

## Advances and challenges in understanding the multifaceted pathogenesis of amyotrophic lateral sclerosis

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### Summary

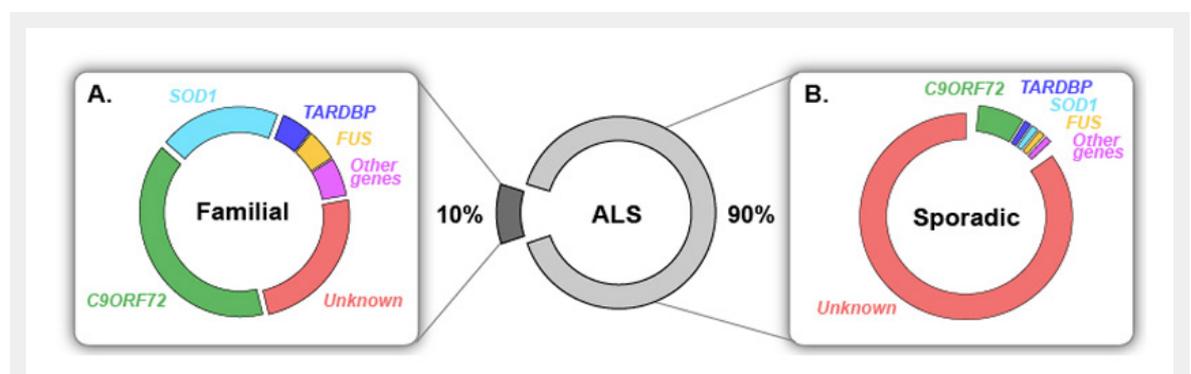
Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease, which primarily affects motor neurons leading to progressive paralysis and death within a few years from onset. The pathological hallmark of ALS is the presence of cytoplasmic ubiquitinated protein inclusions in motor neurons and glial cells primarily in the spinal cord. While the vast majority of ALS occurs sporadically (sALS), in ~10% of cases, called familial ALS (fALS), there is clear indication of genetic inheritance. In the last decade, enormous progress was made in unravelling the aetiology of the disease, with the identification of ALS-causing mutations in new genes, as well as key molecular players involved in the origin or progression of ALS. However, much more needs to be done, as the pathogenic mechanisms triggered by a genetic or sporadic event leading to cytotoxicity and neuronal cell death are still poorly understood. The recent discoveries offer new possibilities for devising experimental animal and cellular models, which will hopefully contribute to the development of new techniques

for early diagnosis and the identification of therapeutic targets for ALS. Here we review the current understanding of the aetiology, genetics, and pathogenic factors and mechanisms of ALS. We also discuss the challenges in deciphering ALS pathogenesis that result from the high complexity and heterogeneity of the disease.

**Key words:** *Amyotrophic lateral sclerosis; frontotemporal lobar degeneration; SOD1; TDP-43; FUS; C9ORF72; RNA processing; prion-like spread; protein misfolding; protein aggregation*

### Introduction

Amyotrophic lateral sclerosis (ALS), also called Charcot's or Lou Gehrig's disease, is an adult-onset neurodegenerative disorder. This motor neuron disease is characterised by rapidly progressive paralysis, atrophy, and respiratory failure leading to death typically within 1 to 5 years after symptom onset. The incidence of ALS is approximately 2 to 3 per 100,000 people each year, and the risk of develop-



**Figure 1**

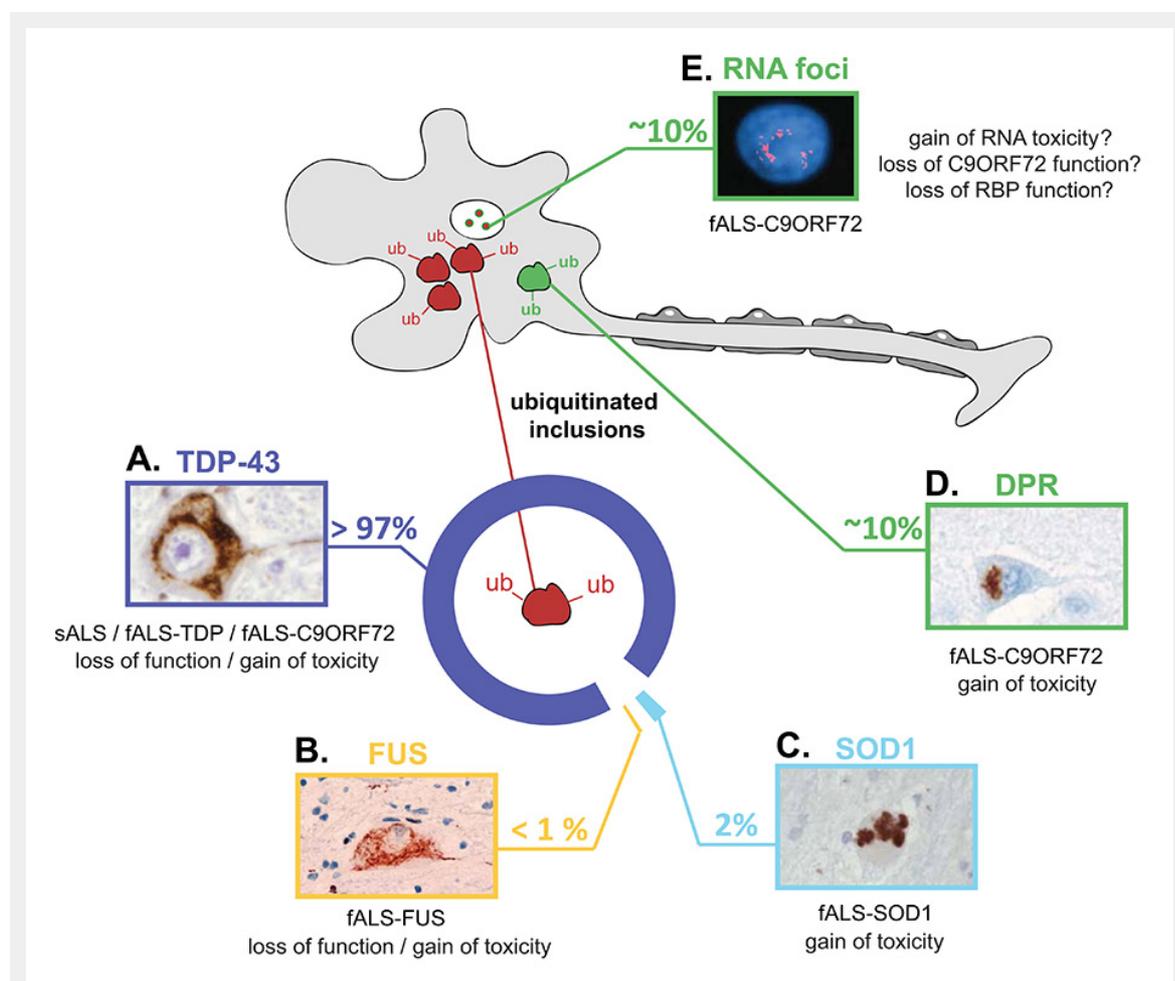
Known genetic causes in familial and sporadic ALS.

Most ALS cases are sporadic (sALS) and only 10% are inherited, called familial (fALS). (A) 20% of fALS are caused by mutations in SOD1, which is the first known ALS-linked gene, identified in 1993. Mutations in the RNA/DNA-binding proteins TDP-43 and FUS cause a small fraction (~5%) of fALS each. Hexanucleotide repeat expansions in C9ORF72 are the most common genetic cause of ALS accounting for almost 40% of fALS in people of European ancestry. Several other genes have been identified as genetic causes of ALS and collectively today over 60% of fALS can be explained by known mutations. (B) While the aetiology of the vast majority of sALS remains unknown, mutations in genes known to cause fALS were identified in a small fraction of apparently sporadic patients. Most strikingly, hexanucleotide repeat expansions in C9ORF72 are causing approximately 7% of sALS without familial history in people of European ancestry.

ing the disease peaks between 50 and 75 years of age and is slightly higher in men. At the time of diagnosis, approximately 50% of motor neurons are already lost, highlighting the tremendous necessity to develop early diagnostic tests. There is no cure for ALS and the only effective treatment is riluzole, a glutamate release inhibitor, which was shown to slow the rate of progression and to prolong survival time, albeit only by approximately three months [1, 2]. Jean-Martin Charcot first described the clinical presentation and lesion profile of ALS almost 150 years ago. The disease is characterised by upper and lower motor neuron degeneration, with the neurological progressive deterioration of the motor cortex, spinal cord and brainstem. ALS is linked to another neurodegenerative disease called frontotemporal lobar degeneration (FTLD), which is the most

common type of dementia below 60 years of age and which is characterised by behavioural and language dysfunction. ALS and FTLD symptoms overlap in a subset of patients or within families [3–6] and the two conditions share common pathological phenotypes and causative gene mutations [7–9].

Approximately 10% of ALS and 30% of FTLD cases, called familial ALS (fALS) or FTLD (fFTLD) respectively, show clear family history indicating genetic inheritance. Superoxide dismutase 1 (*SOD1*) is the first ALS-linked gene that was identified in 1993 [10] and for almost fifteen years ALS research was focused on mutant forms of this protein [11, 12]. Since 2008, starting with the discovery of ALS-linked mutations in the DNA/RNA-binding proteins, TDP-43 [13–17] and FUS [18, 19], we have witnessed an



**Figure 2**

Pathogenic hallmarks of ALS and the underlying factors and mechanisms.

The pathologic hallmark of ALS is the presence of ubiquitinated protein inclusions in motor neurons and glial cells primarily of the spinal cord and motor cortex. The major protein component of these inclusions is different in the various types of ALS, highlighting the heterogeneity of the disease. (A) In the majority of ALS cases, including all sporadic and most familial cases, the ubiquitinated inclusions contain TDP-43. When TDP-43 accumulates in the cytoplasm, it is excluded from the nucleus, where it normally resides in healthy cells. These alterations suggest that either loss of normal TDP-43 function or gain of toxicity (or both) may trigger motor neuron dysfunction and death. (B) In 0.5% of fALS that carry mutations in *FUS*, the cytoplasmic inclusions do not contain TDP-43, but they contain FUS protein. Similar observations to that of TDP-43, with partial nuclear clearance of FUS suggest that its neurotoxicity may be the result of either loss of function or gain of toxicity. (C) In fALS patients with *SOD1* mutations, misfolded SOD1 accumulates and becomes toxic to neurons. These patients do not contain any TDP-43 or FUS inclusions. (D) In contrast, patients with hexanucleotide repeat expansions in *C9ORF72* show typical TDP-43, similar to those found in sALS (A). In addition, C9ORF72 patients accumulate abnormal dipeptide repeat (DPR) proteins, which are produced by an unconventional type of translation of the hexanucleotide repeat RNA, which escapes the nucleus, and binds to ribosomal complexes. In addition to spinal cord motor neurons, DPRs have been observed in the cerebellum, which is typically spared in non-C9ORF72 ALS. (E) In addition to TDP-43 and DPR pathology, C9ORF72 patients accumulate nuclear RNA foci that contain sense and antisense hexanucleotide repeats in neuronal and glial cells. Insert images reproduced with permission from (A) [54], (B) [60], (C) [35], (D) [39], (E) [20].

era of unprecedented genetic discoveries in ALS. In 2011, the most common genetic cause of ALS was identified to be large expansions in an intronic hexanucleotide repeat region of a poorly characterised gene, called *C9ORF72* [20, 21]. Moreover, in addition to *SOD1*, *TARDBP*, *FUS* and *C9ORF72*, many other genes were identified as rare genetic causes of ALS in the last years [22, 23] and collectively today, more than 60% of familial ALS can be explained by known gene mutations (fig. 1A).

In the remaining 90% of ALS, called sporadic ALS (sALS), there is no indication of genetic inheritance. While the cause of most sALS is not understood, recent studies have shown that fALS-linked mutations may also trigger disease in sporadic cases. Indeed a small percentage of sALS patients were shown to carry *de novo* mutations in known ALS-causing genes [24–27]. *C9ORF72* repeat expansions, which were also found in a substantial fraction (~7%) of apparently sporadic ALS patients, most likely are not occurring *de novo* [28, 29], but rather represent cases with insufficient family history or incomplete penetrance [30]. Taken together, ~10% of apparently sporadic ALS cases are caused by known genetic mutations, while the aetiology of the rest ~90% sALS remains enigmatic (fig. 1B). The pathological hallmark of ALS is the presence of ubiquitin-positive inclusions consisting of misfolded protein aggregates in the affected motor neurons and glial cells of the spinal cord and motor cortex. The major protein component of these inclusions in most ALS cases is TDP-43 [31, 32]. Notable exceptions are patients with mutations in *SOD1* and *FUS*, which lack TDP-43 inclusions, but harbour misfolded SOD1 [33–35] or *FUS* [18, 19] protein accumulations, respectively. Moreover, patients with *C9ORF72* expansions exhibit the most complex pathology with typical TDP-43 inclusions, atypical TDP-43 negative inclusions [20, 36, 37] that consist of abnormal dipeptide proteins [38–40] as well as nuclear repeat RNA foci [20, 41] (fig. 2).

The discoveries of the past decade have entirely revolutionised our view of ALS by introducing new key molecular players in pathogenesis. These discoveries have also questioned the current disease classifications with the recognition of the clinical, pathological, and genetic spectrum of ALS/FTLD and the unexpected blurring divide between sporadic and genetic disease. In this review, we first present the heterogeneity of ALS by introducing some of the main genes and proteins that play a role in one or several aspects of this complex disease. Then, we describe the current thinking on how these upstream events lead to the onset and rapid progression of ALS.

### ALS is a heterogeneous disease with diverse genetic causes and complex pathology

Familial ALS is caused by mutations in many different genes that trigger pathogenesis, potentially through one or more common pathways [9]. In this review we focus on, arguably, the most important ALS-linked genes and proteins, namely *SOD1*, TDP-43, *FUS* and *C9ORF72*, which we discuss in the order of their chronological implication in the pathogenesis of ALS.

### SOD1

Mutations in superoxide dismutase 1 (*SOD1*) was the first described genetic cause of ALS [10]. Today more than 170 mutations in the *SOD1* gene have been identified to cause ALS. Interestingly, these mutations span the entirety of the *SOD1* protein sequence [9], suggesting that a single amino acid change in any position of *SOD1* is sufficient to disrupt its physiological conformation, leading to misfolding and accumulation of abnormal forms of the protein. In total, *SOD1* mutations occur in almost 20% of familial and rare sporadic ALS cases (fig. 1).

*SOD1* is a 153 amino acid enzyme that is responsible for scavenging free superoxide radicals in the body. Normally present in the cytoplasm or the mitochondrial intermembrane space as a stable homodimer, *SOD1* misfolds when it carries ALS-causing mutations [42] or in cell stress conditions [43, 44]. Although the misfolded protein is ubiquitinated, and thus directed to degradation [45], *SOD1* escapes the proteolytic process, which results in formation of ubiquitin positive inclusions observed in fALS cases [35].

The crucial role of misfolded *SOD1* in the pathogenesis of familial ALS with *SOD1* mutations is unequivocal. However, the presence of misfolded *SOD1* in sporadic ALS cases remains controversial. While some studies have shown no evidence of misfolded *SOD1* in sALS patients [35, 46], others reported a diffuse cytoplasmic misfolded *SOD1* in motor neurons in several sALS cases, which were not linked to any *SOD1* mutations [47]. This discrepancy may stem from the different anti-misfolded *SOD1* antibodies used in these studies, as well as the inherent challenges in detecting misfolded proteins in postmortem autopsy material. Further studies are needed to resolve this issue that is of great importance for our understanding of the commonalities in disease pathogenesis of ALS cases with seemingly distinct origins.

### TDP-43

An incredible transformation in our perception and understanding of ALS pathogenesis began with the identification of TAR-DNA-binding protein of 43 kDa (TDP-43) as the major component of ubiquitin positive neuronal cytoplasmic inclusions that are the hallmark of ALS and FTLD [31, 32]. This 414 amino acid RNA/DNA binding protein has two RNA recognition motifs (RRM1 and 2) and a glycine-rich region (GRR) that resembles the prion domain of yeast prion proteins. More than 35 mutations in the *TARDBP* gene, which encodes TDP-43, are causes of ALS [13–17]. Most of these mutations are clustered in exon 6 of the *TARDBP* gene encoding the C-terminal GRR [9, 48] and cause approximately 5% of fALS and <1% of sALS (fig. 1).

TDP-43 is a nuclear protein that belongs to the ribonucleoprotein family and is involved in RNA transcription, splicing and transport [9, 48]. It is also able to shuttle between the nucleus and the cytoplasm, where it is associated with granules [49], playing a key role in RNA transport. Under cell stress conditions, TDP-43 translocates to the cytoplasm, where it forms stress granules [50–52]. Stress granules are non-membranous cytoplasmic foci composed of non-translating mRNP that rapidly aggregate in cells exposed to stress conditions such as starvation or

oxidation, allowing selective RNA inactivation [53]. The GRR region is necessary for this function through its prion-like ability to change conformation between a globular and an aggregated form [52].

In ALS, TDP-43 accumulation and aggregation is accompanied by a disturbance in its normal cellular localisation. In healthy cells, TDP-43 is mainly localised in the nucleus, whereas in affected cells, the misfolded form of the protein is redistributed to the cytoplasmic compartment, which leads to depletion/loss of nuclear TDP-43 [31, 32, 54]. Moreover, TDP-43 undergoes biochemical alterations such as hyperphosphorylation, polyubiquitination and C-terminal fragmentation [31, 32]. The mislocalisation and biochemical signature of pathological TDP-43 is characteristic for almost 97% of ALS cases, including most sporadic and familial ALS, with the exception of fALS caused by *SOD1* or *FUS* mutations (fig. 2). The intracellular accumulation and aggregation of TDP-43 in fALS cases with *TARDBP* mutations resembles the pathogenic pattern described in sALS in the absence of mutations [17], suggesting that the same downstream pathways are operating in these two types of ALS.

### FUS

More recently, Fused in Sarcoma (FUS), also called Translocated in Liposarcoma (TLS), has been identified as another protein involved in ALS. Mutations in the *FUS* gene have been described as a cause of ~4% of fALS [18, 19] and rare sALS cases (fig. 1). Interestingly, FUS and TDP-43 show significant structural and functional homology [48]. Indeed, FUS is a 526 amino acid protein that has the ability to bind nucleic acids and comprises a prion-like domain. The nucleic acid binding of FUS is due to the presence of glycine-arginine (R/G)-rich motifs, an RNA recognition motif (RRM), and a zinc finger (ZnF) domain. The first half of the protein, which contains a glutamine-glycine-serine-tyrosine (Q/G/S/Y)-rich region and the R/G-rich motifs, is responsible for the high aggregation propensity of FUS [55] and similarly to TDP-43, FUS is predicted to attain prion-like properties [56]. Moreover, like TDP-43, FUS is mainly nuclear, and utilises a transportin shuttle transport to reach the cytoplasm [57], where it partitions in stress granules [58, 59] and forms intracellular aggregates in fALS cases with *FUS* mutations [60]. In these familial cases, FUS inclusions occur in the absence of TDP-43-positive deposition [18, 19], an observation that in combination with the functional and structural similarities of TDP-43 and FUS [48], suggest that the two proteins trigger ALS by an independent initiating event, but potentially through common downstream pathways. For this reason, we will combine our discussion of TDP-43 and FUS in the rest of this manuscript.

### C9ORF72

In 2011, the identification of a massive GGGGCC hexanucleotide repeat expansion in a poorly characterised gene called *C9ORF72*, as the most common genetic cause of ALS and FTLD [20, 21], was another major breakthrough in the field. In normal healthy controls, this repeat length is shorter than 25 units, whereas in ALS or FTLD patients it can expand up to 800 to 4400 units [20, 61]. In people

of European ancestry, *C9ORF72* hexanucleotide repeat expansions cause 40% of fALS and 25% of fFTLD (fig. 1), while up to 90% of families with concurrent ALS and FTLD have hexanucleotide repeat expansions in *C9ORF72* [22, 23, 62]. Unexpectedly, *C9ORF72* hexanucleotide repeat expansions were also found in approximately 7% of apparently sporadic ALS cases in people of European ancestry, suggesting that the fraction of ALS with genetic origin may be larger than anticipated.

The normal function of *C9ORF72* is not well understood, but recent studies suggest that it may be important for endosomal trafficking [63–65]. *C9ORF72* is homologous to proteins related to Differentially Expressed in Normal and Neoplasia (DENN), which is a GDP/GTP exchange factor (GEF) that activates Rab-GTPases [63, 64]. Furthermore, *C9ORF72* was found in the extracellular space and in cytoplasmic vesicles, where it colocalises with Rab proteins that are implicated in autophagy and endocytic transport in mouse and human neurons [65].

Patients with *C9ORF72* expansions show the most complex pathology of all ALS cases. They not only harbour TDP-43 inclusions, similar to those of sALS patients (fig. 2), but a striking pathological hallmark of *C9ORF72* ALS is the presence of atypical TDP-43-negative ubiquitinated protein inclusions in the hippocampus and cerebellum [20, 36, 37], which is usually spared in non-*C9ORF72* ALS [66, 67]. These inclusions were recently identified to contain five different aggregating dipeptide-repeat (DPR) proteins [40, 68–70], namely poly-(Gly-Ala), poly-(Gly-Pro), poly-(Gly-Arg), poly-(Pro-Arg) and poly-(Pro-Ala) [38–40]. The latter are generated from the hexanucleotide repeat RNA by an unconventional type of translation, called repeat-associated non-ATG (RAN) translation, which operates in both sense [38, 39] and antisense [40] transcripts. Lastly, the hexanucleotide repeat expansions accumulate in nuclear RNA foci in neurons [20] and glial cells [41] of *C9ORF72* ALS patients.

## From human pathology and genetics to experimental ALS models

The major advancements in ALS genetics and pathology have revolutionised the way we understand the disease and they are directing research towards unravelling the underlying pathogenic mechanisms. Just like in any other human disease research field, the first step towards identification of molecular mechanisms is the construction of experimental animal and cellular models, either by mutating or depleting genes encoding for ALS-linked proteins.

### SOD1

Loss of *SOD1* does not appear to cause spontaneous motor neuron degeneration, since homozygote *SOD1* knockout mice are viable and develop without obvious motor abnormalities [71]. In contrast, transgenic mice expressing human *SOD1* with fALS-linked mutations, such as G93A [72] or G85R [42] were shown to develop late-onset, progressive paralysis and aggressive disease with short duration [42]. Strikingly, the G85R mutant provokes disease at protein expression levels below endogenous mouse *SOD1* [73]. Many more mutant *SOD1* animal models have been

created and were shown to faithfully recapitulate the clinical and pathological features of human ALS, as they develop an adult-onset, rapidly lethal motor neuron disease with gliosis and ubiquitinated misfolded SOD1 deposition (reviewed in [12]). The symptom onset, lifespan and rate of progression of the mutant SOD1 models vary depending on the mutation and expression level of human SOD1.

Early reports on transgenic mice overexpressing wild type (WT) SOD1, which were produced to study Down syndrome – as the *SOD1* gene is located on chromosome 21 – showed that increased levels of non-mutated SOD1 protein do not lead to ALS, since they were first described as phenotypically normal, before some complications linked to Down syndrome were identified [74, 75]. Recently, massive overexpression (50-fold) of human WT SOD1 was shown to cause motor neuron degeneration with deposition of misfolded SOD1, reaching terminal stages at ~400 days of age [76], suggesting that SOD1 can become pathologic, even in the absence of mutations.

### TDP-43 and FUS

The effects of mutation or depletion of TDP-43 and FUS have been studied in various models. In mice, homozygous deletion of *TARDBP* causes early embryonic lethality due to defective outgrowth of the inner cell mass prior to implantation [77–79]. Interestingly, aged heterozygous TDP-43 mice develop mild motor dysfunction, despite apparently unchanged TDP-43 protein levels [79]. Two independent studies have shown that selective deletion of TDP-43 from motor neurons induces adult-onset muscle weakness and atrophy [80, 81]. Importantly, in both studies the mice were not reported to develop paralysis, despite motor neuron loss. More recently, constitutive expression of an artificial micro RNA (amiR), targeting TDP-43 resulted in its partial loss, especially in astrocytes [82]. These mice showed a complex phenotype, starting with transient hyperactivity at 40 days, which subsided by 70 days, after which they developed weakness, which progressed to paralysis and death at approximately 150 days. In contrast to the conditional motor neuron TDP-43 depletion [80], which showed more prominent phenotype in males, the amiR-expressing mice showed no gender disparities [82]. Taken together the above studies suggest that while loss of TDP-43 may contribute to motor neuron degeneration, it is not possible to mimic the full spectrum of the ALS clinical presentation by mere depletion of TDP-43.

The alternative hypothesis that mutant or aggregated wild type TDP-43 acquires a new toxic function was tested in a series of transgenic mice and rats. Overexpression of mutant [83–87] or wild type [88] TDP-43 in rodent models induced various abnormalities with aspects of motor neuron disease, but failed to clearly reproduce the ALS symptom spectrum. Disappointingly, in sharp contrast with the mutant SOD1 models, none of the existing transgenic TDP-43 overexpressing rodents developed progressive paralysis or TDP-43 protein aggregation and nuclear clearance.

Disruption of the *Fus* gene in inbred mice is lethal due to major defects in B-lymphocyte development [89], whereas similar disruption in an outbred background produces mice that survive until adulthood and develop male sterility [90].

While the cause of the discrepancy in the phenotypes of these two *Fus* knockout lines is not known, the fact that outbred mice without *Fus* reach adulthood without developing motor neuron dysfunction speaks against the contribution of FUS loss of function in ALS. In rats, overexpression of human FUS carrying the R521C mutation, resulted in loss of cortical, hippocampal and motor neurons, accompanied by denervation and paralysis [91]. Transgenic mice overexpressing human wild type *FUS* show no overt phenotype, unless they are bred to homozygosity, in which case they develop aggressive motor neuron degeneration and death at ~100 days of age [92]. Interestingly, the homozygous mice that express ~2-fold above endogenous FUS levels develop skein-like FUS inclusions in motor neurons.

Nevertheless, the apparent inability of these models to reproduce an ALS-like disease and mimic the associated pathology, questions their relevance for ALS research. The lack of faithful TDP-43 and FUS disease models is currently one of biggest challenges in ALS research and alternative models that mimic all aspects of the disease are urgently needed.

### C9ORF72

The identification of *C9ORF72* hexanucleotide repeat expansions in ALS is very recent, and the generation of relevant animal models are currently underway in several labs worldwide. First reports on mice with heterozygous disruption of the mouse homologue of *C9orf72*, suggested that the mice are born without obvious abnormalities [93, 94], but no data on the consequences of the complete loss of *C9orf72* or expression of expanded hexanucleotide repeats is available at the time of writing. Nevertheless, cellular models based on human motor neurons differentiated from induced pluripotent stem cells (iPSCs) from ALS patients carrying the *C9ORF72* repeat expansion were shown to recapitulate the main pathological aspects, including repeat RNA foci and dipeptide protein inclusions [41, 95–98]. We anticipate that these models will become extremely valuable in understanding *C9ORF72*-ALS pathogenesis.

## From experimental models to molecular ALS pathogenesis

The current experimental models of ALS, allow us to investigate the molecular mechanisms of disease pathogenesis. So, how do those distinct and very diverse genetic mutations and protein misfolding events cause selective motor neuron cytotoxicity and death, leading to the degeneration of an entire part of the central nervous system? And most importantly, what are – if any – the common downstream events that give rise to the characteristic ALS clinical presentation?

### SOD1

Studies on the SOD1 animal models revealed that compromising the enzymatic activity of SOD1 has little or no effect on ALS, but that a toxic property of misfolded SOD1 drives disease. Unexpectedly, this toxic property does not simply operate within the vulnerable motor neurons. Neuronal mutant SOD1 – which is sufficient to cause ALS-like

symptoms in mice [99] – rather directs the onset and early disease [100, 101], while SOD1 expression in glial cells, such as microglia [101] or astrocytes [102], accelerates disease progression. Most recently, mutant SOD1 within oligodendroglia has been shown to cause their dysfunction, which in turn, substantially accelerated disease onset and survival of mutant SOD1 mice [103]. In conclusion, mutant SOD1 toxicity is non-cell autonomous [12] and the convergence of toxicity within motor neurons and their neighbouring glial cells triggers disease.

While the non-cell autonomous nature of ALS is one of the major lessons learned from SOD1 animal models, a statement of concern is that 20 years after the initial discovery, no consensus has emerged as to the exact pathogenic mechanism(s) triggered by misfolded SOD1. Nevertheless, multiple mechanisms have been described, many or all of which are operating in different cell types and are thereby contributing to different aspects of disease (for detailed review see [12]). One of the first identified cytotoxic pathways of mutant SOD1 is excitotoxicity, due to failure in clearing synaptic glutamate, the neurotransmitter that triggers motor neurons to fire. Excitotoxicity in mutant SOD1 patient samples [104] and animal models [42, 105] was shown to originate from deficiency in the glutamate transporter EAAT2, due to decrease in the exosomal transfer of neuronal miR-124a into astrocytes [106]. Mutant SOD1 motor neurons from transgenic mice [107] or human patients [98] also show intrinsic membrane hyperexcitability, which was suggested to contribute to the selective vulnerability of motor neurons in ALS [108]. Mutant SOD1 induces endoplasmic reticulum (ER) stress through multiple mechanisms. Accumulation of misfolded SOD1 aggregates bind to the ER chaperone BiP (Binding immunoglobulin Protein) [109], which mediates the activation of ER stress transducers, such as IRE1, PERK and ATF6. Moreover, mutant SOD1 binds to Derlin-1 [110], a protein that dislocates misfolded proteins from the ER to the cytosol and whose inhibition triggers ER stress. Indeed, ER-related genes are upregulated at pre-symptomatic stages of mutant SOD1 mice [111], as well as in human iPSC-derived motor neurons [112]. Mutant SOD1 was also shown to impair the proteasome [113] and block autophagy [114] leading to dysregulation of protein levels, and to decreased clearance of mutant SOD1. In addition, misfolded SOD1 directly damages mitochondria through deposition on the cytoplasmic face of the outer membrane of the organelles [115, 116]. Other mutant SOD1-mediated neurotoxic mechanisms include extracellular toxicity from aberrant SOD1 secretion [117], excessive production of extracellular superoxide [118], axonal structure and transport dysregulation [119], or even spinal cord microhaemorrhages [120]. The challenge in ALS research is now to define which of the above mechanisms are merely downstream consequences of neuronal dysfunction and which ones occur early in disease, thereby determining the onset or progression of ALS.

#### TDP-43 and FUS

The pathogenic mechanisms of TDP-43 and FUS may result 1) from loss of their normal function due to their nuclear depletion in ALS, 2) from gain of toxic function due to

their cytoplasmic aggregation, or 3) from a combination of the two mechanisms that are not mutually exclusive.

The motor neuron degeneration developed after *in vivo* depletion of TDP-43 in mouse models [80–82] supports the notion that TDP-43 loss of function contributes to ALS pathogenesis. TDP-43 [121, 122] and FUS [123, 124] bind thousands of RNA and are involved in multiple steps of RNA transcription and processing [48]. The two proteins bind mostly to distinct RNAs, but also share a set of approximately 45 common targets that depend on the function of both proteins and whose disturbance contributes to neuronal dysfunction and death in ALS [121, 123]. Most recently, TDP-43 was shown to form cytoplasmic mRNP granules that deliver target mRNAs to distal neuronal compartments, a function that is impaired by ALS-linked mutations [49], suggesting another potential pathogenic mechanism in ALS. Taken together, these findings indicate that alterations in RNA homeostasis and transport play a key role in the pathogenesis of ALS.

The participation of TDP-43 [50–52] and FUS [58, 59] in stress granules, suggest a possible gain of toxic function mechanism in ALS, as these RNA granules might be precursors of the pathological cytoplasmic mislocalisation and aggregation seen in patients. TDP-43 intrinsic aggregative properties have been described, and its propensity for toxic misfolding and deposition is accentuated by ALS-linked mutations [125]. This cytotoxic deposition could sequester RNA and RNA binding proteins, thereby contributing to pathogenesis. While the observation that RNA binding of TDP-43 [126, 127] and FUS [55] is required for their cytotoxicity supports this view, the exact RNAs and/or RNA-binding proteins that are sequestered in the cytoplasmic TDP-43 and FUS aggregates of human neurons remain unknown. Further evidence that stress granule formation is intimately linked to pathogenesis came from a recent study, which reported that genes modulating stress granules are strong modifiers of TDP-43 toxicity and that prolonged stress granule dysfunction in human ALS spinal cord neurons is likely a direct contributor to pathogenesis [128].

#### C9ORF72

Several cytotoxic mechanisms have been described for C9ORF72 hexanucleotide repeat expansions and their relative contribution to ALS pathogenesis is currently under investigation. First of all, patients with C9ORF72 expansions show typical TDP-43 cytoplasmic inclusions with nuclear clearance [20, 36, 37, 61], suggesting that all mechanisms described above are relevant for this type of ALS. Moreover, patients carrying hexanucleotide repeat expansions were shown to produce reduced levels of C9ORF72 RNA [20, 61], due to the inactivation of the repeat-containing allele via promoter hypermethylation [129], or transcriptional abortion of repeat RNAs [130]. Reduced C9ORF72 RNA and, by extension, protein may not be enough to perform its normal function, a mechanism known as haploinsufficiency. The long-term phenotype (or lack thereof) of mice with heterozygous disruption of the mouse C9orf72 homologue [93, 94], could provide a definitive answer for the contribution of haploinsufficiency in C9ORF72–ALS pathogenesis. Another possibility is that the repeat RNA is toxic *per se*. Indeed, the DNA and RNA

hexanucleotide repeats form complex structures, which result in transcriptional hindrance and in accumulation of abortive transcripts that contain the hexanucleotide repeats [20, 40, 41, 130]. Those abortive transcripts form G-quadruplexes and hairpins and bind essential proteins, primarily nucleolin, whose sequestration leads to nucleolar stress and other downstream defects [130]. Moreover, the repeat-containing transcripts can also escape the nucleus and associate with ribosomal complexes in the cytoplasm, where they are subjected to the repeat-associated non-ATG (RAN) translation [38–40], leading to production and accumulation of abnormal dipeptide proteins. Originally, poly-GA aggregates were shown to be the prominent DPRs in patients [69], and in rat primary neurons [68], suggesting a direct role in neurodegeneration. Poly-GA DPRs were indeed very recently shown to cause neurotoxicity *in vitro* by inducing ER stress [131]. However, conflicting results showed that the most common DPRs present in both upper and lower motor neurons are the poly-GP [40], which were most recently shown to be present in the cerebrospinal fluid of C9ORF72 patients [132]. Moreover, the relatively short synthetic sense poly-GR and antisense poly-PR DPRs were reported to be highly toxic, when exogenously applied to human astrocytes, since they entered the nucleus, bound to nucleoli and killed the cells in culture [133]. Finally, expression of “protein-only” poly-GR and poly-PR constructs in *Drosophila* was associated with progressive neuronal death *in vivo*, which is perhaps attributable to the basic nature or a common structural motif of these DPRs [70]. Altogether, DPR toxicity is a central pathogenic mechanism in C9ORF72-ALS.

### Does “prion-like” propagation of ALS proteins drive disease progression?

The physiological function of a protein is often linked to a unique structure and stable three-dimensional conformation, as a result of a normal folding pathway. Some proteins can gain toxic properties upon a disturbance of their structure that can stably exist and be transferred to homologous proteins if their structure allows this pathological conformation. This mechanism underlies the pathogenesis of prion diseases [134], and is suggested to contribute to the progression of many neurodegenerative diseases [135], such as Alzheimer’s (amyloid- $\beta$  [136] and Tau [137]) and Parkinson’s ( $\alpha$ -synuclein) [138] diseases. We recently proposed that a similar mechanism may occur in ALS [56], and many ongoing studies aim now at describing the prion-like pathogenesis of ALS, and the underlying prion-like seeding and spreading mechanisms of the proteins implicated in the disorder. Those could lead, from a single protein-misfolding event, to an actual neurodegenerative disease, following an autocatalytic protein-protein transfer of structural and pathological information.

#### SOD1

SOD1 spontaneously forms fibrils or aggregates *in vitro* and ALS-linked mutations enhance this phenomenon [139, 140]. *In vitro*, the destabilisation of SOD1 structure under experimental specific conditions led to its aggregation, and even triggered a prion-like seeding and increase of aggrega-

tion [140]. Temperature increase, reducing conditions, addition or disruption of disulfide-bonds seem to be the destabilising factors that can initiate the seeded misfolding of SOD1 [141–143]. A major step in that field was the finding that SOD1 misfolding can be transmitted, not only from protein to protein, but also from cell to cell following a prion-like propagation mechanism [142, 144, 145]. *In vitro* formed SOD1 aggregates were actively integrated in neuronal cells through macropinocytosis [142], and triggered aggregation of endogenously expressed SOD1. Once SOD1 aggregation occurs in a cell, the aggregates can be released to the extracellular space and then transferred from cell to cell in a persistent way, heritable after cell passages, supporting a prion-like propagation of aggregation.

#### TDP-43 and FUS

The aggregation properties of TDP-43 and FUS are associated with their physiological role in the formation of stress granules [50–52, 57, 58]. The existence of two distinct conformational states – one of which is highly aggregative – depends on the presence of GRR, also called prion-like or low complexity domain. Indeed, like SOD1, TDP-43 is able to form aggregates *in vitro* [146, 147] and its C-terminal region, where the prion-like domain resides, shows the highest aggregation propensity, while ALS-linked mutations enhance this phenomenon [125, 147]. Compared to TDP-43 and SOD1, FUS shows the highest aggregation potential, as it forms aggregates even in native conditions, without any experimental procedure, such as shaking or sonication [55], but this property is not altered by the presence of ALS-causing mutations [55, 57]. Instead, ALS-linked mutations residing in the nuclear localisation signal of FUS increase its cytoplasmic retention, thereby, possibly facilitating the initiation of cytoplasmic FUS aggregation [57].

A wide step in deciphering prion-like spreading of ALS proteins was made with the very recent description of prion-like properties of TDP-43 extracted from ALS patient brains [148]. In this study, insoluble TDP-43 from ALS brains was introduced as seeds into cells expressing human wild type TDP-43. It induced phosphorylated and ubiquitinated TDP-43 aggregation in a self-templating manner. Furthermore, C-terminal fragments of the insoluble protein acted as seeds, and were shown to be sufficient for templating its self-aggregation. All together, these results indicate that TDP-43 and FUS have prion-like properties that may play a key role in the progression of the respective proteinopathy.

An important contributor to self-propagation of pathologic conformations of TDP-43 and FUS stems from their well-documented autoregulation pathways. Indeed, both TDP-43 [121, 149] and FUS [123, 150] proteins were shown to bind their own RNA in the nucleus, thereby autoregulating their level of expression. This may possibly create a vicious circle of more and more protein substrate production, as a result of sequestration of newly produced TDP-43 and FUS into the cytoplasmic aggregates.

#### C9ORF72

All above mechanisms may contribute to C9ORF72 disease and the spreading of TDP-43 pathology in patients

with hexanucleotide repeat expansions. Moreover, the abnormal dipeptide products [38–40], once made by RAN-translation, may undergo seeded aggregation.

## Conclusions and open questions

In conclusion, ALS is complex and heterogeneous disease that is caused by many, seemingly diverse, upstream events. The great discoveries that we discussed have not only advanced our understanding of ALS, but they have also, inevitably, raised one major question: is ALS one disease, or does each type of ALS described above represent a distinct disease with independent aetiology and pathogenic mechanisms? Alternatively, do these diverse genetic causes of ALS trigger common downstream mechanisms that lead to selective motor neuron degeneration? And if yes, which are these common mechanisms? As we move forward, the answers to these questions will be instrumental, for devising reliable diagnostic tests and most importantly for the identification of therapeutic targets for this devastating and still untreatable disease.

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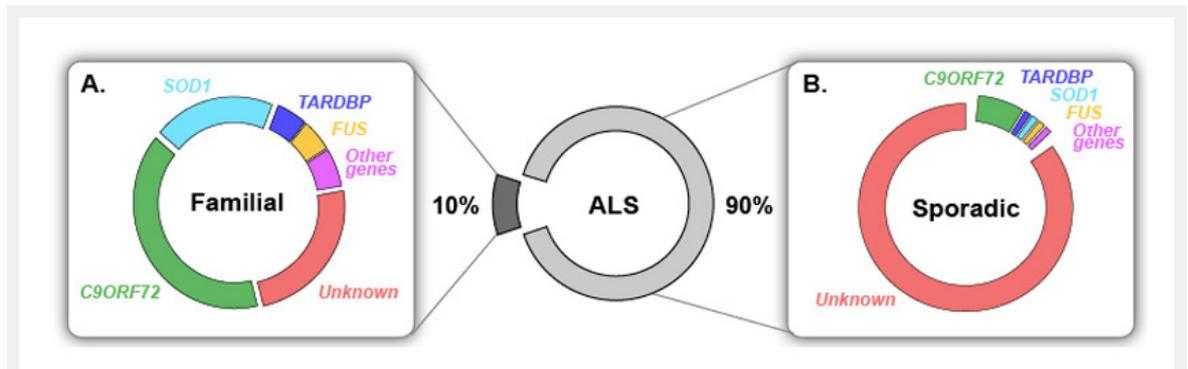
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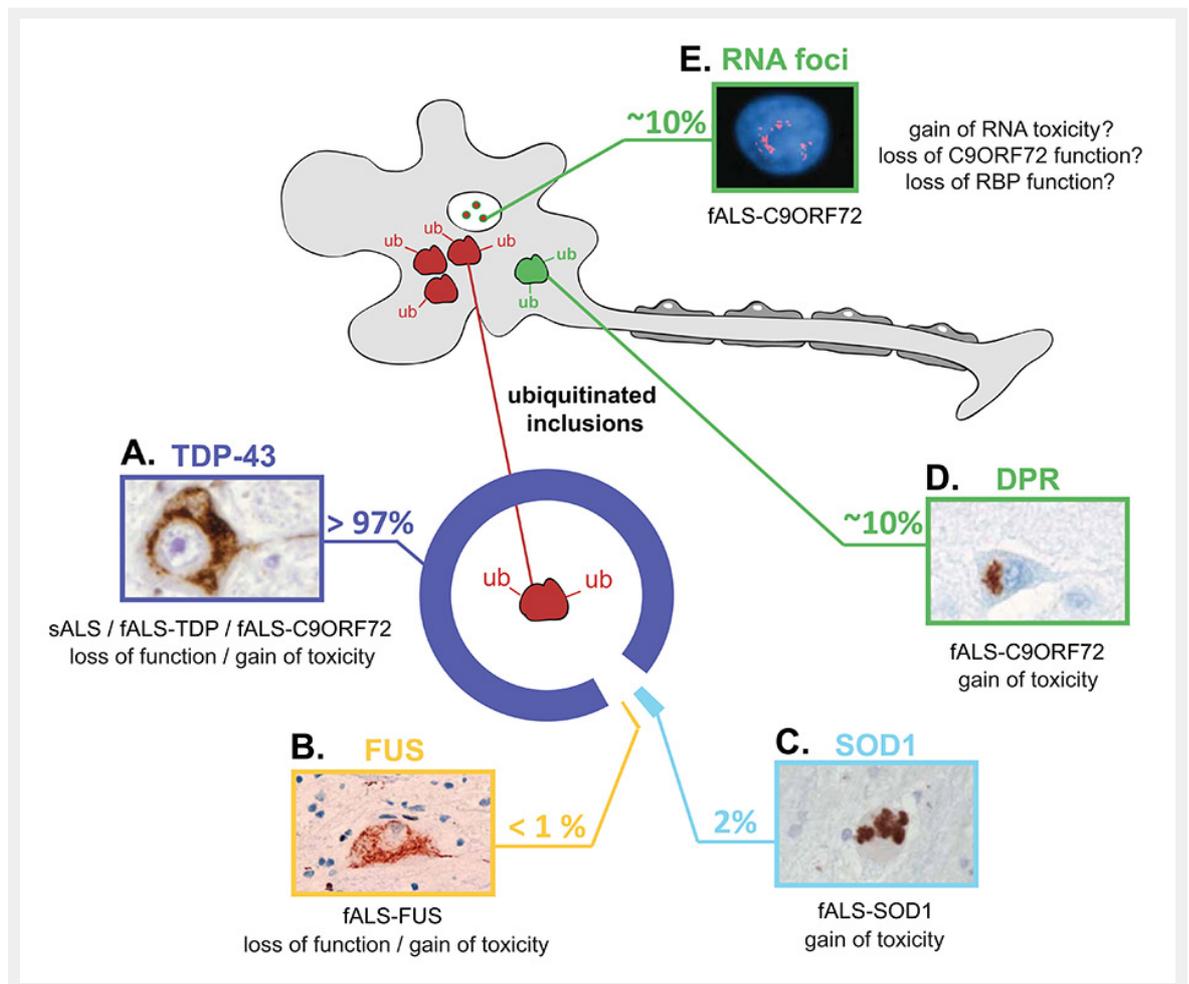
## Figures (large format)



**Figure 1**

Known genetic causes in familial and sporadic ALS.

Most ALS cases are sporadic (sALS) and only 10% are inherited, called familial (fALS). (A) 20% of fALS are caused by mutations in *SOD1*, which is the first known ALS-linked gene, identified in 1993. Mutations in the RNA/DNA-binding proteins TDP-43 and FUS cause a small fraction (~5%) of fALS each. Hexanucleotide repeat expansions in *C9ORF72* are the most common genetic cause of ALS accounting for almost 40% of fALS in people of European ancestry. Several other genes have been identified as genetic causes of ALS and collectively today over 60% of fALS can be explained by known mutations. (B) While the aetiology of the vast majority of sALS remains unknown, mutations in genes known to cause fALS were identified in a small fraction of apparently sporadic patients. Most strikingly, hexanucleotide repeat expansions in *C9ORF72* are causing approximately 7% of sALS without familial history in people of European ancestry.

**Figure 2**

Pathogenic hallmarks of ALS and the underlying factors and mechanisms.

The pathologic hallmark of ALS is the presence of ubiquitinated protein inclusions in motor neurons and glial cells primarily of the spinal cord and motor cortex. The major protein component of these inclusions is different in the various types of ALS, highlighting the heterogeneity of the disease. (A) In the majority of ALS cases, including all sporadic and most familial cases, the ubiquitinated inclusions contain TDP-43. When TDP-43 accumulates in the cytoplasm, it is excluded from the nucleus, where it normally resides in healthy cells. These alterations suggest that either loss of normal TDP-43 function or gain of toxicity (or both) may trigger motor neuron dysfunction and death. (B) In 0.5% of fALS that carry mutations in *FUS*, the cytoplasmic inclusions do not contain TDP-43, but they contain FUS protein. Similar observations to that of TDP-43, with partial nuclear clearance of FUS suggest that its neurotoxicity may be the result of either loss of function or gain of toxicity. (C) In fALS patients with *SOD1* mutations, misfolded SOD1 accumulates and becomes toxic to neurons. These patients do not contain any TDP-43 or FUS inclusions. (D) In contrast, patients with hexanucleotide repeat expansions in *C9ORF72* show typical TDP-43, similar to those found in sALS (A). In addition, *C9ORF72* patients accumulate abnormal dipeptide repeat (DPR) proteins, which are produced by an unconventional type of translation of the hexanucleotide repeat RNA, which escapes the nucleus, and binds to ribosomal complexes. In addition to spinal cord motor neurons, DPRs have been observed in the cerebellum, which is typically spared in non-*C9ORF72* ALS. (E) In addition to TDP-43 and DPR pathology, *C9ORF72* patients accumulate nuclear RNA foci that contain sense and antisense hexanucleotide repeats in neuronal and glial cells. Insert images reproduced with permission from (A) [54], (B) [60], (C) [35], (D) [39], (E) [20].