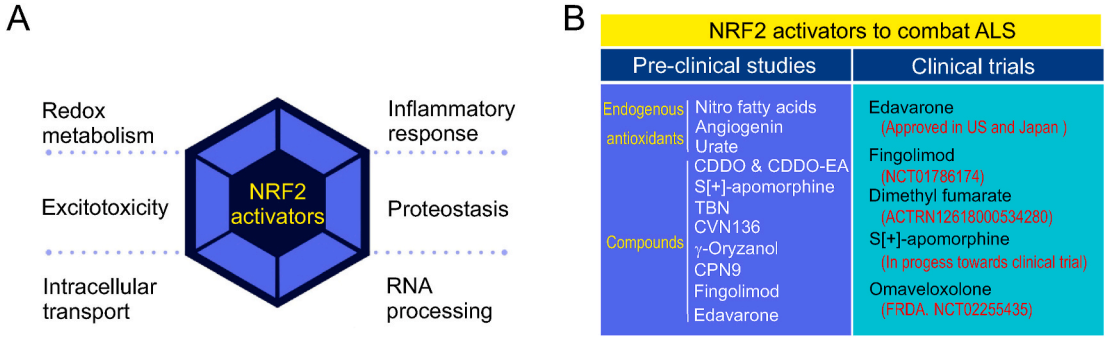

Fig. 5. *NRF2 as a molecular target to combat pathophysiological hallmarks of ALS.* A, development of NRF2 activators for cytoprotection of ALS-damaged cells. B, compounds that have proved cytoprotection through NRF2 activation in preclinical models and those which are currently under development or in clinical trials.

7.2. Clinical trials and therapies aimed at reinforcing NRF2 activity

All the pre-clinical data described above pave the way to analyze whether NRF2 activation might have beneficial disease-modifying effects in human ALS clinical trials. Several ALS clinical trials have explored the therapeutic potential of edavarone. However, this drug only produced a limited decline of the ALSFRS-R score compared with placebo in a highly selected group of ALS patients followed over a short 6-month time period [9,253] (NCT01492686 and NCT00330681). The CSF content of 3NT, a marker for oxidative stress, was markedly reduced in edavarone treated patients to almost undetectable levels at the end of the six-month treatment period, although this biomarker was not included in the final pivotal trial [254]. In addition, the route of administration, intravenous infusion for 10 days each month, is burdensome for patients. Edaravone has been approved for ALS treatment in the US and Japan, but not in Europe. The possibility of developing an oral preparation of edaravone is being explored.

Repurposing the NRF2 activators, fingolimod phosphate and dimethyl fumarate (DMF), should be a feasible strategy since both are already approved by the Food and Drug Administration and the European Medicines Agency for the treatment of multiple sclerosis, with neuroprotective efficacy in animal models of ALS [170,171,247]. The objective of the figolimod (Gilenya TM, Novartis AG) Phase IIA, randomized, controlled trial was to test the short-term safety, tolerability, and target engagement in ALS. No serious adverse events occurred. In line with NRF2 anti-inflammatory properties, circulating lymphocytes decreased significantly and several immune-related genes were downregulated in the fingolimod arm compared with placebo. However, there was no observable benefit of fingolimod on the ALSFRS-R or respiratory function in this Phase 2A trial [255] (NCT01786174). Since October 2017, the TEALS Study (ACTRN12618000534280) has been assessing the efficacy of DMF on disease progression measured by the ALS-FRS-R in SALS patients. The secondary aim of this study is to assess survival, MN dysfunction, respiratory dysfunction, quality of life as well as safety and tolerability. The authors of this study anticipate that the results will be available during 2021 [256]. S [+] apomorphine, which is a potent dual activator of the NRF2 and HSF1 (heat shock factor 1) pathways, with excellent penetration into the CNS across the blood brain barrier, is progressing towards human trials in 2021, and will incorporate imaging and biochemical biomarkers of target engagement and therapeutic efficacy.

Pharmacological activation of NRF2 holds promise for the treatment of other neurodegenerative diseases such as Friedreich ataxia (FRDA). As in the case of ALS, FRDA is a neuromuscular disorders in which NRF2 is downregulated [257–259], and oxidative and inflammatory stress are important drivers of pathology. There are no currently approved therapies for the treatment of FRDA [260] and the role of omaveloxolone, a potent NRF2 activator with a pentacyclic triterpenoid structure, is under clinical development by Reata Pharmaceuticals. The safety and efficacy of omaveloxolone has been tested in a registrational phase II clinical trial for patients with FRDA (MOXIe trial). MOXIe trial provided evidence that omaveloxolone significantly improved neurological function compared to placebo and was generally safe and well tolerated [261] (NCT02255435). The lessons learned from this trial could be certainly translated into novel therapies for ALS, supporting NRF2 as a therapeutic agent (Fig. 5B).

8. Perspectives

The pathophysiological hallmarks of ALS include loss of homeostatic responses such as RNA metabolism, redox control, inflammation, proteostasis and excitotoxicity. The multilayered aspects of ALS heterogeneity could be targeted with one single hit at the transcription factor NRF2 to orchestrate a neuroprotective response. Based on preclinical studies, biopharmaceutical companies are determining whether NRF2 activation could be a feasible strategy for ALS patients. Although NRF2 activation is very promising, we are aware that some questions are still challenging. Pharmacodynamic assessment (i.e. monitoring target engagement and off-target effects) and drug selectivity of NRF2 activators could be improved beyond the state of the art. Undoubtedly, active research conducted on improved preclinical ALS models and the data obtained from the ongoing clinical trials will offer answers to the currently open questions.

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Abbreviations

3NT	3-nitrotyrosine
HNE	4-hydroxynonenal
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ANG	angiogenin
ARE	antioxidant response element
β-TrCP	beta-transducin repeat containing protein
CO	carbon monoxide
CNS	central nervous system
CSF	cerebrospinal fluid
DMF	dimethyl fumarate
FDR	false Discovery Rate
FALS	familiar ALS
UPR	folded protein response
FRDA	Friedreich ataxia
FTD	frontotemporal dementia
Gclm	glutamate-Cysteine Ligase Modifier Subunit
GWAS	genome wide association studies
GPX8	glutathione peroxidase 8
HO1	heme oxygenase-1
HSP70	heat shock protein
HIF1α	hypoxia inducible factor 1α
iNOS	inducible isoform of nitric oxidase
JDP2	jun dimerization protein
KEAP1	kelch-like ECH-associated protein 1
LCM	laser-captured microdissected
miRs	microRNAs
MN	motor neurons
NRF2	nuclear factor (erythroid-derived 2)-like 2
NF-κB	nuclear factor-kappa B
PD	Parkinson's disease
PRX3	perioxiredoxin 3
ROS	reactive oxygen species
SERCA2b	sarco-endoplasmic reticulum ATPase 2b
MAFs	small musculoaponeurotic fibrosarcoma proteins
SALS	poradic ALS
SOD1	superoxide dismutase 1
HDR1	synoviolin E3 ubiquitin ligase
TRX	thioredoxin
UPS	ubiquitin proteasome system
VEGF	vascular-endothelial growth factor

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