Emerging mechanisms of molecular pathology in ALS

Owen M. Peters, …, Mehdi Ghasemi, Robert H. Brown Jr.


Amyotrophic lateral sclerosis (ALS) is a devastating degenerative disease characterized by progressive loss of motor neurons in the motor cortex, brainstem, and spinal cord. Although defined as a motor disorder, ALS can arise concurrently with frontotemporal lobar dementia (FTLD). ALS begins focally but disseminates to cause paralysis and death. About 10% of ALS cases are caused by gene mutations, and more than 40 ALS-associated genes have been identified. While important questions about the biology of this disease remain unanswered, investigations of ALS genes have delineated pathogenic roles for (a) perturbations in protein stability and degradation, (b) altered homeostasis of critical RNA- and DNA-binding proteins, (c) impaired cytoskeleton function, and (d) non-neuronal cells as modifiers of the ALS phenotype. The rapidity of progress in ALS genetics and the subsequent acquisition of insights into the molecular biology of these genes provide grounds for optimism that meaningful therapies for ALS are attainable.
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Department of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts, USA.

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Introduction
Amyotrophic lateral sclerosis (ALS) is a progressive, fatal disorder of motor neurons that begins focally and spreads, leading to weakness of limb, respiratory, and bulbar muscles. Immediately preceding death, there is a near total loss of limb and respiratory function, as well as a loss of the ability to chew, swallow, and speak. ALS is defined as an “orphan disease,” with approximately 2 per 100,000 new cases per year and a prevalence of about 5 per 100,000 total cases each year (1). In the United States (2) and Europe (3, 4), ALS is diagnosed in about 1 in 500 to 1 in 1,000 adult deaths, implying that 500,000 people in the United States will develop this disease in their lifetimes. About 10% of ALS cases are inherited, usually as dominant traits (5, 6). Both familial ALS (fALS) and sporadic ALS (sALS) can develop concurrently with frontotemporal lobar dementia (FTLD). By contrast with the dementia of Alzheimer disease (AD), in which the cardinal finding is memory loss, FTLD is characterized by behavioral changes and progressive aphasia, sometimes accompanied by movement disorders (7, 8). While AD involves prominent pathology in the hippocampus, the essential finding in FTLD is, as the name suggests, early atrophy of the frontal and temporal lobes.

Four recurring themes have emerged from the pathological analysis of autopsied cases with sALS, fALS, or ALS-FTLD with diverse genetic causes. First, the motor neuron death usually entails deposition of aggregated proteins, often ubiquitinated and predominantly cytoplasmic. Second, in ALS, the levels and functions of RNA and RNA-binding proteins are abnormal. Aggregates of protein and RNA are detected both in motor neurons and non-neuronal cells, such as astrocytes and microglia. Third, most cases entail some disturbance of neuronal cytoskeletal architecture and function. Additionally, in almost all cases, motor neuron death is influenced by non-neuronal cells, including oligodendroglia and cells involved in neuroinflammation (e.g., astroglia and microglia).

Protein toxicity: protein aggregation and prion domains
An important theme in ALS pathogenesis is that several species of protein (both WT and mutant) are dysfunctional in both fALS and sALS, as evidenced by the formation of aggregates, abnormal cleavage events, or distinctive posttranslational modifications (e.g., ubiquitination or hyperphosphorylation). These changes occur both as primary consequences of mutations in the affected proteins and as secondary phenomena induced by the underlying disease process.

Protein aggregation and inclusion bodies. It has been apparent for decades that protein pathology is important in ALS, as suggested by an early pathological finding of deposition of threads of ubiquitinated material in motor neurons (9). This has been interpreted as denoting the presence of proteins that are conformationally unstable or modified, and so are destined for degradation. In later stages of motor neuron disease, dense aggregates of ubiquitinated protein are seen, sometimes in association with eosinophilic aggregates described as “Bunina bodies” (10). Whether these deposits are toxic or reflect a cellular response to a more primary pathology remains unclear. Indeed, the possibility that some aggregates may reflect beneficial, compensatory events has also been considered.

Cytosolic superoxide dismutase [Cu/Zn] (SOD1) was the first gene and protein whose mutations were demonstrated to cause ALS (Table 1; ref. 11). Of the more than 160 different missense and 12 truncation mutations detected in SOD1 (12), most impair conformational stability of this abundant protein, triggering its deposition in inclusion bodies within spinal motor neurons (13) both in vitro and in vivo (14, 15). There is a general correlation between the degree of mutation-induced conformational instability and the rate of clinical progression (15). Mutant SOD1 also spontaneously forms oligomers in vivo and in vitro (16, 17) that are submicroscopic and often soluble (18) and may be more toxic than larger, visible aggregates (19, 20).

Aggregates of posttranslationally modified WT SOD1 can also be detected in the spinal cords of many sALS patients (21–23). This
Table 1. Genes whose mutations cause ALS: Part 1 of 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fraction fALS (%)</th>
<th>Locus</th>
<th>Encoded protein</th>
<th>Functionality</th>
<th>Clinical phenotype</th>
<th>Neuropathology</th>
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<tr>
<td>C9ORF72</td>
<td>40–50</td>
<td>9p21.3</td>
<td>C9ORF72</td>
<td>Transcription &amp; pre-mRNA splicing regulation; Membrane traffic via Rab GTPase family</td>
<td>ALS + FTLD</td>
<td>LMN + PLS + HSP + PMA + NCI + NII + DN + GCI + Other</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Intranuclear RNA foci (sense, antisense); cytoplasmic RNA peptide aggregates</td>
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<tr>
<td>SOD1</td>
<td>20–25</td>
<td>21q22</td>
<td>SOD1</td>
<td>Major cytosolic antioxidant</td>
<td>ALS + FTLD</td>
<td>Aggregate; p62, C9ORF72, ubiquilin 2, others; impaired axonal transport, mitochondrial function; disturbed dendritic arborization of neurons; oxidative stress-related neuronal toxicity</td>
</tr>
<tr>
<td>TARDBP</td>
<td>4-5</td>
<td>1p36.2</td>
<td>TARDBP</td>
<td>Transcription &amp; pre-mRNA splicing regulation; miRNA biogenesis; RNA transport &amp; stabilization; translational regulation of ApoE-II &amp; CFTR</td>
<td>ALS + FTLD</td>
<td>LMN + PLS + HSP + NCI + NII + DN + GCI + Other</td>
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<tr>
<td>FUS</td>
<td>4-5</td>
<td>16p11.2</td>
<td>FUS (FUS/TLS)</td>
<td>Transcription &amp; pre-mRNA splicing regulation; miRNA processing; mRNA transport &amp; stabilization; maintenance of genomic integrity; regulating protein synthesis at synapse</td>
<td>ALS + FTLD</td>
<td>LMN + PLS + NCI + NII + DN + GCI + Other</td>
</tr>
<tr>
<td>OPTN</td>
<td>2-3</td>
<td>10p13</td>
<td>Optineurin</td>
<td>Golgi maintenance; exocytosis; vesicular trafficking; regulator of NF-κB signaling pathway; autophagy process</td>
<td>ALS + FTLD</td>
<td>LMN + PLS + NCI + NII + DN + GCI + Other</td>
</tr>
<tr>
<td>PFNY</td>
<td>1-2</td>
<td>17p13</td>
<td>Profilin-1</td>
<td>Regulates ATP-mediated actin polymerization</td>
<td>ALS + FTLD</td>
<td>LMN + NCI + NII + DN + GCI + Other</td>
</tr>
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<td>VCP</td>
<td>1-2</td>
<td>9p13</td>
<td>VCP or p57</td>
<td>Protein degradation via UPS, autophagy, &amp; ER; membrane fusion</td>
<td>ALS + FTLD</td>
<td>LMN + NCI + NII + DN + GCI + Other</td>
</tr>
<tr>
<td>ANG</td>
<td>1-2</td>
<td>14q11.2</td>
<td>Angiogenin</td>
<td>RNA processing &amp; RNA modification; vascularization; PKase activity &amp; assembly of stress granules; neurite outgrowth &amp; pathfinding</td>
<td>ALS + FTLD</td>
<td>LMN + NCI + NII + DN + GCI + Other</td>
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<tr>
<td>TUBA4A</td>
<td>1</td>
<td>2q35</td>
<td>Tubulin α4A</td>
<td>Major component of microtubules; neuronal cell skeleton</td>
<td>ALS + FTLD</td>
<td>Destabilized microtubule network; microtubules repolymerization capability</td>
</tr>
<tr>
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<td>&lt;1</td>
<td>Xp11</td>
<td>Ubiquilin 2</td>
<td>Protein degradation via UPS</td>
<td>ALS + FTLD</td>
<td>Rare +</td>
</tr>
<tr>
<td>TAF5</td>
<td>&lt;1</td>
<td>17q11</td>
<td>TAF5</td>
<td>Transcription initiation; RNA polymerase II gene component</td>
<td>ALS + FTLD</td>
<td>Rare +</td>
</tr>
<tr>
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<td>EWSR1</td>
<td>Transcriptional repressor</td>
<td>ALS + FTLD</td>
<td>Rare +</td>
</tr>
<tr>
<td>hnRNPA1</td>
<td>&lt;1</td>
<td>12q13</td>
<td>hnRNPA1</td>
<td>Packing &amp; transport of mRNA; miRNA biogenesis</td>
<td>ALS + FTLD</td>
<td>Rare +</td>
</tr>
</tbody>
</table>

*As part of multisystem proteinopathy. C9ORF72, chromosome 9 open reading frame 72; DN, dystrophic neurites; GCI, glial cell inclusions; LMN, lower motor neuron disease; miRNA, micro RNA; mutSOD1, mutant superoxide dismutase 1; NCI, neuronal cytoplasmic inclusions; NII, neuronal intranuclear inclusions; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; TLS, translocated in liposarcoma; UMN, upper motor neuron; UPS, ubiquitin-proteasome system; VCP, valosin-containing protein.
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The proximal pathobiology of TDP-43 and SOD1 are fundamentally different. Motor neuron death by mutant SOD1 generally is a consequence of the abundance of the mutant protein: the higher the burden of mutant SOD1^{G93A} protein in mice, the more fulminating the disease. (An exception is a truncated mutant form of SOD1 that causes ALS, although at low levels; ref. 41). To a remarkable degree, cellular function in vivo is relatively unaffected by rather dramatic increases and decreases in levels of the WT protein. SOD1 KO mice demonstrate a late-life, slow motor neuropathy (42) but do not develop fulminant ALS as seen in the transgenic SOD1^{G93A} mice. By contrast, both reduction and elevation of levels of WT TDP-43 can be devastating, leading to frank motor neuron disease (43–46). It thus seems unlikely that mutant TDP-43 toxicity bears a simple relationship to the dose of the protein or its RNA transcript.

Shortly after the identification of TDP-43, a second RNA-binding protein, fused in sarcoma/translocated in liposarcoma (FUS/TLS or just FUS), was implicated through analysis of a fALS pedigree from Cape Verde (47, 48). Structurally comparable to TDP-43 (Figure 1), FUS is localized within the nucleus, where it interacts with DNA and RNA in a broad range of processes. It is able to translocate to the cytoplasm, where it is required for RNA processing. Almost half of the FUS mutations are within the C-terminal nuclear localization sequence, with a smaller cluster found within the glycine-rich domain. Mutant FUS, like mutant SOD1 and TDP-43 — and WT TDP-43 in sALS — is detected in the neuromuscular synaptic structures (6, 33).

Accumulation of ubiquitinated protein is a key characteristic of early ALS pathology (33). A major breakthrough in understanding this was the 2006 discovery by Neumann and colleagues that trans-active response (TAR) DNA-binding protein (TDP-43) is the ubiquitinated target in both sALS and FTLD (35). The TDP-43 gene (TARDBP) encodes a 43-kDa protein containing two RNA recognition domains; a glycine-rich, putative prion-like domain; and both nuclear localization and nuclear export motifs (Figure 1). Under normal conditions, TDP-43 is located in the nucleus, where it is involved in DNA/RNA binding, transcription, RNA splicing, miRNA biogenesis, RNA transport, and RNA transcript stability (reviewed in ref. 36). TDP-43 has a pivotal role in nonsense-mediated decay, the process whereby abnormal mRNA transcripts (e.g., base-pair substituted or truncated) are rapidly degraded.

In most cases of sALS, fALS, and FTLD, hyperphosphorylated, cleaved TDP-43 accumulates diffusely in the cytoplasm of neurons and glia, where it assembles into round and thread-like inclusions. These pathological forms of TDP-43 are first evident within spinal motor neurons but then disperse rostrally into the brain and throughout the CNS (37). TDP-43 has now been identified as a component in cytoplasmic deposits in disparate neurodegenerative diseases (36). The depletion of TDP-43 from the nucleus in these disorders has suggested that TDP-43-mediated toxicity may reflect either loss of its function in the nucleus, an acquired adverse effect of its pathological presence in the cytoplasm (gain-of-function), or both.

Following the identification of TDP-43 protein in inclusion bodies in sALS, several investigators identified ALS-associated mutations in TARDBP in cases of fALS (38), accounting for 4%–5% of dominantly inherited fALS, approximately 1% of sALS (39), and rare cases of FTLD (Table 1; ref. 40). To date, more than 35 dominantly transmitted coding missense mutations have been described in TARDBP; a single premature stop codon was also identified (12). With only three known exceptions, these mutations alter residues encoding the C-terminal glycine-rich domain, the site of a putative prion-like domain.

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In 2011, a trio of teams made the discovery that 40%–50% of fALS and 8%–10% of sALS cases, as well as cases of ALS-FTLD, are caused by mutations in the gene C9orf72 (Figure 2; refs. 54–56). This gene encodes a protein of approximately 54.3 kDa (481 amino acids) whose function remains unknown. Bioinformatic analyses suggest that this protein shares structural features
with differentially expressed in normal and neoplastic (DENN) cells and GDP/GTP exchange factors (GEF), and may regulate membrane cell trafficking (57). The distinctive mutation in this gene is an expansion of an intronic hexanucleotide GGGGCC (G,C) repeat motif. Normally present in 30 or fewer copies, in C9orf72-associated ALS, the repeat domain expands to encompass hundreds of tandem repeats (58). The expanded segment is transmitted as a dominant trait in ALS and ALS-FTLD pedigrees. Affected individuals are heterozygous for the expanded allele; homozygous cases are infrequent (59, 60).
The G\(_4\)C\(_2\) expansion produces both nuclear RNA foci and cytoplasmic protein inclusions. The expansion is located within a normally noncoding intron within the C9orf72 promoter and produces a large RNA transcript containing the motif. At autopsy, C9orf72 mutant brains show widespread intranuclear RNA foci (54, 61) generated from both sense and antisense transcripts across the expanded G\(_4\)C\(_2\) segment. Foci have been described in fibroblasts (62–64) and motor neurons derived from induced pluripotent cells generated from fibroblasts of C9orf72 ALS cases (62–65).

Notably, the expansion shows some instability; its size varies modestly between different cell types in the same individual (65). In addition to these intranuclear RNA foci, histopathological studies have documented that these cases have at least three types of protein inclusions. Two of these are TDP-43-positive inclusions and distinctive spheroidal inclusions that are positive for a 62 kDa protein (discussed below) that is abundant in hippocampus and cerebellum (66). Several components of these p62-positive inclusions have since been identified, including RNA-binding motif 45 (RBM45) (67) and heterogeneous nuclear ribonucleoprotein A3 (hnRNPA3) (68). Unexpectedly, neurons from cases with the C9orf72 intronic expansion also possess a third type of inclusion: intracytosolic aggregates composed of dipeptide repeat proteins encoded by the intronic hexanucleotide repeat that are produced through noncanonical, repeat-associated non-ATG–mediated translation (RAN-mediated) translation. These atypical peptides reflect trans-through noncanonical, repeat-associated non-ATG–mediated encoded by the intronic hexanucleotide repeat that are produced intracytosolic aggregates composed of dipeptide repeat proteins that were structural homologues to fALS-associated FUS: RNase polymerase II, TATA box binding protein–associated (TPB-associated) factor 68 kDa (TAF15) (90), and Ewing sarcoma breakpoint region 1 (EWSR1) (91). Mutational analyses have defined rare coding sequence variants in both TAF15 and EWSR1 in ALS; moreover, mutant TAF15 mislocalizes from the nucleus to form cytoplasmic punctae in spinal cord neurons (90, 92).

More recently, two heterogeneous nuclear ribonucleoproteins (hnRNPA1 and hnRNPA2B1) with prion-like domains have been genetically linked with fALS and the VCP-linked multisystem phenotype described previously (93). Intriguingly, the ribonucleoprotein hnRNPA3 is reported to bind the C9orf72 fALS-associated G\(_4\)C\(_2\) repeat and accumulate in cytoplasmic inclusions unique to C9orf72 patients (68). The prion domain–containing protein CREST has been identified as a sALS risk factor in a trio analysis exome screen (94). CREST is a calcium-activated transcriptional activator and essential component of the nBAF (SWI/SNF) chromatin-remodeling complex.

One caveat arises in considering the model of ALS as a prion-driven disease. In prion disease (e.g., Creutzfeldt-Jakob disease, Kuru, or Gerstmann-Sträussler-Scheinker syndrome), the templated misfolding propagates not only between cells within an individual but also between individuals within a species, and even between species (95, 96). While data support the contention that prion-like misfolding of ALS proteins can propagate from cell to cell, transmission between individuals or species has not been documented in ALS.

Role of prion-like domains in RNP granule formation. Many prion-like domains containing DNA/RNA-binding proteins are components of ribonucleoprotein granules (RNP granules), which maintain RNA homeostasis during cellular stress (reviewed in ref. 89). Stress granules, a form of RNP granule that rapidly assembles in response to a range of stressors, are particularly relevant to ALS pathology. The interaction and self-assembly of the prion-like domains of cytosolic RNA-binding proteins facilitates the rapid assembly of stress granules, allowing the sequestration of RNA into these inclusions—in some cases within minutes of toxic insult (97). The majority of RNA/DNA-binding, prion-like domain–containing proteins associated with ALS can be incorporated into stress granules (88, 97, 98). Thus, in response to stress, mutant forms

**Prion-like domains in ALS proteins.** ALS usually begins focally and spreads in a pattern that implicates contiguous pools of motor neurons. The possibility that a pattern of dissemination of pathology and then motor neuron death in ALS might be prion-like was first suggested more than a decade ago in the context of SOD1-associated disease (83). Indeed, cell-to-cell spread and propagated misfolding of both mutant (84) and WT (22) SOD1 have been reported. The prion hypothesis in ALS has recently been underscored by the observation that TDP-43 and FUS have low-complexity, glycine-rich domains that enhance aggregate formation (Figure 1). Indeed both proteins emerge in an in silico screen for proteins that harbor domains comparable to known yeast prion peptides (85–87). Many proteins contain both RNA-binding and prion-like activities; of approximately 250 human genes predicted to contain prion-like domains (88), 12%–20% are predicted to possess RNA-binding activity (89). Overexpression of DNA/RNA-binding proteins in yeast identified two prion-like proteins that were structural homologues to fALS-associated FUS: RNase polymerase II, TATA box binding protein–associated (TPB-associated) factor 68 kDa (TAF15) (90), and Ewing sarcoma breakpoint region 1 (EWSR1) (91). Mutational analyses have defined rare coding sequence variants in both TAF15 and EWSR1 in ALS; moreover, mutant TAF15 mislocalizes from the nucleus to form cytoplasmic punctae in spinal cord neurons (90, 92).

Mutations in other FALS genes are likely to impair protein degradation. One example is valosin-containing protein (VCP) whose mutations not only cause ALS, but also FTLD, inclusion body myositis, and Paget’s disease (76). VCP is a ubiquitin-dependent segregase that facilitates the segregation and proteosomal degradation of ubiquitinated proteins. Rare mutations in ubiquilin-2 (UBQLN2) and its homolog UBQLN1, which regulate ubiquitination of protein targets, have been associated with a rare, dominantly inherited, X-linked form of fALS (77) and the infantile-onset motor neuron disease Brown-Vialetto-Van Laere syndrome (78), respectively. UBQLN2 is a component of inclusion bodies in the brains and spinal cords of patients harboring UBQLN2 mutations that impair normal proteasome-mediated protein degradation. Polymorphisms in sequestosome-1 (SQSTM1, p62) are also associated with FALS and sALS (79–82). This ubiquitin-binding scaffold protein decorates the surface of inclusion bodies in many neurodegenerative diseases.
Figure 2. Pathogenic mechanisms associated with hexanucleotide repeat-expanded C9orf72 and various DNA/RNA-binding proteins. (A) The mutant C9orf72 gene associated with fALS contains an intronic G4C2 motif often expanded to several hundred (and even several thousand) repeats. This GC-rich domain is transcribed in both the sense and antisense directions, producing mRNA prone to forming large intranuclear foci that are believed to sequester some RNA-binding proteins. The sense or antisense transcripts undergo noncanonical RAN translation in all six possible reading frames, generating five dipeptide-repeat peptides (GA, GR, GP, PR, PA), which form inclusions that are associated with the protein p62. (B) Under normal conditions, the DNA/RNA-binding proteins mutated in fALS, most notably TDP-43 and FUS, are typically located within the nucleus, where they serve multiple functions. These proteins are also able to translocate to the cytoplasm, where they may localize to stress granules under some adverse cellular conditions. When these proteins are defective (e.g., bearing ALS-related mutations) the normal range of interactions with DNA and RNA are disrupted; this can lead to marked changes in transcription, splicing, and translation. Furthermore, the presence of the low-complexity, prion-like domains is thought to facilitate oligomer self-assembly under conditions of cellular stress, thereby promoting prion-like toxicity and intercellular spread.
Table 2. Genes whose mutations cause ALS: Part 2 of 2

<table>
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<tr>
<th>Gene</th>
<th>Fraction FALS (%)</th>
<th>Locus</th>
<th>Encoded protein</th>
<th>Functionality</th>
<th>Clinical phenotype</th>
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<td>&lt;1</td>
<td>7p15</td>
<td>hnRNPA2/B1</td>
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<td>+ +</td>
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<td>9q34.13</td>
<td>Senataxin</td>
<td>DNA/RNA helicase activity; DNA/RNA metabolism</td>
<td>+</td>
<td>↓ neuronal differentiation; ↓ neurite growth</td>
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<tr>
<td>CREST</td>
<td>&lt;1</td>
<td>20q13.3</td>
<td>SS18L1</td>
<td>Ca²⁺-dependent transcriptional activator</td>
<td>+</td>
<td>+ ↓ dendrite outgrowth; ↑ interaction with FUS</td>
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<td>RNA processing; Stabilizing mRNA; gene silencing; chromatin organization</td>
<td>+ Rare</td>
<td>+ +</td>
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<tr>
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<td>12q24</td>
<td>Ataxin-2</td>
<td>RNA processing; regulation of receptor tyrosine kinase endocytosis</td>
<td>+ + + +</td>
<td>↑ Interaction with TDP-43</td>
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<td>5q35</td>
<td>p62 or sequestosome-1</td>
<td>Autophagy &amp; UPS degradation; regulator of NF-κB signaling pathway; immune response</td>
<td>+ + + + + +</td>
<td>+ Disrupted endosomal structure; Aggregates of autophagosomes &amp; multilamellar structures; ↑ TDP-43, p62 &amp; ubiquitin inclusions</td>
</tr>
<tr>
<td>CHMP2B</td>
<td>&lt;1</td>
<td>3p11</td>
<td>CHMP2B</td>
<td>MVBs formation; protein traffic between plasma membrane, trans-Golgi network &amp; lysosome</td>
<td>+ + + +</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>ALS2</td>
<td>&lt;1</td>
<td>2q33.1</td>
<td>Akin</td>
<td>Activation of the small GTPase Rac1 macropinocytosis-associated endosome fusion &amp; trafficking; neurite outgrowth</td>
<td>+</td>
<td>↓ axonal growth; ↓ lysosome-dependent clearance of p62 &amp; LC3-II</td>
</tr>
<tr>
<td>VAPB</td>
<td>&lt;1</td>
<td>20q13</td>
<td>VAPB</td>
<td>Regulation of ER-golgi transport &amp; secretion</td>
<td>+ + + +</td>
<td>↑ TDP-43 toxicity &amp; inclusions; Aberrant synaptic microtubule cytotoxicity; Nuclei mispositioning &amp; aberrant architecture</td>
</tr>
<tr>
<td>SIGMAR1</td>
<td>&lt;1</td>
<td>9p13.3</td>
<td>SIGMAR1</td>
<td>Lipid transport through ER; BDNF &amp; EGF signaling</td>
<td>+ + + +</td>
<td>↑ apoptosis induced by ER stress; ↑ interaction with VAPB</td>
</tr>
<tr>
<td>DCTN1</td>
<td>&lt;1</td>
<td>2p13</td>
<td>Dynactin</td>
<td>ER-to-golgi transport; centripetal movement of lysosomes &amp; endosomes; spindle formation, chromosome movement; nuclear positioning; axonogenesis</td>
<td>+</td>
<td>+ + + + + + + + + +</td>
</tr>
<tr>
<td>SPC11</td>
<td>1</td>
<td>15q21.1</td>
<td>Spatascin</td>
<td>Neuronal cell skeleton; axonal transport; involved in synaptic vesicles</td>
<td>+ + + +</td>
<td>+ ↓ acetylated stabilized tubulin; ↓ synaptic vesicles in neurons; disrupted anterograde axonal transport</td>
</tr>
<tr>
<td>NEFH</td>
<td>&lt;1</td>
<td>22q12.2</td>
<td>NEFH</td>
<td>Maintaining a proper axon diameter</td>
<td>+ + + +</td>
<td>↑ neurofilament aggregates</td>
</tr>
<tr>
<td>PRPH</td>
<td>&lt;1</td>
<td>12q13</td>
<td>Peripherin</td>
<td>Regulating neurite elongation during development &amp; axonal regeneration after injury</td>
<td>+ + + +</td>
<td>+ ↓ ability of the neurofilament network to assemble; + ubiquinated inclusions; Coaggregation with mutSOD1</td>
</tr>
</tbody>
</table>

*As part of multisystem proteinopathy, †Phenotype more similar to Silver syndrome than to ALS, ‡Predominant LMN phenotype, §As part of ALS.

*Predominant UMN phenotype. CHMP2B, charged multivesicular body protein 2B; DN, dystrophic neurites; GCI, glial cell inclusions; LC3-II, microtubule-associated protein 1A/1B-light chain 3-II; LMN, lower motor neuron disease; miRNA, micro RNA; mutSOD1, mutant superoxide dismutase 1; MVBs, multivesicular bodies; NCI, neuronal cytoplasmic inclusions; NEFH, neurofilament heavy chain; NII, neuronal intranuclear inclusions; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; SIGMAR1, Sigma non-opioid intracellular receptor 1; SS18L1, synovial sarcoma translocation gene on chromosome 18-Like 1; UMN, upper motor neuron; UPS, ubiquitin-proteasome system; VAPB, vesicle-associated membrane protein B; VCP, valosin-containing protein.
of TDP-43 (100) and FUS (97) incorporate rapidly into persistent stress granules or form small oligomeric aggregates that interact with the granules. This brings together the prion-like domains of the RNA-binding proteins and thus is potentially permissive for prion assembly and propagation. An abnormally strong interaction of the mutant prion-like domains may prevent the disassembly of these granules, resulting in the persistent sequestration of mRNAs and thereby impeding their translation. Alternatively, their incorporation into granules might facilitate the conversion of pri-on-like domains to amyloid states, seeding larger, fibrillary oligomers, and thus is potentially permissive for the function of the anterograde transport motor complex.

RNA biology

**RNA/DNA binding function.** As noted above, numerous DNA/RNA interacting proteins have now been associated with fALS, most commonly TDP-43 and FUS, with more rare examples, including TAF15, EWSR1, ANG, SETX, ELP3, and ataxin-1 and -2, hnRNPA1 and hnRNPA2B1, and CREST (Tables 1 and 2). TDP-43 and FUS function in almost every aspect of DNA and RNA processing, including transcription, splicing, RNA transport, miRNA processing, and translation. Our understanding of the role of these proteins in ALS pathogenesis is limited; we do not yet know which, if any of the myriad functions served by these proteins, is the Achilles heel in fALS. Under normal physiological conditions, these fALS-associated DNA/RNA-binding proteins are largely nuclear; a fraction of these proteins translocate to the cytoplasm under physiological conditions. The mutant, ALS-associated variant proteins (e.g., TDP-43, FUS, TAF15, hnRNPA1, hnRNPA2B1) are frequently mislocalized to the cytoplasm, often leaving the nucleus entirely depleted of the affected protein (Figure 2; refs. 35, 47). It is unclear whether the primary pathology is loss of function in the nucleus or an acquired cytotoxic effect of the mutant protein in the myriad functions normally served by these proteins. What is clear is that the function of these proteins can be substantially impaired by ALS-associated mutations. As one example, TDP-43 and hnRNPA1 play a role in the recognition of splice sites on RNA transcripts (101, 102). In mice that overexpress the human Q331K mutant, the splicing of over 1,000 different RNA transcripts is altered (103).

**Toxicity of RNA foci.** Both sense and antisense RNA transcripts are present in intranuclear foci in cases of C9orf72-mediated ALS. The possibility that these foci are directly unfavorable is suggested by studies in myotonic dystrophy type 1, wherein RNA foci transcribed from CTG expansions sequester the transcriptional splice factor muscleblind-like 1 (MBNL-1), depleting the pool of the protein in the nucleus (104). The possibility that this model pertains to C9orf72 fALS patients has led to efforts to identify protein-binding partners of the sense and antisense foci generated from the expanded G4C2 repeat domain of C9orf72. Candidates identified to date include ADARB2 (62), hnRNPH, and SF2 (59, 61), though the consequence of this binding is presently unclear. The C9orf72 expansions might compromise cellular viability via mechanisms other than sequestering transcription factors. It is conceivable that the expanded G4C2 domain and/or the foci reduce expression of the normal C9orf72 protein and that this loss of function of the C9orf72 protein impairs motor neuron viability. Another possibility is that the pathological DNA expansions and RNA foci alter transcript splicing. Still another conjecture is that the G4C2 expansions elaborate functional short RNA fragments that act as siRNA or miRNA. Clearly, further studies will be required to characterize the C9orf72 expansion, its RNA and peptide products, and their respective potentials for cytotoxicity of both neuronal and non-neuronal cells.

**Cytoskeletal function**

**Cytoskeletal structure.** A defining feature of motor neurons is the length of their axons, rendering them highly dependent on intra-
Table 3. Genes whose mutations increase ALS risk and/or modify the ALS phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fraction fALS (%)</th>
<th>Locus</th>
<th>Encoded protein</th>
<th>Functionality</th>
<th>Clinical phenotype</th>
<th>ALS</th>
<th>ALS + FTLD</th>
<th>LMN</th>
<th>PLS</th>
<th>HSP</th>
<th>PMA</th>
<th>Neuro-pathology</th>
<th>FTLD</th>
<th>NCI</th>
<th>NII</th>
<th>DN</th>
<th>GCI</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTE</td>
<td>&lt;1</td>
<td>19p13</td>
<td>Neurotrophin</td>
<td>Regulating the neuronal membrane composition</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Disruption of ER; ↑ reticular aggregates; ↑ vacuolization of nerve cell bodies</td>
</tr>
<tr>
<td>PON1-3</td>
<td>&lt;1</td>
<td>7q21</td>
<td>Paraoxonase 1-3</td>
<td>Enzymatic breakdown of nerve toxins</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oxidative stress-related neuronal toxicity</td>
</tr>
<tr>
<td>DAO</td>
<td>&lt;1</td>
<td>12q22</td>
<td>DAO</td>
<td>Regulating levels of D-serine, NMDAR function</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ D-serine levels in motor neurons and glia; ↑ ubiquitinated inclusions</td>
</tr>
<tr>
<td>CHRNA3, CHRMA4, CHRNA4</td>
<td>&lt;1</td>
<td>15q24, 20q13, 15q24</td>
<td>nAChR</td>
<td>Cholinergic neurotransmission</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cationic overloading, Ca^{2+} toxicity in MNS</td>
</tr>
<tr>
<td>ERBB4</td>
<td>&lt;1</td>
<td>2q34</td>
<td>Receptor tyrosine-protein kinase ErbB-4</td>
<td>Neuronal cell mitogenesis and differentiation</td>
<td>+</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mitochondrial fragmentation &amp; DNA instability; mitochondrial crystalloid inclusions</td>
</tr>
<tr>
<td>CHCHD10</td>
<td>&lt;1</td>
<td>22q11</td>
<td>Mitochondrial protein</td>
<td>Mitochondrial genome stability; cristae integrity &amp; mitochondrial fusion</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mitochondrial dysfunction &amp; DNA instability; possible glutamate excitotoxicity</td>
</tr>
<tr>
<td>CY9orf12</td>
<td>&lt;1</td>
<td>9q22</td>
<td>Mitochondrial protein</td>
<td>Unknown</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALS3</td>
<td>&lt;1</td>
<td>18q21</td>
<td>Disulfide redox protein</td>
<td>Unknown</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genes whose variants influence ALS phenotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Functionality</th>
<th>Clinical phenotype</th>
<th>Neuro-pathology</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNC13A</td>
<td>19p13</td>
<td>Regulating neurite outgrowth &amp; synaptic neurotransmission</td>
<td>+</td>
<td></td>
<td>↓ synaptogenesis at neuromuscular junction; possible glutamate excitotoxicity</td>
</tr>
<tr>
<td>CHGB</td>
<td>20p12.3</td>
<td>Involved in ER-Golgi system</td>
<td>+</td>
<td></td>
<td>↓ density of synaptophysin-like immunoreactivity; ↑ interaction with mutSOD1</td>
</tr>
<tr>
<td>SMN</td>
<td>5q33</td>
<td>Regulating biogenesis of small RNPs</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

^Predominant UMN phenotype. CHGB, chromogranin B (secretogranin 1); DAO, D-amino acid oxidase; DN, dystrophic neurites; GCI, glial cell inclusions; KIFAP3, Kinesin-associated protein 3; LMN, lower motor neuron disease; micRNA, micro RNA; mutSOD1, mutant superoxide dismutase 1; nAChR, nicotinic acetylcholine receptor; NCI, neuronal cytoplasmic inclusions; NII, neuronal intranuclear inclusions; NMDAR, N-methyl-D-aspartate receptor; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; UMN, upper motor neuron; UPS, ubiquitin-proteasome system.
cellular transport mechanisms to maintain normal structure and function. Critical in sustaining this extraordinary architecture is the cytoskeleton and associated molecular scaffolds and motors. Early in the genetic studies of ALS, several rare mutations were detected in genes encoding neurofilament heavy chain (105-107) and peripheral (108-110); both components were detected in cytoplasmic inclusions (Table 2; ref. 111). Additional cytoskeletal components have been identified as rare causes or epistatic modifiers of disease onset in ALS. Exome sequencing of FALS DNA identified four mutations in the gene encoding profilin-1 (Table 1), an essential regulatory component in the conversion of monomeric actin (G-actin) to its filamentous form (F-actin) (Figure 3; ref. 112). Components of the cytoskeletal system also act as modifiers of the ALS phenotype. EphA4, an ephrin receptor tyrosine kinase that regulates developmental axon outgrowth, was identified in a zebrafish screen of genes that rescue motor axon abnormalities induced by expression of SOD1 (113). In a Dutch cohort, expression of EphA4 was inversely correlated with both onset and survival in ALS (Table 3). EphA4 normally repels axons, serving as a molecular brake on outgrowth. It is plausible that reduced EphA4 activity through reduced expression or loss-of-function mutations enhances axonal outgrowth in response to injury. It is also possible that this, in turn, accounts for the enhanced survival in ALS mice hemizygous for this gene. Identification of other disease-modifying factors like EphA4 will increase our understanding of molecular events that contribute to ALS and provide targets for therapeutic intervention.

**Axonal transport.** Like the cytoskeleton, efficient axonal transport is critical for axonal function. ALS-related mutations have now been identified in components of the axonal transport system. Dynactin is required for tethering cargos of vesicles and organelles to the retrograde transport motor dynemin. Introduction of mutations in DCTN1 led to dysfunction of dynactin in rodents, with defective vesicular transport, degeneration of motor neurons, and premature death (Table 2; refs. 114, 115). A second component of axonal transport, kinesin-associated protein 3 (KIFAP3), was identified as a modifier of survival in sALS (Table 3). A polymorphism associated with reduced expression of KIFAP3 was found to correlate with an extension of lifespan by over a year in sALS cases (116), although this association is not seen in all populations (117). Forming a trimeric motor complex with KIF3A and KIF3B, KIFAP3 functions in anterograde transport and chromosome cytokinesis. The role of KIFAP3 in disease is unclear; however, increases in its expression have been noted early in pathology in SOD1 transgenic mice (118). Finally, FALS-associated mutations have been identified in vesicle-associated membrane protein/synaptobrevin-associated membrane protein B (VAPB), a ubiquitously expressed protein that is particularly important during golgi and ER vesicle transport and secretion (Table 2; ref. 119). Secreted VAPB is a ligand for Eph receptors; its secretion is blocked by introduction of ALS-associated mutations (120). In drosophila, expression of mutant VAPB induced deficits in synaptic morphology and neurodegeneration (121).

**Non–cell-autonomous influences on motor neuron death**

An important insight from the transgenic SOD1 ALS mice is that cells other than the motor neuron can modify the rate of progression of the disease. This was first convincingly demonstrated by a report that survival of mice expressing mutant SOD1G93A in motor neurons was dramatically improved when the motor neurons were surrounded by WT cells (122). Survival in SOD1G93A transgenic ALS mice is remarkably prolonged by eliminating expression of the SOD1 gene from astrocytes (123), microglia (124), or oligodendroglia (125); reciprocally, it is worsened when mutant SOD1 is largely eliminated from Schwann cells in these transgenic mice (126). The motor neuron death process provokes a neuroinflammatory reaction that recruits and activates astrocytes and microglia. The impact of the microglia, which can be neuroprotective as well as toxic (127, 128), is determined by many factors including the phenotype of incoming reactive T cells (129, 130). That ALS-derived astrocytes can be toxic to motor neurons has been robustly demonstrated in vitro using human astrocytes and motor neurons derived from induced pluripotent stem cells (131-133). The toxic influences derived from cultured ALS astrocytes may involve signaling via prostaglandin receptors (134).

**Conclusion**

Like other neurodegenerative disorders, ALS poses challenges in therapy development. Not least is the inadequacy of our understanding of the basic biology of this disease. Perhaps most importantly, it is unclear what causes sALS. Are there fundamentally distinct mechanisms in motor neuron death yet to be discovered, possibly involving neurotoxic environmental factors? Do genetic factors define susceptibility to such toxins? Why is onset in fALS delayed for many decades when the mutant genes are expressed from the earliest embryonic stages of development? Does this reflect an age-dependent accumulation of injury in post-mitotic motor neurons, an age-dependent loss of capacity to compensate for the toxic genes, a requirement for a concomitant adverse environmental stimulus, or some combination of all of these factors? Will it be possible to define complex, multigenic or epistatic gene-by-gene interactions in ALS? What are the roles of epigenetic factors or disturbances of the microbiome in FALS and sALS?

Another important challenge in developing ALS therapies is the inherent difficulty in accessing the CNS. The brain and spinal cord are protected by the blood-brain barrier, which limits permeation of many small molecules, as well as macromolecular therapies. Two other major challenges in ALS therapy are diagnosing the disease rapidly enough to allow early intervention and the lack of sensitive ALS biomarkers.

These challenges notwithstanding, it is encouraging that there has been substantial progress in delineating genetic factors that modulate ALS risk and phenotype. Each of the more than 40 ALS genes now reported (Tables 1–3) implicates primary events in ALS pathogenesis; the primary events converge on multiple downstream processes (e.g., mitochondrial failure, excitotoxicity, ER stress, or altered axonal transport). Each ALS gene discloses potential therapeutic targets. Most ALS genes are dominantly transmitted and thus are likely to be cytotoxic because of acquired properties that impair motor neuron viability. Of these, some such as SOD1 and C9orf72 show dose dependence of pathology: the more mutant the gene product (whether RNA or protein), the more severe the phenotype. This implies that strategies to silence the offending mutant genes may be beneficial. Fortunately, there are multiple new approaches to gene silencing, such as small, intrathecally delivered antisense
oligonucleotides, and both shRNA and synthetic miRNAs delivered to the CNS via adeno-associated viral vectors. Indeed, a pilot trial of silencing of mutant SOD1 with antisense oligonucleotides has been reported (135). Another approach that has yielded positive pilot data is the use of antibodies to reduce the levels of mutant proteins in the CNS (136). For genes such as TARDBP, for which there is not a clear dose dependence of the mutant gene product to phenotype, other strategies will be needed. It is of interest that forced expression of the regulator of nonsense transcripts 1 (hUPF1) can ameliorate the toxicity of mutant TDP-43 in yeast (137) and in a rat model (138). To the extent that the pathophysiology of fALS and sALS overlap, therapeutic discoveries in the domain of ALS genetics may apply to sALS. These developments and, more generally, the expanding insights into pathophysiology allow optimism that meaningful therapies will be forthcoming, at least for inherited forms of ALS.

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Address correspondence to: Robert H. Brown Jr., Department of Neurology, University of Massachusetts Medical School, 55 Lake Avenue, North, Worcester, Massachusetts 01655, USA. Phone: 617.726.5750; E-mail: robert.brown@umassmed.edu.

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