Trends in Genetics

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Review

ALS Genes in the Genomic Era and their Implications for FTD

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Amyotrophic lateral sclerosis (ALS) is a complex neurodegenerative disease, characterized genetically by a disproportionately large contribution of rare genetic variation. Driven by advances in massive parallel sequencing and applied on large patient–control cohorts, systematic identification of these rare variants that make up the genetic architecture of ALS became feasible. In this review paper, we present a comprehensive overview of recently proposed ALS genes that were identified based on rare genetic variants (*TBK1*, *CHCHD10*, *TUBA4A*, *CCNF*, *MATR3*, *NEK1*, *C21orf2*, *ANXA11*, *TIA1*) and their potential relevance to frontotemporal dementia genetic etiology. As more causal and risk genes are identified, it has become apparent that affected individuals can carry multiple disease-associated variants. In light of this observation, we discuss the oligogenic architecture of ALS. To end, we highlight emerging key molecular processes and opportunities for therapy.

Highlights

High-throughput DNA sequencing, including whole-genome and -exome sequencing, has proven a successful strategy for gene identification in ALS.

Substantial progress in gene identification revealed recurrent key molecular mechanisms in ALS, including proteostasis and autophagy, RNA processing, cytoskeleton dynamics, mitochondrial dysfunction, and DNA damage response.

ALS and FTD are partners of one disease continuum, consequently gene identification in ALS is impacting FTD genetic etiology and vice versa.

The emerging concept of oligogenic inheritance in ALS, and possibly also in FTD, has implications on gene identification, genetic testing, and genetic counseling, as well as therapy development.

ALS and the ALS-FTD spectrum

Amyotrophic lateral sclerosis (ALS) is a devastating progressive adult-onset neurodegenerative disease, affecting both lower and upper motor neurons in the central nervous system. Core clinical symptoms include weakness in limbs and bulbar muscles, respiratory failure, hyperreflexia, and spasticity of arms or legs. Disease onset occurs on average between 40 and 70 years of age, although younger patients have been reported. Disease progression is often aggressive, with patients dying within 3–5 years postdiagnosis. The estimated annual incidence is from one to three cases per 100 000 people worldwide [1]. ALS is most often **sporadic** (see Glossary) but about 5% of patients have a positive family history. Currently, mutations in more than 25 genes have been associated with ALS, with the *C9orf72* **repeat expansion mutation** and *SOD1* mutation as the most common genetic causes (please see Table 1 for list of gene/protein abbreviations used throughout the manuscript).

ALS is closely related to frontotemporal dementia (FTD). Like ALS, FTD is a progressive neurodegenerative disease characterized by degeneration of the frontal and temporal lobes of the brain, resulting in disturbances of behavior, personality, and language. It is estimated that up to 50% of ALS patients show signs of behavioral dysfunction and/or subtle cognitive impairment, resembling dementia, and up to 15% of ALS patients reach the diagnostic criteria of FTD (referred to as ALS-FTD or FTD-ALS patients) [2–4]. Conversely, the same holds true for FTD [5,6]. At the genetic level, mutations in multiple genes contribute to the etiology of both ALS and FTD, as best represented by the *C9orf72* repeat expansion, *TBK1*, *VCP*, and *TARDBP* mutations. By contrast, other genes are specifically associated with only one of the diseases, such as *SOD1* for ALS or *MAPT* and *GRN* for FTD. Although *TARDBP* mutations are rare in ALS and FTD (<1%), pathologically, aggregation of TAR DNA-binding protein 43 (TDP-43) in affected brain regions and motor neurons are found in the majority of ALS (up to 97%) and FTD (up to 50%) patients [7,8]. Owing to this extensive clinical, genetic, and pathological

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Table 1. List of Gene and Protein Abbreviations Used Throughout the Manuscript

Genes	
ANXA1	Annexin A11
ATXN2	Ataxin-2
C9orf72	Chromosome 9 open reading frame 72
C21orf2	Chromosome 21 open reading frame 2 protein
CCNF	Cyclin F
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
FUS	FUS RNA-binding protein
GRN	granulin precursor
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
hnRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
MAPT	Microtubule-associated protein tau
MATR3	Matrin 3
NEK1	NIMA-related kinase 1
PFN1	Profilin 1
SOD1	Superoxide dismutase 1
TARDBP	TAR DNA-binding protein
TBK1	TANK-binding kinase 1
TIA1	T cell-restricted intracellular antigen-1
TUBA4A	Tubulin alpha 4A protein
VCP	Valosin containing protein
Proteins	
ALS2	Alsin
CCS	Copper chaperone for superoxide dismutase
CHCHD3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3
CHCHD6	Coiled-coil-helix-coiled-coil-helix domain-containing protein 6
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
GLE1	Nucleoporin GLE1
IKK	Inhibitor of kappa B kinase
MICOS	Mitochondrial contact site and cristae organizing system
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIMA	Never-in-mitosis A
OPTN	Optineurin
RAB8	Ras-related protein Rab-8
RAB39	Ras-related protein Rab-39
RRM2	Ribonucleoside-diphosphate reductase subunit M2
SCF	Skp1-Cul1-F-box
SMCR8	Smith-Magenis syndrome chromosome region, candidate 8 homolog
SQSTM1/p62	Sequestosome 1
TARDBP/TDP-43	TAR DNA-binding protein
VAPB	Vesicle-associated membrane protein-associated protein B/C
WDR41	WD repeat domain 41

Glossary

CRISPR/Cas9: a gene-editing technology that can target and edit parts of the genome with high accuracy.
Disease anticipation: a phenomenon whereby a genetic disorder presents with earlier disease onset and increased disease severity as it is passed on from one generation to the next. The underlying disease mechanism is a dynamic mutation or repeat expansion in the DNA, such as is seen in Huntington's disease.
DNA damage response: a cellular network involved in detecting, signaling, and repairing DNA damage.
Genetic modifier: a genetic factor that can modify the expression level of a particular gene.
Loss-of-function (LOF) mutation: mutation resulting in the loss or reduction of protein or protein function.
Massive parallel sequencing (MPS): also known as next-generation sequencing (NGS), is a non-Sanger-based high-throughput DNA sequencing approach generating millions to billions of sequence reads in parallel.
Missense mutation: a point mutation in which a single nucleotide change results in a codon for another amino acid.
Nonsense-mediated mRNA decay (NMD): a surveillance pathway that degrades mRNAs carrying premature termination codons.
Oligogenic model: an inheritance model in which mutations in different genes work together to cause disease.
Penetrance: the percentage of individuals carrying a disease-causing genetic variant that expresses the disease phenotype.
Pleiotropy: a genetic phenomenon whereby certain gene mutations or variants can influence more than one distinct clinical phenotype.
Proteostasis: or protein homeostasis is a biological process that regulates the abundance and folding of proteins within the cells.
Repeat-associated non-AUG (RAN) translation: a noncanonical translational process producing homopolymeric expansion proteins in

overlap, ALS and FTD are now considered partners of a disease continuum, referred to as the ALS-FTD spectrum, rather than two separate disease entities.

Novel ALS Genes in the Genomic Era

Mutations in the major established causal ALS genes (*SOD1*, *TARDBP*, *FUS*, *VCP*, *C9orf72*, and *PFN1*) account for approximately 60%–70% of familial ALS (fALS) and about 10% of apparently sporadic ALS (sALS) cases [9], with the GGGGCC hexanucleotide expansion mutation in the 5′ noncoding region of *C9orf72* being by far the biggest contributor (Box 1). However, this also indicates that more genes remain to be uncovered. In recent years, advances in **massive parallel sequencing** approaches such as **whole-genome sequencing** (WGS) and **whole-exome sequencing** (WES), hand in hand with large-scale collaborations [which have previously led to the success of genome-wide association studies (GWAS) in ALS] have facilitated a new wave of gene discovery. These studies are specifically designed to identify rare variants that confer disease risk, variants that are typically not picked up by GWAS, which, by design, target common variants. This has led to the recent identification of at least nine genes carrying such rare causal variants, including *TBK1*, *CHCHD10*, *TUBA4A*, *MATR3*, *CCNF*, *NEK1*, *C21orf2*, *ANXA11*, and *TIA1* (Table 2) [10–19]. Here, we will discuss supportive evidence for their respective impact on the genetic architecture of ALS and FTD and linked molecular pathways.

Box 1. *C9orf72*

In 2011, the discovery of a noncoding hexanucleotide repeat expansion (GGGGCC) mutation in the 5′ noncoding region of the *C9orf72* gene drastically shifted the field, with up to 40% of fALS patients carrying such a repeat expansion. The *C9orf72* gene was identified in multiple multigenerational families presenting with FTD-ALS or ALS-FTD and linked to chromosome 9p21 [86–88]. Also in FTD, the *C9orf72* repeat expansion is the most common genetic cause, explaining 25% of familial FTD and up to 88% of familial patients with both ALS and FTD [118].

Notably, *C9orf72* repeat size is highly polymorphic, and the cut-off to distinguish normal from pathogenic expansions remains somewhat ambiguous (for more on sizing of the repeat and cut-off of pathogenicity, see GeneReviews on C9orf72 [119]). In general, in unaffected individuals repeat size varies from two to 24 repeat units, whereas in both ALS and FTD patients the repeat expands from several hundred to several thousand repeats. The smallest repeat with evidence of cosegregation with disease was 50 repeat units [120]. Currently, the relationship between repeat size and disease phenotype (ALS versus FTD) or onset age is being investigated and some studies provide evidence for **disease anticipation** [120,121].

Little is known about the normal function of the C9orf72 protein, complicating functional characterization, but three major pathological mechanisms have been proposed: (i) loss-of-function and haploinsufficiency. The GGGGCC repeat expansion in the *C9orf72* promoter suppresses gene expression, leading to loss of mutant transcript and protein, as seen for other repeat expansion disorders such as fragile X syndrome and Friedrich's ataxia [88]. However, there is evidence challenging the loss-of-function mechanism hypothesis [104,105,108,122]. (ii) RNA toxicity. Expanded sense (GGGGCC) and antisense (GGCCCC) RNA transcripts form toxic RNA foci, which sequester essential RNA-binding proteins and impair the RNA processing machinery, similar to that of myotonic dystrophy type 1 [87,123]. By contrast, modeling in *Drosophila* argued against an RNA toxicity mechanism [124,125]. Flies with a transgene of 160 GGGGCC repeats expressed it, spliced, and formed many sense RNA foci in the nucleus [125]. Yet, no neurodegeneration was observed, suggesting that the accumulation of RNA foci is not sufficient to trigger neurodegeneration. (iii) Proteotoxicity from dipeptide repeat (DPR) aggregates. **Repeat-associated non-AUG (RAN) translation** of GGGGCC or GGCCCC RNA transcripts generate toxic poly-GA, poly-GP, poly-GR, poly-PA, and poly-PR peptides (in each of the three reading frames), leading to DPR-positive inclusions. These characteristic inclusions are predominant in the cerebellum, hippocampus, and frontotemporal cortex, alongside TDP-43 pathology [126]. Several studies demonstrated that *C9orf72*-derived DPRs are toxic, impair nucleocytoplasmic transport, and can cause neurodegeneration and behavioral deficits [124,127–134]. By contrast, human postmortem studies found no correlation between DPR protein pathology load and distribution and degree of neurodegeneration or phenotype (ALS, FTD, or mixed ALS-FTD), contesting that DPR protein aggregation is the major pathomechanism in *C9orf72* pathogenesis [135]. As outlined above, it is clear that the relative contribution of these three molecular mechanisms is still actively debated and further investigated. Nevertheless, it is very likely that a combination of multiple mechanisms is at play.

multiple reading frames without an AUG initiation codon.

Repeat expansion mutation: a dynamic mutation that results in the expansion (or retraction) of the number of repeat units of a short nucleotide repeat.

RNA foci: the accumulation of expanded RNA repeats which can sequester other critical RNA-binding proteins into toxic nuclear foci.

Sporadic: not inherited, no evidence of affected relatives with same or related disease.

Stress granules: dense aggregations in the cytosol, composed of proteins and RNA molecules that form upon cellular stress.

Ubiquitinated cytoplasmic inclusions: aberrant proteins in the cytosol are poly-ubiquitinated to target them for degradation.

Whole-exome sequencing: a high-throughput DNA sequencing technology of all protein-coding exons and intron–exon boundaries of the genome.

Whole-genome sequencing: a high-throughput DNA sequencing technology of the complete DNA sequence of the genome.

Table 2. Overview of Recent ALS Genes with Relative Mutation Frequencies in Different ALS and FTD Cohorts and Associated Pathways

Gene	Locus	Inheritance	Level of evidence[a]	Mutation frequency							TDP-43 pathology	Implicated disease pathway
				Overall ALS (%)	Familial ALS (%)	Sporadic ALS (%)	Overall FTD (%)	Familial FTD (%)	Sporadic FTD (%)	ALS-FTD (%)		
TBK1[b]	12q14.2	AD[c]	Established	1.3	3	<1	<1	2	1	3–4	+	Autophagy, inflammation
CHCHD10	22q11.23	AD	Established	<1	2	<1	<1	<1	<1	<1	+	Mitochondrial dysfunction, synaptic integrity
TUBA4A	2q35	AD	To be validated	<1	1	<1	<1	<1	–	–	+	Cytoskeletal dynamics, axonal transport
MATR3	5q31.2	AD	Established	<1	1–2	1	–	–	–	–	+	RNA metabolism
CCNF	16p13.3	AD	Established	<1	0.6–3.3	<1	4[d]	–	–	–	+	Proteostasis
NEK1[b]	4q33	n.d.[c]	To be validated	1	1–2	<1	–	–	–	–	n.d.	DNA damage responses, cell cycle control, cytoskeletal organization, mitochondrial membrane regulation
C21orf2[b]	21q22.3	n.d.	To be validated	<1	–	–	–	–	–	–	n.d.	DNA damage response, cytoskeletal organization
ANXA11	10q22.3	AD	To be validated	1.1	1	1.7	–	–	–	–	+	Proteostasis
TIA1	2p13.3	AD	To be validated	<1	2.2	<1	–	–	–	<1	+	RNA metabolism

[a]Level of evidence for the recently identified genes in the ALS-FTD spectrum is classified as 'established' or 'to be validated', based on following criteria: established, genes have been replicated by different studies, including supportive evidence from functional studies; to be validated, gene findings based on single study, further validation in different study populations and/or functional studies required.
[b]Mutation frequencies include only loss-of-function mutations.
[c]Abbreviations: AD, autosomal dominant inheritance; n.d., inheritance mode not determined yet.
[d]Frequency was counted in 99 FTD-TDP patients [15].

TBK1

Large-scale WES studies, followed by rare variant burden analysis, associated *TBK1* with ALS as well as FTD [13,14]. TBK1 is a multifunctional kinase involved in multiple cellular processes, including the innate immune response and inflammation, autophagy, and cell proliferation. It is an important member of the IKK-kinase family involved in the regulation of interferon type 1 and NFκB signal transduction [20]. The majority of *TBK1* mutations are **loss-of-function (LOF) mutations** producing premature termination codons (PTCs), triggering **nonsense-mediated mRNA decay (NMD)**, and resulting in the loss of mutant transcript and subsequent loss or reduction of TBK1 protein [21,22]. In addition to LOF mutations such as frameshift and nonsense mutations, in-frame amino acid deletions were also shown to be pathogenic through loss of TBK1 protein or phosphorylated TBK1 (pTBK1) [21,23]. By contrast, **missense mutations** were observed in both patients and control individuals. Therefore, it was proposed that missense mutations with a verified functional effect on kinase activity or substrate binding, for example, may lead to partial loss of protein function, and in that case should be considered risk alleles rather than causal variants [23].

TBK1 interacts and phosphorylates several protein substrates that were linked to the ALS-FTD spectrum and which participate in autophagic processes, including OPTN and SQSTM1/p62 [24–26]. TBK1 also phosphorylates and likely activates SMCR8 in the C9orf72/SMCR8/WDR41 complex, which acts as a GDP/GTP exchange factor for RAB8 and RAB39, two RAB GTPases involved in autophagy [27] (Figure 1, Key Figure). Notably, the TBK1 Glu696Lys C-terminal domain mutant was shown to abolish interaction with OPTN and prevented recruitment, retention, and activation of both proteins on damaged mitochondria [14,28]. In turn, mutant OPTN reduced *TBK1* expression [22], cementing the relationship between TBK1 and OPTN in the disease pathogenesis of ALS and the ALS-FTD spectrum.

Taken together, *TBK1* LOF mutations account for approximately 1.3% of ALS, 0.4% of FTD, and 3%–4% of ALS-FTD patients (Table 2) [13,14,21–23,29–33]. *TBK1* can be considered an established causal gene, based on conclusive linkage in multiple families, replication in many cohorts from different origin, and supportive functional evidence suggesting a role in autophagy and neuroinflammation.

CHCHD10

CHCHD10 was first linked to ALS in an extended French family with a complex phenotype of ALS, FTD, cerebellar ataxia, and mitochondrial myopathy, resulting from mitochondrial DNA breakage syndrome [11]. The same mutation was found in an additional family with pathology-confirmed FTD-ALS [11]. CHCHD10 forms part of the multiprotein complex MICOS, together with mitofilin, CHCHD3, and CHCHD6, which plays a critical role in the formation and maintenance of cristae structure [34]. Functional characterization showed that the CHCHD10 Ser59Lys mutant led to the fragmentation of the mitochondrial network and the loss of cristae junctions, linking mitochondrial dysfunction to ALS-FTD etiology (Figure 1 and Box 2) [11]. Thus far, 14 patient-only mutations in *CHCHD10*, including 12 missense and two nonsense mutations, were reported in ALS-FTD spectrum patients. Also, two *CHCHD10* missense mutations located in cis, were found to cosegregate in a multigenerational kindred with isolated mitochondrial myopathy [35–37].

Although mutation frequencies across multiple studies reach <1% (Table 2) [11,35–45], there is supportive evidence for a causal role of *CHCHD10* in ALS and FTD, based on cosegregation in multiple families; replication in ALS, FTD, and ALS-FTD patients from different origins; and functional biological data that directly implicates mitochondrial dysfunction.

Key Figure

Inferred Key Molecular Mechanisms in ALS-FTD Spectrum Pathology.

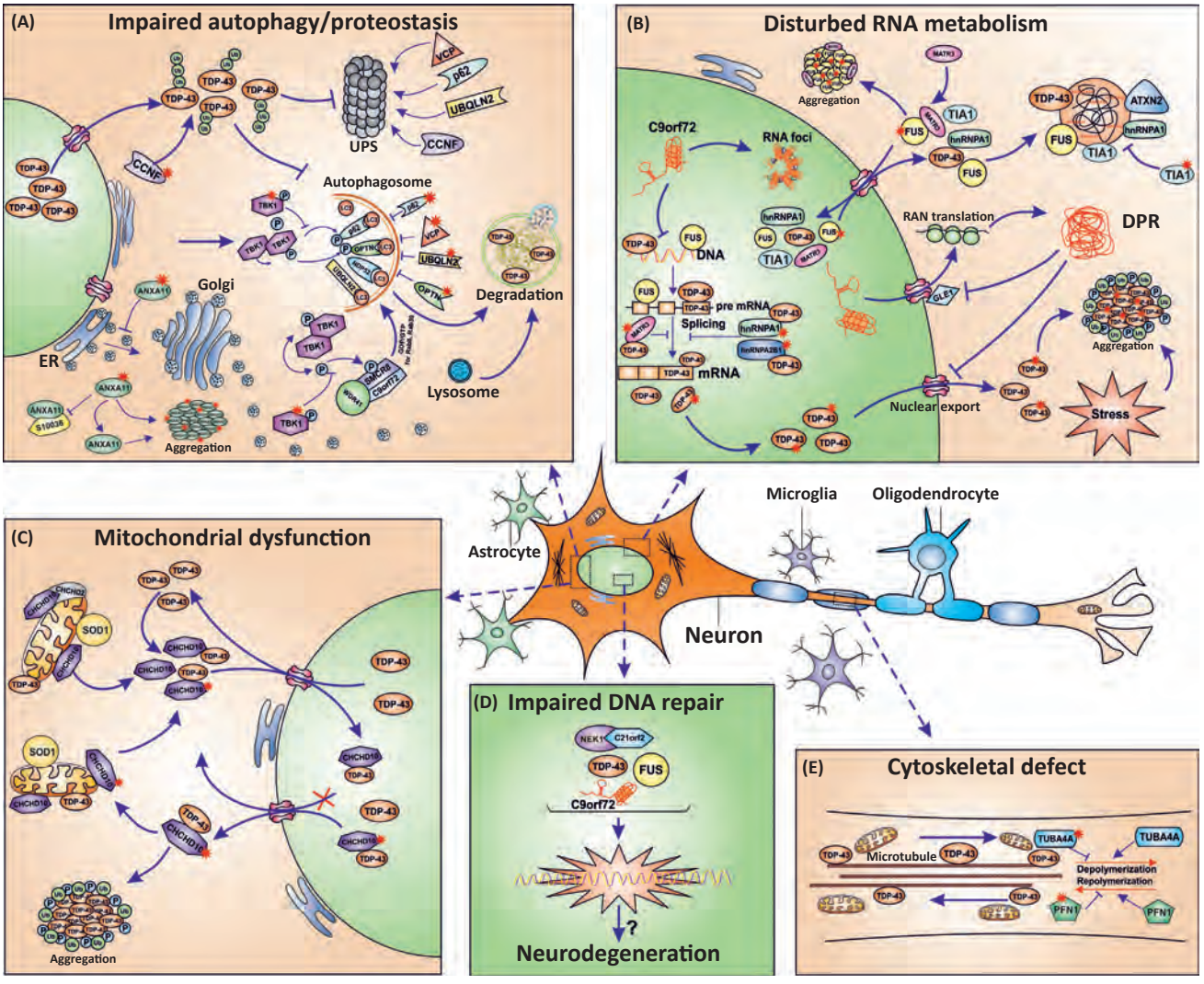

Figure 1. (A) Mutations in TBK1, OPTN, SQSTM1 (p62), UBQLN2, VCP, and CCNF impair protein degradation by affecting the UPS and autophagy pathways. Annexin A11 is involved in vesicular trafficking between the Golgi and ER. Through its binding with calcyclin (S10036) it regulates proteostasis. Mutant annexin A11 aggregates and also sequesters wild type protein, possibly resulting in defective proteostasis and, in turn, TDP-43 accumulation. (B) Disturbances in RNA processing result from mutations in TARDBP, FUS, MATR3, TIA1, hnRNPA1, hnRNA2B1, and C9orf72. Cytoplasmic aggregation of TDP-43 and FUS are common pathological hallmarks in both ALS and FTD. (C) Identification of CHCHD10 mutations has underscored the role of mitochondrial dysfunction in ALS. (D) Similar to TARDBP and FUS, NEK1, C21orf2, and C9orf72 are also associated with impaired DNA damage response. (E) Like PFN1, mutations in TUBA4A alter cytoskeleton dynamics, which affects microtubule integrity and axonal transport; this is also likely for TDP-43. The red stars represent mutated proteins. Abbreviations: ALS, amyotrophic lateral sclerosis; DPR, dipeptide repeat aggregates; ER, endoplasmic reticulum; FTD, frontotemporal dementia; RAN, repeat-associated non-AUG translation; UPS, ubiquitin-proteasome system.

Box 2. Key Molecular Processes in ALS

The seemingly endless discovery of novel ALS genes has highlighted the involvement of multiple cellular mechanisms and molecular pathways in ALS etiology (see Figure 1 in main text). Yet, how dysfunction of these different pathways results in the same disease phenotype is still poorly understood.

TDP-43 aggregation and associated pathology is the pathological hallmark in up to 97% of ALS patients [7], suggesting that TDP-43 is central to the disease process. Wild type TDP-43 is predominantly located in the nucleus, where it regulates RNA splicing of numerous transcripts, including its own. By contrast, mutant TDP-43 aggregates and mislocalizes to the cytoplasm [136]. As for *TARDBP*, mutations in other RNA regulatory genes such as *MATR3*, *hnRNPA1*, and *hnRNPA2B1* are also associated with TDP-43 proteinopathy by impairing RNA processing, likely via direct interaction with TDP-43 [10,137,138]. Together with FUS, ATXN2, TIA1, and hnRNPA1, TDP-43 forms stress granules, which are later degraded by autophagy [7,18,19]. Notably, both FUS and TDP-43 were also linked to DNA damage response [139], as is the case for NEK1 and C21orf2.

Another RNA processing protein, the RNA export mediator GLE1, was associated with ALS [140]. However, the functional relationship between GLE1 and TDP-43 remains ambiguous. One hypothesis is that GLE1 is involved in the nuclear export of RNA targets of TDP-43 and FUS [140]. *C9orf72* repeat expansions carriers also develop TDP-43 proteinopathy in affected brain regions and motor neurons [141]. Recently, the characteristic cytoplasmic DPR inclusions of *C9orf72* repeat expansions carriers (Box 1) were shown to inhibit nuclear import of TDP-43 [142]. Apart from RNA processing genes, mutations in *VCP*, *UBQLN2*, *SQSTM1*, *OPTN*, *CCNF*, *TBK1*, and *ANXA11* can impair protein degradation through the ubiquitin-proteasome system, vesicular transport, and autophagy, which likely regulate the removal of TDP-43 from the cytoplasm [18,136].

For *CHCHD10*, the most recent study in a series of *Caenorhabditis elegans*, mouse, and cell models reported that during mitochondrial stress, CHCHD10 is translocated from the mitochondria and directly interacts with TDP-43. This interaction results in the translocation of this CHCD10-TDP-43 protein complex to the nucleus and subsequently prevents nuclear exit of TDP-43 back to the cytoplasm, which was less efficient in mutant CHCHD10 [143]. It is of interest that, in the mitochondria, CHCHD10 was recently shown to interact with CHCHD2, a protein and gene linked to Parkinson's disease, and that this protein complex is required for efficient mitochondrial respiration [144,145]. Similar to *PFN1*, mutations in *TUBA4A* were demonstrated to destabilize the microtubule network [12]. As microtubule network integrity is a prerequisite for proper axonal transport, TUBA4A seems to indirectly contribute to the transport of target proteins along the axon, including TDP-43 [146,147].

TUBA4A

In a cohort of fALS index patients, an excess of patient variants within the *TUBA4A* gene was found [12]. *TUBA4A* encodes one of eight human α-tubulins, which polymerize with β-tubulins to form the microtubule cytoskeleton. In primary motor neurons, TUBA4A mutants displayed impaired microtubule network assembly and dynamics, and multiple **ubiquitinated cytoplasmic inclusions** (Figure 1 and Box 2). Replication studies identified 11 nonsynonymous and three PTC variants in about 1% of fALS and 0.4% of sALS patients (Table 2) [12,36,46]. Although ALS was the predominant phenotype in *TUBA4A* carriers, a few carriers were diagnosed with cognitive problems or FTD [12,36] (Table 2). However, an extended study in 814 FTD patients ascertained in Spain did not identify *TUBA4A* carriers [47].

While *TUBA4A* variants were identified in multiple studies and modeling of *TUBA4A* mutants demonstrated impaired cytoskeletal dynamics, *TUBA4A* variants are either absent from or very rare in patient cohorts. Also, there is no evidence so far of cosegregating *TUBA4A* variants with disease in affected families. Therefore, for now, there is insufficient evidence to support a causal role for *TUBA4A* in ALS and FTD (Table 2).

MATR3

Exome sequencing in an unresolved kindred identified cosegregating missense mutations in *MATR3* [10]. Across studies, *MATR3* missense mutations were observed in 0.5%–2% of ALS patients (Table 2) [10,48–53]. While in the discovery family, multiple patients were diagnosed with ALS and dementia [10], so far *MATR3* was not screened in FTD patents. MATR3 is a

nuclear matrix protein that binds DNA and RNA through its zinc finger domains and RNA recognition motifs (RRMs) [54], and is assumed to stabilize certain messenger RNA species. Furthermore, MATR3 was shown to function as a direct splicing repressor by binding intronic regions flanking repressed exons [55]. An *in vivo* study demonstrated that overexpressing MATR3 mice develop hindlimb paralysis and forelimb muscle atrophy, suggesting that dysregulation of *MATR3* is involved in neuromuscular functioning [56]. Notably, MATR3 interacts with two other RNA-binding proteins that were genetically linked to ALS, namely TDP-43 and FUS. It was found that MATR3 can be sequestered to cytoplasmic aggregates by mutant FUS (Figure 1 and Box 2) [10,57]. Another study demonstrated that *MATR3* mutations lead to nuclear export defects of *TDP-43* and *FUS* mRNA [58]. Rare *MATR3*-positive cytoplasmic inclusions were observed in an ALS patient carrying a *C9orf72* repeat expansion, potentially unravelling a common cellular pathway for both genes, although these observations need confirmation and further functional studies [10].

The overall data, including cosegregation of MATR3 in multiple ALS families, the presence of rare MATR3 variants in patient groups of different populations, the interaction of MATR3 with TDP-43 and FUS, its involvement in splicing regulation, and the neuromuscular phenotype of an overexpressing MATR3 mouse model, are in favor of a causal role for *MATR3* in ALS (Table 2).

CCNF

Genome-wide linkage analysis identified a *CCNF* missense mutation cosegregating in a large ALS-FTD Australian kindred from British ancestry [15]. Further genetic screening of *CCNF* identified another 10 missense mutations [15]. Across populations from Australia, Europe, America, and Asia, *CCNF* mutations accounted for 0.6%–3.3% of fALS-FTD patients (Table 2) [15,59]. CCNF codes for cyclin F, a member of the cyclin protein family, though it does not bind cyclin-dependent kinases [60,61]. Cyclin F is the founding member of the F-box family of substrate recognition subunits of the SCF ubiquitin ligase complexes, shown to control genome stability through ubiquitin-mediated proteolysis [60]. CCNF catalyzes the transfer of activated ubiquitin to targeted proteins, which are then degraded via the ubiquitin-proteasome system (UPS) [62]. The CCNF mutant, Ser621Gly, impaired this degradation system by disrupting the Lys48-specific ubiquitylation, leading to accumulation of ubiquitinated proteins, including RRM2 and TDP-43, in neuronal cells (Figure 1 and Box 2) [15,63]. Comparable with TBK1, CCNF interacts with SQSTM1, an autophagic receptor that recognizes and transfers ubiquitinated proteins for autophagic degradation [63]. In a recent study in zebrafish, disruption of axonal outgrowth by the mutant Ser621Gly CCNF was observed, suggesting a toxic gain-of-function mechanism for *CCNF* mutations in ALS patients [64].

The genetic findings, together with the functional data obtained in cellular and animal modeling of CCNF mutants displaying impaired ubiquitin-proteasome/autophagy pathways and axonal outgrowth, support a role for CCNF in the ALS-FTD spectrum (Table 2).

NEK1 and *C21orf2*

LOF variants identified in the *NEK1* gene were significantly enriched in 2303 ALS patients compared with 1059 control individuals [16]. Overall, rare variants in *NEK1* were observed in 3%–5% of ALS patients, with LOF variants accounting for nearly 1% (Table 2) [13,16,65,66]. NEK1 belongs to the highly conserved protein family of NIMA-related serine/threonine kinases involved in cell cycle control, ciliogenesis, mitochondrial membrane regulation, and **DNA damage response** [67–70]. In neurons, NEK proteins participate in maintaining the cytoskeleton network [71], linked to ALS etiology by *TUBA4A* (see above) [12] and *PFN1* [72]. Together with the two ALS proteins, vesicle-associated membrane protein-associated protein B/C

(VAPB) and alsin (ALS2), NEK1 interacts with the chromosome 21 open reading frame 2 (C21orf2) in DNA damage repair (Figure 1 and Box 2) [13,73]. *C21orf2* was also recently identified as an ALS gene with an increased rare-variant burden of both LOF and nonsynonymous variants (Table 2) [17]. Remarkably, autosomal recessive mutations in both *NEK1* and *C21orf2* are linked to a skeletal disorder, axial spondylometaphyseal dysplasia, emphasizing the genetic and functional link between these genes and proteins [74,75]. Together, these findings support the role of *NEK1-C21orf2* interaction in DNA damage response in ALS pathogenesis.

Because of the limited replication data in ALS cohorts and the lack of confirmed cosegregation with fALS, *NEK1 and C21orf2* are considered ALS risk genes (Table 2). Also, *NEK1* and *C21orf2* have so far not been investigated in FTD and therefore their contribution to FTD is not clear for now (Table 2).

ANXA11

Exome sequencing in 751 fALS, identified missense mutations in *ANXA11*, including a founder mutation p.Asp40Gly [18]. The annexin A11 protein, belongs to the annexin protein family of calcium-dependent phospholipid-binding proteins, involved in vesicle trafficking, apoptosis, exocytosis, and cytokinesis. Missense variants in this gene had previously been associated with autoimmune disorders [76]. *ANXA11* carriers present clinically with classical ALS with relatively late disease onset of on average 67 years. Postmortem analysis of a p.Asp40Gly carrier showed classic ALS-related p62/SQSTM1 and TDP-43 pathology, as well as unique ANXA11 pathology of abundant skein-like, tubular, filamentous, and basket-like annexin A11-positive aggregates in spinal motor neurons and hippocampal axons. Annexin A11 is involved in vesicular trafficking between the Golgi and endoplasmic reticulum. *In vitro* studies demonstrated that annexin A11 is present in both the nucleus and cytoplasm. In the cytoplasm, annexin A11 was found in vesicle-like structures and foci that were diffusely distributed throughout the soma, axons, and dendrites. Mutant annexin A11 lost association with vesicle-like structures and had a tendency to aggregate. When aggregating, mutant annexin A11 also sequestered wild type protein and interfered in a dominant negative manner with the normal function of annexin A11. Moreover, mutant annexin A11 lost its binding property with interaction partner calcyclin, a protein active in **proteostasis**, rendering annexin A11 less soluble and prone to aggregation. Conversely, increased expression of calcyclin in astrocytes of ALS patients seems to prevent aggregation of mutant annexin A11, possibly by clearance of insoluble annexin A11 by enabling proteasomal degradation (Figure 1 and Box 2).

Mutations in *ANXA11* were observed in about 1% fALS and 1.7% sALS patients and implicate calcium-binding proteins and defective intracellular trafficking in ALS pathogenesis [18]. The identification of an *ANXA11* founder mutation in affected relatives from multiple families and of additional missense mutations in unrelated ALS patients, strongly support *ANXA11* as a causal ALS gene. Further studies in ALS and FTD cohorts and families will help determine the contribution of *ANXA11* mutations to the genetic etiology of these diseases and provide a better understanding of the role of impaired proteostasis in the pathophysiology of ALS-FTD (Table 2).

TIA1

Exome sequencing in an unresolved European ALS-FTD family with TDP-43 brain pathology identified cosegregation of a missense mutation, p.Pro362Leu, in the *TIA1* gene [19]. Similar to several other ALS genes, *TIA1* encodes a RNA-binding protein comprising a prion-like low-complexity sequence domain (LCD). Mutations in this domain were associated with Welander

distal myopathy, a pathology also characterized by aggregates of TDP-43 and p62 [77–79]. Analysis of 1039 ALS or ALS-FTD patients and 3036 control persons uncovered another six carriers of nonsynonymous variants in the LCD of *TIA1*, but none in controls, accounting for ~2% fALS and <0.5% sALS patients [19]. Autopsied brain and spinal cord of *TIA1* carriers confirmed TDP-43 pathology in the extra-motor neocortex, motor cortex, and spinal cord. In addition, frequent hyaline Lewy body-like cytoplasmic inclusions in the lower motor neurons were consistently observed. TIA1 assembles into membraneless organelles like **stress granules**. Mutant TIA1 displayed altered biophysical properties with enhanced liquid–liquid phase separation, believed to precede stress granules formation [19]. TDP-43 is recruited to these TIA1-positive stress granules and becomes rapidly immobile and insoluble, suggesting that mutant TIA1 promotes accumulation of TDP-43 through impaired stress granules dynamics (Figure 1 and Box 2).

Despite the functional evidence of TIA1 in altered stress granule dynamics and TDP-43 aggregation, genetic replication in extended patient cohorts and significant cosegregation data in informative families is missing. Therefore, the true genetic contribution of *TIA1* to risk for ALS or FTD remains to be determined.

Oligogenic Architecture of ALS

For many years, when a pathogenic mutation was present in *SOD1* it was assumed to be the only genetic cause leading to ALS in a patient carrier or affected family. In some families, however, the single *SOD1* mutation did not fully explain disease segregation, since some *SOD1* carriers never developed disease and other patients did not carry the *SOD1* mutation [80]. These observations questioned the **penetrance** of *SOD1* mutations and suggested an **oligogenic model** for ALS, meaning that other mutated genes may be needed to fully express the disease. Subsequently, many studies showed that the frequency of ALS patients and families carrying two or more mutations in ALS-associated genes is higher than expected by chance [66,81–83], providing evidence that ALS is an oligogenic disease caused by multiple rare variants with additive or synergistic effects on disease presentation.

The identification of the *C9orf72* repeat expansion as the most common genetic cause of ALS, explaining 40% of fALS and 10% of sALS cases, has further underscored the influence of oligogenic inheritance in ALS [84,85]. Because of its high prevalence, co-occurrence of multiple mutations is most often observed in combination with a *C9orf72* repeat expansion. Among the 74 reported double (or triple) mutation carriers, 51 (69%) carried a *C9orf72* repeat expansion (Table 3). Furthermore, *C9orf72* repeat expansions were identified in unaffected relatives and were occasionally also detected in unrelated control cohorts [86–88], suggesting incomplete penetrance. Indeed, in *C9orf72* carriers, it appears that additional mutations in genes associated with the ALS-FTD spectrum may drive disease presentation in the direction of ALS, FTD, or a combination of both symptomatologies (i.e., act as **genetic modifiers** of disease phenotype). Looking at Table 3, some striking correlations can be observed. Combined with *FUS* ($n = 3$), *OPTN* ($n = 4$), *ANG* ($n = 3$), or *SOD1* ($n = 2$), *C9orf72* carriers always present with only ALS. By contrast, combined with *GRN* ($n = 6$), all *C9orf72* patients had only FTD. *GRN* mutations are exclusively linked to FTD, which likely explains the latter correlation. The same goes for *C9orf72* with a second mutation in *SOD1* and ALS. A *C9orf72* repeat expansion, together with a *NEK1* mutation, repeatedly resulted in an ALS phenotype ($n = 4$), but once resulted in ALS-FTD. Since *NEK1* mutation screens in FTD cohorts are yet missing, it may be premature to draw conclusions on its potential impact on the FTD phenotype. Inheritance of *C9orf72* with *TARDBP* ($n = 8$) or *TBK1* ($n = 3$), resulted in all three presentations of the ALS-FTD spectrum.

Table 3. Overview of Reported Multiple Gene Rare Variant Carriers with ALS or ALS-FTD[a]

Variant 1	Pathogenicity	Variant 2	Pathogenicity	Variant 3	Pathogenicity[b]	ALS/FTD	Family history	Age of onset (years)	Refs
C9orf2	P	*TARDBP p.Ala382Thr*	P			ALS-FTD	+	43–45	[148]
C9orf2	P	*TARDBP p.Ala321Val*	LP			ALS	+	37	
C9orf2	P	*TARDBP p.Asn352Ser*	LP			ALS	+	42–47	
C9orf2	P	*TARDBP p.Ala382Thr*	P			ALS	+	47	
C9orf2	P	*TARDBP p.Ser292del*	VUS			FTD	+	47–54	
C9orf2	P	*TARDBP p.Ala382Thr*	P			ALS-FTD	+	43.8	
C9orf2	P	*TARDBP p.Asn267Ser*	LP			FTD	+		
C9orf2	P	*FUS p.Gln210His*	VUS			ALS	+	58	
C9orf2	P	*FUS p.Gly174del*	VUS			ALS	+	62	
C9orf2	P	*FUS p.Arg521Cys*	LP			ALS	+	40	
C9orf2	P	*SOD1 p.Asp110Tyr*	VUS			ALS	+	59	
C9orf2	P	*SOD1 p.Asp90Ala*	VUS			ALS	+	51	
C9orf2	P	*GRN p.Tyr294Cys*	VUS			FTD	+	64	
C9orf2	P	*GRN p.Cys466Lysfs*46*	LP			FTD	+	52	
C9orf2	P	*GRN p.Arg493**	P			FTD	+	50	
C9orf2	P	*GRN p.Arg493**	P			FTD		62	
C9orf2	P	*GRN p.Cys31fs*	LP			FTD	+	59.5	
C9orf2	P	*ANG p.Ile46Val*	VUS			ALS		38	
C9orf2	P	*ANG p.Lys17Ile*	VUS			ALS	+	47	
C9orf2	P	*OPTN p.Glu322Lys*	VUS			ALS	+	50	
C9orf2	P	*OPTN p.Asp128Glufs*22*	VUS			ALS	+	46	
C9orf2	P	*SQSTM1 p.Arg212Cys*	VUS			ALS-FTD	+	63	
C9orf2	P	*SQSTM1 p.Val153Ile*	VUS			ALS	+		
C9orf2	P	*UBQLN2 p.Gly502_Ile504del*	VUS			ALS	+	52	
C9orf2	P	*PRPH* p.Arg133Pro	VUS			ALS		70	
C9orf2	P	*PSEN2* p.Ile146Val	VUS			FTD	+	68	
C9orf2	P	*MAPT p.Pro301Leu*	LP			FTD	+	53	
C9orf2	P	*VAPB p.Val234Ile*	VUS			ALS	+	65	
C9orf2	P	*DAO p.Arg38His*	VUS			ALS	+	42	
C9orf2	P	*DCTN1 p.Ile196Val*	VUS			ALS	+		
C9orf2	P	*SETX p.Ile2547Thr*	VUS			ALS	+		

Table 3. (continued)

Variant 1	Pathogenicity	Variant 2	Pathogenicity	Variant 3	Pathogenicity[b]	ALS/FTD	Family history	Age of onset (years)	Refs
TARDBP p.Gly287Ser	LP	*VAPB p.Met170Ile*	VUS			ALS			
TARDBP p.Asn352Ser	LP	*ANG p.Lys17Ile*	VUS			ALS-FTD	+	61	
SOD1 p.Ala5Val	LP	*DAO p.Ser345Phe*	VUS			ALS	+		
SOD1 p.Pro67Ala	LP	*SETX p.Ile2547Thr*	VUS			ALS	+		
SOD1 p.Gly93Asp	P	*ANG p.Arg121Cys*	VUS			ALS		72	
FUS p.Arg485Trp	VUS	*SETX p.Ile2547Thr*	VUS			ALS			
FUS p.Arg521Cys	LP	*ANG p.Lys17Ile*	VUS			ALS	+	53	
DCTN1 p.Arg1049Gln	VUS	*SETX p.Ser323Asn*	VUS			ALS			
DCTN1 p.Thr1249Ile	VUS	*SETX p.Met274Val*	VUS			ALS			
TAF15 p.Arg408Cys	VUS	*SETX p.Ile2547Thr*	VUS	*SETX p.Thr14Ile*	VUS	ALS			
SETX p.Cys1554Gly	VUS	*DCTN1 p.His1270Gln*	VUS	*FIG4* p.Met694Val	VUS	ALS			
OPTN Ala481Val	VUS	*OPTN p.Gln235**	VUS			FTD	+	64	[22]
*OPTN p.Gly538Glufs*27*	LP	*TBK1 p.Arg117**	LP			FTD		68	
C9orf2	P	*TBK1 p.Gly244Val*	VUS			ALS-FTD	+	41	[23]
C9orf2	P	*OPTN p.Glu322Lys*	VUS			ALS		74	[149]
SQSTM1 p.Arg33Val	VUS	*TBK1 p.Met690fs*	LP			ALS		66	[48]
C9orf2	P	*TBK1 p.Glu476fs*	LP			ALS	+	44	[33]
C9orf2	P	*TBK1 p.Leu277Val*	VUS			ALS		26	
C9orf2	P	*NEK1 p.Asn745Lys*	VUS			ALS		65	
OPTN p.Gln314Leu	VUS	*TBK1 p.Ala705fs*	LP			ALS		46	
TARDBP p.Gly287Ser	LP	*NEK1 p.Ala341Thr*	VUS			ALS		64	
C9orf2	P	*NEK1 p.Arg232Cys*	VUS			ALS-FTD			[66]
C9orf2	P	*NEK1 p.Arg261His*	VUS			ALS			
C9orf2	P	*NEK1 p.Arg261His*	VUS			ALS			
C9orf2	P	*NEK1 p.Arg261His*	VUS			ALS			
C9orf2	P	*NEK1 p.Ser1036**	VUS	*TUBA4A* p.Thr38Met	VUS	ALS	+	47	
*TARDBP p.Tyr374**	LP	*NEK1 p.Arg261His*	VUS			ALS	+	47	
SOD1 p.Ile114Thr	LP	*NEK1 p.Asn745Lys*	VUS			ALS+D	+		
C9orf2	P	*VCP p.Arg155His*	P			ALS			[92]
C9orf2	P	*TARDBP p.Ala321Val*	LP			ALS	+		
C9orf2	P	*UBQLN2 p.Thr334Met*	VUS			ALS			

Table 3. (continued)

Variant 1	Pathogenicity	Variant 2	Pathogenicity	Variant 3	Pathogenicity[b]	ALS/FTD	Family history	Age of onset (years)	Refs
C9orf2	P	*ALS2 p.Ser654Gly*	VUS			ALS	+		
C9orf2	P	*ANG p.Lys78Glu*	VUS			ALS	+		
C9orf2	P	*OPTN c.626+1G>T*	VUS			ALS			
SOD1 p.Asp77Tyr	VUS	*FUS p.Ser135Asn*	VUS			ALS	+		
FUS p.Arg269Trp	VUS	*OPTN p.Arg271His*	VUS			ALS	+		
UBQLN2 p.Gln460Arg	VUS	*SOD1 p.Asp91Ala*	VUS			ALS	+		
SOD1 p.Ile114Thr	LP	*ALS2 p.Pro1288Leu*	VUS			ALS	+		
TARDBP p.Met337Val	P	*TARDBP p.Asn179Asp*	VUS			ALS			
C9orf2	P	*TYROBP p.Val47Ala*	VUS	*CSF1R p.Gly747Arg*	VUS	D	+	49	[150]
C9orf2	P	*TREM2 p.Glu151Lys*	VUS			D	+	53	
C9orf2	P	*GSN p.Glu268Lys*	VUS	*SOD1 p.Ala141Ala*	LP	ALS-FTD	+	70	
C9orf2	P	*GRN c.-2C>T*	LP			FTD	+	65	

[a]Abbreviations: ALS, amyotrophic lateral sclerosis; D, dementia unspecified; FTD, frontotemporal dementia; LP, likely pathogenic; P, pathogenic; VUS, variant of unknown significance.
[b]Variant classification according to Sherloc, a comprehensive refinement of the ACMG-AMP guidelines [151].

When *C9orf72* repeat expansions carriers are excluded, mutations in multiple ALS genes appear mostly in pure ALS patients (83%), and double mutations are most often observed with *TARDBP* and *NEK1* (Table 3). In a Belgian cohort of ALS and ALS-FTD patients, additional mutations in ALS genes were detected in over 50% of *NEK1* carriers; the most frequent of these was the *C9orf72* repeat expansion [66], a frequency that was significantly higher than expected by chance. Most striking was the cosegregating of the *NEK1* p.Ser1036* LOF mutation in two affected siblings with fALS and cognitive impairment, who also carried a *C9orf72* repeat expansion and *TUBA4A* p.Thr381Met variant, and presented with early disease onsets of 47 and 52 years (Table 3) [36,66].

In the past, identification of ALS gene mutations made use of single gene-based Sanger sequencing. Consequently, rare ALS genes were not systematically evaluated and mutations in these genes are likely underrepresented in mutation databases. Recently, high-throughput DNA parallel sequencing has proven to be the most effective approach in simultaneously analyzing panels of genes and identifying multiple gene mutation carriers, accounting for 1%–4% of ALS patients [66,89–92]. One study revealed that patients carrying multiple mutations develop ALS 10 years earlier than patients carrying a mutation in a single ALS gene [91]. In line with this observation, another study reported that ALS patients carrying a single or no mutation had longer survival times than patients carrying multiple mutations [93], suggesting that oligogenic variants may also influence disease progression and severity in ALS.

Opportunities for Therapy

To date, there is no effective cure for ALS. Since 1995, the antiglutamatergic agent riluzole has been the only pharmacological treatment administered to ALS patients, with a median increased survival of up to 3 months [94]. This year, the US FDA approved edaravone as an ALS drug, after successful clinical trials in Japan and South Korea [95,96]. Edaravone is a neuroprotective drug that acts as an antioxidant. Treatment reduced functional decline over a period of 6 months when started early in the disease progress [97,98]. Still, these two ALS drugs have only limited beneficial effects, and no drug is available that significantly extends the lifespan of ALS patients, indicating that better therapies are urgently needed. Hereto, the rapid development in genetic studies identifying new ALS genes and related disease pathways hold promises for new therapeutic strategies (Figure 1 and Box 2). For example, targeting ALS genes, genetic modifiers, or related disease molecules with antisense oligonucleotides (ASOs) have shown promising results. In Phase I of a clinical trial, the direct delivery of ASOs against *SOD1* to the CSF of fALS patients by intrathecal infusion was able to eliminate mutant SOD1 without adverse effects [99]. Currently, clinical trials with second generation ASOs against *SOD1* have entered Phase I (NCT02623699).[i] In line with these findings, ASO treatment against *SOD1* improved survival of human fALS iPSC, and decreased expression of apoptotic markers [100]. Further, in *SOD1* p.Gly93Ala transgenic mice, inactivation by ASOs of an important microRNA associated with inflammatory response (miR-155), significantly prolonged the survival and disease duration of the mice [101,102]. Notably, for the first time, the progression of ALS could be controlled (i.e., started and stopped, by treatment of *SOD1* p.Gly93Ala mice with the PET-imaging agent CuATSM) [103]. CuATSM is known to deliver copper into the CNS, and is used here to promote the maturation of SOD1, through the CCS protein which completes SOD1 maturation by inserting copper. When SOD1 lacks this metal cofactor, it tends to misfold and become toxic, often leading to the degeneration of motor neurons. For *C9orf72* (Box 1), the use of ASOs against the regions flanking the GGGGCC repeat prevented the formation of **RNA foci** and dipeptide repeat pathology [104–107]. In adult mouse brain and spinal cord, *C9orf72* RNA levels were reduced to 60%–70% after 3 weeks of intracerebroventricular stereotactic injection with ASOs [108]. Apart from causal genes, genetic modifiers

have also been targeted. Expanded polyglutamine repeats in *ATXN2* cause spinocerebellar ataxia type 2 but, intermediate repeats were associated with an increased risk for ALS [89,109,110]. ATXN2 forms a complex with TDP-43 and is a potent modifier of TDP-43 toxicity in animal and cellular models [111]. Silencing of *ATXN2* mediated by ASOs or crossing *ATXN2* knockout mice with TDP-43 transgenic mice, improved survival of the mice and reduced TDP-43 pathology [112].

Using the **CRISPR/Cas9** technology to target mutant genes and reintroduce wild type copies by adeno-associated viral (AAV) vectors, will be of interest for the development of precision therapy in the future. Although the use of AAVs for gene delivery has its limitations, such as lower gene expression and limited exogenous DNA fragment size, AVVs have been shown to be safe (nonpathogenic), with low immunogenicity, ability to cross the blood–brain barrier, and efficient transfection of DNA to different cell types such as neurons, glia, or astrocytes, with long-term effects [113]. Deletion of the *C9orf72* repeat expansion in patient-derived iPSCs by CRISPR/Cas9 prevented the formation of RNA foci and rescued hypermethylation at the *C9orf72* locus, without changing mRNA or protein expression levels [114]. CRISPR/Cas9 is currently being used to engineer new ALS animal models for multiple ALS genes, which, in turn, will open new avenues for the development of precision therapies in the future [115].

Concluding Remarks

Progress in genome technology and gene discovery in the past few years has drastically improved our understanding of the multiple disease pathways involved in ALS, including autophagy, cytoskeleton dynamics, mitochondrial dysfunction, RNA processing, and DNA damage repair. However, questions remain (see Outstanding Questions). It has become increasingly clear that ALS is genetically complex (Box 3), with an important contribution of

Box 3. ALS: A Complex Disease

ALS is a complex disease with a strong genetic contribution. About 5% of patients have first and second degree affected relatives [152]. Mendelian ALS was linked to rare mutations in genes *SOD1*, *C9orf72*, *TARDBP*, and *FUS*. The Mendelian inheritance can however be obscured by reduced and/or age-related penetrance, and this likely explains why these causal mutations can also be detected in sporadic cases. Furthermore, in apparently sporadic patients, twin studies estimated the heritability to be as high as 60% [153].

Some patients appear to carry more than one rare disease-causing mutation. An increasing number of studies are showing that the number of patients with multiple ALS-associated mutations is higher than what can be expected by chance, based on the individual mutation frequencies of the respective genes [66,81,82,83], underscoring the oligogenic component to ALS.

In addition to rare variants [minor allele frequency (MAF) $< 1\%$], it was estimated that the contribution of common variation (MAF $> 5\%$) to the heritability of sALS is $\sim$12% [154]. Large GWAS have identified multiple risk loci associated with ALS, although not all could be replicated in independent populations, and so most of the heritability of sALS remains unresolved [154].

In addition to genetic predisposition, the role of environmental factors in ALS has been widely investigated. Most studied environmental factors include exposure to heavy metals, electromagnetic fields and electric shocks, pesticides, cyanotoxins, and excessive physical activity. So far, pesticide exposure, and the genetic predisposition to pesticide-induced damage, seems to be the only environmental effect for which the literature evidence is supporting a role in neurodegeneration and ALS. All other investigated environmental factors so far, have given conflicting and inconclusive results (for more on environmental factors in ALS see [155]).

Taken together, these multilevel contributions classify ALS as a complex disorder with a monogenic component of rare high-penetrant variants, an oligogenic component of rare intermediate penetrant variants, and a multifactorial component of common risk variants, possibly all under the influence of gene–environment interactions.

Outstanding Questions

What are the molecular mechanisms that drive disease presentation of ALS-FTD spectrum gene mutations (e.g., in *C9orf72* or *TBK1*) towards an ALS phenotype rather than an FTD phenotype, and vice versa? What are the genetic factors or modifiers that affect disease presentation?

It is remarkable that many ALS genes were associated with distinct clinical phenotypes such as proteinopathies of muscle and bone (*VCP*, *HNRNPA1*, *HNRNPA2B1*, *MATR3*, *TIA1*), (spino) cerebellar ataxia (*ATXN2, CHCHD10*), mitochondrial myopathy (*CHCHD10*), and autoimmune disorders (*ANXA11*). What are the molecular mechanisms that underlie this high degree of **pleiotropy** and can these mechanisms be exploited for disease-modifying therapies?

How do *CHCHD10* mutations trigger TDP-43 aggregation? Why does the interaction between mutant CHCHD10 and TDP-43 fail to keep TDP-43 in the nucleus?

Do *TUBA4A* mutations directly or indirectly alter axonal transport of TDP-43?

MATR3-positive cytoplasmic inclusions were observed in a *C9orf72* repeat expansion carrier. Therefore, the question arises whether there is a direct connection between *C9orf72* and *MATR3* and if, through its DNA/RNA-binding properties, MATR3 can bind the *C9orf72* repeat?

No significant cosegregation of *NEK1* or *C21orf2* mutations has so far been described. What is the degree of penetrance of these variants/mutations and are they strong enough to lead to disease on their own or do they require the burden of additional ALS-associated gene mutations?

How do dipeptide repeats (DPRs) affect nucleocytoplasmic transport of TDP-43 and other proteins? Is there any link with GLE1?

How do *ANXA11* mutations affect proteostasis, calcyclin interaction,

rare genetic variation of high to intermediate penetrance, in addition to common risk variants with small effect sizes. It is important that this complex genetic architecture of ALS is taken into account when proceeding with gene discovery studies, genetic testing and counseling, and therapy development.

The identification of the *C9orf72* repeat expansion exemplifies that noncoding DNA variations, which are not covered by WES, also contribute to ALS. Although WES is now widely used in gene identification studies, there is no doubt that WGS will soon replace WES as the standard method for gene discovery. Thanks to the Project MinE[ii] and the 'Ice bucket challenge', WGS data of more than 15 000 ALS patients and 7500 healthy control individuals from around the world will be released. These large-scale genomics data, together with related omics information obtained from transcriptomics, proteomics, and metabolomics analyses, will shed further light on unresolved genetic causes, postgenomic effects, and involved molecular pathways in ALS, and associated therapeutic opportunities. Hopefully, in the near future, comparable initiatives to generate global full-genome publically available data in FTD, will be set up.

For many years, small model organisms have been used to screen drug targets and develop therapies for ALS. Although the human and animal genome are closely related, post-translational modifications and gene expression among species may differ significantly; consequently some drugs may have beneficial effects in animal ALS models but not in humans [116]. With the introduction of genome editing technology, generating large knock-in animal models or human iPSCs, that more closely mimic the patients' genomic context, will overcome overexpression, off-target, and mosaic effects, which holds great promise for the future [117]. Patient-derived iPSCs can effectively be used for high-throughput screening of FDA approved drugs. Also, the development of modern intrathecal administration of ASOs for silencing of different ALS targets, in combination with safe delivery methods, will enable us to develop more effective clinical therapies.

vesicular transport, and TDP-43 accumulation?

How does TIA1 recruit TDP-43 to stress granules and does mutant TIA1 promote TDP-43 accumulation and aggregation?

Are *TIA1* mutations limited to the prion-like low-complexity domain (LCD) or can genetic variation outside the LCD also predispose to ALS or FTD?

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Resources

[i]https://clinicaltrials.gov

[ii]www.projectmine.com

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