The changing scene of amyotrophic lateral sclerosis

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Abstract | Several recent breakthroughs have provided notable insights into the pathogenesis of amyotrophic lateral sclerosis (ALS), with some even shifting our thinking about this neurodegenerative disease and raising the question as to whether this disorder is a proteinopathy, a ribonucleopathy or both. In addition, these breakthroughs have revealed mechanistic links between ALS and frontotemporal dementia, as well as between ALS and other neurodegenerative diseases, such as the cerebellar atrophies, myotonic dystrophy and inclusion body myositis. Here, we summarize the new findings in ALS research, discuss what they have taught us about this disease and examine issues that are still outstanding.

Frontotemporal dementia
This term refers to the clinical presentation of the behavioural variant of frontotemporal lobe degeneration.

Frontotemporal lobe degeneration
(FTLD). A pathology that is characterized by frontal and temporal lobe atrophy. It presents with behavioural or language abnormalities.

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects both lower motor neurons in brainstem and spinal cord, and the upper motor neurons in the motor cortex. Loss of these neurons leads to muscle atrophy and weakness, fasciculations and spasticity.

Symptoms and signs emerge when axonal connections fail: that is, when the axon retracts and denervation of the lower motor neurons or the muscle occurs. Initially, retraction is compensated by sprouting and collateral re-innervation by axons from neurons that seem to be more resistant to the degenerative process[1]. However, as the disease progresses, this compensational mechanism fails owing to the involvement of even the more resistant neurons. It is only after this initial stage of axonal dysfunction and retraction that the neuronal cell body becomes visibly abnormal and dies, as has been shown in animal models[1–3]. The exact timing of these events in human ALS is less certain, but the rare post-mortem reports on patients who died very early in the disease confirm this sequence[3].

Neurons in the prefrontal and temporal cortex are also affected in ALS but to varying degrees[4]. Degeneration of such neurons results in frontal executive dysfunction in many patients with this disorder and concomitant frontotemporal dementia in about 15% of patients. This is known as ALS with frontotemporal lobe degeneration (FTLD). ALS and FTLD are therefore at opposite ends of the spectrum of a single disease (BOX 1).

ALS is traditionally classified into two categories: familial ALS (FALS) and sporadic ALS (SALS). FALS and SALS are clinically very similar. FALS is predominantly hereditary and then almost always autosomal dominant; X-linked or recessive FALS is rare (for a review, see REF. 5). FALS is caused by mutations in a heterogeneous set of genes (TABLE 1; see Supplementary information S1 (figure)). Patients who do not have affected relatives are said to have the sporadic form. Incomplete family history, non-paternity and incomplete penetrance are some reasons for misclassification.

The age of clinical onset of ALS is highly variable but is almost always after the fourth decade of life; juvenile ALS is rare. When the disease has its biological onset is unknown. In rodent models of FALS, which admittedly are overexpression models, abnormalities in excitability, axonal transport and neuronal architecture are present as early as embryonic development[6–9]. Still, as in humans, these animals develop no clinical abnormalities until late into adulthood. Thus, biologically, the disease may start early in life and become clinically apparent much later. The reasons for this are currently unknown.

ALS is relentlessly progressive, as motor neuron injury gradually spreads. Most patients with ALS die within 3 to 5 years after symptom onset, but, again, the variability in clinical disease duration is large, with some patients dying within months after onset and others surviving for more than two decades. Large differences in survival and age at disease onset exist even between individuals from one family, in whom ALS is caused by exactly the same mutation, suggesting the existence of other factors that modify the phenotype[10].

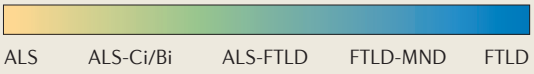
Post-mortem examinations reveal that patients with ALS, as well as rodent models of this disease, exhibit a loss of neurons in the motor nuclei in the brainstem and the ventral horn of the spinal cord that is accompanied

Box 1 | **Amyotrophic lateral sclerosis and frontotemporal lobe degeneration**

Amyotrophic lateral sclerosis (ALS) affects about one-to-two individuals per 100,000 per year, with males being affected somewhat more frequently than females. Most patients are in their fifties or sixties at onset of the disease, but ALS occurs at all ages. ALS usually first affects the limbs (spinal onset), but one-fifth of patients have speech or swallow problems at onset (bulbar onset). Lower motor neurons in the spinal cord and brainstem and upper motor neurons in the cortex are affected in ALS. If clinical signs of only the upper motor neurons are present, the disease is called primary lateral sclerosis (PLS). Many patients with PLS go on to develop evidence of lower motor neuron disease (MND) and then meet the criteria for the diagnosis of ALS. Patients with clinical evidence of lower MND only are said to have progressive muscular atrophy (PMA). Some of these patients develop upper motor neuron signs during disease progression. Most patients with PMA have a disease course that is not much different from that of ALS.

Frontotemporal lobe degeneration (FTLD) affects three-to-four individuals per 100,000 per year and is characterized by abnormalities of behaviour or language. The behavioural variant is denoted as frontotemporal dementia (FTD), and this is the second most common cause of dementia with onset at under 60 years of age. Pathologically, the FTLDs are classified based on the types of inclusions found in cortical neurons in post-mortem examinations (for a review, see REF. 199). In a large proportion of patients with FTLD, these inclusions contain TAR DNA-binding protein 43 (TDP43), which is similar to what is found in motor neurons of patients with ALS[12].

Of patients with ALS, 15% have FTD[4,200] with TDP43-positive inclusions in cortical neurons[201], whereas at least 50% have evidence for more subtle cognitive and/or behavioural dysfunction[4]. Of the patients with FTLD, 15% also have ALS, but many more have some evidence of lower motor neuron involvement[202,203]. This suggests that ALS and FTLD are two ends of the spectrum of one disease (see the figure). The identification of a common cause for ALS and FTLD — the chromosome 9 open reading frame 72 (*C9ORF72*) mutation — confirmed this clinical and pathological concept[107,108]. Patients for which there is clinical evidence for both disorders are said to have ALS-FTLD. Many patients with ALS show some cognitive or behavioural changes but do not meet the criteria for FTLD: they are said to have ALS-Ci/Bi (ALS with cognitive or behavioural impairment). Patients with FTLD can similarly show evidence of mild motor neuron involvement (clinically or on electromyographs) without developing ALS: they are said to have FTLD-MND. Some patients have pure ALS or FTLD.



by a marked neuroinflammatory reaction, consisting of astrocytic activation and microglial proliferation (reviewed in REF. 11). Involvement of the upper motor neurons results in axonal degeneration and secondary loss of myelin in the lateral spinal cord. The remaining motor neurons show inclusions consisting of aggregated proteins. The trigger for this protein aggregation is thought to be protein misfolding caused by mutations, protein damage, such as oxidation, or protein seeding (see below). In many patients with ALS, inclusions contain ubiquitylated and phosphorylated TAR DNA-binding protein 43 (TDP43)[12]. TDP43-positive inclusion-bearing cells often show loss of normal nuclear TDP43 staining[12].

In this Review, we begin by summarizing the initiating events by which mutant proteins may induce disease (FIG. 1). Then, we discuss some of the factors that determine the vulnerability of motor neurons to these events and what role non-neuronal cells have in establishing the ALS phenotype. Last, we consider the pathways that are involved in motor axon failure and retraction.

Unfolded protein response (UPR). This denotes a stress reaction of the cell in response to the accumulation of misfolded proteins in the endoplasmic reticulum. It aims to stall protein translation and increase the production of chaperone molecules to restore proper folding. If it is not successful, the UPR gives way to the apoptotic machinery.

ALS: a failure of proteostasis

Aggregates of disease-causing mutant proteins are hallmarks of neurodegenerative diseases. The current thinking is that in ALS these aggregates or, more likely, their oligomeric complex precursors, disturb normal protein homeostasis and induce cellular stress[1,13]. By interfering with various cellular functions, such as intracellular transport, cytoskeletal architecture and mitochondrial function, cellular stress gives rise to axonal retraction and ultimately cell death (FIG. 1).

SOD1 ***mutations paved the way.*** Superoxide dismutase 1 (*SOD1*) mutations underlie about 20% of familial ALS. SOD1 is an enzyme composed of 153 amino acids and is involved in free radical scavenging, in which more than 150 different mutations have been reported to be pathogenic (Supplementary information S1 (figure)), as has been reviewed recently in REF. 5. Almost all are dominant, but in Scandinavian populations, the D90A mutation is recessive, and the reasons for this are poorly understood. Most are missense mutations, but a few truncation mutations at the very C-terminal part of the protein have been reported, suggesting that most of the molecule must be present for it to have its pathogenic effect.

Mutant SOD1 misfolds and is targeted for degradation through ubiquitylation[14]; however, the misfolded protein seems to escape this regulatory process in the cell (FIG. 2). In addition, mutant SOD1 seems to have toxic effects on the cell's degradation machinery, impairing its two major components: the proteasomal pathway and autophagy[13,15]. In fact, an increase in autophagosomes has been detected in motor neurons in the spinal cord of patients with ALS and of rodent models[16,17], suggesting that the effect of mutant SOD1 on protein degradation observed *in vitro* is also seen *in vivo*, and not only in rodents but also in humans.

Mutant SOD1 accumulates as oligomers and later as aggregates, which lead to a stress response. Then, either because of further accumulation of the mutant protein or because of an additional stressor such as ageing, this response mounts to an unfolded protein response, which is followed in a time-locked fashion by microglial activation[1,18]. In the mutant SOD1 mouse model (BOX 2), various cellular functions, such as mitochondrial energy production, axonal transport and others (as reviewed in REF. 19), have been found to subsequently fail, resulting in axonal retraction and denervation followed by cell death. However, in spite of a wealth of experimental data obtained *in vitro* and in animal models, evidence for what happens in humans is far less overwhelming.

Wild-type SOD1 that is oxidized also misfolds, it is aggregation-prone and toxic to motor neurons, much like mutant SOD1 (REFS 20–22). Interestingly, using a conformation-specific antibody, some[21] but not all[23] investigators found misfolded SOD1 in motor neurons in a subset of patients with SALS that did not have *SOD1* mutations. This finding suggests a role for wild-type SOD1 in SALS, possibly after secondary (oxidative) modification (see below).

Table 1 | **Genetic causes of amyotrophic lateral sclerosis**

Mutant molecule	Gene locus	Inheritance	Predominant phenotype					Estimated% of FALS	Refs
			ALS	ALS±FTLD	PLS	PMA	FTLD		
Enzyme									
Superoxide dismutase 1 (SOD1)	21q22.1	Dominant*	+	Rare		+		20%	231
RNA-binding and/or processing protein dysfunctions									
TAR DNA-binding protein 43 (TDP43)	1p36.2	Dominant	+	+			Rare	1–5%	49,50
FUS	16p11.2	Dominant	+‡	+			Rare	1–5%	84,87
TATA-binding protein associated factor 15 (TAF15)	17q11.1–q11.2	Unknown	+					Unknown	98
Ewing sarcoma breakpoint region 1 (EWSR1)	Unknown	Unknown	+					Unknown	99
Angiogenin (ANG)	14q11.2	Dominant	+	+				<1%	232
Senataxin (SETX)	9q34	Dominant	(+)§					Unknown	233
Repeat expansions									
Chromosome 9 open reading frame 72 (C9ORF72)	9p21.3–p13.3	Dominant	+	+			+	40–50%	107,108
Ataxin 2 (ATXN2)	12q24	Dominant	+			+		<1%	68
Proteostatic proteins									
Ubiquilin 2 (UBQLN2)	Xp11	Dominant		+				<1%	24
Optineurin (OPTN)	10p15–p14	Dominant‖	+	+				<1%	35
Sequestosome (SQSTM1)	5q35	Dominant	+	+			+	Unknown	29
Valosin-containing protein (VCP)	9p13	Dominant	+	+			+	<1%	30
Charged multivesicular body protein 2b (CHMP2B)	3p11	Dominant	+	+			+	Unknown	34
Phosphatidylinositol 3,5-bisphosphate 5-phosphatase (encoded by *FIG4*)	6q21	Dominant	+		+			Unknown	36
Excitotoxicity									
D-amino-acid oxidase (DAO)	12q24	Dominant	+					<1%	146
Cytoskeleton/cellular transport deficits									
Vesicle-associated membrane protein-associated protein B and C (VAPB)	20q13.3	Dominant	+		+	+		<1%	184
Peripherin	12q13.12	Sporadic	+					Unknown	176
Dynactin 1 (DCTN1)	2p13	Dominant	+					Unknown	180
Neurofilament heavy chain (NFH)	22q12.2	Dominant?	+					Unknown	177
Profilin 1 (PFN1)	17p13.2	Dominant	+					Unknown	181
Uncertain									
Spatacsin	15q21.1	Recessive	+‡					Unknown	234
Alsin	2q33.2	Recessive	+‡		+			<1%	185,235
Awaits identification	18q21	Dominant	+					Unknown	236
Awaits identification	20ptel–p13	Dominant	+					Unknown	237
Awaits identification	15q15.1–q21.1	Recessive	+					Unknown	238

ALS, amyotrophic lateral sclerosis; FALS, familial ALS; FTLD, frontotemporal lobe degeneration; NA, not applicable; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy. *Recessive for D90A in Scandinavian population. ‡Juvenile onset possible. §Phenotype more similar to Silver syndrome than to ALS. ‖May be recessive in Japanese population.

Impaired protein degradation and ALS. More evidence for a failure of ubiquitin-dependent protein degradation as a factor in the pathogenesis of ALS comes from the discovery of FALS-linked mutations affecting proteins that are directly involved in proteostasis (TABLE 1; see Supplementary information S1,S2 (figures)).

Mutations in ubiquilin 2 (*UBQLN2*) cause X-linked FALS or FALS-FTLD[24]. UBQLN2 is one of the four members of the ubiquitin-like protein family and has an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin-association (UBA) domain (Supplementary information S1 (figure)). A link

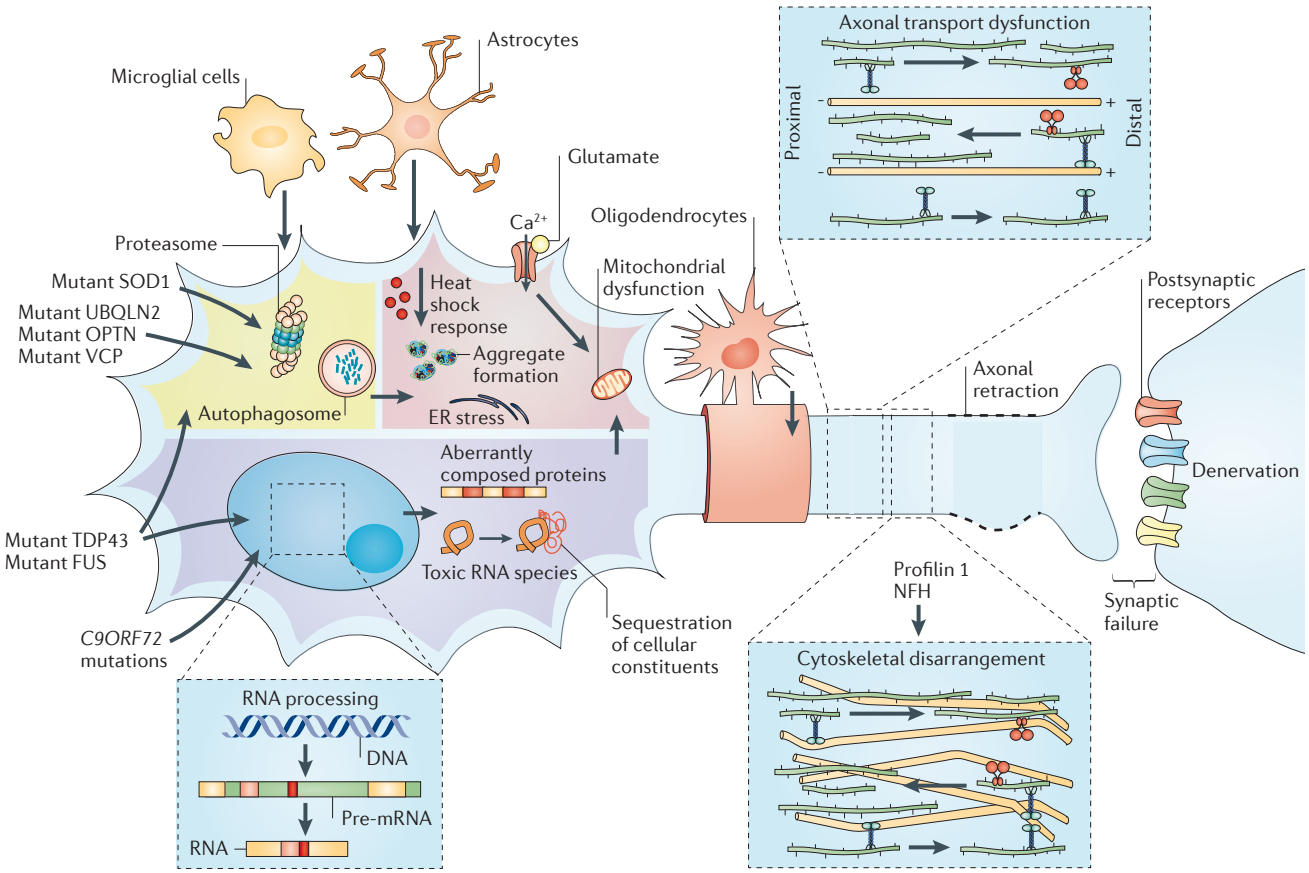


Figure 1 | **Overview of events in the pathogenesis of amyotrophic lateral sclerosis.** Interference with normal proteasomal or autophagic protein degradation is caused by mutations in superoxide dismutase 1 (SOD1), valosin-containing protein (VCP), ubiquilin 2 (UBQLN2), charged multivesicular body protein 2b (CHMP2B), optineurin (OPTN) and, potentially, TAR DNA-binding protein 43 (TDP43) and FUS (amyotrophic lateral sclerosis (ALS) as a proteinopathy; shaded yellow). Disturbance of normal RNA processing, which yields erroneously assembled proteins and toxic RNA species, is caused by mutations in chromosome 9 open reading frame 72 (*C9ORF72*) and potentially *TARDBP*, which encodes TDP43, and *FUS* (ALS as an RNopathy; shaded purple). Through both gain- and loss-of-function mechanisms, these primary pathogenic changes result in progressive cellular failure (shaded light red) that is characterized by protein clumping, aggregate formation, endoplasmic reticulum (ER) stress and Golgi and mitochondrial failure. Axonal architecture (cytoskeleton) and function (transport) fail, and axonal retraction results in denervation of neurons (such as the lower motor neuron) or muscle. Non-neuronal cells modify this process through the loss of their normal effect on the neuron and/or the gain of a toxic effect. Vulnerability factors such as stress response capacity (for example, by the capability to activate heat shock proteins) and susceptibility to excitotoxicity (for example, through the permeability characteristics of the glutamate receptor) determine (or co-determine) which neurons are (mostly) susceptible to these processes. Some ALS-causing mutant proteins may act more downstream in this model (for example, profilin 1 and neurofilament heavy chain (NFH) through a direct effect on the cytoskeleton and D-amino-acid oxidase on excitotoxicity). Axonal attraction systems (for example, semaphorin and vascular endothelial growth factor) and repellent systems (for example, NOGO and ephrins) appear to modify the processes of axonal retraction and denervation.

between the ubiquilin family and neurodegenerative had been demonstrated already, as *UBQLN1* has been genetically associated with Alzheimer's disease[25].

UBQLN2 delivers ubiquitylated proteins to the proteasomal degradation machinery by binding these proteins through its UBA domain and to the proteasome through its UBL domain[26] (Supplementary information S1 (figure)). A region with 12 PXX repeats located N-terminally from the UBA domain harbours the mutations P497H, P497S, P506T, P509S and P525S, which have been associated with FALS[24] (Supplementary information S1 (figure)). Recent studies also identified mutations outside but in the vicinity of the PXX domain[27,28]. UBQLN2 is found in skein-like inclusions (which often also contain other proteins, such as TDP43) in motor neurons in the spinal cord of humans with ALS[24]. Furthermore, it has been detected in inclusions in neurons of patients with SALS or FTLD (in the absence of disease-linked *UBQLN2* mutations)[24] and of patients with *FUS* mutations[27], suggesting that UBQLN2 may have a role in ALS that is not associated with the mutations mentioned above.

On the basis of the identification of mutations in *UBQLN2*, another gene encoding a receptor for ubiquitylated proteins, sequestosome 1 (SQSTM1; also known as p62), was studied as a possible candidate ALS gene. Interestingly, mutations in *SQSTM1* were found

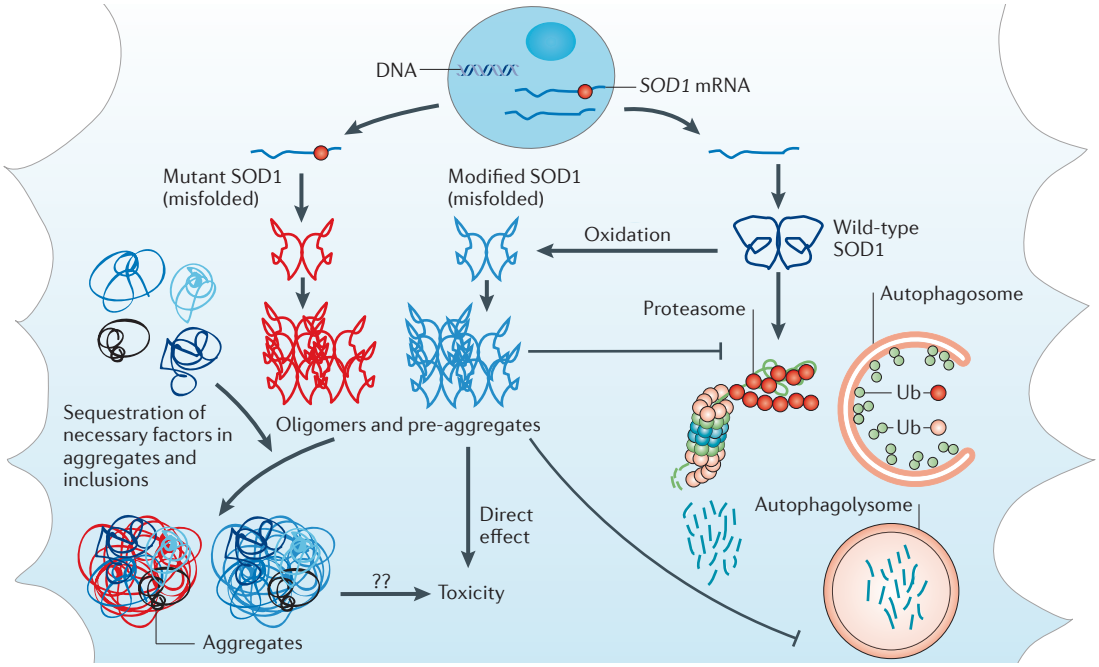


Figure 2 | **Possible pathogenic mechanism of misfolded SOD1-induced toxicity.** Mutant superoxide dismutase 1 (SOD1) and modified wild-type SOD1 misfold and adopt a similar conformation. This misfolded protein seems to escape the normal degradation machinery and forms oligomers, pre-aggregates and then aggregates, and impairs the proteasome and autophagy. Toxicity arises directly through the effects of the misfolded (perhaps oligomerized) protein on processes such as mitochondrial respiration and axonal transport. Toxicity may also arise indirectly through disturbing normal proteostasis. Whether the aggregates or inclusions exert toxic effects — for example, by sequestering factors that are essential for cellular function — is uncertain. Ub, ubiquitin.

in patients with FALS and in individuals with SALS[29], but the pathogenic relevance of these changes awaits further study.

Mutations in the gene encoding valosin-containing protein (VCP; also known as p97) also cause ALS[30]. *VCP* mutations were first identified in patients with inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD)[31]. Exome sequencing subsequently identified mutations in *VCP* in a small set of patients with ALS[30]. VCP is a multifunctional ubiquitin-sensitive chaperone that unfolds proteins and disassembles complexes. It has key roles in endoplasmic reticulum-associated and proteasomal protein degradation and in autophagy[32] (Supplementary information S2 (figure)). In autophagy, VCP establishes the fusion of lysosomes with autophagosomes. The N-terminal region of VCP binds polyubiquitylated substrates[33], and the mutations identified in ALS and IBMPFD are all clustered in this N-terminal part of the protein (Supplementary information S1 (figure))[30,31].

Mutations in the genes encoding charged multivesicular body protein 2b (CHMP2B)[34], optineurin (OPTN)[35] and phosphatidylinositol 3,5-bisphosphate 5-phosphatase (PI(3,5)P2; which is encoded by *FIG4*)[36] are rare causes of ALS (TABLE 1). CHMP2B is involved in the sorting of integral membrane proteins in multivesicular bodies and their subsequent clearance by autophagy[37]. Mutations in *CHMP2B* are dominant and cause ALS-FTLD or FTLD[34,38]. OPTN is an inhibitor of nuclear factor-κB (NF-κB) regulation and is involved in vesicular trafficking, the immune response and transcription regulation. It binds to ubiquitylated receptor-interacting protein (RIP; also known as RIPK1) to prevent NF-κB activation[39]. Dominant missense, recessive deletion and nonsense mutations of OPTN have been identified in both patients with FALS and with SALS[35]. OPTN inclusions can be found in the spinal cord of patients with SALS[40]. *FIG4* is located in vacuolar membranes, regulates the phosphorylated state of PI(3,5)P2 and is essential for normal lysosome function[41]. Dominant *FIG4* mutations have been found in a small subset of patients with ALS, and heterozygosity for a deleterious allele of *FIG4* has been found to be a risk factor for ALS[36].

Whether the proteasomal system or the autophagic machinery is at the heart of the degradation failure in ALS remains to be seen. The intense crosstalk between the two systems makes it difficult to dissect the two experimentally[42]. In mice, motor neuron-specific knockout of the proteasome subunit RPT3 (also known as PSMC4) resulted in motor neuron loss and the formation of aggregates containing TDP43, RNA-binding protein FUS and OPTN, whereas knockout of the autophagy gene *Atg7* did not[43]. This may be circumstantial evidence for a greater sensitivity of the motor neurons to proteasomal dysfunction than to autophagic failure.

Despite this evidence, the effect of the mutations described above in an *in vivo* cell-specific context remains to be elucidated. Indeed, however appealing, the presence of mutations affecting proteins that normally contribute to proteasome function or autophagy does not necessarily

Box 2 | **Animal models for amyotrophic lateral sclerosis**

Various mutant superoxide dismutase 1 (SOD1) rodent models have been generated (for a review, see REF. 204). These animals develop an adult-onset fatal phenotype that is characterized by muscle denervation, astrocytic and microglial activation, and a loss of motor neurons in the ventral spinal cord. Overexpression of mutant SOD1 is needed to induce the phenotype and is a caveat of this model. It can induce experimental artefacts and renders the use of wild-type SOD1-overexpressing controls necessary. In spite of being an excellent model, it remains to be demonstrated that the findings obtained in the mutant SOD1 model are also applicable to other forms of familial amyotrophic lateral sclerosis (ALS) or sporadic ALS. In particular, this mouse model has been criticized for its poor predictive value in terms of potential therapies. However, differences in the design of mouse versus human trials may explain most of the discrepancies. The timing of the start of treatment (in the mouse, this is sometimes as early as postnatal day 7 and, in humans, it can be months or even years after the onset of weakness) and heterogeneity of types of ALS and of patients are only some of these differences.

The effect of the loss of TAR DNA-binding protein 43 (TDP43) has been studied in various models. In flies, knockout of TDP43 induced a neuromuscular phenotype[205] and, in zebrafish, Tdp43 knockdown gave rise to shortened axons with aberrant branching[206]. In mice, homozygous deletion of *Tardbp*, which encodes TDP43, is lethal, but, interestingly, heterozygous knockout mice develop mild motor dysfunction[207]. Selective deletion of TDP43 from motor neurons induces motor neuron loss[208]; the latter finding provides strong support of a loss-of-function in the mechanism of motor neuron degeneration.

Several yeast, *Caenorhabditis elegans*, *Drosophila melanogaster* and zebrafish models have been generated to study the effects of mutant TDP43. In *C. elegans*, yeast and zebrafish, overexpression of mutant TDP43 induces greater abnormalities than overexpression of the wild-type protein[66,206,209], but the generation of reliable rodent models of TDP43 toxicity has proven to be difficult (reviewed recently in REF. 210). Overexpression of mutant or wild-type TDP43 in rodents both induces a phenotype mainly consisting of cortical abnormalities and, in some instances, (minor) lower motor neuron involvement[67,211–214]. Their significance as ALS models is uncertain. In some rodent models, overexpression of even wild-type TDP43 caused extremely aggressive disease that resulted in early death[213]. Interestingly, adeno-associated virus-mediated expression of TDP43 in the cervical spinal cord of cynomolgus monkeys induced progressive motor neuron loss[215]. A conditional TDP43-expressing rat model in which mutant TDP43 is expressed at the adult stage for only a limited time period, showed a fast, progressive decline in motor function. Remarkably, there was a dramatic recovery upon cessation of TDP43 expression at the paralytic stage, suggesting that motor neuron disease is reversible, at least in this specific experimental condition[216].

Deletion of *Fus* in mice is lethal or induces a phenotype unrelated to neurodegeneration, depending on the genetic background[217,218]. Hippocampal neurons generated from these FUS-knockout mice have reduced spine density and defects in spine morphology[219]. In rats, conditional overexpression of FUS^{R521C}, more so than expression of wild-type FUS, resulted in loss of cortical, hippocampal and motor neurons, with denervation and paralysis[220]. Transgenic mice overexpressing wild-type human FUS develop an aggressive motor neuron degeneration, which is characterized by globular and skein-like FUS-positive inclusions in motor neurons[221]. The validity of these models to study ALS remains to be demonstrated.

indicate a deficit of protein turnover as the pathogenic mechanism. These proteins have many other functions that may be more important for the disease mechanism. A protein can have been co-opted during evolution to perform a specific function in motor neurons (or their dendritic or axonal compartments) that is (more or less) different from its canonical function. One example for this comes from another motor neuron disease, spinal muscular atrophy (SMA; as reviewed in REFS 44,45). This disease is caused by a recessive deletion in the gene encoding the protein survival of motor neuron (SMN). Although this protein has an essential function in splicing in the nucleus, it has axonal and synaptic effects in motor neurons[45]. The absence of SMN in mice is lethal[46]. The effect of a shortage of SMN is limited to the motor neuron and induces SMA[45]. Similar findings have been reported for VCP. Through its N-terminal domain and the D1 domain, VCP interacts with various cofactors in a cell- and context-dependent way. One such cofactor is neurofibromin 1 (NF1), and the VCP–NF1 complex affects neuronal morphology and spine formation[47]. Mutations in *VCP* disrupt the protein's interaction with NF1, leading to impaired spinogenesis and synaptogenesis[47].

In conclusion, there is evidence that dysproteostasis caused by malfunction of the proteasomal and autophagic degradation machinery contributes to the cellular failure that characterizes motor neurons in certain forms of ALS. This dysfunction is likely to be multifactorial in origin, involving both gain-of-function and loss-of-function mechanisms.

Mutations in RNA-binding proteins cause ALS

A surprising number of proteins linked to ALS are directly or indirectly involved in RNA processing (for reviews, see REFS 5,48) (TABLE 1). However, it was the identification of ALS-causing mutations in the genes encoding TDP43 and the RNA-binding protein FUS, both of which are involved in pre-mRNA splicing, RNA transport and RNA translation, that led to the idea that aberrant RNA metabolism contributes to ALS pathogenesis.

TARDBP *mutations change the theme: RNA processing and ALS.* Mutations in *TARDBP*, which encodes TDP43, are a rare cause of dominant ALS[49,50]. This gene was investigated as a candidate gene for ALS because TDP43 is present in neuronal inclusions in most patients with ALS or FTLD without tau inclusions[12]. Almost all of these mutations are located in exon 6 of *TARDBP*, which encodes the C-terminal glycine-rich part of the protein (Supplementary information S1 (figure)). Most are missense mutations, but there are a few deletion mutations that give rise to a protein truncated at the very C-terminal[5,51]. Notably, *TARDBP* mutations give rise to ALS with or without FTLD but only rarely to FTLD[5].

TDP43 is part of the family of heterogenous ribonucleoproteins and is normally located in the nucleus. It binds DNA and RNA and is a multifunctional protein involved in transcription, RNA splicing and transport. Its role in mRNA splicing has gained the most attention[51–53]. TDP43 contains two RNA recognition motifs (RRMs) and binds to intronic GU-rich sequences that often lie far away from the intron–exon boundaries[54,55]. As many pre-mRNAs contain such repeats, it is not surprising that TDP43 targets more than 6,000 mRNAs[54]. Knockdown of TDP43 changes the abundance of more than 600 mRNAs and the splicing of 965 mRNAs[54] and affects the splicing and stability of many non-coding RNAs and microRNAs (miRNAs)[54–57]. TDP43 has a bipartite nuclear localization signal (NLS) between the N terminus and RRM1, and one nuclear export signal (NES) in RRM2 (Supplementary information S1 (figure)). TDP43 shuttles between the nucleus and the cytoplasm. In the cytoplasm, this protein associates with granules in which RNA is translationally silenced

and transported to a target site (for example, the spine), where translation is resumed. In response to stressors such as starvation or oxidative stress, TDP43 is detectable mainly in the cytoplasm, where it is incorporated into stress granules[58,59] (FIG. 3a). In these, mRNA is translationally inactive, which allows the cell to prioritize protein synthesis and adequately respond to the stressor. The glycine-rich domain of TDP43 is necessary for its accumulation into stress granules through its prion-like properties[58]. When stress resolves, stress granules disaggregate with the help of disaggregases and chaperones, allowing TDP43 to relocate to the cell nucleus, although this process is poorly understood[60].

It is still uncertain whether and how this function relates to the mechanism through which mutant TDP43 causes ALS. It is thought that stress granules constitute a pro-aggregation environment and go on to give rise to the inclusions that are typically seen in ALS[61] (FIG. 3). Stress granule-associated factors such as T cell intracellular antigen 1 (TIA1), nucleolysin TIAR and eukaryotic translation initiation factor 3 subunit (eIF3) are indeed present in TDP43-containing aggregates[62–65]. TDP43 is therefore sequestered in these aggregates in the cytoplasm, which leaves the nucleus devoid of it. In the brain, cytoplasmic TDP43 undergoes secondary modifications such as hyperphosphorylation, ubiquitylation and processing into smaller fragments[12] (reviewed in REF. 51). Whether these secondary modification steps of TDP43 are necessary for ALS pathogenicity is not clear.

These data imply that TDP43 causes pathogenesis in a two-step manner (FIG. 3). The first step involves the exit of TDP43 from the nucleus and the second step involves the irreversible formation of stress granule-based aggregates. Disease-associated mutations cause a shift of TDP43 location from nucleus to cytoplasm and increase its aggregation propensity[66], but it is uncertain whether these changes are sufficient to initiate this vicious circle *in vivo*. Indeed, in a recently developed mouse model, the expression of mutant TDP43 induced a phenotype in which there was no formation of aggregates or abnormal processing of the mutant protein and even no loss of TDP43 from the nucleus[67].

This sequence of events allows for both a gain-of-function and a loss-of-function mechanism of pathogenicity of ALS. The increased cytoplasmic aggregation (gain-of-function) results in nuclear depletion of TDP43, which induces abnormalities of RNA processing (loss-of-function)[54,55] (FIG. 1,3b). The finding that mutant TDP43 enhances normal TDP43 splicing function for some RNA targets but loss of function for others[67] brings the two mechanisms together at the molecular level. Furthermore, the sequestration of TDP43 in the cytoplasm may be pathogenic, as it acts as a sink for proteins or RNAs (FIG. 3). Transgenic animals have been generated

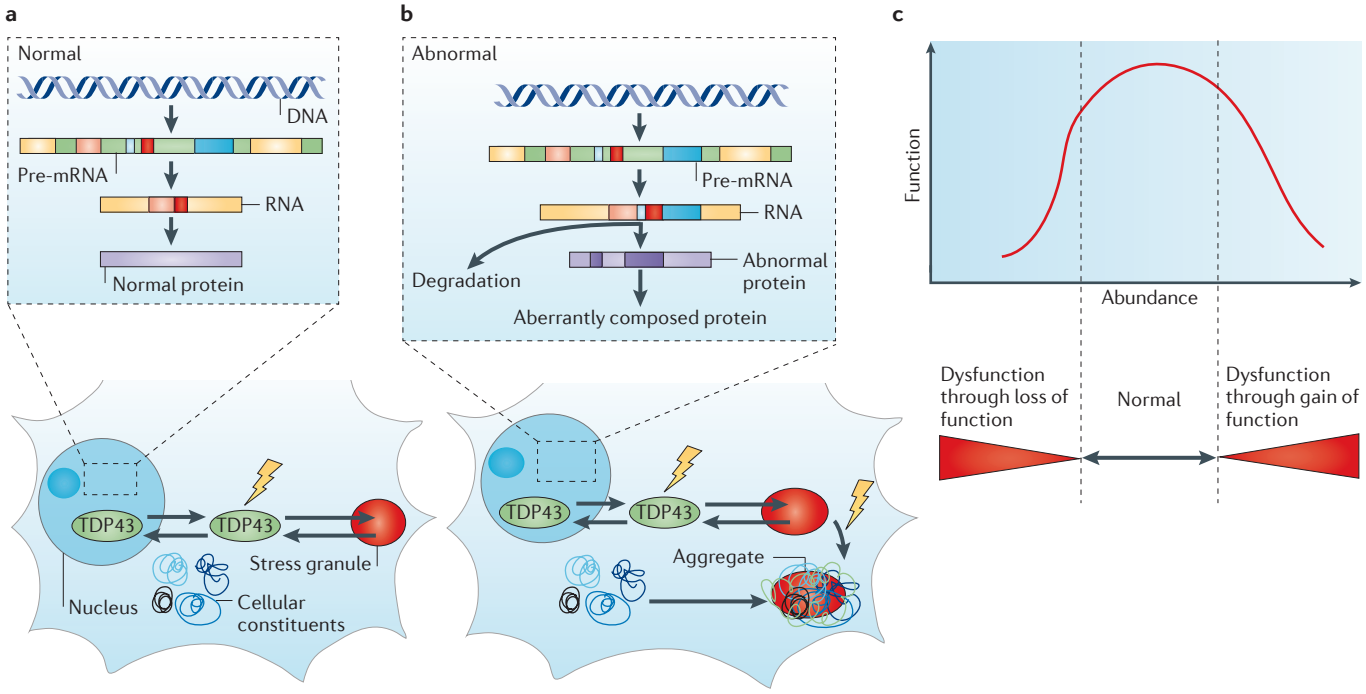


Figure 3 | **Possible pathogenic mechanism of mutant TDP43.** **a** | Normal function of TAR DNA-binding protein 43 (TDP43). TDP43, together with other factors, is essential for normal RNA processing in the nucleus. Upon stress (represented by the lightning symbol), it leaves the nucleus and accumulates in stress granules. If stress resolves, TDP43 is released from these granules and re-enters the nucleus. **b** | Mutations favour TDP43 leaving the nucleus and its irreversible accumulation in stress granules; additional stress factors may determine this irreversibility. As a result, mRNA splicing fails. RNAs are degraded (resulting in the absence of certain proteins) or erroneously spliced RNAs give rise to proteins containing aberrant sequences. Toxic RNA fragments may leave the nucleus and have hazardous effects in the cytoplasm. Other proteins (and RNAs) may be sequestered in the TDP43 accumulations. **c** | The abundance of TDP43 in the cell is tightly regulated; slight disturbances in one direction or the other may induce cellular dysfunction.

to study this gain- versus loss-of-function that is mediated by TDP43 (BOX 2). Studies using yeast, flies and chickens suggest that RNA binding but not mislocalization is necessary for TDP43 to be toxic[68,69]. In addition, it is notable that TDP43 binds and modulates the function of VCP, indicating that interfering with the physiological function of TDP43 can result in the dysfunction of proteins involved in protein degradation[70]. The interaction between TDP43 and VCP may therefore bring together these two important pathogenic mechanisms in ALS.

All cellular and animal models that have been developed so far suggest that both TDP43 deficiency and TDP43 (wild-type and mutant) overexpression are hazardous to the cell (BOX 2; FIG. 3c). Thus, it seems as if a tight regulation of the abundance of TDP43 is necessary. Of note in this regard is the finding that TDP43 autoregulates its own expression: it binds to its own 3′ untranslated region (3′ UTR) and induces degradation of its mRNA, most likely through nonsense-mediated RNA decay or exosome-mediated degradation[54,71]. The sequestration of TDP43 in the cytoplasmic granules therefore increases the expression of TDP43 and the toxicity related to it. This means that factors that slightly affect TDP43 abundance may have significant hazardous effects, even in the absence of mutations in this protein. The notion that simply too much TDP43 is sufficient to induce neurodegeneration would be greatly supported by finding duplication mutations of *TARDBP* in FALS, similar to what has been described for α-synuclein in Parkinson's disease[72] or amyloid precursor protein in Alzheimer's disease[73]. Such mutations, however, have yet to be identified.

The hazardous effects of TDP43 dysregulation may be partially mediated through the NF-κB pathway. TDP43 acts as a co-activator of p65 and increases the vulnerability of motor neurons to toxic inflammatory mediators. Treatment with withaferin A — albeit not a very specific inhibitor of NF-κB activity — attenuatued denervation in TDP43-overexpressing mice[74].

Studies in yeast have identified several modifiers of toxicity of mutant TDP43, including ataxin 2 (ATXN2), which enhances TDP43 toxicity[68]. ATXN2 causes spinocerebellar ataxia type 2 (SCA2) through the presence of a polyglutamine (polyQ) repeat, which is encoded by a CAG trinucleotide repeat expansion (≥33 repeats) in its first exon[75]. On the basis of findings in yeast, a genetic study was performed in humans, which showed intermediate-length repeats (27–33 repeats) in *ATXN2* to be associated with ALS[68,76–79]. PolyQ expansions in ATXN1 (REF. 77), but not ATXN3 (REF. 79) were also found to be associated with ALS. Subsequently, even pathogenic *ATXN2* mutations (≥33 repeats) were identified in patients with FALS, adding *ATXN2* to the list of genes that cause ALS[76]. The repeats associated with ALS are interrupted by one-to-three CAA codons (which also encode a glutamine residue), and the number of interruptions affects ALS disease onset[80]. At least *in vitro*, intermediate-length ATXN2 polyQ expansions enhance the processing of TDP43 (REF. 81).

Another protein that enhances the toxicity of TDP43 is debranching enzyme homologue 1 (DBR1). In yeast, *DBR1* deletion abrogates TDP43 toxicity[82]. DBR1 is a 2′-5′ phosphodiesterase that debranches circular lariat introns that are formed during pre-mRNA splicing. These linearized RNAs are then degraded by RNases. Surprisingly, in the absence of DBR1, these intronic sequences accumulate in the cytoplasm, where they may sequester TDP43 and thus keep it from hazardous interactions with other cellular constituents, such as RNAs and RNA-binding proteins. Knockdown of *DBR1* in a human neuronal cell line and in primary rat neurons similarly rescued TDP43 toxicity[82]. These findings demonstrate the usefulness of models such as yeast. It is expected that yeast screening will identify more genes that cause ALS or genes that modify the toxicity of genes that are already known to cause ALS.

FET *gene mutations.* FUS belongs to the FET family of proteins, to which TAF15 (TATA-binding protein associated factor 15) and EWSR1 (Ewing sarcoma breakpoint region 1) also belong[83]. Mutations in *FUS* cause ALS and almost all are dominant, although the first one was identified in a Cape Verdian recessive pedigree (FUS^{H517Q})[84]. Like TDP43, FUS is an RNA-binding protein that contains a prion-like domain. FUS has multiple RNA-binding domains with RGG motifs, an RRM domain and a zinc finger domain that mediates RNA binding (Supplementary information S1 (figure)). The N-terminal QGSY-rich region, together with the glycine-rich region, establishes a prion-like domain that determines the aggregation characteristics of FUS[85].

Almost all mutations in *FUS* that cause ALS are missense in nature and affect the C-terminal NLS-containing part of the protein or the glycine-rich region (reviewed in REFS 5,86; see Supplementary information S1 (figure)). Some of these mutations result in a C-terminal-truncated protein[86]. *FUS* mutations have been found in patients with ALS who may or may not also have FTLD, but not unequivocally in patients with 'pure' FTLD[86]. In the brains and spinal cord of patients with *FUS* mutations, FUS-positive inclusions are present[84,87]. FUS^{P525L}, $FUS^{c.1554–1557delACAG}$ and, to a lesser degree, FUS^{R495X} are associated with an aggressive phenotype of ALS[88–90], which affects patients in the first or second decade of life and is fatal after 1–2 years. The motor neurons of these patients are characterized by the presence of basophilic inclusions[89,90].

Although FUS is a nuclear protein, it is also present in the cytoplasm and is concentrated at the postsynaptic density[91,92]. It uses transportin as a carrier to shuttle between the nucleus and cytoplasm[92,93]. Most mutations in *FUS* disrupt transportin-mediated nuclear import and cause mislocalization of the FUS protein in the cytoplasm, where it is recruited into stress granules that may form inclusions[93]. Interestingly, arginine methylation (in the most distal arginine- and glycine-rich domain) by protein N-arginine methyltransferase 1 modulates nuclear import of FUS, and its inhibition rescues the transport deficit[94].

Different FUS transgenic animal models have been generated (BOX 2). Genetic screening studies in yeast showed that genes involved in stress granule

assembly and RNA metabolism can modify FUS toxicity[85]. Surprisingly, mutations do not seem to enhance the aggregation propensity or toxicity of FUS[85].

FUS binds to more than 5,500 genes through a GUGGU-binding motif, and depletion of FUS alters the splicing of nearly 1,000 mRNAs[95]. FUS binds to very long intron sequences, as does TDP43 (REFS 54,55,95). It seems to stabilize these very long pre-mRNAs to which it binds co-transcriptionally and regulates their splicing. Most targets of FUS are different from those of TDP43 (REF. 95). Interestingly, 41 genes were upregulated and 45 genes were downregulated upon FUS depletion as well as upon TDP43 depletion, and the ones that were downregulated typically contained long introns and binding sites for both proteins[95]. Several of these genes encode known neuronal proteins and decreased levels of three of these (PARK2, SMYD3 and KCNIP4) were found in the remaining motor neurons of patients with SALS.

It is currently unknown whether mutant FUS causes toxicity owing to a toxic gain-of-function mechanism, a loss-of-function mechanism or both. A two-hit model, which is similar to the one mentioned above for TDP43, has been proposed to underlie FUS toxicity[96]. However, TDP43 and FUS do not colocalize in cytoplasmic aggregates, and their binding partners are different[85,95], suggesting that their pathogenic mechanisms may be quite disparate. Of notice, similar to what is seen with TDP43 (see above), ATXN2-containing intermediate-length repeats enhance FUS toxicity in cellular models[97].

RNA-binding and prion-like domains establish a theme. TDP43 and FUS are both RNA-binding proteins that contain a prion-like domain (which is necessary for stress granule formation). Therefore, proteins with these two characteristics were considered as candidates for the underlying cause of ALS and were identified using bioinformatics tools in combination with a yeast toxicity study[98]. One such candidate, the FET protein TAF15, exhibits similar toxicity and aggregation propensity as TDP43 and FUS in yeast and in flies, and cytoplasmic TAF15-positive puncta have been observed in motor neurons of patients with SALS[98]. Sequencing of the third FET member, EWSR1, also yielded mutations in patients with ALS, but again, segregation in families remains to be shown[99].

Prion-like domains and the progressive nature of ALS. In addition to its role in the accumulation of TDP43 and FUS in stress granules, the prion-like domain in these proteins may also contribute to the progressive, spreading nature of ALS (reviewed in REF. 100). Proteins harbouring a prion-like domain promote aggregation by acting as a template to induce the conversion of natively folded proteins and thus trap the normal protein in the aggregate. Proteins different from the mutant one can also be recruited in these aggregates through a process called cross-seeding, which may explain why multiple proteins are found in the aggregates in patients with ALS[101]. SOD1, TDP43 and FUS have been shown to have such prion-like characteristics[102–104]. It has been suggested that this complex of aggregated and aggregation-inducing proteins is taken up by neighbouring cells in which they induce further aggregation. In this way, the disease is propagated from cell to cell. If this model is correct, ALS is expected to propagate or spread through contiguity. This may fit with the clinical observation that ALS usually affects regions in contiguity with the site of onset; that is, it does not skip anatomical regions[105]. Definite proof for this hypothesis in ALS is still awaited, but α-synuclein fibrils have been demonstrated to be able to recruit α-synuclein into Lewy bodies and induce spreading neurodegeneration *in vivo*[106].

The identification of *TARDBP* and *FUS* mutations in ALS challenged the proteocentric thinking about the pathogenesis of this disease and suggested that ALS and FTLD are biologically related diseases. However, it was the identification of the mutation in chromosome 9 open reading frame 72 (*C9ORF72*) as a cause for ALS that really meant a leap forward for the field, as it established a molecular link between ALS and FTLD. It meant a breakthrough for clinical ALS genetics because of the frequency of this mutation in both ALS and FTLD, and placed RNA toxicity in the spotlight.

C9ORF72 mutations: is ALS a ribonucleopathy?

The mutations in *C9ORF72* that cause ALS are nucleotide repeat expansion mutations[107–109]. *C9ORF72* contains a GGGGCC hexanucleotide (G_4C_2) sequence that is located between two transcription initiation sites (Supplementary information S1 (figure)). In most normal individuals, the G_4C_2 sequence is repeated mostly two-to-five times. It is rarely repeated more than five times and is never repeated more than 30 times. An abnormal expansion of this sequence is found in about 40% of families with ALS and in about 7% of patients with supposed SALS[110]. The length of the expanded repeat is still uncertain but may consist of several hundreds or even thousands of repeats. This expansion is also found in patients with FTLD without ALS (that is, in 25% of patients with familial FTLD and 6% of patients with supposed sporadic FTLD)[107–110]. Many, but not all, patients with this mutation carry a founder haplotype[110,111].

The normal function of the presumably cytoplasmic protein C9ORF72 is unknown, but it is structurally related to DENN domain proteins, which are highly conserved GDP–GTP exchange factors for RAB GTPases[112]. Preliminary findings suggest that two isoforms are generated: a 481-amino-acid protein encoded by exons 2–11 and a shorter one of 222 amino acids encoded by exons 2–5 (Supplementary information S1 (figure))[107].

Bulbar onset is frequent in C9ORF72-associated ALS, as is frontotemporal involvement, which is usually of the behavioural variant[108,109,113–116]. Adult-onset psychosis, Parkinsonism and ataxia can be part of the presenting clinical picture[117,118]. The penetrance of the mutation is probably 50% around the age of 60 years and nearly 100% above the age of 80 years[110]. Many questions about the genotype–phenotype correlation remain to be answered. One question concerns the intergenerational instability of the repeat. The age-of-onset has been reported to be 7 years younger in the subsequent generation, but this may reflect ascertainment bias[113]. Somatic instability certainly occurs and explains the difference in repeat length found in brain

Bulbar onset
The onset of ALS in the bulbar region: that is, the muscles of pharynx, tongue and larynx. The patient usually presents with speech and swallowing problems.

Intergenerational instability
This refers to the mechanism through which certain repeat expansions can increase in length during gametogenesis.

Somatic instability
Repeat expansions can grow longer in dividing cells. This explains why the length of an expansion can be variable between tissues and within cells within a tissue.

versus blood cells[107]. Another question concerns a possible effect of length of expansion and of homozygosity on disease severity, and the possibility that the normal allele influences the pathogenicity of the repeats. Last, it needs to be elucidated whether intermediate-length repeats represent pre-mutations. Intriguingly, in several patients with *C9ORF72* mutations, a second mutation has been found in another gene (*TARDBP*, for example)[119]. The biological significance of this finding is unknown, but it suggests that in some families, ALS may be oligogenic in aetiology[120]. Such additional mutations may also contribute to the phenotypic variability seen with *C9ORF72* mutations.

Patients with ALS who carry *C9ORF72* hexanucleotide expansions have features that are typical of ALS, with TDP43-positive inclusions in the remaining motor neurons, in the cortex and in the hippocampus[107]. In the hippocampus and cerebellum, pathognomonic inclusions are found that are TDP43-negative but SQSTM1-positive and also contain ubiquitin and UBQLN2 (REF. 115). In the cerebellum, these may be related to the ataxia that occurs in some patients with ALS[115,121].

Is RNA toxicity causing ALS? How repeat expansions in *C9ORF72* cause ALS remains to be elucidated (FIG. 4). In the few ALS patients with a *C9ORF72* expansion that have been studied, the levels of *C9ORF72* mRNA were reduced by 50%[107,109], suggesting that the expanded allele does not generate mature mRNA; confirmation of this assertion at the protein level is difficult as the available antibodies for this protein are far from reliable. Thus, the *C9ORF72* expansion may be a loss-of-function mutation. This would be similar to what is seen in Friedreich's ataxia. This spinocerebellar degeneration is caused by a GAA repeat in the first intron of the gene encoding frataxin, resulting in absence of this protein[122]. Of note, Friedreich's ataxia is a recessive disorder, whereas ALS caused by *C9ORF72* mutations is dominantly inherited. One therefore has to invoke haplo-insufficiency. Again, examples of neurodegenerative diseases that are caused by haplo-insufficiency are known to exist, such as FTLD caused by dominant loss-of-function mutations in progranulin (*PGRN*)[123,124].

Fluorescence *in situ* hybridization experiments using a $(G_4C_2)_4$ probe have demonstrated that the expanded hexanucleotide repeat forms nuclear RNA foci in neurons in the frontal cortex and spinal cord in patients with *C9ORF72* mutations[107]. Pre-mRNA containing the expansion may thus exert a deleterious gain-of-function effect (FIG. 4) that is similar to what is seen in myotonic dystrophy. Myotonic dystrophy is a dominant muscle disease, which has two forms: myotonic dystrophy type 1 and myotonic dystrophy type 2. Both are caused by expanded repeats that have toxic gain-of-function effects. Myotonic dystrophy type 1 is caused by a CTG repeat located in what is transcribed as the 3′ UTR of the mRNA for myotonic dystrophy protein kinase (DMPK). Myotonic dystrophy type 2 is caused by a CCTG repeat in the first intron of the gene encoding zinc finger protein 9 (ZNF9; also known as CNBP)[125–127]. These repeats are a sink for nuclear RNA-binding proteins that then are unavailable for the correct splicing of other mRNAs, such as troponin, the muscle chloride channel and tau[128–130]. This results in the expression of proteins that erroneously contain or lack certain sequences. Their impaired function underlies the cardiac abnormalities, myotonia, cognitive dysfunction and the many other problems characteristic of this disease. Musclebind-like 1 (MBNL1) is one of the RNA-binding proteins that are sequestered by the repeat[131]. MBNL1 appears to have a central role in the generation of the phenotype, as the clinical phenotype of mice deficient in this factor mimics the myotonic dystrophy phenotype[132,133]. The binding of proteins to the expanded G_4C_2 may be facilitated by the fact that the repeat forms G-quadruplexes[134]. If *C9ORF72* pre-mRNA containing the expansion gains a toxic function, the question arises whether the expansion in itself is toxic or whether the rest of the *C9ORF72* mRNA needs to be present: in other words, whether the toxicity of the repeat is 'gene context-dependent'.

These two mechanisms (haplo-insufficiency versus gain-of-function) are not mutually exclusive and do not exclude other mechanisms for the repeat expansion to cause FALS. One mechanism that may also need to be taken into account is repeat-associated non-ATG (RAN) translation[135]. RAN translation occurs across long, hairpin-forming repeats. In myotonic dystrophy

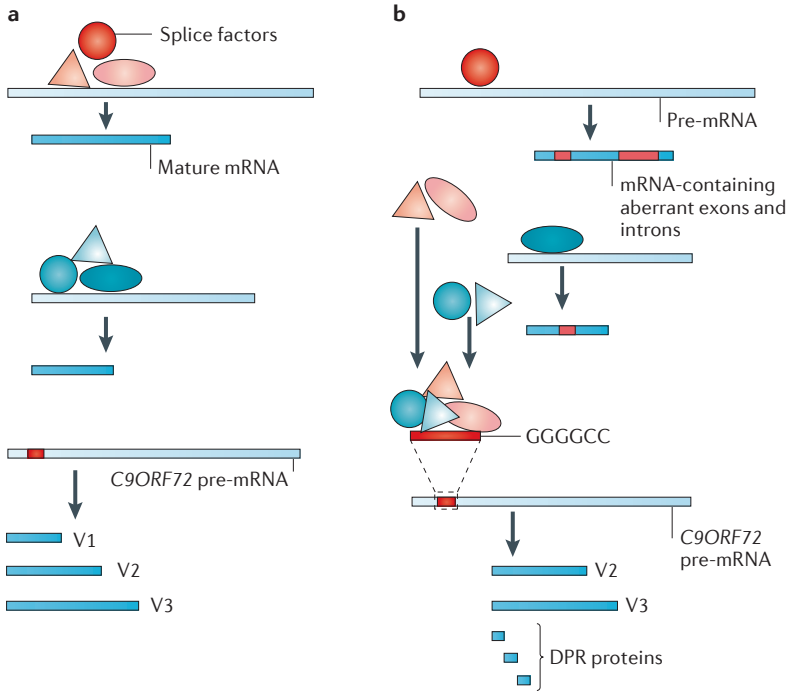


Figure 4 | **Possible pathogenic mechanism of motor neuron degeneration induced by the *C9ORF72* repeat expansion.** **a** | RNA-binding proteins are pivotal for normal splicing. Different splicing factors (in shades of red and blue) associate with a whole range of pre-mRNAs, enabling their correct splicing. Transcription from non-expanded chromosome 9 open reading frame 72 (*C9ORF72*) leads to the generation of at least three different RNAs (V1–V3). **b** | In *C9ORF72* repeat expansion carriers, abnormal recruitment of these splice factors to the repeat expansion results in aberrant splicing of a whole range of genes, generating transcripts containing exons that are meant to be spliced out or lacking exons that are meant to be retained. The expansion is likely to interfere with the generation of V1 but may generate normal V2 and V3 transcripts. Alternatively, the expansion may generate insoluble and potentially toxic dipeptide repeat (DPR) proteins through repeat-associated non-ATG (RAN) translation.

type 1, it results in the accumulation of homopolymeric polyQ proteins that may contribute to the pathogenesis of this disorder[136]. Two recent reports have indeed identified dipeptide repeat (DPR) proteins, which are most likely generated through RAN translation from expanded G_4C_2 RNA, in aggregates of patients with C9ORF72$^{(G4C2)exp}$-associated ALS and/or FTLD[137,138]. The pathogenic significance of these DPR proteins remains to be demonstrated.

ALS affects motor neurons and a subpopulation of neurons in the frontal and temporal cortex. Nevertheless, mutant proteins that cause FALS are expressed ubiquitously. The question therefore arises: why are these neurons are more susceptible to the pathogenic effects induced by these mutant proteins? This issue of neuronal vulnerability is a central to neurodegenerative disorders, and below, we consider some of the factors that are thought to contribute to its mechanism.

Neuronal vulnerability in ALS

Several levels of selective neuronal vulnerability need to be considered. First, sensory and autonomic neurons as well as cerebellar neurons are almost always either preserved or affected to a very mild and subclinical degree. Second, motor neurons and frontotemporal neurons are affected to variable degrees. Even within one family, some patients with a *C9ORF72* mutation may mainly have ALS, whereas others, harbouring the same mutation, mainly have FTLD[107–109]. Third, not all types of motor neurons are affected in ALS. Oculomotor neurons and neurons in Onuf's nucleus (sacral motor neurons innervating sphincter muscle) are unaffected in this disease, except in rare patients who have exceptionally long survival because of ventilatory support[139,140]. Last, there are remarkable differences in vulnerability even among spinal ventral horn neurons, with large motor neurons being more affected than smaller ones. These large motor neurons have large fields of innervation, limited sprouting capacity and physiologically behave as fast fatiguable units. Fast fatigue-resistant motor neurons are less vulnerable. Slow, tonic motor neurons are most resistant in ALS, surviving in the mutant SOD1 mouse until the very late stages of disease[141]. These are small motor neurons, innervating small neuromuscular fields, with notable sprouting capacity[2,141] (reviewed in REFS 1,140). In mutant SOD1 mice, large fast fatigable motor neurons generate an unfolded protein response before fatigue-resistant motor neurons do and long before small slow, tonic motor neurons[18]. This sequence of events may reflect a difference in the stress-coping capacity of these different types of neurons. Furthermore, large motor neurons express higher levels of the ephrin receptor EPHA4 than small motor neurons, and deletion of EPHA4 increases the regeneration capacity of motor neurons[142]. Blockade of this receptor in the mutant SOD1 mouse rescues large motor neurons to a greater extent than small ones, suggesting that the ephrin system may contribute to differences in neuronal vulnerability.

Vulnerability that is due to limited stress-coping capacity and regeneration potential may be further enhanced by the high susceptibility of motor neurons to glutamate-induced excitotoxicity (reviewed in REF. 143). Permeability of a subtype of glutamate receptors to calcium is a major determinant of this susceptibility. Furthermore, in humans with ALS and in rodent models of the disease, there is a well-documented loss of excitatory amino acid transporter 2 (EAAT2; also known as SLC1A2)[144,145]. Insufficient clearance of glutamate by this glial glutamate transporter may result in increased or prolonged glutamatergic stimulation of the motor neuron. Interestingly, a mutation in the gene encoding D-amino-acid oxidase (DAO) has been reported to segregate with disease in a family with ALS[146] (TABLE 1). D-serine, a substrate for this enzyme, is a modifier of glutamate transmission[147]. Dysregulation of the serine biosynthetic pathway has also been reported in the mutant SOD1 mouse[148]. Excitotoxic motor neuron death is mediated by calcium entry through AMPA-type glutamate receptors. The glutamate receptor 2 (GluR2) subunit of such receptors determines calcium permeability[149]. Motor neurons express this subunit to a limited extent only and are thus more prone to lethal calcium-overload upon glutamatergic stimulation[150]. Furthermore, in contrast to oculomotor neurons, the calcium-buffering capacity of spinal motor neurons is limited, increasing their susceptibility to excitotoxicity even further[151].

Finally, another mechanism that may contribute to motor neuron vulnerability was suggested in studies carried out on TDP43 and FUS. TDP43 and FUS bind to long intronic stretches of RNA. Interestingly, nervous tissue contains relatively longer intron-containing transcripts than other tissues. This may be one explanation for why neurons are particularly susceptible to TDP43 and FUS abnormalities[54,55,95].

Although motor neuron loss is the major characteristic of ALS, sustained activation of a neuroinflammatory response executed by a diverse range of glial cells is commonly found in the spinal cords of patients with ALS and of rodent models. As these cells contribute to the progressive motor neuron degeneration phenotype, the mechanism of neurodegeneration in ALS is said to be non-cell autonomous.

Glial cells in the pathogenesis of ALS. Studies of mutant SOD1 mice have shown that cell death in ALS is non-cell autonomous, as the astrocytes and microglial cells that surround motor neurons contribute to disease onset and progression[19,152–156]. These glial cells become increasingly activated as the disease progresses in both animal models and patients: a phenomenon called neuroinflammation. This reaction can have both deleterious and protective consequences (as reviewed in REF. 11). Recent evidence has greatly strengthened this concept.

The fact that ALS astrocytes can induce motor neuron death has been demonstrated *in vivo* and *in vitro*. Of notice, this toxic effect was not only shown for astrocytes from the mutant SOD1 mouse but also for astrocytes from patients with SALS[157]. Interestingly, wild-type SOD1 was found to elicit the toxic effect of astrocytes taken from patients with SALS, as knocking down SOD1 in these astrocytes abrogated toxicity[157].

This remarkable but puzzling discovery adds to the mounting evidence that wild-type SOD1 has a crucial role in SALS.

The dual (protective and toxic) effects of microglial cells in ALS are well established but certainly not fully elucidated and have been reviewed recently in REF. 158. Already at early disease stages, microglia recruit peripheral monocytes to the CNS[159]. These monocytes are polarized to a macrophage phenotype in ALS mice and in patients with ALS and promote neuronal loss[159]. The role of T cells in ALS is only beginning to emerge. T cells infiltrate the spinal cord of patients with ALS and SOD1 mutant mice[160–162]. $CD4^+$ T helper cells appear to modulate the inflammatory response beneficially, as deletion of these cells promotes neurotoxic action of microglia and astrocytes and a worsened disease outcome in mutant SOD1 mice[160,161]. Regulatory T cells that infiltrate the spinal cord at the early symptomatic disease stages seem to have a beneficial influence by slowing disease progression, but their neuroprotective influence ultimately fails[163]. Likewise, in patients with ALS, regulatory T cells influence disease progression rates: an early reduction in the expression of the regulatory T cell transcription factor FOXP3 was found to be predictive of rapid disease progression[164].

Recent evidence has revealed a role for yet another glial player in ALS: the oligodendrocyte. Oligodendrocytes play a pivotal part in axonal maintenance by providing the neuron with lactate through the monocarboxylate lactate transporter MCT1 (REF. 165). Loss of oligodendrocytic MCT1 is toxic to motor neurons *in vitro* as well as *in vivo*. In the mutant SOD1 mouse, oligodendrocytes degenerate but are continuously replaced by newly differentiated NG2 cells[166]. However, these newly differentiated cells appear to be insufficient in terms of metabolic support, as suggested by the finding that MCT1 expression is decreased in patients with ALS as well as in the mutant SOD1 mice[165,166]. This lowered MCT1 expression and the ensuing decrease in trophic support may at least contribute to the motor neuron loss in ALS.

Deletion of mutant SOD1 in myocytes does not influence motor neuron degeneration in mutant SOD1 mice[167]. However, this finding does not mean that myocytes do not contribute to the disease phenotype that is observed in these animals. Indeed, miR-206, a muscle-specific miRNA, is highly upregulated in the mutant SOD1 mouse, and deleting miR-206 from this mouse model accelerates disease progression[168]. Similarly, deletion of mutant SOD1 from endothelial cells did not affect disease phenotype in mutant SOD1 mice in spite of the endothelial damage and microhaemorrhages that were found in the spinal cord[169,170]. Therefore, the issue of whether and how a vascular factor contributes to ALS remains an area of intense research.

Overall, these data indicate that glial cells mainly affect disease progression, whereas disease onset is determined within the motor neuron itself. However, it should be noted that not all data are consistent with this hypothesis. A study using chimeric mice in which all neurons and oligodendrocytes expressed mutant SOD1 but all other cell types were a mixture of mutant and non-transgenic cells showed that disease onset was determined by the level of chimerism of the glial cells rather than the mutant SOD1 expression in neurons and oligodendrocyte lineage cells[171]. Furthermore, although all data mentioned above were obtained with the $SOD1^{G37R}$ mouse, deleting the mutant protein from astrocytes in the dismutase-inactive $SOD1^{G85R}$ mouse affected disease onset[155]. Thus, the biological reality may be more complex than what can be explained by a simple dichotomy.

Axon failure in ALS

Studies in rodents and humans have demonstrated that axonal retraction and denervation occur long before the loss of cell bodies and that intrinsic axonal mechanisms actively determine the phenotype in ALS[2,3]. A number of factors involved in axonal architecture and/or function has been shown to have a role in ALS (reviewed in REF. 172). We mention some of them here.

It is generally accepted that disruption of axonal transport contributes to the pathogenesis of ALS. Decreased abundance of the neurofilament light chain (NFL), which is encoded by *NEFL*, is well documented in ALS[173]. Interestingly, TDP43 and FUS bind to *NEFL* mRNA[54,95,174], which is sequestered in stress granules in ALS motor neurons[175]. Mutations in the genes encoding neurofilament heavy chain (NFH) and peripherin have also been identified in a very small number of patients with ALS[176–178]. Mutations in dynactin cause a lower motor neuron syndrome with vocal cord paralysis[179] and may be associated with ALS in some populations[180]. The most compelling evidence for a mechanistic involvement of the cytoskeletal network in ALS comes from the recent finding that mutations in profilin 1 (*PFN1*) can cause this disorder[181] (TABLE 1). PFN1 is essential for the polymerization of actin[181]. Mutations in *PFN1* inhibit axonal outgrowth through decreasing actin polymerization in embryonic motor neurons *in vitro*[181]. This inhibitory effect on axonal outgrowth may enhance retraction and denervation in the adult neuromuscular system. Evidence for this also comes from the finding that genetic or pharmacological inhibition of EPHA4 in fish and rodents attenuates the mutant SOD1- and mutant TDP43-induced phenotype[142]. EPHA4 is a receptor of the ephrin axonal repellent system and induces cytoskeletal rearrangements through RHOA GTPase[182]. Low EPHA4 expression in human ALS is associated with later onset and longer survival. Interestingly, the N-terminal cleavage product of vesicle-associated membrane protein-associated protein B and C (VAPB) normally interacts with EPHA4, modulating ephrin-induced signalling[183]. Mutations in VABP are a rare cause of ALS[184] (TABLE 1). Mutant VAPB is aberrantly processed, which results in increased ephrin signalling[183]. Of note, alsin, in which recessive (loss-of-function) mutations are a rare cause of ALS[185] (TABLE 1), also belongs to the RHO-family GTPases[186]. NOGO-A, an axonal outgrowth inhibitor, is upregulated in muscle of patients with ALS and of mutant SOD1 mice[187,188]. The effect of genetically deleting

RTN4, which encodes NOGO-A, is equivocal, as this affects the activity of this protein both in the spinal cord and at the neuromuscular junction[188], but it is hoped that antibodies that only inhibit its effect at the junction may have a beneficial effect on the disease in humans.

By contrast, at least in animal models, factors positively affecting axonal outgrowth appear to favour re-innervation and attenuate ALS. Vascular endothelial growth factor (VEGF), which is a known angiogenic factor and has recently been found to be a trophic factor for motor neurons and an axon guidance molecule[189–191], attenuates the phenotype when overexpressed or when administered intracerebroventricularly in mutant SOD1 rodent models. It is possible that it may have a similar effect in humans[191].

Genome-wide association studies have found an association between SALS and single-nucleotide polymorphisms (SNPs) in genes encoding axonal proteins. SNPs that reduce the expression of kinesin-associated protein 3 (KIFAP3), a protein that forms a complex with motor proteins KIF3A and KIF3B, have been found to be associated with increased survival of patients with this disease, although not in all studies[192,193]. By contrast, an SNP in *UNC13A*, which encodes a protein involved in synaptic glutamate release, is associated with shorter survival times in ALS[194,195]. Finally, a polymorphism in the gene encoding elongator acetyltransferase complex subunit 3 (ELP3) was found to be associated with ALS[196]. ELP3 is a subunit of the RNA elongator complex, and it acetylates histones and modifies the wobble base of tRNA[197]. In flies, ELP3 affects glutamatergic vesicle release through acetylation of the active zone protein BRP[198]. Whether this function of ELP3 in flies is the same in humans remains to be determined. Together, these data suggest that factors involved in the cytoskeletal organization, cellular transport and synaptic vesicle release are involved in the pathogenesis of ALS. This is of interest, as some of them are thought to be potential therapeutic targets for both familial and sporadic forms of the disorder.

Box 3 | **Therapeutic implications and opportunities**

Reducing the abundance of the pathogenic mutant protein is an obvious strategy for the causal treatment of neurodegenerative diseases. In amyotrophic lateral sclerosis (ALS), this has been attempted using antimutant superoxide dismutase 1 (SOD1) antibodies or antisense oligonucleotides targeting mutant *SOD1* mRNA. Both approaches attenuated the disease course of the mutant SOD1 rodent model[222,223]. The use of antisense oligonucleotides is being investigated in early clinical studies. This approach is obviously very appealing for the treatment of patients with chromosome 9 open reading frame 72 (*C9ORF72*) expansions (assuming they are directly toxic). The therapeutic effect of these cause-specific strategies is expected to be substantial, as is evident in the case of myotonic dystrophy treatment[224], but they will only be applicable to a small subset of patients. At this moment, this causal approach is only feasible in genetically determined ALS. If SOD1 contributes to the pathogenesis of sporadic ALS as mentioned above[20,21,157], it obviously becomes a target in a much larger population.

Conversely, interfering with more downstream-mediating pathways may be beneficial for a larger group of patients, as these pathways appear to be common to several forms of ALS. Many targets have become apparent upon gradual understanding of the disease. However, in view of the multiplicity of contributing factors, it is likely that only a cocktail-like combination of drugs interfering with these factors will significantly affect the disease process.

Unconventional methods of delivery of therapeutic factors may need to be considered in ALS (such as continuous intracerebroventricular or intrathecal administration). This may be necessary to either bypass the blood–brain barrier or to avoid systemic side effects. The coupling of carrier molecules to potential therapeutic substances is also being explored[225]. The use of cells and viral vectors to deliver substances directly to motor neurons are alternative approaches[226,227]. The lack of control or reversibility of substance release or expression in such systems requires complicated molecular control systems.

The use of precursor cells or stem cells for the treatment of ALS has attracted great interest over the past decade. The discovery that differentiated cells, even from elderly patients with ALS, can be reprogrammed into pluripotent cells, which in turn can be differentiated into neural cells has further increased this interest[228]. Some assume that such cells, when injected into the spinal cord, can differentiate into motor neurons, replace the diseased motor neurons and re-innervate muscle. It may be more realistic to hypothesize that transplantation of such cells into the ventral horn can result in a trophic effect, supporting the failing motor neurons. Several trials in which human neural stem cells are transplanted in the spinal cord of patients with ALS have been initiated (ClinicalTrials.gov identifiers: NCT01348451, NCT01640067)[229]. As mentioned before, astrocytes contribute to the motor neuron degeneration in ALS, and transplantation of glial precursor cells affects disease progression in rodent models[230]. Therefore, early clinical trials evaluating the effect of the transplantation of glial restricted precursor cells in the spinal cord are being designed.

The more complex, the more opportunities

Textbook neurology addresses ALS as a very homogenous disorder. This may explain why some expected this disease to have a single cause, a single pathogenesis and a single phenotype. However, ALS turns out to be a very heterogeneous disorder (TABLE 1). Moreover, each one of these ALS-causing genes can induce various phenotypes. Mutations in *SOD1* can give rise to progressive muscular atrophy in some family members and classical ALS in others. Similarly, a *C9ORF72* expansion mutation can give rise to an ALS phenotype in some family members, a frontotemporal dementia phenotype in others, and Parkinsonism or even psychosis in others. This has led to the concept that ALS is a syndrome and not just one disease. In addition, the discovery that not only mutant protein-related toxicity but mutant RNA toxicity may also contribute to the pathogenesis of ALS adds to the complexity. Lastly, the mechanism underlying the axonal withdrawal, synaptic dysfunction and, in the end, neuronal loss appears multifactorial. A strict dichotomy of loss-of-function versus gain-of-function probably does not respond to a biological reality — both effects seem to be at play. This complexity is a challenge for ALS research, b oth clinical and basic, but also offers many opportunities for therapeutic intervention (BOX 3).

Progress in our understanding of the mechanism underlying ALS over the past decade has been substantial, but many essential questions remain. The cause of SALS and of the factors that establish the vulnerability of motor neurons are only some of them. Even obvious questions await an answer: when does the disease start biologically in humans? Why do mutant proteins that are present from the beginning of an individual's life only cause a disease after several decades, which is then fatal within a couple of years? It is hoped that the recently acquired insights into the biology of motor neuron degeneration in ALS can be translated into disease-modifying treatments that make a substantial difference for patients.

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Competing interests statement
The authors declare no competing financial interests.

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