The genetics of amyotrophic lateral sclerosis: current insights

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Abstract: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that results in loss of the upper and lower motor neurons from motor cortex, brainstem, and spinal cord. While the majority of cases are sporadic, approximately 10% show familial inheritance. ALS is usually inherited in an autosomal dominant manner, although autosomal recessive and X-linked inheritance do occur. To date, 24 of the genes at 26 loci have been identified; these include loci linked to ALS and to frontotemporal dementia-ALS, where family pedigrees contain individuals with frontotemporal dementia with/without ALS. The most commonly established genetic causes of familial ALS (FALS) to date are the presence of a hexanucleotide repeat expansion in the \textit{C9ORF72} gene (39.3% FALS) and mutation of \textit{SOD1}, \textit{TARDBP}, and \textit{FUS}, with frequencies of 12%–23.5%, 5%, and 4.1%, respectively. However, with the increasing use of next-generation sequencing of small family pedigrees, this has led to an increasing number of genes being associated with ALS. This review provides a comprehensive review on the genetics of ALS and an update of the pathogenic mechanisms associated with these genes. Commonly implicated pathways have been established, including RNA processing, the protein degradation pathways of autophagy and ubiquitin–proteasome system, as well as protein trafficking and cytoskeletal function. Elucidating the role genetics plays in both FALS and sporadic ALS is essential for understanding the subsequent cellular dysregulation that leads to motor neuron loss, in order to develop future effective therapeutic strategies.

Keywords: protein degradation, protein trafficking, \textit{C9ORF72}, TDP-43, RNA processing, autophagy

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder with an incidence of two to three per 100,000 and a lifetime risk of one per 400 individuals.\(^1\) Usually with an adult onset, initial clinical symptoms, such as loss of dexterity in the fingers or a mild limp in limb onset ALS, or slurring of speech in bulbar onset ALS, are caused by loss of the upper motor neurons in the motor cortex and brainstem and lower motor neurons in the spinal cord. Over time, the progressive nature of the disease is associated with further muscle wasting, weight loss, fasciculations, and eventually death due to respiratory failure, 32 months following symptom onset.\(^1\) During disease progression, cognitive impairment may develop in up to 40% of patients, and \(\sim \)5% will go on to develop frontotemporal dementia (FTD).\(^1\)

While the majority of cases are sporadic ALS (SALS) with no familial history of disease, \(\sim \)10% of cases are familial ALS (FALS) and are clinically indistinguishable from SALS cases. Generally, in adult onset ALS, the disease is inherited as an autosomal...
dominant (AD) trait, although rare cases of juvenile ALS are more commonly associated with autosomal recessive (AR) inheritance. However, there are also instances of AR inheritance of AD genes in specific populations (SOD1 in Scandinavia and FUS in Cape Verde). In addition, there is evidence of reduced penetrance of disease-associated mutations (including p.I114T SOD1 and G4C2 C9ORF72) and oligogenic inheritance, illustrating that ALS is a highly complex genetic disorder.

The first genetic cause of ALS was identified in 1993 through linkage analysis to the Chr21 marker DS21S223. Subsequent analysis of the nearby gene, SOD1, identified multiple pathogenic mutations in these FALS families. Over the following 16 years, linkage analysis and candidate gene sequencing of ALS families have identified further genes associated with AD or AR ALS and one instance of X-linked inheritance (Table 1). With the advent of next-generation sequencing, whole exome sequencing (WES) has allowed an exponential increase in the identification of disease-associated genes (Figure 1). Following the first use of WES to identify that the VCP gene was associated with the disease, WES has been used on many FALS samples and assisted in identifying seven further genes in the last 3 years.

To date, >22 ALS and four ALS + FTD (FTDALS) loci have been established, with the causative genes identified in the majority of cases. This review summarizes the current insights that have been gained from the four most common causes of FALS: SOD1, TARDBP, FUS, and C9ORF72. These genes have highlighted the roles of oxidative stress and RNA processing as contributory pathogenic mechanisms in ALS. In addition, rarer genetic variants have implicated additional biological pathways such as the ubiquitin–proteasome system (UPS), protein trafficking, and impaired cytoskeletal function. We have used the numbering of loci as described in the Online Mendelian Inheritance in Man Phenotypic Series for ALS (PS105400) and FTDALS (PS105550) in this review.

**ALS1: Cu–Zn superoxide dismutase (SOD1)**

Mutation of Cu–Zn superoxide dismutase 1 (SOD1) was the first described genetic cause for familial FALS. The majority of SOD1 mutations are AD, with a highly penetrant pattern of inheritance, and are primarily associated with limb onset ALS. An exception to this rule is the D90A mutation, predominantly found in Scandinavian populations, where it is inherited in an AR manner. Frequency of SOD1 mutations varies depending on populations, from 23.5% in Scandinavia.

**Figure 1** Gene frequencies in ALS.

**Notes:** Each gene is plotted against the year it was found; the size of the circles signifies the frequency of mutations in FALS or ALS as cited in the literature. Where gene frequencies were not available, these have been given a circle size equivalent to 1%, for illustrative purposes.

**Abbreviations:** ALS, amyotrophic lateral sclerosis; FALS, familial ALS.
Table 1: Overview of key information available for ALS and FTDALS loci

| Loci        | Method                        | Chromosomal location | Gene       | FALS/ALS frequency (%) | Year | Onset     | Inheritance | Implicated pathogenic mechanisms                              | Reference                      |
|-------------|-------------------------------|----------------------|------------|------------------------|------|-----------|-------------|---------------------------------------------------------------|--------------------------------|
| ALS1        | Linkage                       | 2q12.2q11            | SOD1       | Range 12–23.5           | 1993 | Adult     | AD (AR)     | Oxidative stress, UPS, autophagy                              | Rosen et al.                   |
| ALS2        | Linkage                       | 2q33.1               | ALS2       | Not available           | 2001 | Juvenile  | AD          | Endosomal trafficking                                         | Hadano et al.                  |
| ALS3        | Linkage                       | 18q21                | Unknown    | Not available           | 2002 | Juvenile  | AD          | RNA processing                                                | Chen et al.                    |
| ALS4        | Linkage, candidate gene, WES  | 9q34.13              | SETX       | Not available           | 2010 | Juvenile  | AR          | Intracellular cargo transport, axonal growth                 | Orlacchio et al.               |
| ALS5        | Linkage                        | 15q21.1              | SPG1L      | 40                      | 2010 | Juvenile  | AR          | RNA processing stress granule function                        | Takahashi et al.              |
| ALS6        | Linkage, candidate gene       | 16p11.2              | FUS        | 4.1                    | 2009 | Adult     | AD (AR)     | RNA processing, stress granule function                       | Vance et al.                   |
| ALS7        | Linkage                       | 20p13                | Unknown    | Not available           | 2004 | Adult     | AD          | UPR, ER stress, intracellular membrane trafficking            | Khoury et al.                  |
| ALS8        | Linkage                       | 20q13.32             | VAPB       | *0.6                    | 10   | Adult     | AD          | RNA processing                                                | Nishimura et al.               |
| ALS9        | Candidate gene                | 14q11.2              | ANG        | 1.5                    | 2006 | Adult     | AD          | RNA processing                                                | Greenway et al.                |
| ALS10       | Linkage, candidate gene       | 1p36.22              | TARDBP     | 0.5% ALS, *4%          | 2008 | Adult     | AD          | RNA processing                                                | Sreedharan et al.              |
| ALS11       | Candidate gene                | 6q21                 | FIG4       | 2.8                    | 2009 | Adult     | AD          | Endosomal trafficking                                         | Kirby et al.                   |
| ALS12       | Family study, homozygosity mapping | 10p13              | OPTN       | 2.6                    | 2010 | Adult     | AD (AR)     | Autophagy                                                    | Maruyama et al.                |
| ALS13       | Candidate gene                | 12q24.12             | ATXN2      | 4.7% ALS               | 2011 | Adult     | AD          | RNA processing                                                | Eiden et al.                   |
| ALS14       | Family study, WES             | 9p13.3               | VCP        | 2.4                    | 2010 | Adult     | AD          | UPR, autophagy                                                | Johnson et al.                 |
| ALS15       | Linkage                       | Xp11.21              | UBQLN2     | 2.1                    | 2011 | Adult     | X-LD        | UPR, ER stress, proteasome function                           | Deng et al.                    |
| ALS16       | Family study, homozygosity mapping | 9p13.3             | SIGMAR1    | Not available           | 2011 | Juvenile  | AD, AR      | Endosomal trafficking                                         | Luty et al.                    |
| ALS17       | Linkage, candidate gene       | 3p11.2               | CHMP2B     | *1% ALS                | 2006 | Adult     | AD          | Endosomal trafficking, autophagy                              | Parkinsson et al.             |
| ALS18       | Family study, WES             | 17p13.2              | PFN1       | 2.5                    | 2012 | Adult     | AD          | Cytoskeleton, axonal growth                                   | Wu et al.                      |
| ALS19       | Family study, WGS             | 2q34                 | ERBB4      | 0.5                    | 2013 | Adult     | AD          | Neuronal development                                         | Takahashi et al.               |
| ALS20       | WES, candidate gene           | 12q13.13             | HnRNP A1   | 0.5                    | 2013 | Adult     | AD          | RNA processing                                                | Kim et al.                     |
| ALS21       | Family study, WES             | 5q31.2               | MATR3      | 1.5                    | 2014 | Adult     | AD          | RNA processing                                                | Johnson et al.                 |
| ALS22       | Family study, WES             | 2q35                 | TUBA4A     | 1.4                    | 2014 | Adult     | AD          | Cytoskeleton                                                  | Smith et al.                   |
| FTDALS1     | GWAS, linkage, targeted NGS   | 9p21.2               | C9orf72    | *39.3                   | 2011 | Adult     | AD          | RNA processing, endosomal trafficking, autophagy              | Renton et al.                  |
| FTDALS2     | Family study, WES             | 2q21.23              | CHCHD10    | *3.6                    | 2014 | Adult     | AD          | Mitochondrial function                                       | DeJesus-Hernandez et al.       |
| FTDALS3     | Candidate gene                | 5q35.3               | SQSTM1     | 1.8                    | 2011 | Adult     | AD          | Protein degradation, autophagy                                | Bannwarth et al.               |
| FTDALS4     | Family study, WES             | 12q14.2              | Tbk1       | 5.2                    | 2015 | Adult     | AD          | Autophagy, neuroinflammation                                  | Fecto et al.                   |

**Notes:** ALS and FTDALS loci numbering is determined by the phenotypic series information available within Online Mendelian inheritance in Man, OMIM®. The frequency of mutations within FALS originates from the original paper or from the reference marked with an asterisk (*). In some cases, frequency in ALS, rather than FALS, is provided.

**Abbreviations:** ALS, amyotrophic lateral sclerosis; FTDALS, frontotemporal dementia and ALS; FALS, familial ALS; WES, whole-exome sequencing; WGS, whole genomic sequencing; UPS, ubiquitin-proteasome system; UPR, unfolded protein response; AD, autosomal dominant; AR, autosomal recessive; X-LD, X-linked dominant; ER, endoplasmic reticulum; GWAS, genome-wide association study; NGS, next generation sequencing.
to 12% in Germany; mutations have also been identified in apparently sporadic cases. The ALSoD database (http://alsod.iop.kcl.ac.uk) reports that there are 183 mutations in SOD1 associated with disease (accessed November 2015), the majority of which are point mutations. Given that SOD1 encodes a 153 amino acid protein, this number is remarkable, with the mutations distributed throughout the gene and impacting upon a variety of domains within the protein. This is in contrast to some of the other ALS-associated mutations, which are more often located within a particular motif of the protein product, particularly as it is unclear whether all the reported SOD1 “mutations” are indeed pathogenic.

The multiple mutations throughout the protein have also resulted in challenges determining how they are responsible for the disease phenotype. SOD1 is a ubiquitously expressed antioxidant protein, which catalyzes free radical superoxide to hydrogen peroxide and oxygen. As the majority of mutant proteins retain this enzymatic function, the pathogenicity is proposed to act through a toxic gain of function, although the precise nature of this toxicity remains to be determined. Many mutually compatible pathogenic mechanisms have been proposed including oxidative stress, excitotoxicity, protein aggregation, neuroinflammation, apoptosis, mitochondrial dysfunction, axonal transport dysregulation, and endoplasmic reticulum (ER) stress. Mutant SOD1 proteins (mtSOD1) show variable states of metallation and disulfide bond formation, which leads to the ability of the demetalled and unfolded apoform to enter the intermembrane space of the mitochondrion, thereby causing mitochondrial dysfunction. In addition, demetallation leads to increased instability and mtSOD1 shows a higher aggregation propensity than wild-type SOD1. More recently, mtSOD1, along with misfolded wild-type SOD1, has been shown to move from cell to cell and initiate a prion-like seeded aggregation of SOD1. While initial work demonstrated the propagation of misfolded protein in cell culture models, spinal homogenates from paralyzed mutant G93A SOD1 mice injected into 6-month-old G85R-SOD1:YFP mice (who do not usually get disease within 3 months) resulted in challenges determining how they are responsible for the disease phenotype. SOD1 is a ubiquitously expressed antioxidant protein, which catalyzes free radical superoxide to hydrogen peroxide and oxygen. As the majority of mutant proteins retain this enzymatic function, the pathogenicity is proposed to act through a toxic gain of function, although the precise nature of this toxicity remains to be determined. Many mutually compatible pathogenic mechanisms have been proposed including oxidative stress, excitotoxicity, protein aggregation, neuroinflammation, apoptosis, mitochondrial dysfunction, axonal transport dysregulation, and endoplasmic reticulum (ER) stress. Mutant SOD1 proteins (mtSOD1) show variable states of metallation and disulfide bond formation, which leads to the ability of the demetalled and unfolded apoform to enter the intermembrane space of the mitochondrion, thereby causing mitochondrial dysfunction. In addition, demetallation leads to increased instability and mtSOD1 shows a higher aggregation propensity than wild-type SOD1. More recently, mtSOD1, along with misfolded wild-type SOD1, has been shown to move from cell to cell and initiate a prion-like seeded aggregation of SOD1. While initial work demonstrated the propagation of misfolded protein in cell culture models, spinal homogenates from paralyzed mutant G93A SOD1 mice injected into 6-month-old G85R-SOD1:YFP mice (who do not usually get disease till 20 months) produced a progressive motor neuron disease within 3 months.

Normally, misfolded proteins are removed from the cell via the UPS. However, in SOD1-ALS, and also in SALS, the UPS has been shown to be impaired. In addition, mahogunin ring finger 1, an E3 ubiquitin ligase, which catalyzes monoubiquitination of proteins and marks them for degradation via a UPS-independent mechanism, has been shown to be decreased in the G93A mouse model. Interestingly, however, overexpression of this protein reduced SOD1 toxicity by suppressing the aggregation of SOD1. Thus, therapeutic strategies for ALS include increasing clearance of misfolded SOD1, and the heat-shock protein inducer, arimoclomol, is one such drug currently under investigation.

While initially oxidative stress was thought to be one of the primary mechanisms of mutant SOD1, the continued research on SOD1 pathogenic mechanisms has implicated UPS, protein aggregation, and degradation, as well as other aspects of protein trafficking. These pathways are also implicated by the discovery of additional FALS genes (“Protein trafficking and degradation-related genes” section).

**ALS10: TAR DNA-binding protein (TARDBP)**

The transactive response DNA-binding protein 43 (TDP-43) is encoded by TARDBP gene on chr1p36.22. TARDBP is responsible for 4%–5% of FALS and nearly 1% of SALS. Mutations in TARDBP are inherited in an AD manner and are associated with a classic ALS clinical phenotype. TARDBP encodes several isoforms of which TDP-43 is considered the most prevalent. TDP-43 is a heterogeneous nuclear ribonucleoprotein (hnRNP) with a nuclear localization signal (NLS) and nuclear export signal, which allows shuttling of the protein between the nucleus and cytoplasm. The TDP-43 protein contains three further domains, two RNA recognition motifs (RRM1 and RRM2), involved in RNA and DNA binding, and a glycine-rich domain, which is essential for interactions with other proteins and is the location of the majority of mutations.

TDP-43 was initially identified as a transcription repressor that binds to TAR DNA in human immunodeficiency virus-1. Subsequently, TDP-43 has been shown to play a role in RNA metabolism, including RNA transcription, alternative splicing, pre-microRNA processing, RNA transport, and messenger RNA (mRNA) stability. TDP-43 has the ability to autoregulate its own gene expression by binding to the 3′ untranslated region (3′UTR) of its mRNA, leading to instability and decay. TDP-43 also binds to UG-rich sequences in multiple mRNA sequences to regulate splicing. In addition, a novel function has recently been described, with TDP-43 able to repress the splicing of nonconserved exons known as cryptic exons. Removal of TDP-43 allowed these cryptic exons to be incorporated into mRNA sequences, which subsequently disrupted translation and induced nonsense mediated decay. Finally, TDP-43 is also known to be a component of stress granules (SGs), although it is unclear whether this role contributes to neurodegeneration.
TDP-43 is a prominent protein in the characteristic ubiquitinated cytoplasmic inclusions found in patients with ALS and FTD. Approximately 97% of patients with FALS and SALS are positive for TDP-43 inclusions in the motor cortex and spinal cord, thereby establishing TDP-43 as a major protein signature for disease, not just those carrying TARDBP mutations. The loss of TDP-43 nuclear localization in ALS is well documented, and the resulting splicing deficits in ALS cellular and animal models and in patient samples have been reported. In addition to a loss of nuclear function, a cytoplasmic gain of function may also contribute to neurodegeneration. A mouse model with a mutation in the NLS of human TARDBP, thereby limiting TDP-43 to the cytoplasm, showed increased expression of transcription-related and chromatin assembly genes and histone 3’UTR processing genes. Importantly, these transcriptional changes were not seen when an antisense oligomer was added to knockdown TDP-43 expression, thereby supporting a cytoplasmic toxic gain of function. Finally, similar to the prion-like propagation of disease described in SOD-ALS, there is also evidence that wild-type TDP-43 oligomers can spread horizontally from cell to cell via microvesicles, including the brain lysates of patients with ALS, as well as vertically along axons. Thus, reducing the aggregation of these mutant proteins is becoming a more widely applicable therapeutic strategy.

**ALS6: fused in sarcoma (FUS)**

The FUS gene on chr16p11.2 was first identified as a fusion oncogene in liposarcoma. FUS belongs to the FET protein family and has been shown to be an hnRNP due to its involvement in the transcription process, transport, trafficking, alternative splicing, and microRNA processing. Similar to TDP-43, it is also present in SGs. Structurally, FUS is composed of 526 amino acids that form an N-terminal domain rich in glutamine–glycine–serine–tyrosine (QGSY), three arginine–glycine–glycine (RGG)-rich domains, an RRM, and a zinc finger motif, as well as nuclear export signal and NLS that enable nucleocytoplasmic shuttling of the protein.

Mutations in the FUS gene were initially identified in an AR Cape Verde family, although subsequent screening established FUS to be causative also in AD ALS. FUS mutations account for ~4% of FALS and 1% of SALS, with the majority of mutations located either within exons 3–6, encoding the QGSY-rich and first RGG region, or in exons 12–15, which encode the zinc finger domain, the other two RGG domains, and NLS. While these in the C-terminal have been shown to be functional, those in exons 3–6 are more commonly found in SALS or do not always segregate with disease, suggesting incomplete penetrance or nonpathogenic variations.

Previously, depletion of RNA polymerase II from the nucleus had been shown to lead to an increase of cytoplasmic FUS, suggesting that FUS had a role in transcription. It has subsequently been shown that FUS mediates the interaction between RNA polymerase II and the splicing factor U1 snRNP, thereby coupling transcription to splicing. Mutations in FUS lead to mislocalization of both FUS and U1 snRNP to the cytoplasm, and other RNA-binding proteins, including SMN1, hnRNPA1, and hnRNPA2, also colocalize in mtFUS aggregates. The consequence of these mtFUS interactions includes dysregulated splicing and an increased binding of FUS with SMN, leading to a reduction in Gem bodies, thereby representing both a loss and a gain of function by the mtFUS.

**FUS** mutations may also confer pathogenicity via additional interactions. FUS has been shown to bind mRNAs and facilitate their transport down dendrites and subsequently has been shown to bind to the polyA tail of AMPA receptor GluA1, regulating its stability, with the loss of FUS leading to a reduction of GluA1. FUS has also been shown to translocate to the mitochondria through interaction with the mitochondrial chaperone heat shock protein 60 (HSP60) leading to mitochondrial damage. Finally, mtFUS interacts with Pur-alpha in SGs and increases phosphorylation of the elongation initiation factor 2-alpha, consequently inhibiting protein synthesis. However, the contribution of each of these interactions on disease pathogenesis remains to be determined.

**FTDALS1: C9ORF72 (C90RF72)**

The most common cause of FALS to date is the expansion of an intronic GGGGCC repeat in C9ORF72. The region was originally identified through genome-wide association studies of SALS cases, as well as within the Finnish ALS population, while initial sequencing of the gene failed to identify any point mutations, targeted next-generation sequencing of the region established an intronic repeat region between the noncoding exons 1a and 1b. While healthy controls most commonly have <10 hexanucleotide repeats, individuals with ALS usually carry 400–2,000 repeats. The repeat expansion has been identified in 37.6% of FALS and 6.3% of SALS, as well as in up to 25.1% of familial FTD cases. Therefore, it is not surprising that the most significant clinical phenotype associated with this genetic subtype is an increased incidence of a family history of FTD. In addition, there is evidence that there are more bulbar onset cases...
associated with C9ORF72-related ALS (up to 44%, compared to 25%–26% in non-C9ORF72 ALS), with some studies also reporting an earlier age of onset (by 1.8–5.0 years) and shorter disease duration (by 5.7–12.0 months).52

The function of the C9ORF72 gene is currently being determined, though structural analysis has established that there is similarity to “differentially expressed in normal and neoplastic-like proteins”, which are GDP/GTP exchange factors regulating Rab-GTPases involved in vesicular trafficking.53 Further work demonstrated how C9ORF72 colocalized with Rab proteins involved in autophagy and endosomal trafficking.54 While the function is being established, several hypotheses have been proposed to explain how the intronic hexanucleotide repeat may cause neurodegeneration: 1) haploinsufficiency, 2) RNA toxicity, and 3) dipeptide repeat (DPR) protein toxicity.

Haploinsufficiency

Lower levels of C9ORF72 transcript were seen in patients with the repeat expansion compared to controls, and the haploinsufficiency hypothesis was supported when knockdown of the C9orf72 homolog in zebrafish caused an axonal degeneration.55 In contrast, in a conditional C9orf72 knockout mouse, where C9orf72 was specifically ablated in neuronal cells, there was no evidence of a neurodegenerative phenotype.56 However, a systematic investigation of the expression levels of the three C9ORF72 transcripts (variant 1 = exon 1a, 2–5; variant 2 = exon 1b, 2–11; variant 3 = exon 1a, 2–11) demonstrated significantly reduced expression of variants 1 and 2 in the cerebellum and frontal cortex of C9ORF72 expansion carriers, with a correlation between a higher level of expression of variant 1 and survival.57 This suggests that antisense oligomer strategies should avoid reducing C9ORF72 expression levels.

RNA toxicity

RNA foci were identified to be located primarily in the nucleus and occasionally in the cytoplasm of motor neurons and were found to be composed of both sense and antisense RNA.58 The presence of antisense RNA foci has been shown to be correlated with TDP-43 mislocalization59 but not DPRs.58 The repeat sequence is thought to form G-quadruplex structures within the cell. Many RNA-binding proteins colocalize with the RNA foci, potentially sequestering them from the cell and disrupting their RNA-processing functions.60,61 This may underlie the significant dysregulation of RNA splicing that is seen in the presence of the expansion, where greater disruption is present in patients with a shorter survival.62,63 However, RNA foci have been observed in fibroblasts from asymptomatic patients60,64 and in BAC transgenic mice containing an expanded allele; while RNA foci and DPRs recapitulate the neuropathology of ALS, there is no evidence of neurodegeneration.65,66 This is in contrast to a mouse expressing a 66-repeat G4C2 expansion specifically in the central nervous system, which exhibited neuropathological, behavioral, and motor deficits at 6 months.67

DPR proteins

Finally, it was demonstrated that the GGGGCC repeat expansion was subject to repeat-associated non-ATG translation.58 Both the sense and the antisense RNA are translated, forming DPR proteins composed of poly-GA, -GP, -GR, -PA, and -PR (with poly-GP generated from both antisense and sense RNA).58,68 These DPR proteins are found aggregated within the neuronal cytoplasmic inclusions and neuronal intranuclear inclusions in the motor cortex, cerebellum, hippocampus, and spinal cord, which also stain positive for ubiquitin and p62. Recently, antibodies raised against each of the DPR proteins have demonstrated that there is little correlation between DPR distribution/burden and clinical phenotype,69,70 which the authors suggest is evidence against DPR aggregation being a major pathogenic mechanism. This is in contrast to work using a Drosophila model, in which DPR expression caused neurodegeneration in the fly eye.71

In summary, there appears to be growing evidence supporting some form of RNA dysregulation as a contributing mechanism to C9ORF72-ALS. However, while different cellular and animal models using different constructs are generating conflicting results on the contribution of each of the three hypotheses, the precise mechanisms still remain to be fully elucidated. Disruption of C9ORF72 protein function in endosomal trafficking may also be a contributory factor.

Other RNA-processing genes associated with ALS

Prior to the identification of TARDBP and FUS as ALS-associated genes, two RNA-processing genes had already been implicated in ALS: angiogenin (ANG) and senataxin (SETX). Subsequently, mutations in hnRNPA1 and matrin 3 (MATR3) have been identified through WES, and ataxin 2 (ATXN2) was identified as a risk factor.

ALS9: angiogenin (ANG)

Following the identification of the ANG single-nucleotide polymorphism rs11701 as overrepresented in ALS cases
from Scotland and Ireland, screening of \( \text{ANG} \) identified seven missense mutations in 15 ALS cases, of which four were FALS (1.54%) and eleven were SALS (0.80%).\(^{73}\) \( \text{ANG} \) is a member of the pancreatic ribonuclease superfamily and is neuroprotective, while in mtANG this ability is impaired.\(^{74}\)

While multiple mutations have been identified, p.K17I has not always shown disease segregation. However, a meta-analysis has demonstrated that Caucasian individuals carrying this allele have a 1.65 greater risk of ALS, which is increased to tenfold in FALS.\(^{75}\) \( \text{ANG} \) has also been shown to induce the assembly of SGs.\(^{76}\) Interestingly, this induction is inhibited by G-quadruplex structures, which are formed by the G4C2 expansions exacerbate the FUS mutant phenotype in cellular models.

### ALS: senataxin (\( \text{SETX} \))

Mutations in \( \text{SETX} \) are associated with the juvenile onset of ALS with distal muscle weakness and absence of bulbar or sensory symptoms. Patients typically have a long and slow disease progression with a relatively normal life span.\(^{77,78}\)

Rare, AD mutations in \( \text{SETX} \) occur in ALS, while recessive \( \text{SETX} \) mutations are associated with ataxia-oculomotor apraxia-2.\(^{78}\) The mechanisms by which \( \text{SETX} \) variants lead to ALS is unknown; however, \( \text{SETX} \) encodes a DNA/RNA helicase protein proposed to play a role in DNA repair in response to oxidative stress. \( \text{SETX} \) also interacts with RNA-processing proteins regulating transcription and pre-mRNA processing suggesting that the cause of motor neuron degeneration through \( \text{SETX} \) mutations is as a result of abnormal RNA processing.\(^{79}\)

### ALS13: ataxin 2 (\( \text{ATXN2} \))

More than 36 repeats of CAG within \( \text{ATXN2} \) causes spinocerebellar ataxia 2; however, intermediate repeats of 27–33 were found to strongly associate with ALS having established that \( \text{ATXN2} \) modifies TDP-43 toxicity in yeast.\(^{80}\) \( \text{ATXN2} \) is an RNA-binding protein that is involved in RNA processing and localized to the ER, Golgi apparatus, and SGs; \( \text{ATXN2} \) also interacts with FUS and intermediate expansions exacerbate the FUS mutant phenotype in cellular models.\(^{81}\) A recent meta-analysis of >6,000 ALS and 7,000 controls has identified that repeat lengths of 25–28 were actually protective, whereas the significant risk was associated with CAG repeats of 31–33.\(^{82}\) This finding is supported by an Italian study, where additionally <31 repeats were associated with spinal onset ALS and a shorter survival.\(^{83}\)

### ALS20: heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)

Following the identification of \( \text{hnRNPA1} \) and \( \text{hnRNPA2B1} \) mutations as causative in multisystem proteinopathy families using exome sequencing, these genes were specifically analyzed in 212 FALS for which exome sequencing was available.\(^{84}\) A single case was identified with a mutation in \( \text{hnRNPA1} \). Interestingly, \( \text{hnRNPA1} \) and A2/B1 are known interacting partners of TDP-43, and \( \text{hnRNPA1} \) also interacts with ubiquilin-2.\(^{85}\) In ALS motor neurons, there is a loss of intense hnRNPA1 nuclear staining, which also correlates with nuclear loss of TDP-43, although \( \text{hnRNPA1} \) was not seen to colocalize with TDP-43 in the skin-like inclusions.\(^{86}\)

However, screening of 113 Italian FALS and 135 Dutch FALS and 1,084 Dutch SALS failed to find any \( \text{hnRNPA1} \) mutations, suggesting that this is a very rare cause of FALS.

### ALS21: matrin 3 (\( \text{MATR3} \))

Exome sequencing of a large pedigree identified a mutation in the \( \text{MATR3} \) gene; a previous family carrying a mutation in \( \text{MATR3} \), originally diagnosed with AD distal, asymmetrical myopathy with vocal cord paralysis was reassessed and rediagnosed as having ALS.\(^{87}\) Future screening of Italian and British ALS cases identified a further two mutations: one FALS and one SALS. \( \text{MATR3} \) is an RNA/DNA-binding protein that interacts with TDP-43; while the p.S85C mutation enhances this interaction, two other mutations, p.F115C and p.T22A, do not. However, this difference may underlie the slow progression of the disease in the family carrying the p.S85C mutation. While no further mutations were found in 372 FALS cases from France, Taiwan, Australia, and French Canada, four mutations were found in apparent SALS cases (three in French Canadians and one in a Taiwanese).\(^{88-91}\)

### FET family genes

The TATA box-binding protein-associated factor 15 (\( \text{TAF15} \)) and Ewing sarcoma breakpoint region 1 (\( \text{EWSR1} \)) are RNA-binding proteins and form the FET family of proteins along with FUS. All three contain prion-like domains, a feature used to rank potential RNA-binding proteins as being involved in ALS following a functional yeast screen.\(^{92}\) Screening of \( \text{TAF15} \) identified five missense variants in 1,262 ALS cases, while screening of \( \text{EWSR1} \) identified two potential mutations among 817 ALS cases.\(^{93}\) While these variants were absent from controls, they were identified in patients with SALS, so segregation could not be demonstrated. However, both \( \text{TAF15} \) and \( \text{EWSR1} \) proteins show cytoplasmic mislocalization
in SALS. Recently, whole-genome sequencing (WGS) identified an EWSR1 mutation in a set of monozygote twins discordant for ALS, suggesting that additional factors may influence disease.94

Protein trafficking and degradation-related genes

From the identification of the first gene for AR ALS, ALS2, combined with the presence of characteristic ubiquitinated inclusions in ALS motor neurons, dysregulation of protein trafficking and protein degradation has been implicated in the disease process. Mutations in genes involved in endosomal transport include alsin (ALS2), vesicle-associated membrane protein (VAMP)-associated protein B (VAPB), chromatin modifying protein 2B (CHMP2B), and phosphoinositide 5-phosphatase (FIG4); those involved in the UPS include ubiquitin 2 (UBQLN2), sequestosome 1 (SQSTM1), and sigma nonopioid intracellular receptor 1 (SIGMAR1), and autophagy is primarily implicated by mutations in optineurin (OPTN), valosin-containing protein (VCP), and tank-binding kinase 1 (TBK1). There is some overlap between these three biological pathways, which have also been implicated in SOD1- and C9orf72-related ALS.

ALS2: alsin (ALS2)

The ALS2 gene was originally identified through linkage analysis in consanguineous families from Tunisia and Saudi Arabia.95,96 The majority of the mutations lead to protein truncations, leading to a proposed loss of function. Alsin is thought to play a role in activating Rab5 GTPases. Rab5 is essential for endosomal trafficking, and in alsin knockout mice, neurons showed increased endosomal fusion and degradation but reduced mobility.97 One of the endosome components is the AMPA receptor GluR2, levels of which are reduced in alsin knockout mice.98

ALS8: vesicle-associated membrane protein (VAMP)-associated protein B (VAPB)

Linkage analysis of a large Brazilian family first established VAPB as a causative gene for ALS,99 and the p.P56S mutation has been identified in multiple Brazilian pedigrees, indicative of a common founder.100 Additional mutations have been reported, though not all the variations found segregated with disease.101–104 VAPB is a type II integral ER membrane protein, involved in intracellular trafficking and the unfolded protein response105 and in regulating ER–mitochondria interactions.106 However, the p.P56S mutant protein was unable to initiate the unfolded protein response, altered calcium uptake into the mitochondria, and disrupted anterograde axonal transport of mitochondria.107–109

ALS17: chromatin modifying protein 2B (CHMP2B)

Mutations in CHMP2B were initially identified in two probable FALS and a further three SALS cases; the majority had a predominant lower motor neuron phenotype.110,111 CHMP2B is a component of the ESCRT-III endosomal trafficking system, sorting cargos into multivesicular bodies. More recently, four novel mutations were identified in apparently sporadic ALS cases, and these were located in the domain required to form the multivesicular bodies.2 In cellular models, mutant CHMP2B led to the formation of large vacuoles and an increase in autophagy marker LC3-II, implicating dysregulation of autophagy as a mechanism in ALS.

ALS11: phosphoinositide 5-phosphatase (FIG4)

Mutations in FIG4 were originally identified as causative in Charcot–Marie–Tooth disease type 4J, though one family had a clinical phenotype resembling ALS. Screening of FALS and SALS cases identified nine variants, with six showing impaired function in yeast.112 FIG4, also known as SAC3, regulates PI(3,5)P2 levels and thereby controls retrograde trafficking of endosomal vesicles to the Golgi. The mutant proteins showed loss of phosphatase activity, mislocalization, and inability to bind to the PI(3,5)P2 complex. Further screening in Italian and Taiwanese populations failed to find any novel variants, though only 80 SALS and 15 FALS were screened in each study.113,114 No pathological assessment was available on the mutation carriers; however, FIG4 was not shown to be mislocalized in SALS.115

ALS15: ubiquilin 2 (UBQLN2)

UBQLN2 was identified by linkage analysis in a large multigenerational family. Screening of additional FALS cases with no male-to-male transmission found four further mutations; these were all located within the PXX repeat region of the protein.116 Additional screening identified further variants adjacent or within the PXX repeat region.117,118 Mutations have been shown to disrupt the protein degradation pathway through defective binding to the proteasome119 and causing mislocalization of OPTN from Rab-11-positive endosomal vesicles,120 as well as potentially impairing RNA metabolism,
through loss of binding of UBQLN2 to hnRNP proteins, including hnRNPA1.85

FTDALS3: sequestosome 1 (SQSTM1)
SQSTM1 or p62 is a ubiquitin-binding protein that plays a role in protein degradation via the proteasome and autophagy and is found within the characteristic ubiquitinated inclusions in patients with ALS. Screening of this gene found multiple mutations in both FALS and SALS cases.121 Further mutations were found in patients with ALS, some in association with Paget disease of bone, which is also known to be caused by SQSTM1 mutations.122,123 In a zebrafish model, where endogenous SQSTM1 was knocked-down, the fish showed behavioral and axonal abnormalities, as well as disrupted autophagy, as demonstrated by increased mTOR levels.124 Human SQSTM1 was able to rescue the phenotype, but the frequently found mutation, p.P392L, was unable to do so.

ALS16: sigma nonopioid intracellular receptor 1 (SIGMAR1)
Initially 3-UTR variants were identified within several AD FTDALS or FTD families and suggested that pathogenicity occurred through alteration of mRNA stability.125 However, using homozygosity mapping, a missense mutation in SIGMAR1 was subsequently found to segregate in a large consanguineous family with AR juvenile ALS (ARJALS).126 SIGMAR1 is an ER chaperone, a subunit of the ligand-regulated potassium channel, and enables mitochondrial calcium transport via the IP3 receptor; mutation of SIGMAR1 causes the formation of cytoplasmic aggregations, a reduction in ATP production and subsequent decrease in proteasome activity.127 However, whether SIGMAR1 contributes to AD ALS is yet to be fully elucidated.

ALS12: optineurin (OPTN)
Mutations in OPTN were originally identified through homozygosity mapping of consanguineous Japanese AR ALS families, which identified a homozygous exonic deletion and a homozygous nonsense mutation.128 Further screening of FALS cases identified two AD families heterozygous for a missense mutation. OPTN mediates its function through protein–protein interactions; it binds to ubiquitin and UBQLN2, is an autophagy receptor (facilitating the recruitment of cargos to autophagosomes), is required for Golgi organization (as demonstrated by the Golgi fragmentation seen in postmortem spinal motor neurons),129 and also regulates NF-κB signaling.130 Subsequent screening has identified additional heterozygote mutations in SALS131 and AR ALS cases.132,133

ALS14: valosin-containing protein (VCP)
Exome sequencing of a four-generation Italian family initially suggested mutation of VCP as causative in ALS.134 Subsequently, a further four variants were identified in FALS cases, thereby providing further evidence that VCP is associated with ALS. VCP is an AAA+ (extended family of ATPases associated with various cellular activities) ATPase protein involved in a variety of cellular functions including mediating the proteasomal degradation of ubiquitinated protein in multimeric complexes and the targeting of substrates to autophagosomes.135 While screening failed to find any VCP mutations in some populations,136–138 potential mutations were found in others and also in SALS cases.139,140 VCP mutations are also associated with inclusion body myopathy with early onset Paget Disease and frontotemporal dementia (IBMPFD), and patient fibroblasts have shown mitochondrial uncoupling and a reduction in ATP production,141 a feature also seen with SIGMAR1 mutations.

FTDALS4: tank-binding kinase (TBK1)
Mutations in TBK1 were initially identified through exome sequencing of 2,874 ALS cases; dominant variants were found in 1.097% of cases and loss of function mutations in 0.382%.142 This was shortly followed by a second paper in which screening of 252 FALS cases identified nine loss of function and four missense mutations.143 Mutations have subsequently been found in ALS, FTDALS, and FTD cases.144,145 TBK1 has a role in both innate immunity and NF-κB signaling, as well as in autophagy. TBK1 binds and phosphorylates ALS-related proteins OPTN and SQSTM1, whereas TBK1 mutants have been shown to no longer bind OPTN.143

Impaired axonal transport and cytoskeletal dysfunction
Neurons are extremely large cells that require transport of organelles, proteins, and RNA from the cell body down the axons. Molecular motors such as kinesins and dynein guide these cargos to microtubules to mediate anterograde and retrograde transport, respectively. While mutation of the p150 dynactin subunit in mice generated a neurodegenerative phenotype, mutations in this gene were not found in human ALS.146,147 However, exome sequencing has identified several cytoskeletal genes with mutations reported to be causative in ALS.

ALS5: spatacsin (SPG11)
WES of two affected siblings from a nonconsanguineous family diagnosed with ARJALS identified only one gene,
SPG11, in which variants were found in a compound heterozygous state. The involvement of the hereditary spastic paraparesis gene, normally associated with HSP with thin corpus callosum, had previously been implicated by a candidate gene screening of SPG11, in which mutations in 10 families with ARJALS were identified. While the exact function of the protein is unknown, iPSC-derived neuronal cells with SPG11 mutations demonstrated the protein colocalized with the cytoskeleton, and mutations caused axonal instability and impaired axonal transport.

ALS18: profilin 1 (PFN1)
Two multigenerational ALS families were determined to carry mutations in the PFN1 gene following WES. Extending the screen to additional FALS cases identified a further three mutations in five FALS cases and a p.E117G variant that was identified at very low frequency in controls. Further screening of ALS cases identified additional mutations and the variant. A meta-analysis subsequently determined that the p.E117G was associated with ALS and proposed this variant as a risk factor. The function ofPFN1 is to convert monomeric actin to filamentous actin, and it is also found localized to SGs. It has been demonstrated that PFN1 mutations destabilize the protein, thereby leading to a loss of function, while the mutant protein is misfolded, thereby leading to a gain of function through aberrant protein interactions. However, the effect of the mutant protein on actin formation and SG dynamics is yet to be elucidated.

ALS22: tubulin alpha 4A (TUBA4A)
WES of 363 FALS index cases followed by rare variant burden identified five ALS cases with rare variants in TUBA4A; these included four missense mutations and one nonsense mutation, all of which were encoded in exon 4, in highly conserved amino acids, and absent from 4,300 exome variant server (EVs) controls. While further sequencing of ALS cases only identified one further variant, functional studies demonstrated that the p.W407X nonsense mutant failed to localize to the microtubules, instead forming cytoplasmic inclusions, leading to disrupted microtubule assembly and stability, through a dominant-negative mechanism. Subsequent screening in the Chinese ALS population failed to identify any variants; data from other populations will undoubtedly emerge as further WES experiments are completed.

Intermediate filament variants
Cytoskeletal dysfunction is further implicated in the pathogenesis of ALS through rare variants being identified in intermediate filament genes. Neurofilaments (light, medium, and heavy) are the major structural components of the neuronal cytoskeleton and are present within the characteristic ubiquitinated protein inclusions. Candidate gene screening identified rare insertion/deletion variants in the KSP repeat domains of neurofilament heavy (NEFH) gene in SALS cases, while a single frameshift mutation has been identified in peripherin (PRPH1). However, the absence of mutations in known familial cases and ability to show segregation with disease has reduced the creditability of these genes as ALS loci.

Additional loci
Several additional ALS loci have been identified. Two loci are yet to have their associated genes identified: ALS3 on chr18q21 and ALS7 on chr20q13. A further two genes have been identified in FALS pedigrees, though their functional effects are currently predicted to disrupt neuronal development and mitochondrial function.

ALS19: Erb-b2 receptor tyrosine kinase 4 (ERBB4)
WGS of a Japanese AD ALS family identified a missense mutation in ERBB4. Additional screening identified the same mutation in an unrelated Canadian family and a further mutation in a SALS case. ERBB4 is a receptor tyrosine kinase that is activated by neuregulin, resulting in autophosphorylation of the C-terminal. Mutations in ERBB4 reduced the level of autophosphorylation. ERBB4 was found to localize to C-boutons, arising from interneurons, which synapse with spinal motor neurons. Interestingly, C-boutons are not found in oculomotor neurons, which are spared in ALS, while increases in neuregulin levels in C-boutons increase during disease progression of the SOD1 G93A transgenic mice.

FTDALS2: coiled-coil helix coiled-coil helix domain-containing protein 10 (CHCHD10)
CHCHD10 was initially associated with ALS through WES of a family exhibiting clinical features including ALS, FTD, cerebellar ataxia, and myopathy. This led to ALS and ALS-FTD families being screened. Several additional mutations were found, though it became evident that the p.P34S mutation was nonpathogenic, as it was also found at similar frequencies in controls. The function of CHCHD10 is unknown; it localizes to the mitochondria. Fibroblasts from family members of the original pedigree showed multiple mitochondrial DNA deletions, respiratory chain defects, and structurally abnormal mitochondria, suggesting that CHCHD10 may have a role in the respiratory chain and/or in mitochondrial genome stability. This has been supported
by additional work by Genin et al., which found not only a loss of cristae and mitochondrial genome repair in patient fibroblasts but also a failure of apoptosis due to an inability to release cytochrome C.

**Conclusion**

ALS genetics is having a significant impact on our understanding of the disease and the mechanisms implicated in neurodegeneration. The majority of genes encode proteins involved in RNA processing and the protein degradation pathways, UPS, and autophagy. However, neither of these nor the other pathways implicated work in isolation but impact on other cellular processes. The proposed mechanisms are mutually compatible, and it is most likely that multiple dysregulated pathways contribute to the loss of motor neurons. This is clearly demonstrated by TDP-43, an RNA-binding protein, that is mislocalized from the nucleus, thereby causing loss of nuclear function, and is aggregated in the cytoplasm as a component of the characteristic ubiquitinated inclusions.

Along with multiple genetic causes, it is clear that these genes are also implicated in additional disorders, not only in other neurodegenerative disorders such as FTD, HSP, and ataxia, but also in myopathies, Paget disease of bone, and glaucoma (Table 2). The use of WES or WGS, in projects such as the 100,000 Genomes Project in the UK (www.genomicsengland.co.uk), or Project Mine among the International ALS community (www.projectmine.com), will potentially enable a greater understanding of why mutations in a gene in one family present with a specific clinical phenotype, while another family shows a different disease.

WES and WGS will also further our understanding of the impact of oligogenic inheritance in ALS. While family pedigrees clearly show inheritance in an AD manner for classical ALS, the move away from analyzing a single gene at a time has highlighted evidence of mutations in multiple ALS genes in some patients. Screening of a large ALS cohort demonstrated that 14% of FALS and 2.6% of SALS cases had more than one potential pathogenic mutation in a known ALS gene, and these cases had a significantly earlier onset of disease. This also highlights the fact that apparent SALS cases also carry genetic mutations, as has been evidenced by the identification of de novo mutations in SALS cases following WES of trios comprising of an ALS patient and their two unaffected parents. While in some cases, these may actually be rare AR mutations, additional WES and WGS sequencing of cases will allow the genetic contribution in SALS, estimated to be 61%, to be elucidated.

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Table 2 Clinical phenotypes also associated with ALS genes

| ALS loci | Gene name | Alternative clinical phenotypes | Inheritance |
|----------|-----------|---------------------------------|-------------|
| ALS2     | ALS2      | Primary lateral sclerosis, juvenile (PLSJ) Spastic paralysis, infantile onset ascending (IAHSP) | AR          |
| ALS4     | SETX      | Spinocerebellar ataxia, autosomal recessive 1 (SCAR1) | AR          |
| ALS5     | SPG11     | Spastic paraplegia 11 (SPG11) Charcot–Marie–Tooth disease, axonal, type 2X (CMT2X)| AR          |
| ALS6     | FUS       | Tremor, hereditary essential 4 (ETH4) | AD          |
| ALS8     | VAPB      | Spinal muscular atrophy (SMA), late onset. Finkel type (SMAFK) | AD          |
| ALS11    | FIG4      | CMT4 polyneuropathy, autosomal recessive (PNAAR) | AR          |
| ALS12    | OPTN      | Glaucome, primary open angle (POAG) | AD          |
| ALS13    | ATXN2     | Spinocerebellar ataxia 2 (SCA2) | AD          |
| ALS14    | VCP       | Inclusion body myopathy with early onset Paget disease and frontotemporal dementia 1 (IBMPFD1) | AD          |
| ALS16    | SIGMAR1   | SMA, distal, autosomal recessive 2 (DSMA2) | AR          |
| ALS17    | CHMP2B    | Frontotemporal dementia, Chr 3-linked (FTD3) | AD          |
| ALS20    | hnRNPA1   | IBMPFD3 | AD          |
| FTDALS2  | CHCHD1    | SMA, Jokela type (SMAJ) | AD          |
| FTDALS3  | SQSTM1    | Paget disease of bone 3 (PDB3) | AD          |

Notes: Data obtained from gene–phenotype relationship data available in Online Mendelian Inheritance in Man, OMIM®. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University; Baltimore, MD, USA. Available at: http://omim.org. Accessed March 16, 2016.

Abbreviations: ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; AR, autosomal recessive; AD, autosomal dominant.

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