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Decoding ALS: From Genes to Mechanism

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Preface

Amyotrophic lateral sclerosis (ALS) is a progressive and uniformly fatal neurodegenerative disease. A plethora of genetic factors underlying ALS have now been identified that drive motor neuron degeneration, increase susceptibility to the disease, or influence the rate of progression. Emerging themes include dysfunction in RNA metabolism and protein homeostasis, with specific defects in nucleocytoplasmic trafficking, induction of endoplasmic reticulum stress, and impaired dynamics of ribonucleoprotein bodies such as RNA granules that assemble through the process of liquid-liquid phase separation. Extraordinary recent progress in understanding the biology of ALS provides new grounds for optimism that meaningful therapies for ALS will be identified.

ALS is a prototypical neurodegenerative disease characterized by progressive degeneration of motor neurons in the brain and spinal cord. First described by the neurologist Jean-Martin Charcot, the name reflects both the degeneration of corticospinal motor neurons, whose descending axons in the lateral spinal cord appear scarred (“lateral sclerosis”), and the demise of spinal motor neurons, with secondary denervation and wasting of muscle (“amyotrophy”). Corticospinal neurons make direct or indirect connections onto spinal motor neurons, which in turn innervate skeletal muscles and trigger their contractions (Fig. 1a). This review summarizes clinical and pathological features in ALS and describes how discoveries in ALS genetics have illuminated major themes in the molecular pathophysiology of this disease.

ALS is known in the United States as *Lou Gehrig's disease* and in the United Kingdom as *motor neuron disease*. While onset is commonly in mid-adulthood (mean of 55 years of age), ALS may begin as early as the first or second decade or in late life. Like most neurodegenerative diseases, it begins focally and spreads: symptoms that begin as subtle cramping or weakness in the limbs or bulbar muscles progress to paralysis of nearly all skeletal muscles. Some subsets of motor neurons, such as those that innervate extraocular muscles or sphincters, are spared until late in the disease. However, the disease is invariably

fatal. Death typically results within 3–5 years, although some forms demonstrate protracted survival.

ALS is an orphan disease, newly afflicting 1–2 individuals per 100,000 each year in most countries; however, the prevalence of ALS is much lower, reflecting the rapid lethality of the disease¹. In the United States and United Kingdom, ALS causes more than 1 in 500 adult deaths, a statistic that predicts that more than 15 million people now alive worldwide will succumb to this disease.

The clinical manifestations of ALS

Significant heterogeneity exists within the general rubric of ALS. Distinct clinical subsets are distinguished by involvement of different sets of motor neurons or different regions of the body. Depending on the location of primary pathology, patients may develop weakness with flaccidity and atrophy of the limbs (*progressive muscular atrophy*, affecting mainly the spinal or lower motor neurons), prominent hyperreflexia and spasticity with increased limb tone but relatively little muscle atrophy (*primary lateral sclerosis*, affecting corticospinal motor neurons with limited involvement of spinal motor neurons), tongue atrophy with thickness of speech and difficulty swallowing (*bulbar ALS*, affecting brainstem motor neurons that serve muscles of tongue movement, chewing, swallowing and articulation), or slow and highly dysfunctional speech and swallowing in the absence of tongue atrophy, often accompanied by accentuation of emotional reflexes (*pseudobulbar palsy*, affecting cortical frontobulbar motor neurons). Importantly, ALS shows clinical overlap with several other adult-onset degenerative disorders, most frequently frontotemporal dementia (FTD), that may constitute a clinical spectrum (see Box).

Genetic contributions to ALS

About 10% of cases of ALS are transmitted within families, almost always as dominant traits, frequently with high penetrance. The first ALS gene, cytosolic superoxide dismutase or *SOD1*, was reported in 1993², with more than 50 additional potential ALS genes published since, although validating the causality of specific variants remains a challenge. Applying relatively rigorous criteria, one can generate a list of genes whose mutations are unequivocally implicated in ALS pathogenesis (Table 1). These genes may be grouped loosely into (1) those that alter proteostasis and protein quality control; (2) those that perturb aspects of RNA stability, function and metabolism; and (3) those that disturb cytoskeletal dynamics within the motor neuron axon and distal terminal. The offending mutations are largely missense substitutions, although the genetic lesion in *C9ORF72* is an enormous expansion of an intronic hexanucleotide repeat.

Strictly speaking, the term *sporadic ALS* refers to disease that presents without a family history of the disease, although this term is sometimes mistakenly thought to refer to ALS that occurs without a genetic basis. Technological advances have permitted broad DNA sequencing in sporadic ALS patients and revealed that genetic variants in established ALS genes are not infrequent. For example, it is now evident that 1–3% of sporadic ALS cases reflect missense mutations in *SOD1*³; another 5% or more are caused by intronic expansions

in *C9ORF72*⁴. Pathogenic mutations in other ALS genes (e.g., *TARDBP*, *FUS*, *HNRNPA1*, *SQSTM1*, *VCP*, *OPTN*, *PFN1*) also have been identified in the sporadic ALS population, although they are rare.

Of immense interest are genetic variants that enhance ALS susceptibility or modify the clinical phenotype, even if the variants do not themselves cause ALS. Robustly confirmed in this category is *ATXN2*. Large trinucleotide repeat (CAG) expansions in the coding sequence of *ATXN2* cause spinocerebellar ataxia type 2, which sometimes displays motor weakness as an early presentation. It is therefore striking that mid-range CAG expansions of *ATXN2* increase the risk of developing ALS⁵. In contrast, variants that reduce expression of axonal guidance gene *EPHA4* improve overall survival of ALS patients⁶.

The pathology of ALS

At autopsy, ALS cases reveal degeneration of motor neurons in the motor cortex, brainstem motor nuclei, and anterior horns in the spinal cord. In the initial phases of the disease, ALS spares the motor neurons that innervate extraocular muscles, as well as those that control bowel and bladder function. As spinal motor neurons degenerate, their target muscles become atrophied. Degeneration of the spinal processes of corticospinal neurons results in scarring in the lateral tracts of the spinal cord. As the disease progresses, affected spinal motor neurons shrink and accumulate rounded or thread-like deposits of aggregated proteins that are collectively referred to as inclusions (Fig. 1b). The cytoplasmic inclusions in ALS often become ubiquitinated; it is now recognized that an early target for ubiquitination is TAR-DNA binding protein (TDP43), which forms the major component of ubiquitinated inclusions in most ALS cases⁷.

Some additional pathological features are associated with specific genes. For example, cases of ALS caused by microsatellite expansions in *C9ORF72* reveal intranuclear RNA foci⁸, distinctive cytoplasmic inclusions derived from dipeptide repeat proteins (DPRs, discussed below)^{9,10}, as well as p62-positive, largely TDP43-negative neuronal cytoplasmic inclusions predominantly in the cerebellum and hippocampus¹¹. Cases of ALS caused by mutations in *SOD1* and *FUS* are pathologically distinct in that they do not exhibit TDP-43 pathology, but rather inclusions of abnormal SOD1 and FUS proteins, respectively. In addition to these pathological findings in motor neurons, there is abundant evidence for significant pathology in non-neural cell types (e.g., insidious astrogliosis and microgliosis). As reviewed below, it is likely that both forms of non-cell-autonomous cellular reactivity adversely influence progression in ALS.

Pathogenic mechanisms of ALS

The molecular era of discovery in ALS began in 1993 with the identification of dominant mutations in *SOD1*, the gene encoding an abundant, ubiquitously expressed cytoplasmic superoxide dismutase². A major antioxidant, the normal function of SOD1 is to catalytically convert highly reactive superoxide (most frequently produced by errors within mitochondria) to either hydrogen peroxide or oxygen.

An important initial realization arose from expression of mutant *SOD1* in mice, which demonstrated that motor neuron degeneration is driven by one or more acquired toxicities of the mutant protein^{12,13} and is independent of dismutase activity¹⁴. The >170 ALS-causing mutations now identified (<http://alsod.iop.kcl.ac.uk/>) lie in virtually every region of the 153-amino acid *SOD1* polypeptide. Moreover, while many variants retain partial or full dismutase activity, there is no correlation between reduction in activity and age of disease onset or rapidity of disease progression¹⁵. These findings led to the consensus view that disease arises from one or more toxic properties of the many *SOD1* mutants and not from reduced dismutase activity.

A sobering reality, however, is that 23 years after the initiating discovery, no consensus has yet emerged as to the primary toxicity of mutant *SOD1*. Instead, a plethora of toxic mechanisms have been proposed to mediate the degeneration and death of motor neurons (Fig. 2). One prominent finding is that a proportion of each ALS-causing *SOD1* mutant fails to fold properly, thus implicating accumulation of misfolded *SOD1* as a possible toxic contributor in ALS (Fig. 2a). The misfolded *SOD1* forms ubiquitinated cytoplasmic inclusions that can occur early in disease and escalate as disease progresses¹⁶.

The accrual of ubiquitinated *SOD1* aggregates in patients with *SOD1* mutations is paralleled by accrual of ubiquitinated TDP-43 aggregates in patients with *TARDBP* mutations (and in the vast majority of patients with sporadic ALS as described in greater detail below), highlighting a correlation between protein aggregation and disease. Nevertheless, as has been demonstrated in other neurodegenerative diseases, large aggregates of the disease-causing mutant *SOD1* are not sufficient to drive disease, as their elimination fails to affect any aspect of fatal disease developed in mice¹⁷.

Non-cell-autonomous toxicity: the neighborhood matters

Like the other major neurodegenerative diseases, all of the genes whose mutations cause ALS are expressed in multiple cell types. Indeed, it is now clear that ALS arises in part by non-cell-autonomous mechanisms. In other words, disease arises from a combination of mutant damage within motor neurons and their glial partners, rather than from damage exclusively in neurons.

For mutant *SOD1*, this concept is underscored by studies in mice revealing that high expression levels of mutant *SOD1* in all motor neurons is not sufficient for early-onset disease¹⁸. Conversely, reduction of mutant *SOD1* synthesis in motor neurons is of no benefit in slowing the rate of disease progression after onset even when applied pre-symptomatically^{19–21}. Thus, ALS is a disease not just of the motor neuron but the motor system, which is comprised of the motor neuron and intimately associated cells of several types.

The critical role of glia in ALS

The importance of glial cells in motor neuron degeneration and death emerged from studies in which mutant *SOD1* synthesis was silenced in microglia, astrocytes or oligodendrocyte precursors. Microglia, the innate immune cells of the nervous system, become activated in all instances of ALS (Fig. 2b). Synthesis by microglia of mutant *SOD1* is a key determinant

of rapid disease progression, as determined by selectively silencing the mutant gene within microglia²¹ or using cell grafting to replace mutant-expressing microglia with normal ones²². Consistent with these findings, inhibiting nuclear factor kappa B (NFκB) suppresses this neuroinflammatory component of microglial toxicity to co-cultured motor neurons²³.

An additional mechanism of mutant SOD1 damage produced by microglia is counterintuitive: stimulation of excessive *extracellular production* of superoxide (O_2^-)²⁴. Misfolded mutant SOD1 can associate with Rac1, a small GTPase that controls the activation of NADPH oxidase, a complex that produces superoxide (Fig. 2b). Thus, instead of its normal function of removing intracellular superoxide, mutant SOD1 may drive microglia to produce high levels of extracellular superoxide.

Disturbance in microglial function has also recently emerged as a potential contributor to the subtype of ALS associated with mutations in *C9ORF72*. Recognition that *C9ORF72* mutation results in decreased expression levels in ALS patients⁸ has led to speculation that loss of C9ORF72 protein function may contribute to disease. The protein encoded by *C9ORF72* is a probable guanine exchange factor for one or more not-yet-identified G proteins. Its inactivation in mice results in abnormal microglia and age-related neuroinflammation, providing further evidence that non-cell-autonomous, microglial-mediated inflammation may contribute to ALS²⁵⁻²⁷.

A critical contribution to pathogenesis from mutant SOD1 is driven by **oligodendrocytes**, the cells that myelinate the axons of upper motor neurons and the initial axonal segments of lower motor neurons. Reduction in mutant SOD1 synthesis early in oligodendrocyte maturation produced a more striking delay in disease onset²⁸ than did similar suppression of mutant synthesis by motor neurons^{18,21}. Oligodendroglia also support motor neuron function by direct supply to the axon of the energy metabolite lactate through the action of the monocarboxylate transporter 1 (MCT1) (Fig. 2c). Mutant SOD1 impairs expression of MCT1 by oligodendrocytes in mouse models of ALS²⁹. Similar reduction in MCT1 accumulation is found in sporadic ALS²⁹, consistent with a non-cell-autonomous role of reduced oligodendroglial energy supply as a general component of ALS pathogenesis.

A third glial cell type, the **astrocyte**, provides nutrients, buffering ions, recycling of the neurotransmitter glutamate, and the final layer of the blood brain barrier. Selective reduction in mice of mutant SOD1 synthesis by astrocytes slowed disease onset³⁰ or progression²⁰. This delayed progression was accompanied by delayed microglial activation, demonstrating a functional crosstalk between mutant astrocytes and microglia.

One of the early-proposed mechanisms underlying ALS was glutamate excitotoxicity, the excessive firing of motor neurons derived from failure to rapidly remove synaptic glutamate (Fig. 2d). Astrocytes limit motor neuron firing through their rapid recovery of glutamate, a function mediated by the EAAT2 glutamate transporter (Fig. 2d). Loss of EAAT2 has been observed in both SOD1 mutant rodent models^{16,31} and familial and sporadic ALS patient samples³². The resulting failure to quickly clear synaptic glutamate triggers repetitive action potential firing, a corresponding increase in calcium influx, and endoplasmic reticulum (ER) and mitochondrial stress whose calcium storage abilities become overwhelmed.

Astrocytes also protect motor neurons from excitotoxic damage via release of an unidentified soluble factor(s) that induces motor neurons to upregulate the glutamate receptor subunit GluR2³³. Incorporation of GluR2 subunits into glutamate receptors reduces their calcium permeability, providing protection from excitotoxicity by decreasing influx of calcium into neurons. Astrocytes expressing mutant SOD1 fail to regulate GluR2 expression in co-cultured neurons, thereby increasing their vulnerability to excitotoxic damage³³.

Beyond excitotoxicity, multiple teams have used *in vitro* co-cultures of motor neurons and astrocytes (or astrocyte-conditioned media) to show that astrocytes expressing ALS-linked mutations produce a diffusible toxicity to motor neurons^{34–38} (Fig. 4d). There is, however, no consensus as to the identity of the toxic species. Even more provocatively, astrocytes from familial or sporadic ALS patients (produced from autopsy samples by direct isolation of astrocytes³⁸ or isolation of neuronal precursor cells that can subsequently be converted into astrocytic precursors and then into astrocytes³⁷) are toxic to co-cultured normal motor neurons. The latter finding³⁷ is especially provocative as it indicates that neuronal precursor cells in the affected portions of sporadic ALS patient tissue have already acquired damage and this damage is retained following multiple divisions in cell culture and subsequent differentiation. A crucial unsettled controversy remains as to whether toxicity from sporadic ALS-derived astrocytes is³⁷ or is not³⁸ mediated by changes in SOD1.

Perhaps most importantly, a non-cell-autonomous contribution of astrocytes to ALS-like disease has been demonstrated in rodents expressing mutant SOD1, in which cervical transplantation of lineage-restricted astrocyte precursors resulted in delayed progression of disease³⁹.

Multiple ALS-linked genes induce ER stress or impair protein degradation machinery

ER stress has been implicated broadly in ALS (Fig. 2a). Initial evidence arose from studies of mutant SOD1, with evidence for misfolded SOD1 binding to the cytoplasmic surface of the integral membrane ER protein derlin-1⁴⁰. This binding led to inhibition of ER-associated degradation (ERAD), the pathway for degradation of misfolded proteins extracted from the ER. Moreover, relieving ER stress delays disease progression in an animal model of disease⁴¹.

There is now overwhelming evidence that disruption of the two major protein clearance pathways, the ubiquitin-proteasome system and autophagy, can be central components of disease mechanism in ALS (Fig. 2a). Multiple ALS-causing mutations are in genes whose products are directly involved in protein degradation, including ubiquilin 2⁴² and p62 (encoded by *SQSTM1*)⁴³, both of which function as adapters to bring polyubiquitinated proteins to the proteasome or autophagosome for degradation. Mutations have also been reported in optineurin⁴⁴, a proposed receptor for autophagy⁴⁵, and valosin-containing protein (VCP)⁴⁶, which functions in ERAD and sorting of endosomal proteins. Additional studies have reported FTD-linked mutations in CHMP2B⁴⁷, which has been implicated in autophagosome maturation and/or endosomal cargo sorting/degradation. ALS-linked mutations are also found in VAPB⁴⁸, which functions in the unfolded protein response for delivery of ER-ejected substrates to the proteasome. A preponderance of biochemical evidence from mice expressing mutant SOD1 has demonstrated decreased activities of the

proteasome pre-symptomatically in lumbar spinal cords⁴⁹ or after sustained expression of mutant SOD1 in a cultured neuronal line⁵⁰.

Axonal disorganization and disrupted transport in ALS

Axonal cytoskeletal disorganization, especially of neurofilaments, has long been a conspicuous feature of both familial and sporadic ALS (Fig. 2e). As the most asymmetric cells in nature (with some axons more than a meter long), motor neurons have a crucial requirement for axonal transport to deliver the components synthesized in the cell bodies to axons and synapses. ALS-linked SOD1 mutants have been demonstrated to slow both anterograde⁵¹ and retrograde^{52,53} routes months before neurodegeneration. Indeed, reduction in retrograde transport by mutation in dynactin⁵⁴, an activator of the retrograde motor cytoplasmic dynein⁵⁵, provokes human motor neuron disease.

The peculiar architecture of neurons also presents a challenge for these cells to change local gene expression at the synapse in response to neuronal input or change in the synaptic environment. To meet this challenge, neurons transport all of the necessary components for translation (e.g., mRNA, ribosomes and translation factors) to distal sites for local protein synthesis⁵⁶. The spatial distribution of mRNAs depends upon proper microtubule-dependent transport of neuronal RNA transport granules and other factors, and is regulated by several RNA-binding proteins associated with ALS, including TDP-43, FUS and hnRNPA1. ALS-causing mutations in TDP-43 impair the axonal transport of RNA granules in *Drosophila* and cultured neurons, including motor neurons derived from ALS patients⁵⁷.

A prion-like spread in inherited ALS

Prion-like, templated conversion of a natively folded protein into a misfolded version of itself is now recognized as a prominent feature for cell-to-cell spread of aggregates in neurodegenerative disease. Examples include templated α -synuclein, A β , and tau misfolding in Parkinson's and Alzheimer's diseases and in chronic brain injury (reviewed in ref⁵⁸). Evidence for similar templated toxicity has emerged for misfolded SOD1^{59,60}, with wild type SOD1 exacerbating toxicity from mutant SOD1 in mice⁶¹. Prion-like propagation and disease development initiated focally has been demonstrated after injection of mutant SOD1-containing lysates into mutant SOD1-expressing mice⁶². This finding replicates the correlation of focal initiation and spreading in both familial and sporadic ALS patients⁶³.

To date, prion-like propagation has not been achieved in rodents without co-existence of a pre-existing, weakly active mutant ALS gene; it is unclear whether this challenges a prion-like spread model for sporadic ALS or raises the possibility that there must be a pre-existing sensitivity in these patients to facilitate such spreading. Coupled with recognition that misfolded mutant SOD1 can be secreted by motor neurons or astrocytes⁶⁴, potentially by the newly discovered pathway of unconventional secretion of misfolded proteins as an adaptation to proteasome dysfunction⁶⁵, stochastic focal initiation provides a plausible mechanism for age-dependent disease onset and subsequent spread. With most instances of ALS marked by aggregated TDP-43 rather than SOD1, an unresolved question is whether TDP-43 also exhibits templated misfolding that can spread cell-to-cell.

The intersection of RNA biology with ALS pathogenesis

In 2006 Virginia Lee and colleagues reported mislocalization of the RNA-binding protein TDP-43 from its predominantly nuclear location to ubiquitin-positive cytoplasmic inclusions in affected areas of the brain and spinal cord of ALS patients⁷. Within just a few years, TDP-43 mislocalization became widely recognized as the hallmark pathology of sporadic and most familial ALS. This seminal discovery has implications beyond ALS, since TDP-43 mislocalization to cytoplasmic inclusions is also the hallmark pathology of tau-negative FTD (roughly half of all FTD cases) and inclusion body myopathy (IBM)^{7,66}, diseases that show genetic overlap with ALS. Moreover, TDP-43 pathology is also found as secondary pathology in a subset of patients with Alzheimer's and Parkinson's diseases^{67,68}. The importance of TDP-43 in pathogenesis was cemented by the identification of ALS-causing mutations in this RNA-binding protein⁶⁹. The subsequent identification of ALS-causing mutations in related RNA-binding proteins, including FUS and hnRNPA1, focused substantial attention on the role of RNA biology in pathogenesis⁷⁰⁻⁷³ (Fig. 2f).

TDP-43, FUS and hnRNPA1 are heterogeneous nuclear ribonucleoproteins (hnRNPs), a family of proteins that regulates RNA metabolism at every stage of the RNA life cycle. These three hnRNPs bind to thousands of RNA targets⁷⁴⁻⁷⁷; thus, disturbance in the function of one or more of these proteins has the potential to impact RNA metabolism on a broad scale. Further links between ALS pathogenesis and RNA biology came from the identification of ALS-causing mutations in the RNA-binding protein matrin-3⁷⁸, appreciation of increased risk of ALS in association with certain alleles of the RNA-binding protein ataxin-2⁵, and recognition of RNA-related mechanisms of disease associated with mutations in *C9ORF72* (see below)^{8,79}.

Phase separation of RNA-binding proteins gives rise to membrane-less organelles

RNA metabolism transpires in complex RNA-protein assemblies, which themselves may coalesce into a variety of membrane-less organelles, such as nucleoli and stress granules. Interestingly, these membrane-less organelles behave as complex liquids that arise by phase separation, a process in which protein-laden RNAs separate from the surrounding aqueous nucleoplasm or cytoplasm in a manner akin to the separation of oil and vinegar⁸⁰. It was recently discovered that phase separation is mediated by low complexity sequence domains (LCDs) present in RNA-binding proteins such as TDP-43, FUS and hnRNPA1⁸¹⁻⁸³ (Fig. 3a-b). Whereas the assembly of membrane-less organelles is biologically advantageous (e.g., stress granule formation), phase transition also presents a risk, since RNA-binding proteins within the liquid phase are highly concentrated, with fibrillization-prone LCDs placed in close proximity. Indeed, ALS-causing mutations are frequently found in the LCDs of TDP-43⁸⁴, FUS⁸⁵ and hnRNPA1⁷². As a consequence, these mutations alter the dynamics of membrane-less organelles and also accelerate fibrillization, resulting in the formation of pathological amyloid-like fibrils that deposit in the cell bodies and neuropil^{81-83,86} (Fig. 3c).

Mutations in the LCDs of at least six different hnRNPs result in a clinicopathological spectrum that ranges from ALS/FTD to IBM (Fig. 3a and Box 1). It should be noted that some disease-causing mutations in RNA-binding proteins do not impact the LCD. For example, some ALS-causing mutations in FUS and hnRNPA1 disturb the nuclear

localization sequence (NLS) and result in accumulation of these proteins in the cytoplasm^{70,71,73}. Phase transition by LCD-containing RNA-binding proteins is exquisitely concentration dependent⁸², and it is likely that increased accumulation of FUS and hnRNPA1 in the cytoplasm as a consequence of mutations impacting their respective NLSs is sufficient to drive excess phase separation, as evidenced by hyperassembly of stress granules in cells derived from patients with the respective mutations^{72,87}.

RNA metabolism defects in ALS

There are multiple potential consequences of disturbed phase transitions by disease-associated RNA-binding proteins, including (1) altered material properties of RNA granules, which can impair the biological processes served by these structures; (2) the generation of oligomers or amyloid-like fibrils that have toxic properties; and (3) a partial or complete loss of the normal function of key RNA-binding proteins⁸⁸. With respect to this last point, a long-recognized feature of ALS histopathology is redistribution of TDP-43 from the nucleus to the cytoplasm⁷. Similar redistribution is observed for FUS and hnRNPA1 when disease mutations occur in these genes^{70,71,73}. This redistribution may reflect a cytoplasmic sink produced by poorly dynamic cytoplasmic RNA granules, the deposition of amyloid-like fibrils, defects in nucleocytoplasmic trafficking, and potentially other mechanisms.

The culmination of this redistribution is depletion of RNA-binding proteins from the nucleus that has the potential to cause a relative loss of nuclear function. A long-recognized nuclear function of TDP-43 is regulation of alternative splicing⁸⁹. Experimental depletion of TDP-43 in rodents was found to alter hundreds of splicing events in the brain, resulting in depletion of several RNAs encoding synaptic proteins⁷⁵. Additionally, loss of nuclear TDP-43 generates usage of cryptic splice sites⁹⁰ that, in general, may lower levels of correctly spliced protein-encoding mRNAs. Furthermore, the fact that TDP-43 autoregulates its synthesis⁷⁵ establishes the possibility of a feed-forward mechanism amplifying the impact of partial loss of TDP-43 function.

Loss of FUS or hnRNPA1 from the adult nervous system produces defects analogous to that of loss of TDP-43, although different subsets of mRNAs are associated with depletion of each of these RNA-binding proteins^{76,77}. An important unanswered question is to what extent does ALS caused by other genetic perturbations, especially *C9ORF72*-related ALS (see below), also involve disturbances in RNA biology that intersect mechanistically with mutations in TDP-43, FUS and hnRNPA1.

Regulatory RNA biogenesis and function in ALS

Both TDP-43 and FUS are components of macromolecular complexes that generate small non-coding RNAs (microRNAs) that function in RNA silencing. Loss of TDP-43 or FUS results in reduced expression of microRNAs in model systems, including *Drosophila* models and iPS-derived motor neurons from patients with TDP-43 mutations, suggesting a possible role for altered RNA silencing in ALS⁹¹. microRNAs contribute to the maintenance of neuromuscular junctions, suggesting that motor neurons may be especially sensitive to disturbances in microRNA biogenesis^{92,93}. Indeed, global downregulation of microRNAs has been reported in motor neurons from patients with sporadic ALS⁹⁴, although the role of

reduced microRNAs in pathogenesis remains to be established⁹⁴. Nonetheless, the expression of regulatory RNAs appears to be robustly and consistently altered in the serum of ALS patients, and this may present an opportunity for biomarker development⁹⁵.

The curious case of C9ORF72-related ALS/FTD

Although the identification of ALS-causing mutations impacting SOD1 and RNA-binding proteins highlighted pathophysiological pathways by which disease might arise, the majority of the genetic burden of ALS remained unaccounted for until 2011. Genetic linkage studies^{96,97} followed by several large genome-wide association studies^{98–101} identified a location in the chromosome 9p21 region for a gene whose mutations caused both ALS and FTD. In the course of sequencing the non-coding regions of candidate genes within chromosome 9p21, Rosa Rademakers and colleagues identified pathogenic GGGGCC repeat expansion in *C9ORF72* as the basis for C9-ALS/FTD⁸. In unaffected individuals, the hexanucleotide GGGGCC was present in 2–23 repeats, whereas in affected individuals this was expanded to hundreds or thousands of repeats. Working in parallel, Brian Traynor and colleagues independently discovered pathogenic GGGGCC repeat expansion in *C9ORF72*⁹. Similar to numerous other neurodegenerative diseases caused by microsatellite expansion, there appears to be an inverse relationship between repeat expansion length and disease severity^{102,103}. Affected individuals apparently inherit hundreds of repeat copies, but these undergo expansion to 3000–5000 copies in cells of the nervous system¹⁰⁴.

A contribution to disease from reduced C9ORF72 function?

Three non-exclusive mechanisms by which expanded GGGGCC repeats may cause C9-ALS/FTD have been proposed (Fig. 4). Decreased *C9ORF72* expression levels in C9-ALS/FTD patients^{104,105} has led to speculation that loss of *C9ORF72* protein may contribute to disease. The function of the *C9ORF72* protein is poorly understood, but it contains a conserved DENN domain and can function as a guanine nucleotide exchange factor for several Rab proteins in experimental systems¹⁰⁶. In cultured cells and in zebrafish, depletion of endogenous *C9orf72* may exacerbate the toxicity of aggregation-prone proteins such as polyglutamine-expanded ataxin-2¹⁰⁷. However, knockdown of endogenous *C9orf72* mRNA with antisense oligonucleotides was well tolerated in mice and did not result in behavioral or motor impairments¹⁰⁸. Furthermore, conditional knockout of *C9orf72* in mouse brain did not cause appreciable motor neuron or other degenerative phenotypes, with no evidence of hallmark ALS/FTD pathologies¹⁰⁹. Mice with complete *C9orf72* knockout in all tissues also developed and aged normally without motor neuron disease, but, interestingly, developed progressive splenomegaly and lymphadenopathy^{25–27,110}. These mice were found to have abnormal macrophages and microglia, as well as age-related neuroinflammation^{25,110} and signs of autoimmunity²⁷, raising the possibility of a non-cell-autonomous inflammatory contribution to C9-ALS/FTD.

Nevertheless, the dominant inheritance pattern of C9-ALS/FTD, the absence of ALS/FTD patients with null alleles or missense mutations in *C9ORF72*, and the absence of neurodegeneration in *C9orf72* knockout mice argue against loss of function of *C9ORF72* as the sole driver of disease. Indeed, most empirical evidence points to gain-of-toxic-function

as the primary mechanism driving neurodegeneration in C9-ALS/FTD. For example, AAV-mediated delivery to the brain of a construct expressing expanded GGGGCC repeats elicits neurodegeneration¹¹¹, although the nature of the toxic species in C9-ALS/FTD remains unclear.

Toxic gain of function from accumulation of repeat-containing RNA

The initial description of the *C9ORF72* mutation included evidence that RNA foci containing the GGGGCC repeat accumulate in the brain and spinal cord of C9-ALS/FTD patients⁸ (Fig. 1b). Soon thereafter it was appreciated that *C9ORF72* is bidirectionally transcribed, and that both sense- (GGGGCC) and antisense- (CCCCGG) containing foci accumulate in affected cells^{112–114}. The accrual of pathological RNA foci in C9-ALS/FTD is reminiscent of pathological RNA foci observed in myotonic dystrophy types 1 and 2 and fragile X tremor ataxia syndrome, which are also caused by pathological repeat expansions in non-coding regions¹⁰². In these diseases, the accumulated repeat-containing RNA sequesters RNA-binding proteins involved in splicing, leading to defects in RNA splicing that underlie some aspects of pathogenesis¹⁰². Similarly, a number of RNA-binding proteins bind to expanded GGGGCC and GGCCCC repeats *in vitro*, and rare co-localization with RNA foci has been observed for several of these proteins in patient tissue^{115–119}.

Simple model systems have illustrated functional consequences of sequestration of some hexanucleotide repeat-binding proteins, for example Pura and RANGAP, but the contribution of these interactions to disease remains uncertain^{118,120}. Notably, GGGGCC (but not CCCCCG) repeats can adopt a stable secondary structure known as a G-quadruplex, which may contribute to the persistence of this RNA species as well as its ability to reach distal neurites and associate with RNA-binding proteins in transport granules and potentially interfere with local translation^{121–123}.

Toxic gain of function from accumulation of dipeptide repeats

Substantial evidence has now accrued implicating a second mode of gain-of-toxic-function in C9-ALS/FTD; specifically, toxicity from DPR proteins produced by repeat-associated non-AUG (RAN) translation. This type of unconventional translation occurs in the absence of an initiating AUG codon¹²⁴ and may rely upon secondary structure formed by repeat-expanded RNA^{124,125}. In C9-ALS/FTD, this unconventional translation occurs in all reading frames from both sense and antisense transcripts, and results in the production of five DPR proteins: glycine-alanine (GA) and glycine-arginine (GR) from sense GGGGCC transcripts, proline-arginine (PR) and proline-alanine (PA) from antisense GGCCCC transcripts, and glycine-proline (GP) from both sense and antisense transcripts^{112–114,126}. It is now appreciated that all of these DPRs are produced in C9-ALS/FTD patients and account for the ubiquitin- and p62-positive, but TDP-43-negative neuronal cytoplasmic and intranuclear inclusions that are found widely in the brain and spinal cord^{112,114,117,126,127} (Fig. 1b).

Issues of when, where, and how much expression of each DPR species occurs in patient brain remain to be clarified. Multiple reports have described DPR deposition in C9-ALS/FTD patient brain, and in some instances an inverse relationship between the burden of DPRs and neurodegeneration has been described^{126,128,129}. These studies have been based

on examination of post-mortem brain at end-stage disease and have relied upon detection of large inclusions by immunohistochemistry, an approach that is likely to under-represent the pathological burden of soluble DPRs. Nevertheless, the apparent discrepancy between the burden of DPR deposition (greatest in the cerebellum) and the severity of neurodegeneration (greatest in the motor cortex and spinal cord) need to be resolved in order to understand the role of DPRs in disease.

Experiments conducted in model systems have provided evidence that at least some DPR species are toxic in cultured cells and animal models of disease, although the DPR expression levels used to produce short-term toxicity have sometimes been quite high. The arginine-containing peptides (GR and PR) in particular appear to be most toxic. For example, the addition of GR or PR peptides to cultured cells results in their uptake and accumulation in nucleoli, defects in RNA processing, and subsequent cell death¹³⁰. Similarly, independent expression of each of the five DPR species in cultured neurons revealed GR and PR to be very toxic, whereas similar levels of PA, GA and PG were well tolerated. Consistent with these observations, *Drosophila* engineered to express each of the five DPR species have consistently shown that GR and PR are very toxic in neuronal tissue, whereas GA is modestly toxic and GP and PA appear to be non-toxic^{131–133}. That said, other investigations have reported toxicity associated with GA expression in cell culture^{134–136} and in AAV-mediated delivery to mouse brain¹³⁶, albeit in the latter instance the GA-mediated toxicity accompanied its accumulation to just under 2% of total brain protein. Additionally, the efforts reported thus far to model DPR toxicity have used short repeat (<100) lengths. Unsettled too is how the properties of those short DPRs compare with the RAN translation products in patients, which may be much larger.

A defect in nucleocytoplasmic trafficking

Whereas the nature of the toxic gain of function remains an open question, there is converging evidence that one downstream consequence of the *C9ORF72* mutation is impaired nucleocytoplasmic trafficking. A comprehensive, unbiased screen for genetic modifiers of GGGGCC repeat-expanded toxicity in *Drosophila* identified 18 genes centered on the nuclear pore complex and nucleocytoplasmic trafficking¹³³. A separate unbiased screen for genetic modifiers of PR toxicity in yeast also identified numerous genes encoding components of the nuclear pore complex and effectors of nucleocytoplasmic trafficking¹³⁷. A third study focused on the nucleocytoplasmic transport factor RANGAP, which binds GGGGCC RNA. In these experiments, genes encoding RANGAP and other nucleocytoplasmic transport factors were identified as modifiers of GGGGCC repeat-expanded toxicity in *Drosophila*¹²⁰. Consistent with these results, morphological abnormalities were found in nuclear envelope architecture in cell and animal models of disease as well as in the brain of C9-ALS/FTD patients. Moreover, defects in nucleocytoplasmic transport of RNA and proteins were found in induced pluripotent stem cell (iPSC)-derived neurons from C9-ALS/FTD patients^{120,133,137}.

Approaches to therapy for C9-ALS/FTD

The relative contribution of the various proposed modes of toxicity to the eventual development of C9-ALS/FTD remains an important unanswered question that will influence

strategies for therapeutic intervention. For example, efforts are underway to impede RAN translation with small molecules, but the success of such an approach will depend on the role of DPRs in disease. Irrespective of the primary basis for toxic gain of function, however, the mutant *C9ORF72* gene itself presents an attractive target for therapeutic intervention. For example, antisense oligonucleotides are able to reverse pathological features present in iPSC-derived neurons^{108,138,139} or fibroblasts¹⁰⁸ from C9-ALS/FTD patients. Indeed, iPSC-derived neuronal and glial cells may prove to be a useful model system in which to develop approaches to mitigate mutant *C9ORF72*-related toxicity even before the basis of toxicity is fully elucidated.

Therapeutic efforts will also be aided by the recent development of transgenic mouse models expressing human *C9ORF72* with ~450 hexanucleotide repeats that recapitulate aspects of the molecular pathology^{26,140–142}, neuropsychological deficits^{142,26} and the motor phenotype¹⁴² of C9-ALS/FTD. Particularly promising is the finding that pathological abnormalities can be reversed, and development of neuropsychological deficits delayed, by single-dose infusion of an antisense oligonucleotide that induces catalytic degradation of hexanucleotide-containing RNAs, without exacerbating reduction in RNAs encoding the *C9ORF72* protein²⁶.

Looking forward

Clearly there has been dramatic recent progress in defining the genetic topography and molecular biology of ALS. There is little doubt that the pace of discovery will continue or accelerate in at least three domains.

First, it is certain that our understanding of the *genetic basis of ALS* will continue to evolve. Programs are already in place to accumulate thousands of fully sequenced whole ALS genomes. More ALS genes are likely to be defined, both via conventional Mendelian genetics and through enhanced association studies that identify increased burdens of rare variants, including those in non-coding DNA. In parallel, enhanced scoring and recording of quantifiable clinical parameters will permit definition of genetic variants that modify the phenotype of ALS. The existence of extensive ALS genome databases will for the first time allow comprehensive studies of epistasis, characterizing the interactions of multiple genes to perturb motor neuron viability.

Second, while the last two decades have witnessed extraordinary progress in understanding familial ALS, it is likely that insights will be acquired to *elucidate sporadic ALS*. One view is that all sporadic ALS will ultimately be shown to reflect multiple genetic determinants. Alternatively, there is increasing interest in exogenous factors that might trigger sporadic neurodegeneration. It has been proposed that atypical infections or activation of endogenous retroviruses¹⁴³ may be critical. While external environmental factors have been elusive in ALS, there is new interest in the intrinsic environment represented by the microbiome.

Third, and perhaps most importantly, there will be significant achievements in the *development of ALS therapies*. Though daunting, the complexity of the molecular pathology of ALS is promising as a roadmap to defining therapeutic targets. Moreover, for

categories of ALS arising from well-defined genetic defects, advances in gene silencing and editing technologies will permit personalized therapeutic programs. When combined with improved methods of delivering therapy to the central nervous system, these approaches will lead to strategies to attenuate the lethal course of this disease.

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Box**ALS shows frequent clinical and genetic overlap with related diseases**

Whereas ALS historically had been viewed as a disease with purely motor manifestations, careful observations of ALS patients over the last 30 years has revealed that ALS overlaps clinically, pathologically and genetically with several other degenerative disorders. In particular, it has been appreciated that motor neuron loss may be accompanied by loss of cortical neurons in the frontal and temporal cortices. The clinical correlate is frontotemporal dementia (FTD), an entity that, unlike Alzheimer disease, does not entail major memory loss but instead impairs judgment and executive skills, often leading to behavioral disturbances. About 20% of ALS patients meet the clinical criteria for a concomitant diagnosis of FTD, although as many as 50% of patients experience cognitive impairment. Less frequently ALS occurs concomitantly with Paget's disease of bone (PDB) or inclusion body myopathy (IBM). Similar to ALS, both FTD and IBM are characterized by inclusions of TDP-43 and related RNA-binding proteins. The relationship between ALS, FTD, PDB and IBM has been extended by genetic evidence, potentially placing them within a continuum of a broader degenerative disorder. The relationship between these diseases is further substantiated by the syndrome multisystem proteinopathy, a genetically heterogeneous, dominantly inherited disorder characterized by ALS/FTD occurring concomitantly with IBM and/or PDB.

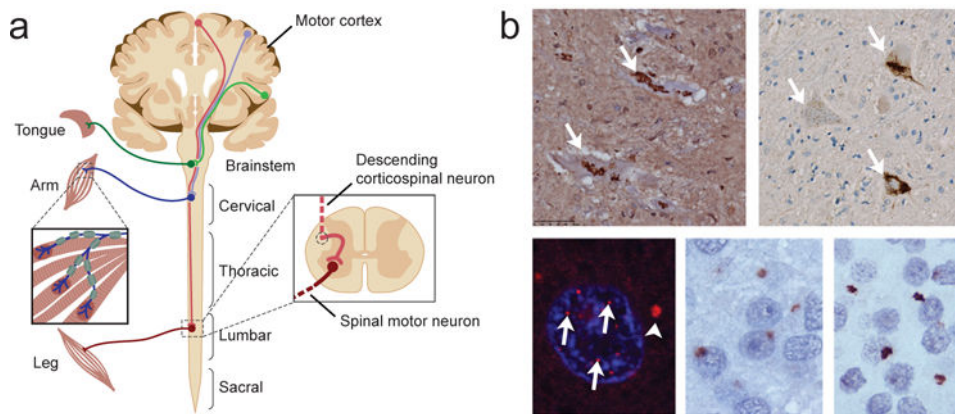


Figure 1. The components of the nervous system impacted in ALS

(a) ALS primarily impacts descending corticospinal motor neurons (*upper motor neurons*) that project from the motor cortex to synapses in the brainstem and spinal cord, and bulbar or spinal motor neurons (*lower motor neurons*) that project to skeletal muscles. (b) Typical pathology of different ALS subtypes. Clockwise from top left: SOD1 aggregates in spinal motor neurons in SOD1-related familial ALS; TDP-43 redistribution to cytoplasmic inclusions in spinal motor neurons in sporadic ALS; GR dipeptide repeat pathology in the dentate nucleus of C9-ALS/FTD; GA dipeptide repeat pathology in the dentate nucleus of C9-ALS/FTD; RNA foci in the nucleus (arrows) and cytoplasm (arrowhead) of a cortical neuron in C9-ALS/FTD.

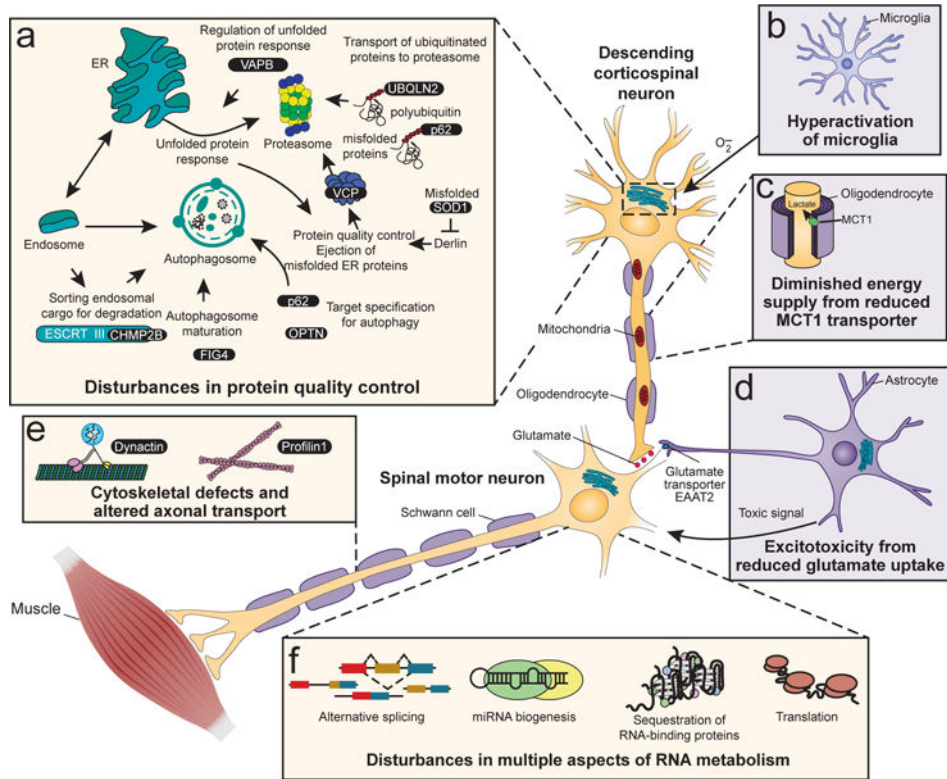


Figure 2. Schematic representation of the major cellular disease mechanisms implicated in ALS (a) Disturbances in protein quality control and ER stress. (b) Microglial activation and production of extracellular superoxide. (c) Reduced energy supply from oligodendrocytes to the underlying motor axons following reduced levels of the MCT1 lactate transporter. (d) Release from astrocytes of an as-yet unidentified species toxic to motor neurons. (e) Disruption of the cytoskeleton and impaired axonal transport. (f) Disturbance of multiple aspects of RNA metabolism are implicated in ALS.

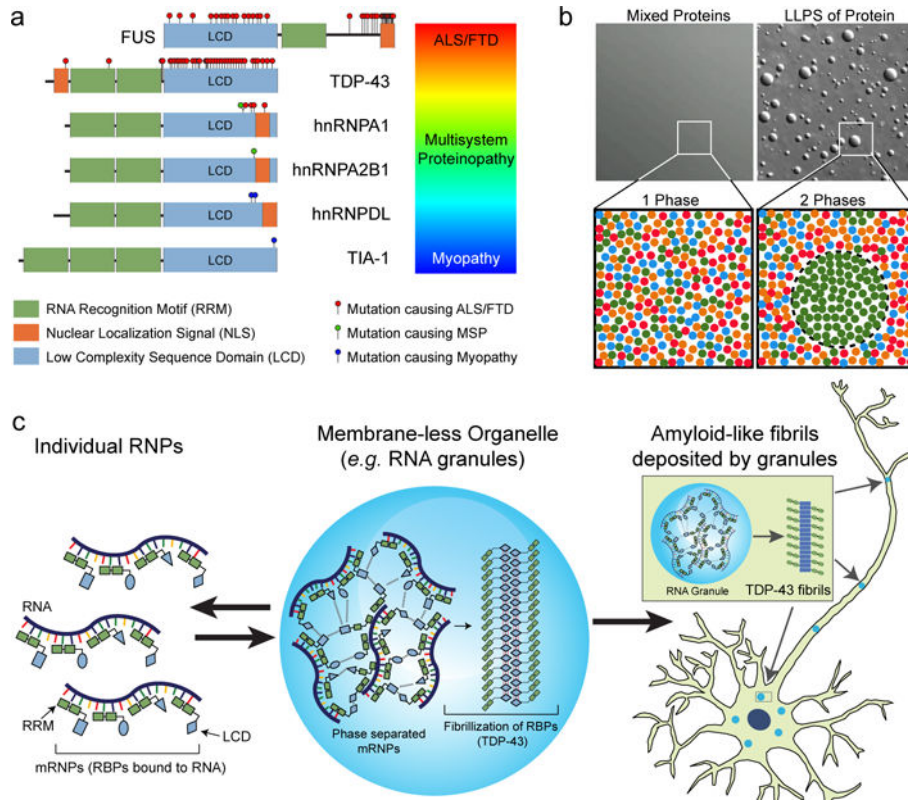


Figure 3. ALS mutations impair the assembly, dynamics and function of membrane-less organelles such as RNA granules
 (a) Schematic representation of six hnRNPs that harbor mutations causative of a disease spectrum that ranges from ALS/FTD to myopathy. (b) Phase separation by the RNA-binding protein hnRNPA1. RNA-binding proteins with a LCD can transition from a single mixed phase to two distinct phases, one of which is a concentrated liquid droplet. (c) Phase separation contributes to the assembly, dynamics and liquid properties of membrane-less organelles such as RNA granules. Within RNA granules, the high concentration and close apposition of LCDs risks transition to amyloid-like fibrils, which likely provides the source for pathological deposition of proteins particularly prone to fibrillization, such as TDP-43.

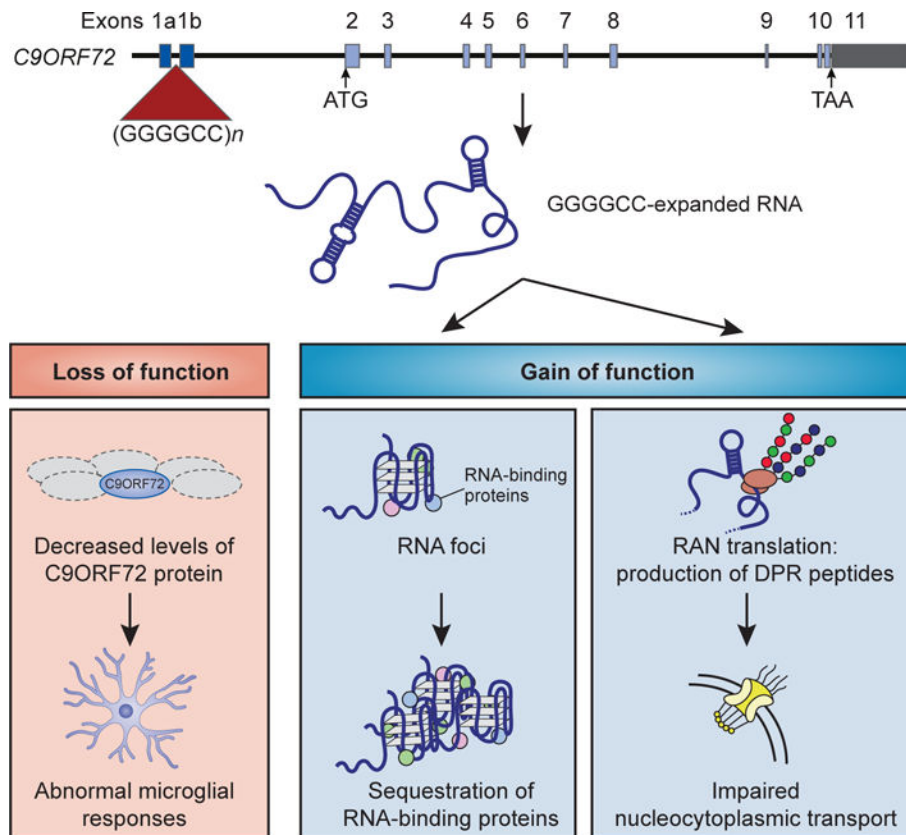


Figure 4. Three non-exclusive mechanisms have been proposed for C9-ALS/FTD

The offending mutation is expansion of a hexanucleotide repeat (GGGGCC) from fewer than 23 to hundreds or thousands in C9-ALS/FTD patients. This mutation results in modest reduction of C9ORF72 protein that appears insufficient to cause disease but may contribute to disease progression. Expression of sense and antisense transcripts containing the expanded repeat likely drive a toxic gain of function. The two major modes of toxic gain of function implicated are (1) toxicity of the mutant transcript, perhaps through sequestration of RNA-binding proteins, and (2) unconventional translation to produce dipeptide repeat proteins, some of which may be toxic.

Table 1

The genetics of ALS.

Locus	Gene	Protein	Protein Function	Mutations	Proportion of ALS familial	Proportion of ALS sporadic	Discovery Date
21q22.1	<i>SOD1</i>	Cu/Zn superoxide dismutase	Superoxide dismutase	>150	20%	2%	1993 ²
2p13	<i>DCTN1</i>	Dynactin	Component of dynein motor complex	10	1%	<1%	2003 ^{3,4}
14q11	<i>ANG</i>	Angiogenin	Ribonuclease	>10	<1%	<1%	2006 ^{1,44}
q36	<i>TARDBP</i>	TDP-43	RNA-binding protein	>40	5%	<1%	2008 ^{69,145}
16p11.2	<i>FUS</i>	FUS/TLS	RNA-binding protein	>40	5%	<1%	2009 ^{70,71}
9p13.3	<i>VCP</i>	Valosin-containing protein	Ubiquitin segregase	5	1–2%	<1%	2010 ⁴⁶
10p15-p14	<i>OPTN</i>	Optineurin	Autophagy adaptor	1	4%	<1%	2010 ⁴⁴
9p21-22	<i>C9ORF72</i>	C9ORF72	Possible guanine nucleotide exchange factor	Intronic GGGGCC repeat	25%	10%	2011 ^{8,79}
Xp11.23-Xp13.1	<i>UBQLN2</i>	Ubiquilin 2	Autophagy adaptor	5	<1%	<1%	2011 ⁴²
5q35	<i>SQSTM1</i>	p62	Autophagy adaptor	10	<1%	<1%	2011 ^{43,146}
17p13.2	<i>PFN1</i>	Profilin-1	Actin-binding protein	5	<1%	<1%	2012 ¹⁴⁷
12q13.1	<i>HNRNPA1</i>	hnRNPA1	RNA-binding protein	3	<1%	<1%	2013 ^{72,73}
5q31.2	<i>MATR3</i>	Matrin 3	RNA-binding protein	4	<1%	<1%	2014 ⁷⁸
2q36.1	<i>TUBA4A</i>	α -tubulin 4a	Microtubule subunit	7	<1%	<1%	2014 ⁴⁸
22q11.23	<i>CHCHD10</i>	Coiled-coil-helix-coiled-coil-helix domain containing 10	Mitochondrial protein of unknown function	2	<1%	<1%	2014 ⁴⁹
12q14.1	<i>TBK1</i>	TANK-binding kinase 1	Regulates autophagy and inflammation	10	?	?	2015 ¹⁵⁰