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Review Article

Molecular Mechanisms of Neurodegeneration Related to *C9orf72* Hexanucleotide Repeat Expansion

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Two clinically distinct diseases, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), have recently been classified as two extremes of the FTD/ALS spectrum. The neuropathological correlate of FTD is frontotemporal lobar degeneration (FTLD), characterized by tau-, TDP-43-, and FUS-immunoreactive neuronal inclusions. An earlier discovery that a hexanucleotide repeat expansion mutation in chromosome 9 open reading frame 72 (*C9orf72*) gene causes ALS and FTD established a special subtype of ALS and FTLD with TDP-43 pathology (C9FTD/ALS). Normal individuals carry 2–10 hexanucleotide GGGGCC repeats in the *C9orf72* gene, while more than a few hundred repeats represent a risk for ALS and FTD. The proposed molecular mechanisms by which *C9orf72* repeat expansions induce neurodegenerative changes are *C9orf72* loss-of-function through haploinsufficiency, RNA toxic gain-of-function, and gain-of-function through the accumulation of toxic dipeptide repeat proteins. However, many more cellular processes are affected by pathological processes in C9FTD/ALS, including nucleocytoplasmic transport, RNA processing, normal function of nucleolus, formation of membraneless organelles, translation, ubiquitin proteasome system, Notch signalling pathway, granule transport, and normal function of TAR DNA-binding protein 43 (TDP-43). Although the exact molecular mechanisms through which *C9orf72* repeat expansions account for neurodegeneration have not been elucidated, some potential therapeutics, such as antisense oligonucleotides targeting hexanucleotide GGGGCC repeats in mRNA, were successful in preclinical trials and are awaiting phase 1 clinical trials. In this review, we critically discuss each proposed mechanism and provide insight into the most recent studies aiming to elucidate the molecular underpinnings of C9FTD/ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two clinically distinct entities. ALS, also known as motor neuron disease (MND), Lou Gehrig's disease, and Charcot disease, affects both upper and lower motor neurons leading to hyperreflexia, spasticity, fasciculations, and muscular atrophy [1]. The disease onset is mostly after 60 years of age with a prevalence of around 5 cases per

100,000 [2]. ALS patients can be divided into subgroups according to neuropsychological deficits: ALS with cognitive impairment (ALS-ci), ALS with behavioral impairment (ALS-bi), and ALS with combined cognitive and behavioral impairment (ALS-cbi) [3]. In FTD, neurodegeneration affects the frontal and temporal lobes causing frontotemporal lobar degeneration (FTLD) and is associated with changes in behavior and personality, deficits in frontal executive functions, and language impairment [3]. FTD is considered to

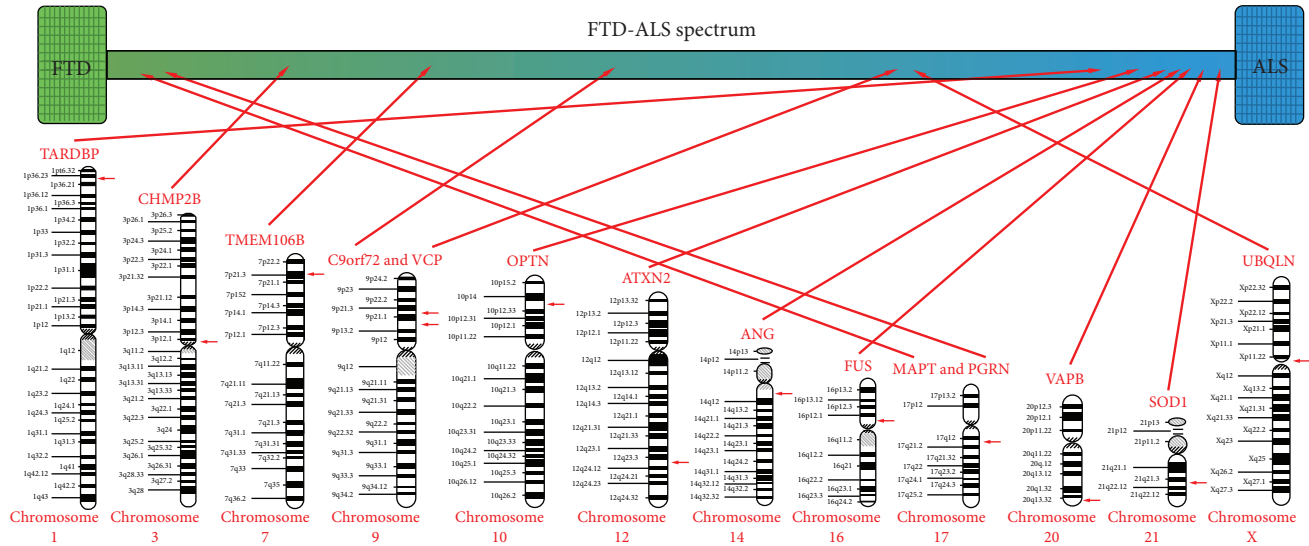


FIGURE 1: Influence of different genes on FTD/ALS clinical spectrum.

be one of the most common forms of dementia in the population under 65 years of age with the prevalence around 10 cases per 100,000 [4]. FTD patients are divided in three clinical syndromes according to their symptomatology: two language variants (progressive nonfluent aphasia (PNFA) and semantic dementia (SD)), and behavioral variant of frontotemporal dementia (bvFTD) [3]. Based on the clinical, genetic, and neuropathological overlap between ALS and FTD, these two diseases are now considered as two extremes of the FTD/ALS spectrum (Figure 1) [1]. About 15% of FTD patients show symptoms of ALS, and up to 50% of ALS patients have symptoms of FTD [5].

2. Main Features of ALS and FTLT-TDP Neuropathology

The vast majority of ALS cases (ALS-TDP) and the most common FTD pathological subtype (FTLD-TDP) show TDP-43 (TAR DNA-binding protein 43) immunoreactive aggregates forming characteristic inclusions (for overview see [6, 7]). The hallmarks of ALS with TDP-43 pathology (ALS-TDP) are skein-like, granular, and compact inclusions in motor neurons, whereas in FTLD-TDP, there are four distinct morphological types (A–D) with characteristic distribution and morphology of dystrophic neurites, cytoplasmic, and intranuclear inclusions. There is a good correlation with both clinical phenotype and genetic alterations [8]. For example, cases with hexanucleotide repeat expansions in *C9orf72* are typically associated with type A and type B pathology, whereas mutations in the progranulin gene *GRN* and valosin-containing protein gene (*VCP*) are associated with type A and type D, respectively. Regarding the correlation with clinical phenotypes, for example, PNFA is associated with type A, SD with type C, and inclusion body myopathy with frontotemporal dementia (IBMF) with type D. In both ALS and FTLD, there is glial pathology mainly affecting oligodendroglia, with TDP-43-immunoreactive cytoplasmic inclusions [6, 7]. While most of the ALS cases

are sporadic, about half of all FTD patients show a familial pattern of inheritance linked to mutations in several different genes [6, 9–11].

3. Hexanucleotide Repeat Expansion in *C9orf72* Gene

A mutation in chromosome 9 open reading frame 72 (*C9orf72*) has been identified as a common genetic cause of both ALS and FTD. The link of ALS and FTD to chromosome 9 was first reported in 2006 in two independent studies [12, 13]. It was later shown that the mutation is an expansion of GGGGCC (G4C2) hexanucleotide repeat in a noncoding region of the *C9orf72* gene [14–16], which is the most common genetic cause of ALS and FTD (so called C9FTD/ALS) in certain populations.

In humans, three transcript variants of *C9orf72* have been identified. Transcript variants 2 and 3 give rise to identical protein isoforms of 481 amino acids, while the 3' site of transcript variant 1 is truncated and encodes an isoform of 222 amino acids. The hexanucleotide repeat is located within the promoter region for transcript variant 2, which corresponds to the position of the first intron in transcript variants 1 and 3 depending on the transcription start site used [17, 18].

While the number of G4C2 repeat units in the DNA of healthy individuals is up to 25, the number of repeats in the DNA of ALS and FTD patients is usually 400 to several thousand [14–16, 19–21]. A small percentage of patients have shorter expansions, from 45–80 repeats [19], and an even shorter expansion, around 30 repeats, has been associated with the disease [15]. Notably, there is an apparent gap between short pathogenic repeat sizes of 45 to 80 and long expansions from 400 to several thousand units. This is likely due to high genomic instability of the intermediate long repeats, which may have a tendency to either expand or contract [19]. Interestingly, longer expansions have been

recently correlated with an earlier onset of disease [19]. In contrast, other studies detected either positive correlation [22–25] or no association [22, 25] between disease severity and expansion size. Additionally, the results of these studies were variable depending on the tissue in which expansion size was measured [22, 25].

4. C9orf72 Protein

The function of C9orf72 protein is still unclear. Bioinformatics predictions suggest that C9orf72 belongs to the family of DENN (differentially expressed in normal and neoplastic cells) proteins, which function as RabGEF (guanine exchange factor) regulators of membrane trafficking by activating RabGTPases [26, 27]. RabGEF facilitates a release of GDP from Rab and exchanges it for GTP. To support the role of C9orf72 as RabGEF, a study conducted on neuronal cell lines and human spinal cord tissue revealed that it was colocalized and coprecipitated with Rab proteins [28]. Therefore, C9orf72 could be involved in Rab-mediated cellular trafficking and protein degradation. Additionally, C9orf72 protein was detected at presynaptic sites, and as it interacts with Rab proteins, it is believed that C9orf72 could regulate synaptic vesicles as RabGEF for RAB3 proteins [29]. Xiao et al. detected interactions of C9orf72 with different components of the nuclear pore complex (NPC) and nuclear receptors in human brain tissue, indicating a possible role of C9orf72 in nucleocytoplasmic trafficking [30]. Nuclear transport is compromised in different C9FTD/ALS models (such as *Drosophila*, induced pluripotent stem cells (iPSC) derived neurons, human brain tissue, yeast cells, mouse primary neurons, and primary human dermal fibroblasts) [31–33]. As knockdown of C9orf72 leads to disruption in endosomal trafficking and formation of autophagosome [28], it is proposed that C9orf72 may be also involved in the regulation of endosomal trafficking and autophagy. Additional studies established the involvement of C9orf72 in the regulation of autophagy [34–36]. In studies conducted on cell lines, it was also shown that C9orf72 could be involved in the regulation of lysosomal function [37, 38] and in the formation of stress granules [39]. In mice, C9orf72 is required for the normal macrophage and microglial function [40]. It was shown that decreased levels of C9orf72 cause dysfunctional microglia that are related to neurodegeneration [40].

5. Neuropathological Features of C9FTD/ALS

Cases with hexanucleotide repeat expansion in C9orf72 are typically associated with type A and B FTLT-DTP pathology, as previously mentioned. The neuropathology of C9FTD/ALS shows pathognomonic ubiquitin- and p62-positive and, rarely, TDP-43-containing inclusions in the cerebellum (Purkinje cells and granular cells) and hippocampus [41, 42]. A unique feature is the presence of aggregating dipeptide repeat proteins within a proportion of inclusion bodies, some of which may not show phosphorylated TDP-43 (pTDP-43) immunoreactivity [43, 44]. Ultrastructurally, the characteristic inclusions in FTLT and ALS with C9orf72 mutation are granular and filamentous [45].

6. Potential Mechanisms of C9orf72 Hexanucleotide Repeat Expansion-Mediated Neurodegeneration

Three mechanisms have been proposed for G4C2 hexanucleotide repeat expansion (HRE) in C9orf72 to induce neurodegenerative changes: (1) loss of C9orf72 function through haploinsufficiency, (2) toxic gain-of-function due to the generation of aberrant HRE-containing RNA, and (3) toxic gain-of-function through the accumulation of dipeptide repeat proteins (DPR) translated from hexanucleotide repeat RNA. Potential mechanisms by which C9orf72 repeat expansions result in neurodegeneration are summarized in Figure 2. Microphotograph with characteristic histopathological changes is given in Figure 3. Studies investigating these mechanisms are summarized in Table 1.

6.1. Loss of C9orf72 Function through Haploinsufficiency. Carriers of C9orf72 HRE have decreased levels of C9orf72 transcripts [14, 16], which are also reflected in the decreased C9orf72 protein levels in the frontal and temporal cortex [20], presumably due to the loss of transcription from the mutant allele carrying the HRE. A systematic study of C9orf72 levels in patients with HRE revealed decreased levels of C9orf72 transcripts in comparison to non-HRE patients and controls [46]. Interestingly, the levels of the long C9orf72 protein isoform were decreased in the brain, while levels of the short C9orf72 protein isoform were increased (as detected by Western blot). Immunohistochemical analysis of spinal motor neurons showed decreased expression of short C9orf72 protein isoform on nuclear membrane in C9ALS cases compared to controls, while subcellular localization of long C9orf72 protein isoform was unchanged [30]. Another study detected 80% reduction of long C9orf72 protein isoform in the cerebellum of C9orf72 HRE carriers in comparison to controls [29]. C9orf72 hexanucleotide repeat expansion might induce DNA hypermethylation and consequently lead to decreased C9orf72 transcription [47]. HRE is methylated when the number of repeats is larger than 90 [48]. Moreover, the DNA from a fraction of C9orf72 HRE carriers is also methylated in the 5' CpG island [47, 49, 50]. The repeat expansion-induced DNA methylation in the C9orf72 promoter region, which results in down-regulated C9orf72 transcription, therefore provides a likely explanation for the association between the size of repeat expansion and the age of onset of the disease [19]. More precisely, the authors tested C9orf72 promoter activity in human kidney and neuroblastoma cell lines and observed reduced C9orf72 transcription in cells with larger repeats and increased methylation. Thus, they proposed that higher methylation of C9orf72 promoter may be an explanation of how repeat expansion could lead to loss-of-function, without excluding the possibility that repeat expansion could also lead to toxic gain-of-function [19]. There is also a possibility that higher-order DNA structures formed on G4C2 repeats could lead to abortive transcription of C9orf72 and therefore be the cause for decreased C9orf72 transcription [51].

C9orf72 loss-of-function in *C. elegans* and zebrafish models results in motor neuron degeneration, indicating a

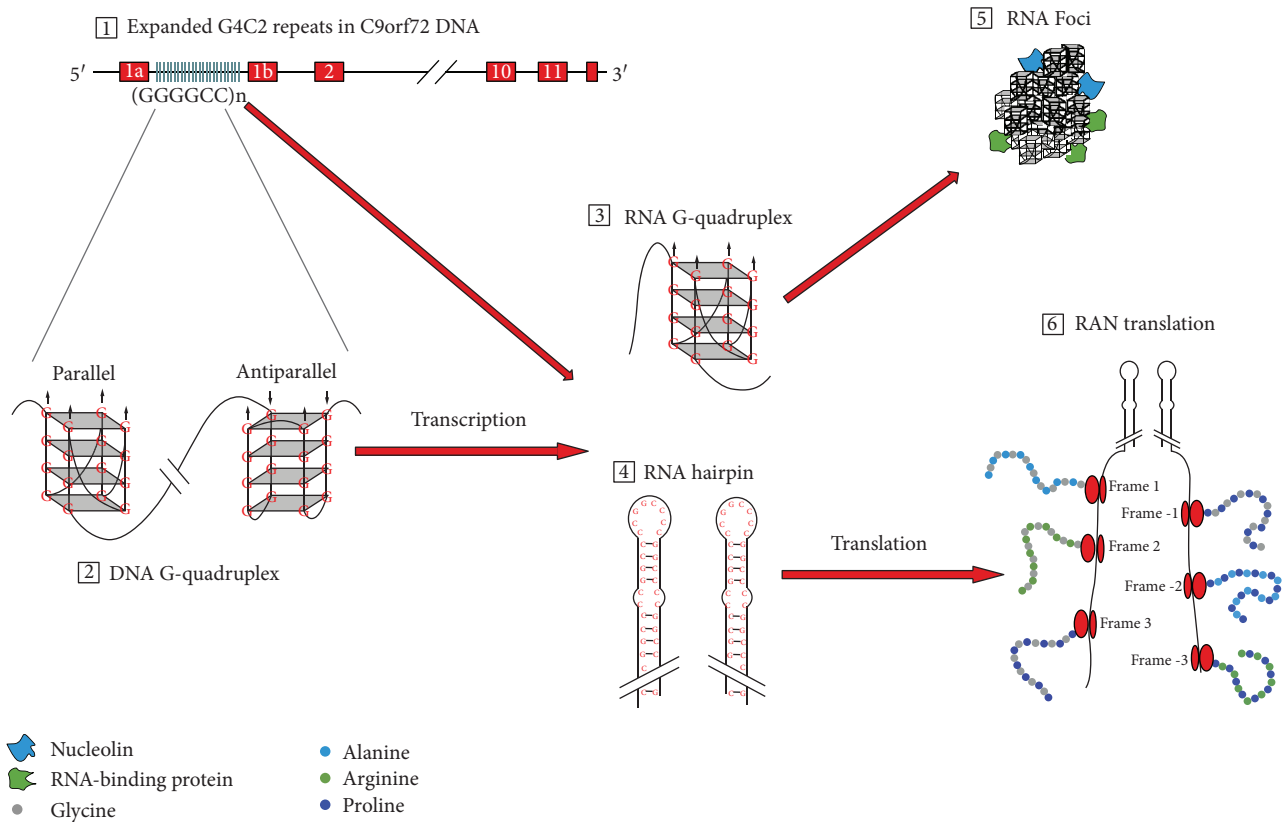


FIGURE 2: Potential mechanisms of *C9orf72* hexanucleotide repeat expansion (HRE)-mediated neurodegeneration. Pathology due to repeats in *C9orf72* gene may emerge from *C9orf72* haploinsufficiency, RNA toxicity, and DPR accumulation. HRE in the noncoding region of the *C9orf72* gene (1) form G-quadruplex structures (2). RNA transcribed from HRE DNA region can form different structures including G-quadruplexes (3) and RNA hairpins (4). HRE-containing RNA form RNA foci (5), which bind RNA-binding proteins. The last possible mechanism underlying pathology in C9FTD/ALS is through the repeat-associated non-ATG (RAN) translation, in which five different dipeptide repeat proteins can be formed—poly-GA, poly-GP, and poly-GR from the sense strand and poly-GP, poly-PA, and poly-PR from the antisense strand (6).



FIGURE 3: TDP-43-immunoreactive cytoplasmic inclusions, finely granular aggregates, and lack of nuclear labelling in a spinal cord motoneuron of a patient with ALS caused by *C9orf72* hexanucleotide repeat expansion.

possible disease mechanism [52, 53]. However, human *C9orf72* gene has only partial homology with its ortholog in *C. elegans* and zebrafish. Thus, loss-of-function mechanisms were assessed in *C9orf72* knockout mice as the mouse ortholog of *C9orf72* gene is more similar to the human *C9orf72*

gene [18]. Various knockout and knockdown models have been developed, yet none of the mouse models showed phenotypes characteristic for ALS or FTD [35, 40, 54–57], indicating that *C9orf72* loss-of-function is not the true cause of disease. The observation that patients homozygous for *C9orf72* repeat expansion do not have more severe symptoms of disease compared to heterozygotes further supports these findings [58, 59] (Table 2).

6.2. Toxic Gain-of-Function due to Aberrant HRE-Containing RNA

6.2.1. Higher-Order Structures of DNA and RNA Formed by *C9orf72* HRE Sequence.

Due to the uniformity of the G4C2 sequence and the abundance of guanine nucleotides, expanded hexanucleotide repeats in the *C9orf72* gene form higher-order DNA structures called G-quadruplexes [51, 60]. G-quadruplexes formed in the DNA can adopt both parallel- and antiparallel-stranded conformations, and increasing the length of the repeats creates a heterogeneous mixture of these structures. Both C-rich sense and antisense strands can assemble as i-motifs and hairpin structures [61]. G-quadruplex structures formed in the *C9orf72* HRE region

TABLE 1: Summary of studies on the mechanisms by which *C9orf72* repeat expansions cause neurodegeneration.

Reference	Molecular mechanism supported by the study			Experimental model
	Loss-of-function	Gain-of-function RNA-mediated gain-of-function	Gain-of-function Protein-mediated gain-of-function	
Renton et al. [15]		+		Human DNA Human brain tissue Cell culture
DeJesus-Hernandez et al. [14]	+	+		Human DNA Human brain tissue Cell culture
Gijssels et al. [16]	+			Human brain tissue Human DNA
FratTA et al. [58]		+	+	Human DNA
Therrien et al. [52]	+			<i>Caenorhabditis elegans</i>
Belzil et al. [135]	+			Human brain tissue Cell culture
Gendron et al. [67]		+	+	Human DNA Human brain tissue Cell lines
Zu et al. [93]		+	+	Human brain tissue Human RNA Cell culture
Mizielinska et al. [68]		+		Human brain tissue
Lee et al. [70]		+		Human brain tissue Cell culture Zebrafish embryos Rat brain
Sareen et al. [71]		+		iPSC-derived neurons
Xu et al. [72]		+		Cell culture <i>Drosophila</i>
Lagier-Tourenne et al. [54]		+		Human brain tissue Cell culture
Donnelly et al. [69]		+		Human brain tissue Cell culture iPSC-derived neurons
Ciura et al. [53]	+			Zebrafish Human brain tissue Cell culture
Wen et al. [94]		+	+	Cell culture <i>Drosophila</i>
Liu et al. [50]		+	+	Human autopsy tissue Human DNA Cell culture
May et al. [138]			+	Human brain samples Cell culture
Zhang et al. [102]			+	Human brain samples Cell culture
Cooper-Knock et al. [73]		+		Human brain samples Cell culture
Su et al. [134]		+	+	Cell culture
Kwon et al. [95]			+	Cell culture
Haesler et al. [51]		+		Human brain tissue iPSC-derived neurons Cell culture

TABLE 1: Continued.

Reference	Molecular mechanism supported by the study			Experimental model
	Loss-of-function	Gain-of-function RNA-mediated gain-of-function	Gain-of-function Protein-mediated gain-of-function	
Mizielinska et al. [87]		+	+	<i>Drosophila</i>
Russ et al. [139]		+	+	Human DNA
Prudencio et al. [113]		+	+	Human brain tissue
Chew et al. [140]		+	+	Mouse model expressing 66 G4C2 repeats
Freibaum et al. [32]	+		+	<i>Drosophila</i>
Yang et al. [101]			+	<i>Drosophila</i> Human brain samples Cell culture iPSC-derived neurons
Jovičić et al. [33]			+	Yeast cells Cell culture
Yamakawa et al. [99]			+	Cell culture Mice brains
Hu et al. [141]		+		Cell culture
Gendron et al. [110]			+	Human brain samples
Tao et al. [98]			+	Cell culture
Tran et al. [86]			+	<i>Drosophila</i> iPSC-derived neurons Human brain tissue
Koppers et al. [55]		+	+	Conditional C9orf72 knockout mouse model
Cooper-Knock et al. [83]		+		Human brain tissue
Zhang et al. [31]		+	+	<i>Drosophila</i> iPSC-derived neurons Human brain tissue
Rossi et al. [84]		+		Cell culture
Cooper-Knock et al. [116]		+		Cell culture
Schweizer Burguete et al. [85]		+		Cell culture <i>Drosophila</i> iPSC-derived neurons
Liu et al. [92]		+	+	BAC mouse model of C9FTD/ALS
Kanekura et al. [96]			+	Human brain samples Cell culture
Boeynaems et al. [142]			+	<i>Drosophila</i>
Chang et al. [103]			+	Cell culture
Flores et al. [143]			+	Cell culture
Lin et al. [126]			+	Cell culture
Lopez-Gonzalez et al. [144]			+	iPSC-derived neurons
Liu et al. [145]	+			Human DNA and RNA
Dodd et al. [146]		+		
Zhang et al. [147]			+	Mice that exhibit poly(GA) pathology
Westergard et al. [148]			+	iPSC-derived neurons Cell culture
Sellier et al. [36]	+			Cell culture Zebrafish
Mori et al. [149]		+	+	Human brain samples Cell culture

TABLE 1: Continued.

Reference	Molecular mechanism supported by the study			Experimental model
	Loss-of-function	Gain-of-function RNA-mediated gain-of-function	Gain-of-function Protein-mediated gain-of-function	
Gijssels et al. [19]	+			Human DNA
Ugolino et al. [150]	+			C9orf72 knockout mice C9orf72 knockdown cell models
Lee et al. [125]			+	<i>Drosophila</i> Cell culture
O'Rourke et al. [40]		+	+	C9orf72 +/- and C9orf72 -/- mice
Atanasio et al. [56]		+	+	C9orf72-deficient mouse
Jiang et al. [91]		+	+	C9orf72 +/- and C9orf72 -/- mice Mice expressing BAC with the human expanded C9orf72 gene
Webster et al. [34]	+			Cell culture iNeurons (obtained by differentiation of iNPCs)
Ji et al. [151]	+			C9orf72 knockout mouse model Cell culture
Ohki et al. [152]			+	Zebrafish
Liu et al. [153]		+		Cell culture
Hu et al. [154]		+		Cell culture
Green et al. [155]			+	Cell culture
Schludi et al. [156]			+	Transgenic mice expressing codon-modified (GA) ₁₄₉
Gupta et al. [157]			+	Cell culture
Khosravi et al. [158]			+	Cell culture
Shi et al. [159]			+	Cell culture
Saberi et al. [160]			+	Human brain samples
Kramer et al. [105]			+	Cell culture
Herranz-Martin et al. [161]		+	+	Mice that overexpress 10 or 102 interrupted G4C2 repeats
Zhou et al. [162]			+	Cell culture Human brain samples
Lehmer et al. [163]			+	Human CSF
Hautbergue et al. [82]		+	+	<i>Drosophila</i> Cell culture
Boeynaems et al. [100]			+	Cell culture
Maharjan et al. [39]	+	+	+	Cell culture
Moens et al. [88]		+	+	<i>Drosophila</i>
Cheng et al. [164]			+	Cell culture
Swinnen et al. [165]		+		Zebrafish
Shi et al. [59]	+		+	Cell culture iPSC-derived neurons Human brain samples
Simone et al. [137]		+	+	iPSC-derived neurons <i>Drosophila</i>
Tabet et al. [166]			+	Human brain sections Cell culture
Corrionero and Horvitz [167]	+			<i>Caenorhabditis elegans</i>

TABLE 1: Continued.

Reference	Molecular mechanism supported by the study		Experimental model
	Loss-of-function	Gain-of-function RNA-mediated gain-of-function Protein-mediated gain-of-function	
Zamiri et al. [168]		+	CD spectroscopy UV melting Gel electrophoresis
Swaminathan et al. [104]		+	Zebrafish
Yeh et al. [169]	+		Zebrafish
Meeter et al. [170]		+	Human brain samples
Nonaka et al. [171]		+	Cell culture
Frick et al. [29]	+		Human brain samples Cell culture <i>C9orf72</i> knockout mice

BAC: bacterial artificial chromosome; CSF: cerebrospinal fluid; iNPCs: induced neural progenitor cells; iPSC: induced pluripotent stem cells. Studies investigating pathological mechanisms of *C9orf72* HRE but not clearly supporting any of the three proposed disease mechanisms are not included in this table. Search for these studies was completed on May 20, 2018. The literature search was performed in Google Scholar using the keywords: “*C9orf72*”, “mechanism”, “pathological”, “loss-of-function”, “gain-of-function”, “haploinsufficiency”, “RNA foci”, and “DPR”. The literature search was conducted by two independent researchers.

TABLE 2: Evaluation of potential mechanisms underlying pathology in C9FTD/ALS.

Molecular mechanism underlying pathology in C9FTD/ALS	Pros	Cons
Loss-of-function	<i>C9orf72</i> loss-of-function models in <i>C. elegans</i> and zebrafish result in motor neuron degeneration Carriers of <i>C9orf72</i> HRE have decreased levels of <i>C9orf72</i> mRNA and proteins in the brain	<i>C9orf72</i> loss-of-function mouse models do not show phenotype characteristic for ALS and FTD Patients homozygous for <i>C9orf72</i> repeat expansion do not have more severe symptoms of disease
RNA-mediated gain-of-function	HRE-containing RNA transcripts accumulate and form nuclear aggregates, or RNA foci, in the brain of patients with mutated <i>C9orf72</i> Sequestration of RNA-binding proteins into RNA foci can disrupt RNA processing, translation, nucleocytoplasmic transport, and granule transport and lead to nucleolar stress Higher abundance of RNA foci in patients carrying C9FTD/ALS HRE is associated with earlier disease onset	<i>Drosophila</i> models of RNA toxic gain-of-function fail to produce neurodegeneration The results on RNA toxic gain-of-function mouse models are conflicting and need to be further investigated
Protein-mediated gain-of-function	<i>Drosophila</i> model of protein-mediated gain-of-function develops neurodegeneration DPR disrupt nucleocytoplasmic transport, RNA processing, translation, ubiquitin proteasome system, formation of stress granule, and Notch signalling pathway and can lead to nucleolar stress	Amounts of DPR in the brain do not correlate with clinical phenotype, severity of diseases, and neurodegeneration Abundance of DPR is low in the brain regions most affected by ALS and FTD

ALS: amyotrophic lateral sclerosis; C9FTD/ALS: hexanucleotide repeat expansion in *C9orf72* causing ALS and FTD; DPR proteins: dipeptide repeat proteins; FTD: frontotemporal dementia; HRE: hexanucleotide repeat expansion.

cause generation of truncated RNA transcripts that are aborted in the hexanucleotide repeat region [51]. These aberrant RNA transcripts containing repetitive hexanucleotide sequence can also form G-quadruplexes and RNA hairpin structures. Additionally, HRE-containing RNA can form hybrids with HRE-containing DNA called R-loops [51, 62–64]. Together, these higher-order structures of DNA and RNA are thought to act as promoters and

regulatory elements affecting replication, transcription, and translation of the surrounding region [65, 66], and to exert deleterious effect on cells by causing nucleolar stress and impeding RNA processing [51], as discussed below.

6.2.2. *Sequestration of RNA-Binding Proteins into Nuclear Aggregates by the C9orf72 HRE-RNA.* HRE-containing RNA transcripts accumulate and form nuclear aggregates, or

RNA foci, in the brain of patients with *C9orf72* HRE [14]. As the transcription of *C9orf72* HRE DNA region can also occur in the antisense direction, antisense RNA is also found within foci in the brains of patients with *C9orf72* HRE [67, 68]. Several proteins specifically bind to *C9orf72* HRE-containing RNA, including ADARB2, ALYREF, hnRNP H, hnRNP A1, nucleolin, Pur α , and SRSF2 [51, 69–73]. A number of RNA-binding proteins (RBP) are implicated in the HRE-induced neurodegeneration.

(1) *Nucleolin*. One of the main constituents of the nucleolus, nucleolin, has a high affinity for *C9orf72* RNA containing G-quadruplex structures of G4C2 repeat [51]. Nucleolin bound to RNA G-quadruplexes dislocates from the nucleoli and disperses through the nucleus. This results in impaired rRNA processing, followed by decreased maturation of ribosomes, and finally, accumulation of untranslated mRNA in the neuronal cytoplasm [51].

(2) *Pur α* . Another protein that has been identified as a HRE-RNA binding is Pur α [72]. Involvement of Pur α in the pathogenesis of ALS/FTD is supported by experiments showing that Pur α overexpression alleviates HRE-mediated toxicity. In other words, Pur α overexpression prevents cell death both in mammalian cells and in *Drosophila* [72]. Pur α is a component of the RNA-transport granules—particles that carry mRNAs to the nerve fibers where translation of those mRNAs into proteins occurs [74, 75]—and is involved in the regulation of the cell cycle [76] and in cell differentiation [77, 78]. Sequestration of Pur α by the *C9orf72* hexanucleotide RNA repeats could impair neuronal mRNA transport thus leading to neurodegeneration [79].

(3) *hnRNPs*. The largest group of proteins sequestered by the *C9orf72* HRE-containing RNA are heterogeneous nuclear ribonucleoproteins (hnRNPs) [44, 51]. It was shown that *C9orf72* interacts with hnRNP-U, hnRNP-F, hnRNP-K [51], hnRNP-A2/B1, and hnRNP-A1 [28], while hnRNP A1 and hnRNP H colocalize with RNA foci [70, 71]. HnRNP H is involved in the regulation of RNA processing [80]; thus, hnRNP H sequestration in RNA cause aberrant RNA processing and may enhance neurodegeneration [70].

(4) *ADARB2*. An RNA-editing enzyme ADARB2 also interacts with the HRE-containing RNAs and colocalizes with RNA foci in C9ALS cases [69]. Although the mechanism by which ADARB2 sequestration in RNA foci could contribute to HRE-mediated toxicity is unclear, it was proposed that this protein may be important for RNA foci formation because ADARB2 knockdown results in the reduction of neurons that contain RNA foci [69].

(5) *SRSF1 and SRSF2*. It was shown that serine-arginine-rich splicing factor 1 (SRSF1) and SRSF2 colocalize with RNA foci [70]. Given that SRSF2 is a marker of nuclear speckles, nuclear regions important for storage of splicing factors [81], its accumulation in RNA foci could disrupt the function of these speckles and lead to aberrant RNA processing [73]. Additionally, binding of the nuclear export

adaptor SRSF1 to *C9orf72* repeats promotes export of *C9orf72* repeats from the nucleus. As it was demonstrated that *SRSF1* knockdown in C9FTD/ALS *Drosophila* model blocks neurodegeneration, *SRSF1* could be a potential therapeutic target [82].

(6) *ALYREF*. Because aberrant nuclear transport is observed in different C9FTD/ALS models [31–33], the observation that ALYREF (Aly/REF export factor) also colocalizes with RNA foci in C9FTD/ALS cases [70, 73, 83] is of interest. However, although knockdown of nuclear export adaptor *SRSF1* blocks neurodegeneration in *Drosophila*, only a modest decrease in neurodegeneration was observed after knockdown of *ALYREF* [82].

In conclusion, toxic gain-of-function due to aberrant HRE-containing RNA can lead to dysfunctional RNA processing [73], aberrant translation [84], nucleolar stress [51], disrupted nucleocytoplasmic transport [31], and dysfunction in granule transport [85]. Additionally, it was observed that higher abundance of RNA foci in the frontal cortex of C9FTD patients is associated with earlier disease onset [68]. However, several experimental studies failed to detect neurodegeneration in *in vivo* C9FTD/ALS models displaying RNA foci (Table 2). Neurodegeneration was not observed in *Drosophila* models carrying 160 G4C2 repeats [86], 288 G4C2 repeats [87], and 1000 G4C2 repeats [88]. More precisely, *Drosophila* models carrying “RNA-only” repeats that formed RNA foci but not DPRs failed to produce neurodegeneration, while *Drosophila* models carrying “pure repeats” that could form both RNA foci and DPRs displayed neurodegeneration [87, 88]. However, two transgenic C9FTD/ALS mouse models with both RNA and protein toxic gain-of-function failed to produce neurodegeneration [89, 90], while two other transgenic mouse models showed signs of neurodegeneration [91, 92]. Regarding the latter two C9FTD/ALS mice models, whether neurodegeneration was caused by toxic HRE-containing RNAs or DPRs translated from hexanucleotide repeat RNA remains to be assessed [91, 92].

6.3. *Toxic Gain-of-Function through Accumulation of Dipeptide Repeat Proteins Translated from C9orf72 HRE-Containing RNA*. RNA transcripts of *C9orf72* HRE region can undergo repeat-associated non-ATG (RAN) translation in which different DPR proteins are synthesized [43, 44, 93]. Poly-Gly-Ala, poly-Gly-Pro, and poly-Gly-Arg are translated from different open reading fragments on the sense transcript and poly-Gly-Pro, poly-Pro-Arg, and poly-Pro-Ala from the antisense transcript. Toxicity of DPR proteins seems to be mainly dependent on arginine-containing DPR proteins, particularly poly-Pro-Arg [33, 87, 94, 95]. Arginine-rich DPRs appear to disrupt primarily nucleocytoplasmic transport [33] and RNA processing [33, 95], which can cause dysregulation of translation [96, 97], nucleolar stress [98], disturb ubiquitin proteasome system [99], affect the formation of stress granules [100], and influence the Notch signalling pathway [101]. Additionally, it was shown that poly-Gly-Ala DPR proteins can disturb the ubiquitin proteasome system and cause endoplasmic reticulum stress [102], and enhance the formation of toxic amyloid fibrils [103].

Although there is molecular evidence of DPR proteins' toxicity in *in vitro* and *in vivo* C9FTD/ALS models [104, 105], post mortem analyses of human brain revealed inconsistent results (Table 2). No correlation of DPR proteins with clinical phenotype, severity of disease, and neurodegeneration was observed, and the abundance of DPR proteins was low in the brain regions most affected in ALS and FTD [106–109]. This lack of correlation may signal that neurons carrying toxic DPR proteins are possibly dead at the time of autopsy [18]. Additionally, there were discrepancies in the outcome of the studies comparing the distribution of DPR proteins between ALS and FTD cases [107, 110, 111].

7. Cellular Processes Affected in C9FTD/ALS

Although many cellular processes are affected by *C9orf72* repeat expansion, including translation [96], ubiquitin proteasome system [99], Notch signalling pathway [101], granule transport [85], and normal function of TDP-43 (for review see [17]), here we discuss the cellular processes affected by *C9orf72* repeat expansion for which sufficient information is available.

7.1. Effects of DPR Proteins and HRE-Containing RNAs on Nucleocytoplasmic Transport. A hallmark morphological feature of TDP-43 proteinopathies is the cytoplasmic accumulation and aggregate formation of TDP-43. We have demonstrated earlier that impaired nucleocytoplasmic transport plays a major role in this process [112]. Importantly, *C9orf72* repeat expansion compromises nucleocytoplasmic transport of proteins and RNA through the nuclear pores. A study in yeast expressing arginine-rich DPR constructs demonstrated that one of the main targets of toxic DPR proteins is nucleocytoplasmic transport [33]. In support of these findings, genetic screens in *Drosophila* designed to identify genes linked to HRE-induced toxicity also identified components of the nuclear pore complex and nucleocytoplasmic transport machinery [31, 32]. Furthermore, RCC1, which is a human protein required for nucleocytoplasmic transport, is mislocalized in cells derived from patients with the *C9orf72* HRE, providing preliminary evidence that C9FTD/ALS HRE might affect nucleocytoplasmic transport in human cells as well [33]. Taken together, the studies in yeast and *Drosophila* provided the first evidence that *C9orf72* repeat expansion causes degeneration by impairing nucleocytoplasmic transport of proteins and RNA [31–33]; however, the effect of C9FTD/ALS HRE on nucleocytoplasmic transport in human cells needs to be further investigated. These findings open a possibility of targeting nucleocytoplasmic transport as a potential new therapeutic target in ALS and FTD [31, 32]. Additionally, as mRNA export factor ALYREF [70, 73, 83] and Ran GTPase-activating protein (RanGAP, regulator of nucleocytoplasmic transport) [31] colocalize with RNA foci (observed in C9FTD/ALS brains) and their function is disrupted, it seems that not only DPRs can lead to aberration of nucleocytoplasmic transport but also HRE-containing RNAs.

7.2. *C9orf72* Hexanucleotide Repeat Expansion and RNA Processing. Several studies reported different transcriptional profiles between C9FTD/ALS patients and controls [54, 69, 71, 113–115]. Additionally, Cooper-Knock et al. observed an enrichment in RNA splicing factors in C9FTD/ALS patients [116], further supporting the observation of aberrant RNA processing caused by pathological processes in C9FTD/ALS. The main mechanism that leads to disturbed RNA processing in C9FTD/ALS patients is through sequestration of RBP in RNA foci [14, 73], but it could be also caused by DPR proteins as they can bind to RBP too [33, 95].

7.3. HRE-Containing RNAs and Dipeptide Repeat Proteins Can Lead to Nucleolar Stress. The way by which nucleolin binding to RNA foci disrupts the normal function of the nucleolus [51] was discussed above. It was also observed that DPR proteins can cause nucleolar stress in cell lines [98]. Enlargement of the nucleolus was observed in *in vitro* [94, 98] and *in vivo* [101] C9FTD/ALS models expressing arginine-rich DPR proteins. Enlargement of the nucleolus in these models leads to its fragmentation and decreased maturation of rRNA [98].

7.4. The Effect of DPRs on Formation and Function of Membraneless Organelles. Cells possess several RNA and protein-containing membraneless organelles, collectively referred to as ribonucleoprotein (RNP) granules, which separate from the cytoplasm or nucleoplasm into a distinct liquid phase-like state that is typically slightly denser than the surrounding [117]. Examples of such organelles are nucleoli and Cajal bodies in the nucleus and processing bodies (P-bodies), stress granules, and transport granules in the cytoplasm [117–119]. Formation of such compartments is triggered by intrinsically disordered low complexity polypeptide sequences present within RBP, which have the ability to undergo phase transitions [120–122]. Furthermore, it has been shown that nuclear membraneless suborganelles, such as Cajal bodies and nuclear speckles, can be nucleated by several coding and noncoding RNAs through the recruitment of proteins residing in these nuclear bodies [123]. Stress induces the formation of many membraneless compartments [124]. Lee et al. reported that arginine-rich DPRs bind low complexity polypeptide sequences of RBP that are components of membraneless organelles. Arginine-rich DPRs alter phase separation of those proteins and, in that way, disturb the function of membraneless organelles [125]. Liquid-liquid phase separation of RBP is very important for the normal formation of membraneless organelles. Other authors also showed that arginine-rich DPRs disturb the function and dynamics of membraneless organelles [126], more precisely, stress granules [98, 100]. Because it has been proposed that stress granules could be seeding points where aggregation of pathological proteins in FTD and ALS begins [127, 128], understanding the influence of DPR proteins on dynamics of membraneless organelles is highly relevant in this context.

7.5. *C9orf72* HRE Affects Autophagy and Apoptosis. Adulterations in the autophagic pathway can alter protein

homeostasis, causing detrimental effects on neurons that have been implicated in neurodegenerative diseases like Parkinson's disease and tauopathies. Autophagy prevents proteotoxic cell death by removing damaged, misfolded, and unwanted proteins [129]. Hindrance of *C9orf72* function leads to a consequential decrease in Rab GTPase activity, a protein involved in membrane fusion, vesicle formation, and vesicle trafficking, which impairs the endocytosis process and autophagy. This causes an increased amount of p62/SQSTM1 and TDP-43, known markers of ALS-FTD [36]. Using *C9orf72*-deficient mice and cell lines, Sullivan et al. demonstrated that *C9orf72* forms a binding complex with SMCR8 and WDR41, which allow it to interact with FIP200/Ulk1/ATG13/ATG101 complex and initiate autophagy [35]. Additionally, *C9orf72* HRE have been implicated in increased endoplasmic reticulum stress by causing dysregulation of its calcium channels that leads to neuron apoptosis. This has been quantified by observing a decrease in antiapoptotic genes Bcl-2 and Bcl-X_L, while causing an increased expression of proapoptotic gene BAK [130].

7.6. *C9orf72* HRE and Neuroinflammation. Experimental studies investigating *C9orf72* loss-of-function in *C9orf72*-deficient mice revealed an increase in proinflammatory cytokines, lymphadenopathy, splenomegaly, alterations in myeloid cells from spleen, and in some cases of autoimmunity [40, 56, 57]. Additionally, a human autopsy study showed that C9ALS cases had more severe microglial pathology in the medulla and motor cortex than ALS cases without *C9orf72* HRE [131]. These findings raised questions about increased neuroinflammation in C9FTD/ALS patients and possible involvement of activated microglia in disease pathogenesis. It was proposed that microglia could represent a link between three potential pathological mechanisms of *C9orf72* HRE, whereby microglia is being most affected by loss of *C9orf72* function, while neurons are most affected by a gain-of-function mechanism. Altogether, increased neuroinflammation, accumulation of RNA foci, and DPRs could lead to neuronal death (reviewed in [132]).

8. Potential Therapeutic Approaches

Potential therapeutics most commonly target HRE-containing RNA, because its degradation abolishes RNA toxicity and the formation of DPR proteins by RAN translation. The following approaches have been considered so far.

8.1. Antisense Oligonucleotides. Antisense oligonucleotides (ASOs) targeting HRE-containing RNAs were tested in different studies [54, 69, 91]. After treatment of C9FTD/ALS cells in *in vitro* [54, 69, 71] and *in vivo* models with ASOs (mice expressing bacterial artificial chromosome (BAC) with the human expanded *C9orf72* gene [91] and adeno-associated virus (AAV) (G₄C₂)₆₆ mice [133]), a reduction in RNA foci was observed. Additionally, treatment with ASOs also led to a decrease in DPRs [91, 133]. Although ASOs used in these studies did not affect the levels of normal *C9orf72* protein [54, 71, 91], the possibility of total silencing *C9orf72* cannot be confirmed, so these approaches need to

be considered with caution. A clinical trial for ASO targeting HRE-containing RNAs (WVE-3972-01; <https://adisinight.springer.com/trials/700291284>) is expected in the fourth quarter of 2018.

8.2. Small Molecules. The advantage of using small molecules as therapeutics for C9FTD/ALS is that these compounds could not only target HRE-containing RNAs or DPR proteins but also cellular processes affected by pathology underlying C9FTD/ALS. Su et al. developed three small molecules that target hexanucleotide repeat region of RNAs and could stop RAN translation in (G₄C₂)₆₆-expressing COS7 cells [134]. As epigenetic alterations were observed in C9FTD/ALS cases, epigenetic changes in the *C9orf72* gene were also targeted. Usage of G-quadruplex-binding small molecules yielded increased expression of *C9orf72* protein [135–137].

9. The Mechanisms of *C9orf72* HRE-Mediated Neurodegeneration Are Not Mutually Exclusive

It is possible that the proposed mechanisms of C9FTD/ALS coexist and act in a combined manner. Maharjan et al. suggested that HRE in *C9orf72* gene affects normal expression of *C9orf72*, diminishes levels of *C9orf72* protein, and consequently impairs the formation of stress granules during the cellular stress (caused by formation of RNA foci and DPRs). In other words, *C9orf72* loss-of-function made cells more sensitive to toxicity caused by gain-of-function mechanisms [39]. Additionally, Lall and Baloh [132] proposed a model that unifies all three pathological mechanisms mentioned above. Other authors stressed the importance of generation of rodent experimental models in which all three mechanisms coexist, for example, by crossing *C9orf72* knockout mouse and mouse carrying BAC with human expanded *C9orf72* gene [18]. Although it was shown that ASO targeting HRE-containing RNAs do not affect the levels of normal *C9orf72* protein [54, 71, 91], the possibility of silencing *C9orf72* totally should not be overlooked. As ASO targeting HRE-containing RNAs moves into phase 1 of clinical trials, the development of novel cellular and animal experimental models that exhibit all three pathological mechanisms is highly relevant.

10. Conclusions

Seven years after the discovery of hexanucleotide repeat expansion in *C9orf72* gene, a lot of progress has been made in the clarification of molecular mechanisms through which *C9orf72* repeat expansions cause neurodegeneration. Three possible, not mutually exclusive, mechanisms could together contribute to the pathogenesis of disease [39]. The majority of the studies investigating the molecular mechanisms of pathological processes in C9FTD/ALS support either toxic HRE-RNA or DPR-dependent gain-of-function, with many studies supporting both mechanisms (Table 1). Hence, to better identify potential therapeutic targets, further studies are needed to fully understand molecular events underlying pathological processes in C9FTD/ALS.

Abbreviations

ALS:	Amyotrophic lateral sclerosis
ALS-bi:	ALS with behavioral impairment
ALS-ci:	ALS with cognitive impairment
ALS-cbi:	ALS with combined cognitive and behavioral impairment
ALYREF:	Aly/REF export factor
ASOs:	Antisense oligonucleotides
BAC:	Bacterial artificial chromosome
bvFTD:	Behavioral variant of frontotemporal dementia
C9orf72:	Chromosome 9 open reading frame 72
C9FTD/ALS:	Hexanucleotide repeat expansion in C9orf72 causing ALS and FTD
CSF:	Cerebrospinal fluid
DENN proteins:	Differentially expressed in normal and neoplasia proteins
DPR proteins:	Dipeptide repeat proteins
FTD:	Frontotemporal dementia
FTLD:	Frontotemporal lobar degeneration
FUS:	Fused in sarcoma
G4C2:	GGGGCC
GEF:	Guanine exchange factor
hnRNPs:	Heterogeneous nuclear ribonucleoproteins
HRE:	Hexanucleotide repeat expansion
IBMFD:	Inclusion body myopathy with frontotemporal dementia
iNPCs:	Induced neural progenitor cells
iPSC:	Induced pluripotent stem cells
MND:	Motor neuron disease
NCL:	Nucleolin
NPC:	Nuclear pore complex
P-bodies:	Processing bodies
PNFA:	Progressive nonfluent aphasia
pTDP-43:	Phosphorylated TDP-43
RAN translation:	Repeat-associated non-ATG translation
RanGAP:	Ran GTPase-activating protein
RBP:	RNA binding proteins
RNP:	Ribonucleoprotein
SD:	Semantic dementia
SRSF:	Serine-arginine-rich splicing factor
TDP-43:	TAR DNA-binding protein 43
VCP:	Valosin-containing protein.

Conflicts of Interest

The authors declare no conflict of interest.

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References

- [1] T. van Langenhove, J. van der Zee, and C. van Broeckhoven, "The molecular basis of the frontotemporal lobar degeneration–amyotrophic lateral sclerosis spectrum," *Annals of Medicine*, vol. 44, no. 8, pp. 817–828, 2012.
- [2] P. M. Worms, "The epidemiology of motor neuron diseases: a review of recent studies," *Journal of the Neurological Sciences*, vol. 191, no. 1-2, pp. 3–9, 2001.
- [3] M. J. Strong, S. Abrahams, L. H. Goldstein et al., "Amyotrophic lateral sclerosis - frontotemporal spectrum disorder (ALS-FTSD): revised diagnostic criteria," *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, vol. 18, no. 3-4, pp. 153–174, 2017.
- [4] E. Ratnavalli, C. Brayne, K. Dawson, and J. R. Hodges, "The prevalence of frontotemporal dementia," *Neurology*, vol. 58, no. 11, pp. 1615–1621, 2002.
- [5] A. S. L. Ng, R. Rademakers, and B. L. Miller, "Frontotemporal dementia: a bridge between dementia and neuromuscular disease," *Annals of the New York Academy of Sciences*, vol. 1338, no. 1, pp. 71–93, 2015.
- [6] T. Hortobágyi and N. Cairns, "Amyotrophic lateral sclerosis and frontotemporal dementia," in *Neuropathology of Neurodegenerative Diseases: A Practical Guide*, G. Kovacs, Ed., pp. 209–248, Cambridge University Press, 2015.
- [7] T. Hortobágyi and N. J. Cairns, "Chapter 26 - Amyotrophic lateral sclerosis and non-tau frontotemporal lobar degeneration," *Handbook of clinical neurology*, vol. 145, pp. 369–381, 2017.
- [8] I. R. A. Mackenzie, M. Neumann, A. Baborie et al., "A harmonized classification system for FTLD-TDP pathology," *Acta Neuropathologica*, vol. 122, no. 1, pp. 111–113, 2011.
- [9] T. W. Chow, B. L. Miller, V. N. Hayashi, and D. H. Geschwind, "Inheritance of frontotemporal dementia," *Archives of Neurology*, vol. 56, no. 7, pp. 817–822, 1999.
- [10] S. M. Rosso, L. D. Kaat, T. Baks et al., "Frontotemporal dementia in the Netherlands: patient characteristics and prevalence estimates from a population-based study," *Brain*, vol. 126, no. 9, pp. 2016–2022, 2003.
- [11] M. J. Strong, T. Hortobágyi, K. Okamoto, and S. Kato, "Amyotrophic lateral sclerosis, primary lateral sclerosis and spinal muscular atrophy," in *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders*, D. Dickson and R. Weller, Eds., pp. 418–433, Wiley-Blackwell, 2011.
- [12] C. Vance, A. al-Chalabi, D. Ruddy et al., "Familial amyotrophic lateral sclerosis with frontotemporal dementia is linked to a locus on chromosome 9p13.2–21.3," *Brain*, vol. 129, no. 4, pp. 868–876, 2006.
- [13] M. Morita, A. al-Chalabi, P. M. Andersen et al., "A locus on chromosome 9p confers susceptibility to ALS and frontotemporal dementia," *Neurology*, vol. 66, no. 6, pp. 839–844, 2006.
- [14] M. DeJesus-Hernandez, I. R. Mackenzie, B. F. Boeve et al., "Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS," *Neuron*, vol. 72, no. 2, pp. 245–256, 2011.
- [15] A. E. Renton, E. Majounie, A. Waite et al., "A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome

- 9p21-linked ALS-FTD,” *Neuron*, vol. 72, no. 2, pp. 257–268, 2011.
- [16] I. Gijselinck, T. van Langenhove, J. van der Zee et al., “A *C9orf72* promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study,” *The Lancet Neurology*, vol. 11, no. 1, pp. 54–65, 2012.
- [17] T. W. Todd and L. Petrucelli, “Insights into the pathogenic mechanisms of chromosome 9 open reading frame 72 (*C9orf72*) repeat expansions,” *Journal of Neurochemistry*, vol. 138, pp. 145–162, 2016.
- [18] X. Wen, T. Westergard, P. Pasinelli, and D. Trotti, “Pathogenic determinants and mechanisms of ALS/FTD linked to hexanucleotide repeat expansions in the *C9orf72* gene,” *Neuroscience Letters*, vol. 636, pp. 16–26, 2017.
- [19] I. Gijselinck, on behalf of the BELNEU CONSORTIUM13, S. Van Mossevelde et al., “The *C9orf72* repeat size correlates with onset age of disease, DNA methylation and transcriptional downregulation of the promoter,” *Molecular Psychiatry*, vol. 21, no. 8, pp. 1112–1124, 2016.
- [20] A. J. Waite, D. Bäumer, S. East et al., “Reduced *C9orf72* protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the *C9ORF72* hexanucleotide repeat expansion,” *Neurobiology of Aging*, vol. 35, no. 7, pp. 1779.e5–1779.e13, 2014.
- [21] O. Dols-Icardo, A. Garcia-Redondo, R. Rojas-Garcia et al., “Characterization of the repeat expansion size in *C9orf72* in amyotrophic lateral sclerosis and frontotemporal dementia,” *Human Molecular Genetics*, vol. 23, no. 3, pp. 749–754, 2014.
- [22] M. van Blitterswijk, M. DeJesus-Hernandez, E. Niemantsverdriet et al., “Association between repeat sizes and clinical and pathological characteristics in carriers of *C9ORF72* repeat expansions (Xpansize-72): a cross-sectional cohort study,” *The Lancet Neurology*, vol. 12, no. 10, pp. 978–988, 2013.
- [23] J. Beck, M. Poulter, D. Hensman et al., “Large *C9orf72* hexanucleotide repeat expansions are seen in multiple neurodegenerative syndromes and are more frequent than expected in the UK population,” *The American Journal of Human Genetics*, vol. 92, no. 3, pp. 345–353, 2013.
- [24] A. Hübers, N. Marroquin, B. Schmoll et al., “Polymerase chain reaction and Southern blot-based analysis of the *C9orf72* hexanucleotide repeat in different motor neuron diseases,” *Neurobiology of Aging*, vol. 35, no. 5, pp. 1214.e1–1214.e6, 2014.
- [25] A. Nordin, C. Akimoto, A. Wuolikainen et al., “Extensive size variability of the GGGGCC expansion in *C9orf72* in both neuronal and non-neuronal tissues in 18 patients with ALS or FTD,” *Human Molecular Genetics*, vol. 24, no. 11, pp. 3133–3142, 2015.
- [26] D. Zhang, L. M. Iyer, F. He, and L. Aravind, “Discovery of novel DENN proteins: implications for the evolution of eukaryotic intracellular membrane structures and human disease,” *Frontiers in Genetics*, vol. 3, p. 283, 2012.
- [27] T. P. Levine, R. D. Daniels, A. T. Gatta, L. H. Wong, and M. J. Hayes, “The product of *C9orf72*, a gene strongly implicated in neurodegeneration, is structurally related to DENN RabGEFs,” *Bioinformatics*, vol. 29, no. 4, pp. 499–503, 2013.
- [28] M. A. Farg, V. Sundaramoorthy, J. M. Sultana et al., “*C9ORF72*, implicated in amyotrophic lateral sclerosis and frontotemporal dementia, regulates endosomal trafficking,” *Human Molecular Genetics*, vol. 23, no. 13, pp. 3579–3595, 2014.
- [29] P. Frick, C. Sellier, I. R. A. Mackenzie et al., “Novel antibodies reveal presynaptic localization of *C9orf72* protein and reduced protein levels in *C9orf72* mutation carriers,” *Acta Neuropathologica Communications*, vol. 6, no. 1, p. 72, 2018.
- [30] S. Xiao, L. MacNair, P. McGoldrick et al., “Isoform-specific antibodies reveal distinct subcellular localizations of *C9orf72* in amyotrophic lateral sclerosis,” *Annals of Neurology*, vol. 78, no. 4, pp. 568–583, 2015.
- [31] K. Zhang, C. J. Donnelly, A. R. Haeusler et al., “The *C9orf72* repeat expansion disrupts nucleocytoplasmic transport,” *Nature*, vol. 525, no. 7567, pp. 56–61, 2015.
- [32] B. D. Freibaum, Y. Lu, R. Lopez-Gonzalez et al., “GGGGCC repeat expansion in *C9orf72* compromises nucleocytoplasmic transport,” *Nature*, vol. 525, no. 7567, pp. 129–133, 2015.
- [33] A. Jovičić, J. Mertens, S. Boeynaems et al., “Modifiers of *C9orf72* dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS,” *Nature Neuroscience*, vol. 18, no. 9, pp. 1226–1229, 2015.
- [34] C. P. Webster, E. F. Smith, C. S. Bauer et al., “The *C9orf72* protein interacts with Rab1a and the ULK1 complex to regulate initiation of autophagy,” *The EMBO Journal*, vol. 35, no. 15, pp. 1656–1676, 2016.
- [35] P. M. Sullivan, X. Zhou, A. M. Robins et al., “The ALS/FTLD associated protein *C9orf72* associates with SMCR8 and WDR41 to regulate the autophagy-lysosome pathway,” *Acta Neuropathologica Communications*, vol. 4, no. 1, p. 51, 2016.
- [36] C. Sellier, M. L. Campanari, C. Julie Corbier et al., “Loss of *C9ORF72* impairs autophagy and synergizes with polyQ ataxin-2 to induce motor neuron dysfunction and cell death,” *The EMBO Journal*, vol. 35, no. 12, pp. 1276–1297, 2016.
- [37] J. Amick, A. Roczniak-Ferguson, and S. M. Ferguson, “*C9orf72* binds SMCR8, localizes to lysosomes, and regulates mTORC1 signaling,” *Molecular Biology of the Cell*, vol. 27, no. 20, pp. 3040–3051, 2016.
- [38] J. Amick and S. M. Ferguson, “*C9orf72*: at the intersection of lysosome cell biology and neurodegenerative disease,” *Traffic*, vol. 18, no. 5, pp. 267–276, 2017.
- [39] N. Maharjan, C. Künzli, K. Buthey, and S. Saxena, “*C9ORF72* regulates stress granule formation and its deficiency impairs stress granule assembly, hypersensitizing cells to stress,” *Molecular Neurobiology*, vol. 54, no. 4, pp. 3062–3077, 2017.
- [40] J. G. O’Rourke, L. Bogdanik, A. Yanez et al., “*C9orf72* is required for proper macrophage and microglial function in mice,” *Science*, vol. 351, no. 6279, pp. 1324–1329, 2016.
- [41] S. Al-Sarraj, A. King, C. Troakes et al., “p62 positive, TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus define the pathology of *C9orf72*-linked FTL and MND/ALS,” *Acta Neuropathologica*, vol. 122, no. 6, pp. 691–702, 2011.
- [42] M. E. Murray, M. DeJesus-Hernandez, N. J. Rutherford et al., “Clinical and neuropathologic heterogeneity of c9FTD/ALS associated with hexanucleotide repeat expansion in *C9ORF72*,” *Acta Neuropathologica*, vol. 122, no. 6, pp. 673–690, 2011.
- [43] P. E. A. Ash, K. F. Bieniek, T. F. Gendron et al., “Unconventional translation of *C9ORF72* GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS,” *Neuron*, vol. 77, no. 4, pp. 639–646, 2013.

- [44] K. Mori, S. M. Weng, T. Arzberger et al., "The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTL/ALS," *Science*, vol. 339, no. 6125, pp. 1335–1338, 2013.
- [45] C. Troakes, S. Maekawa, L. Wijesekera et al., "An MND/ALS phenotype associated with C9orf72 repeat expansion: abundant p62-positive, TDP-43-negative inclusions in cerebral cortex, hippocampus and cerebellum but without associated cognitive decline," *Neuropathology*, vol. 32, no. 5, pp. 505–514, 2012.
- [46] M. van Blitterswijk, T. F. Gendron, M. C. Baker et al., "Novel clinical associations with specific C9ORF72 transcripts in patients with repeat expansions in C9ORF72," *Acta Neuropathologica*, vol. 130, no. 6, pp. 863–876, 2015.
- [47] Z. Xi, L. Zinman, D. Moreno et al., "Hypermethylation of the CpG island near the G4C2 repeat in ALS with a C9orf72 expansion," *American Journal of Human Genetics*, vol. 92, no. 6, pp. 981–989, 2013.
- [48] Z. Xi, M. Zhang, A. C. Bruni et al., "The C9orf72 repeat expansion itself is methylated in ALS and FTL/ALS patients," *Acta Neuropathologica*, vol. 129, no. 5, pp. 715–727, 2015.
- [49] Z. Xi, I. Rainero, E. Rubino et al., "Hypermethylation of the CpG-island near the C9orf72 G4C2-repeat expansion in FTL/ALS patients," *Human Molecular Genetics*, vol. 23, no. 21, pp. 5630–5637, 2014.
- [50] E. Y. Liu, J. Russ, K. Wu et al., "C9orf72 hypermethylation protects against repeat expansion-associated pathology in ALS/FTD," *Acta Neuropathologica*, vol. 128, no. 4, pp. 525–541, 2014.
- [51] A. R. Haeusler, C. J. Donnelly, G. Periz et al., "C9orf72 nucleotide repeat structures initiate molecular cascades of disease," *Nature*, vol. 507, no. 7491, pp. 195–200, 2014.
- [52] M. Therrien, G. A. Rouleau, P. A. Dion, and J. A. Parker, "Deletion of C9ORF72 results in motor neuron degeneration and stress sensitivity in *C. elegans*," *PLoS One*, vol. 8, no. 12, article e83450, 2013.
- [53] S. Ciura, S. Lattante, I. le Ber et al., "Loss of function of C9orf72 causes motor deficits in a zebrafish model of amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 74, no. 2, pp. 180–187, 2013.
- [54] C. Lagier-Tourenne, M. Baughn, F. Rigo et al., "Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 47, pp. E4530–E4539, 2013.
- [55] M. Koppers, A. M. Blokhuis, H. J. Westeneng et al., "C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits," *Annals of Neurology*, vol. 78, no. 3, pp. 426–438, 2015.
- [56] A. Atanasio, V. Decman, D. White et al., "C9orf72 ablation causes immune dysregulation characterized by leukocyte expansion, autoantibody production and glomerulonephropathy in mice," *Scientific Reports*, vol. 6, no. 1, p. 23204, 2016.
- [57] E. Sudria-Lopez, M. Koppers, M. de Wit et al., "Full ablation of C9orf72 in mice causes immune system-related pathology and neoplastic events but no motor neuron defects," *Acta Neuropathologica*, vol. 132, no. 1, pp. 145–147, 2016.
- [58] P. Fratta, M. Poulter, T. Lashley et al., "Homozygosity for the C9orf72 GGGGCC repeat expansion in frontotemporal dementia," *Acta Neuropathologica*, vol. 126, no. 3, pp. 401–409, 2013.
- [59] Y. Shi, S. Lin, K. A. Staats et al., "Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons," *Nature Medicine*, vol. 24, no. 3, pp. 313–325, 2018.
- [60] P. Šket, J. Pohleven, A. Kovanda et al., "Characterization of DNA G-quadruplex species forming from C9ORF72 G4C2-expanded repeats associated with amyotrophic lateral sclerosis and frontotemporal lobar degeneration," *Neurobiology of Aging*, vol. 36, no. 2, pp. 1091–1096, 2015.
- [61] A. Kovanda, M. Zalar, P. Šket, J. Plavec, and B. Rogelj, "Anti-sense DNA d(GGCCCC)_n expansions in C9ORF72 form i-motifs and protonated hairpins," *Scientific Reports*, vol. 5, no. 1, article 17944, 2015.
- [62] P. Fratta, S. Mizielinska, A. J. Nicoll et al., "C9orf72 hexanucleotide repeat associated with amyotrophic lateral sclerosis and frontotemporal dementia forms RNA G-quadruplexes," *Scientific Reports*, vol. 2, no. 1, p. 1016, 2012.
- [63] K. Reddy, B. Zamiri, S. Y. R. Stanley, R. B. Macgregor Jr., and C. E. Pearson, "The disease-associated r(GGGGCC)_n repeat from the C9orf72 gene forms tract length-dependent uni- and multimolecular RNA G-quadruplex structures," *Journal of Biological Chemistry*, vol. 288, no. 14, pp. 9860–9866, 2013.
- [64] K. Reddy, M. H. M. Schmidt, J. M. Geist et al., "Processing of double-R-loops in (CAG)_n(CTG)_n and C9orf72 (GGGGCC)_n(GGCCCC)_n repeats causes instability," *Nucleic Acids Research*, vol. 42, no. 16, pp. 10473–10487, 2014.
- [65] S. Kendrick and L. H. Hurley, "The role of G-quadruplex/i-motif secondary structures as cis-acting regulatory elements," *Pure and Applied Chemistry*, vol. 82, no. 8, pp. 1609–1621, 2010.
- [66] T. A. Brooks, S. Kendrick, and L. Hurley, "Making sense of G-quadruplex and i-motif functions in oncogene promoters," *The FEBS Journal*, vol. 277, no. 17, pp. 3459–3469, 2010.
- [67] T. F. Gendron, K. F. Bieniek, Y. J. Zhang et al., "Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS," *Acta Neuropathologica*, vol. 126, no. 6, pp. 829–844, 2013.
- [68] S. Mizielinska, T. Lashley, F. E. Norona et al., "C9orf72 frontotemporal lobar degeneration is characterised by frequent neuronal sense and antisense RNA foci," *Acta Neuropathologica*, vol. 126, no. 6, pp. 845–857, 2013.
- [69] C. J. Donnelly, P. W. Zhang, J. T. Pham et al., "RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention," *Neuron*, vol. 80, no. 2, pp. 415–428, 2013.
- [70] Y.-B. Lee, H. J. Chen, J. N. Peres et al., "Hexanucleotide repeats in ALS/FTD form length-dependent RNA foci, sequester RNA binding proteins, and are neurotoxic," *Cell Reports*, vol. 5, no. 5, pp. 1178–1186, 2013.
- [71] D. Sareen, J. G. O'Rourke, P. Meera et al., "Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion," *Science Translational Medicine*, vol. 5, no. 208, article 208ra149, 2013.
- [72] Z. Xu, M. Poidevin, X. Li et al., "Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration," *Proceedings*

- of the National Academy of Sciences of the United States of America, vol. 110, no. 19, pp. 7778–7783, 2013.
- [73] J. Cooper-Knock, M. J. Walsh, A. Higginbottom et al., “Sequestration of multiple RNA recognition motif-containing proteins by *C9orf72* repeat expansions,” *Brain*, vol. 137, no. 7, pp. 2040–2051, 2014.
- [74] M. A. Kiebler and G. J. Bassell, “Neuronal RNA granules: movers and makers,” *Neuron*, vol. 51, no. 6, pp. 685–690, 2006.
- [75] Y. Kanai, N. Dohmae, and N. Hirokawa, “Kinesin transports RNA,” *Neuron*, vol. 43, no. 4, pp. 513–525, 2004.
- [76] M. K. White, E. M. Johnson, and K. Khalili, “Multiple roles for Puralpha in cellular and viral regulation,” *Cell Cycle*, vol. 8, no. 3, pp. 1–7, 2009.
- [77] K. Khalili, L. del Valle, V. Muralidharan et al., “Puralpha is essential for postnatal brain development and developmentally coupled cellular proliferation as revealed by genetic inactivation in the mouse,” *Molecular and Cellular Biology*, vol. 23, no. 19, pp. 6857–6875, 2003.
- [78] M. Mishra, L. Del Valle, J. Otte, N. Darbinian, and J. Gordon, “Pur-alpha regulates RhoA developmental expression and downstream signaling,” *Journal of Cellular Physiology*, vol. 228, no. 1, pp. 65–72, 2013.
- [79] B. Wolozin and D. Apicco, “RNA binding proteins and the genesis of neurodegenerative diseases,” *Advances in Experimental Medicine and Biology*, vol. 822, pp. 11–15, 2015.
- [80] A. Chaudhury, P. Chander, and P. H. Howe, “Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: focus on hnRNP E1’s multifunctional regulatory roles,” *RNA*, vol. 16, no. 8, pp. 1449–1462, 2010.
- [81] D. L. Spector and A. I. Lamond, “Nuclear speckles,” *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 2, article a000646, 2011.
- [82] G. M. Hautbergue, L. M. Castelli, L. Ferraiuolo et al., “SRSF1-dependent nuclear export inhibition of *C9ORF72* repeat transcripts prevents neurodegeneration and associated motor deficits,” *Nature Communications*, vol. 8, article 16063, 2017.
- [83] J. Cooper-Knock, A. Higginbottom, M. J. Stopford et al., “Antisense RNA foci in the motor neurons of *C9ORF72*-ALS patients are associated with TDP-43 proteinopathy,” *Acta Neuropathologica*, vol. 130, no. 1, pp. 63–75, 2015.
- [84] S. Rossi, A. Serrano, V. Gerbino et al., “Nuclear accumulation of mRNAs underlies G4C2-repeat-induced translational repression in a cellular model of *C9orf72* ALS,” *Journal of Cell Science*, vol. 128, no. 9, pp. 1787–1799, 2015.
- [85] A. S. Burguete, S. Almeida, F.-B. Gao, R. Kalb, M. R. Akins, and N. M. Bonini, “GGGGCC microsatellite RNA is neuritically localized, induces branching defects, and perturbs transport granule function,” *eLife*, vol. 4, article e08881, 2015.
- [86] H. Tran, S. Almeida, J. Moore et al., “Differential toxicity of nuclear RNA foci versus dipeptide repeat proteins in a *Drosophila* model of *C9ORF72* FTD/ALS,” *Neuron*, vol. 87, no. 6, pp. 1207–1214, 2015.
- [87] S. Mizielinska, S. Gronke, T. Niccoli et al., “*C9orf72* repeat expansions cause neurodegeneration in *Drosophila* through arginine-rich proteins,” *Science*, vol. 345, no. 6201, pp. 1192–1194, 2014.
- [88] T. G. Moens, S. Mizielinska, T. Niccoli et al., “Sense and antisense RNA are not toxic in *Drosophila* models of *C9orf72*-associated ALS/FTD,” *Acta Neuropathologica*, vol. 135, no. 3, pp. 445–457, 2018.
- [89] J. G. O’Rourke, L. Bogdanik, A. K. M. G. Muhammad et al., “*C9orf72* BAC transgenic mice display typical pathologic features of ALS/FTD,” *Neuron*, vol. 88, no. 5, pp. 892–901, 2015.
- [90] O. M. Peters, G. T. Cabrera, H. Tran et al., “Human *C9ORF72* hexanucleotide expansion reproduces RNA foci and dipeptide repeat proteins but not neurodegeneration in BAC transgenic mice,” *Neuron*, vol. 88, no. 5, pp. 902–909, 2015.
- [91] J. Jiang, Q. Zhu, T. F. Gendron et al., “Gain of toxicity from ALS/FTD-linked repeat expansions in *C9ORF72* is alleviated by antisense oligonucleotides targeting GGGGCC-containing RNAs,” *Neuron*, vol. 90, no. 3, pp. 535–550, 2016.
- [92] Y. Liu, A. Pattamatta, T. Zu et al., “*C9orf72* BAC mouse model with motor deficits and neurodegenerative features of ALS/FTD,” *Neuron*, vol. 90, no. 3, pp. 521–534, 2016.
- [93] T. Zu, Y. Liu, M. Banez-Coronel et al., “RAN proteins and RNA foci from antisense transcripts in *C9ORF72* ALS and frontotemporal dementia,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. E4968–E4977, 2013.
- [94] X. Wen, W. Tan, T. Westergard et al., “Antisense proline-arginine RAN dipeptides linked to *C9ORF72*-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death,” *Neuron*, vol. 84, no. 6, pp. 1213–1225, 2014.
- [95] I. Kwon, S. Xiang, M. Kato et al., “Poly-dipeptides encoded by the *C9orf72* repeats bind nucleoli, impede RNA biogenesis, and kill cells,” *Science*, vol. 345, no. 6201, pp. 1139–1145, 2014.
- [96] K. Kanekura, T. Yagi, A. J. Cammack et al., “Poly-dipeptides encoded by the *C9ORF72* repeats block global protein translation,” *Human Molecular Genetics*, vol. 25, no. 9, pp. 1803–1813, 2016.
- [97] H. Hartmann, D. Hornburg, M. Czuppa et al., “Proteomics and *C9orf72* neuropathology identify ribosomes as poly-GR/PR interactors driving toxicity,” *Life Science Alliance*, vol. 1, no. 2, article e201800070, 2018.
- [98] Z. Tao, H. Wang, Q. Xia et al., “Nucleolar stress and impaired stress granule formation contribute to *C9orf72* RAN translation-induced cytotoxicity,” *Human Molecular Genetics*, vol. 24, no. 9, pp. 2426–2441, 2015.
- [99] M. Yamakawa, D. Ito, T. Honda et al., “Characterization of the dipeptide repeat protein in the molecular pathogenesis of c9FTD/ALS,” *Human Molecular Genetics*, vol. 24, no. 6, pp. 1630–1645, 2015.
- [100] S. Boeynaems, E. Bogaert, D. Kovacs et al., “Phase separation of *C9orf72* dipeptide repeats perturbs stress granule dynamics,” *Molecular Cell*, vol. 65, no. 6, pp. 1044–1055.e5, 2017.
- [101] D. Yang, A. Abdallah, Z. Li, Y. Lu, S. Almeida, and F.-B. Gao, “FTD/ALS-associated poly(GR) protein impairs the notch pathway and is recruited by poly(GA) into cytoplasmic inclusions,” *Acta Neuropathologica*, vol. 130, no. 4, pp. 525–535, 2015.
- [102] Y.-J. Zhang, K. Jansen-West, Y. F. Xu et al., “Aggregation-prone c9FTD/ALS poly(GA) RAN-translated proteins cause neurotoxicity by inducing ER stress,” *Acta Neuropathologica*, vol. 128, no. 4, pp. 505–524, 2014.
- [103] Y.-J. Chang, U.-S. Jeng, Y.-L. Chiang, I.-S. Hwang, and Y.-R. Chen, “The glycine-alanine dipeptide repeat from *C9orf72* Hexanucleotide expansions forms toxic amyloids possessing cell-to-cell transmission properties,” *Journal of Biological Chemistry*, vol. 291, no. 10, pp. 4903–4911, 2016.

- [104] A. Swaminathan, M. Bouffard, M. Liao et al., "Expression of C9orf72-related dipeptides impairs motor function in a vertebrate model," *Human Molecular Genetics*, vol. 27, no. 10, pp. 1754–1762, 2018.
- [105] N. J. Kramer, M. S. Haney, D. W. Morgens et al., "CRISPR-Cas9 screens in human cells and primary neurons identify modifiers of C9ORF72 dipeptide-repeat-protein toxicity," *Nature Genetics*, vol. 50, no. 4, pp. 603–612, 2018.
- [106] I. R. Mackenzie, T. Arzberger, E. Kremmer et al., "Dipeptide repeat protein pathology in C9ORF72 mutation cases: clinico-pathological correlations," *Acta Neuropathologica*, vol. 126, no. 6, pp. 859–879, 2013.
- [107] Y. S. Davidson, H. Barker, A. C. Robinson et al., "Brain distribution of dipeptide repeat proteins in frontotemporal lobar degeneration and motor neuron disease associated with expansions in C9ORF72," *Acta Neuropathologica Communications*, vol. 2, no. 1, p. 70, 2014.
- [108] I. R. A. Mackenzie, P. Frick, F. A. Grässer et al., "Quantitative analysis and clinico-pathological correlations of different dipeptide repeat protein pathologies in C9ORF72 mutation carriers," *Acta Neuropathologica*, vol. 130, no. 6, pp. 845–861, 2015.
- [109] J. Gomez-Deza, Y. B. Lee, C. Troakes et al., "Dipeptide repeat protein inclusions are rare in the spinal cord and almost absent from motor neurons in C9ORF72 mutant amyotrophic lateral sclerosis and are unlikely to cause their degeneration," *Acta Neuropathologica Communications*, vol. 3, no. 1, p. 38, 2015.
- [110] T. F. Gendron, M. van Blitterswijk, K. F. Bieniek et al., "Cerebellar c9RAN proteins associate with clinical and neuropathological characteristics of C9ORF72 repeat expansion carriers," *Acta Neuropathologica*, vol. 130, no. 4, pp. 559–573, 2015.
- [111] German Consortium for Frontotemporal Lobar Degeneration, Bavarian Brain Banking Alliance, M. H. Schludi et al., "Distribution of dipeptide repeat proteins in cellular models and C9orf72 mutation cases suggests link to transcriptional silencing," *Acta Neuropathologica*, vol. 130, no. 4, pp. 537–555, 2015.
- [112] A. L. Nishimura, V. Župunski, C. Troakes et al., "Nuclear import impairment causes cytoplasmic trans-activation response DNA-binding protein accumulation and is associated with frontotemporal lobar degeneration," *Brain*, vol. 133, no. 6, pp. 1763–1771, 2010.
- [113] M. Prudencio, V. V. Belzil, R. Batra et al., "Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS," *Nature Neuroscience*, vol. 18, no. 8, pp. 1175–1182, 2015.
- [114] H. V. Barker, M. Niblock, Y. B. Lee, C. E. Shaw, and J. M. Gallo, "RNA misprocessing in C9orf72-linked neurodegeneration," *Frontiers in Cellular Neuroscience*, vol. 11, p. 195, 2017.
- [115] V. Kumar, G. M. Hasan, and M. I. Hassan, "Unraveling the role of RNA mediated toxicity of C9orf72 repeats in C9-FTD/ALS," *Frontiers in Neuroscience*, vol. 11, p. 711, 2017.
- [116] J. Cooper-Knock, J. J. Bury, P. R. Heath et al., "C9ORF72 GGGGCC expanded repeats produce splicing dysregulation which correlates with disease severity in amyotrophic lateral sclerosis," *PLoS One*, vol. 10, no. 5, article e0127376, 2015.
- [117] C. P. Brangwynne, "Phase transitions and size scaling of membrane-less organelles," *The Journal of Cell Biology*, vol. 203, no. 6, pp. 875–881, 2013.
- [118] J. Berry, S. C. Weber, N. Vaidya, M. Haataja, and C. P. Brangwynne, "RNA transcription modulates phase transition-driven nuclear body assembly," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 38, pp. E5237–E5245, 2015.
- [119] A. Aguilera-Gomez and C. Rabouille, "Membrane-bound organelles versus membrane-less compartments and their control of anabolic pathways in Drosophila," *Developmental Biology*, vol. 428, no. 2, pp. 310–317, 2017.
- [120] T. W. Han, M. Kato, S. Xie et al., "Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies," *Cell*, vol. 149, no. 4, pp. 768–779, 2012.
- [121] M. Kato, T. W. Han, S. Xie et al., "Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels," *Cell*, vol. 149, no. 4, pp. 753–767, 2012.
- [122] Y. Lin, D. S. W. Protter, M. K. Rosen, and R. Parker, "Formation and maturation of phase-separated liquid droplets by RNA-binding proteins," *Molecular Cell*, vol. 60, no. 2, pp. 208–219, 2015.
- [123] S. P. Shevtsov and M. Dundr, "Nucleation of nuclear bodies by RNA," *Nature Cell Biology*, vol. 13, no. 2, pp. 167–173, 2011.
- [124] C. Rabouille and S. Alberti, "Cell adaptation upon stress: the emerging role of membrane-less compartments," *Current Opinion in Cell Biology*, vol. 47, pp. 34–42, 2017.
- [125] K.-H. Lee, P. Zhang, H. J. Kim et al., "C9orf72 dipeptide repeats impair the assembly, dynamics, and function of membrane-less organelles," *Cell*, vol. 167, no. 3, pp. 774–788.e17, 2016.
- [126] Y. Lin, E. Mori, M. Kato et al., "Toxic PR poly-dipeptides encoded by the C9orf72 repeat expansion target LC domain polymers," *Cell*, vol. 167, no. 3, pp. 789–802.e12, 2016.
- [127] O. D. King, A. D. Gitler, and J. Shorter, "The tip of the iceberg: RNA-binding proteins with prion-like domains in neurodegenerative disease," *Brain Research*, vol. 1462, pp. 61–80, 2012.
- [128] M. Ramaswami, J. P. Taylor, and R. Parker, "Altered ribostasis: RNA-protein granules in degenerative disorders," *Cell*, vol. 154, no. 4, pp. 727–736, 2013.
- [129] B. Loos, D. J. Klionsky, and E. Wong, "Augmenting brain metabolism to increase macro- and chaperone-mediated autophagy for decreasing neuronal proteotoxicity and aging," *Progress in Neurobiology*, vol. 156, pp. 90–106, 2017.
- [130] R. Dafinca, J. Scaber, N. Ababneh et al., "C9orf72 hexanucleotide expansions are associated with altered endoplasmic reticulum calcium homeostasis and stress granule formation in induced pluripotent stem cell-derived neurons from patients with amyotrophic lateral sclerosis and frontotemporal dementia," *Stem Cells*, vol. 34, no. 8, pp. 2063–2078, 2016.
- [131] J. Brettschneider, J. B. Toledo, V. M. van Deerlin et al., "Microglial activation correlates with disease progression and upper motor neuron clinical symptoms in amyotrophic lateral sclerosis," *PLoS One*, vol. 7, no. 6, article e39216, 2012.
- [132] D. Lall and R. H. Baloh, "Microglia and C9orf72 in neuroinflammation and ALS and frontotemporal dementia," *The Journal of Clinical Investigation*, vol. 127, no. 9, pp. 3250–3258, 2017.
- [133] T. F. Gendron, J. Chew, J. N. Stankowski et al., "Poly(GP) proteins are a useful pharmacodynamic marker for

- C9ORF72-associated amyotrophic lateral sclerosis,” *Science Translational Medicine*, vol. 9, no. 383, article eaai7866, 2017.
- [134] Z. Su, Y. Zhang, T. F. Gendron et al., “Discovery of a biomarker and lead small molecules to target r(GGGGCC)-associated defects in c9FTD/ALS,” *Neuron*, vol. 83, no. 5, pp. 1043–1050, 2014.
- [135] V. V. Belzil, P. O. Bauer, M. Prudencio et al., “Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic event detectable in blood,” *Acta Neuropathologica*, vol. 126, no. 6, pp. 895–905, 2013.
- [136] Z. Zeier, R. Esanov, K. C. Belle et al., “Bromodomain inhibitors regulate the C9ORF72 locus in ALS,” *Experimental Neurology*, vol. 271, pp. 241–250, 2015.
- [137] R. Simone, R. Balendra, T. G. Moens et al., “G-quadruplex-binding small molecules ameliorate C9orf72 FTD/ALS pathology *in vitro* and *in vivo*,” *EMBO Molecular Medicine*, vol. 10, no. 1, pp. 22–31, 2018.
- [138] S. May, D. Hornburg, M. H. Schludi et al., “C9orf72 FTL/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal toxicity and Unc119 sequestration,” *Acta Neuropathologica*, vol. 128, no. 4, pp. 485–503, 2014.
- [139] J. Russ, E. Y. Liu, K. Wu et al., “Hypermethylation of repeat expanded C9orf72 is a clinical and molecular disease modifier,” *Acta Neuropathologica*, vol. 129, no. 1, pp. 39–52, 2015.
- [140] J. Chew, T. F. Gendron, M. Prudencio et al., “Neurodegeneration. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits,” *Science*, vol. 348, no. 6239, pp. 1151–1154, 2015.
- [141] J. Hu, J. Liu, L. Li, K. T. Gagnon, and D. R. Corey, “Engineering duplex RNAs for challenging targets: recognition of GGGGCC/CCCCGG repeats at the ALS/FTD C9orf72 locus,” *Chemistry & Biology*, vol. 22, no. 11, pp. 1505–1511, 2015.
- [142] S. Boeynaems, E. Bogaert, E. Michiels et al., “Drosophila screen connects nuclear transport genes to DPR pathology in c9ALS/FTD,” *Scientific Reports*, vol. 6, no. 1, p. 20877, 2016.
- [143] B. N. Flores, M. E. Dulchavsky, A. Krans et al., “Distinct C9orf72-associated dipeptide repeat structures correlate with neuronal toxicity,” *PLoS One*, vol. 11, no. 10, article e0165084, 2016.
- [144] R. Lopez-Gonzalez, Y. Lu, T. F. Gendron et al., “Poly(GR) in C9ORF72-related ALS/FTD compromises mitochondrial function and increases oxidative stress and DNA damage in iPSC-derived motor neurons,” *Neuron*, vol. 92, no. 2, pp. 383–391, 2016.
- [145] F. Liu, Q. Liu, C. X. Lu et al., “Identification of a novel loss-of-function C9orf72 splice site mutation in a patient with amyotrophic lateral sclerosis,” *Neurobiology of Aging*, vol. 47, pp. 219.e1–219.e5, 2016.
- [146] D. W. Dodd, D. R. Tomchick, D. R. Corey, and K. T. Gagnon, “Pathogenic C9ORF72 antisense repeat RNA forms a double helix with tandem C:C mismatches,” *Biochemistry*, vol. 55, no. 9, pp. 1283–1286, 2016.
- [147] Y.-J. Zhang, T. F. Gendron, J. C. Grima et al., “C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins,” *Nature Neuroscience*, vol. 19, no. 5, pp. 668–677, 2016.
- [148] T. Westergard, B. K. Jensen, X. Wen et al., “Cell-to-cell transmission of dipeptide repeat proteins linked to C9orf72-ALS/FTD,” *Cell Reports*, vol. 17, no. 3, pp. 645–652, 2016.
- [149] K. Mori, Y. Nihei, T. Arzberger et al., “Reduced hnRNPA3 increases C9orf72 repeat RNA levels and dipeptide-repeat protein deposition,” *EMBO Reports*, vol. 17, no. 9, pp. 1314–1325, 2016.
- [150] J. Ugolino, Y. J. Ji, K. Conchina et al., “Loss of C9orf72 enhances autophagic activity via deregulated mTOR and TFEB signaling,” *PLoS Genetics*, vol. 12, no. 11, article e1006443, 2016.
- [151] Y. J. Ji, J. Ugolino, N. R. Brady, A. Hamacher-Brady, and J. Wang, “Systemic deregulation of autophagy upon loss of ALS- and FTD-linked C9orf72,” *Autophagy*, vol. 13, no. 7, pp. 1254–1255, 2017.
- [152] Y. Ohki, A. Wenninger-Weinzierl, A. Hruscha et al., “Glycine-alanine dipeptide repeat protein contributes to toxicity in a zebrafish model of C9orf72 associated neurodegeneration,” *Molecular Neurodegeneration*, vol. 12, no. 1, p. 6, 2017.
- [153] J. Liu, J. Hu, A. T. Ludlow et al., “C9orf72 disease-related foci are each composed of one mutant expanded repeat RNA,” *Cell Chemical Biology*, vol. 24, no. 2, pp. 141–148, 2017.
- [154] J. Hu, F. Rigo, T. P. Prakash, and D. R. Corey, “Recognition of c9orf72 mutant RNA by single-stranded silencing RNAs,” *Nucleic Acid Therapeutics*, vol. 27, no. 2, pp. 87–94, 2017.
- [155] K. M. Green, M. R. Glineburg, M. G. Kearse et al., “RAN translation at C9orf72-associated repeat expansions is selectively enhanced by the integrated stress response,” *Nature Communications*, vol. 8, no. 1, p. 2005, 2017.
- [156] M. H. Schludi, L. Becker, L. Garrett et al., “Spinal poly-GA inclusions in a C9orf72 mouse model trigger motor deficits and inflammation without neuron loss,” *Acta Neuropathologica*, vol. 134, no. 2, pp. 241–254, 2017.
- [157] R. Gupta, M. Lan, J. Mojsilovic-Petrovic et al., “The proline/arginine dipeptide from hexanucleotide repeat expanded C9ORF72 inhibits the proteasome,” *eNeuro*, vol. 4, no. 1, pp. ENEURO.0249–ENEU16.2017, 2017.
- [158] B. Khosravi, H. Hartmann, S. May et al., “Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in C9orf72 ALS/FTLD,” *Human Molecular Genetics*, vol. 26, no. 4, pp. 790–800, 2017.
- [159] K. Y. Shi, E. Mori, Z. F. Nizami et al., “Toxic PR_n polypeptides encoded by the C9orf72 repeat expansion block nuclear import and export,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 7, pp. E1111–E1117, 2017.
- [160] S. Saberi, J. E. Stauffer, J. Jiang et al., “Sense-encoded poly-GR dipeptide repeat proteins correlate to neurodegeneration and uniquely co-localize with TDP-43 in dendrites of repeat-expanded C9orf72 amyotrophic lateral sclerosis,” *Acta Neuropathologica*, vol. 135, no. 3, pp. 459–474, 2018.
- [161] S. Herranz-Martin, J. Chandran, K. Lewis et al., “Viral delivery of C9orf72 hexanucleotide repeat expansions in mice leads to repeat-length-dependent neuropathology and behavioural deficits,” *Disease Models & Mechanisms*, vol. 10, no. 7, pp. 859–868, 2017.
- [162] Q. Zhou, C. Lehmer, M. Michaelsen et al., “Antibodies inhibit transmission and aggregation of C9orf72 poly-GA dipeptide repeat proteins,” *EMBO Molecular Medicine*, vol. 9, no. 5, pp. 687–702, 2017.
- [163] C. Lehmer, P. Oeckl, J. H. Weishaupt et al., “Poly-GP in cerebrospinal fluid links C9orf72-associated dipeptide repeat expression to the asymptomatic phase of ALS/FTD,” *EMBO Molecular Medicine*, vol. 9, no. 7, pp. 859–868, 2017.

- [164] W. Cheng, S. Wang, A. A. Mestre et al., "C9ORF72 GGGGCC repeat-associated non-AUG translation is upregulated by stress through eIF2 α phosphorylation," *Nature Communications*, vol. 9, no. 1, p. 51, 2018.
- [165] B. Swinnen, A. Bento-Abreu, T. F. Gendron et al., "A zebrafish model for C9orf72 ALS reveals RNA toxicity as a pathogenic mechanism," in *Acta Neuropathologica*, vol. 135, no. 3, pp. 427–443, 2018.
- [166] R. Tabet, L. Schaeffer, F. Freyermuth et al., "CUG initiation and frameshifting enable production of dipeptide repeat proteins from ALS/FTD C9ORF72 transcripts," *Nature Communications*, vol. 9, no. 1, p. 152, 2018.
- [167] A. Corriero and H. R. Horvitz, "A C9orf72 ALS/FTD ortholog acts in endolysosomal degradation and lysosomal homeostasis," *Current Biology*, vol. 28, no. 10, pp. 1522–1535.e5, 2018.
- [168] B. Zamiri, M. Mirceta, R. Abu-Ghazalah, M. S. Wold, C. E. Pearson, and R. B. Macgregor Jr, "Stress-induced acidification may contribute to formation of unusual structures in C9orf72-repeats," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1862, no. 6, pp. 1482–1491, 2018.
- [169] T.-H. Yeh, H. F. Liu, Y. W. Li et al., "C9orf72 is essential for neurodevelopment and motility mediated by cyclin G1," *Experimental Neurology*, vol. 304, pp. 114–124, 2018.
- [170] L. H. H. Meeter, T. F. Gendron, A. C. Sias et al., "Poly(GP), neurofilament and grey matter deficits in C9orf72 expansion carriers," *Annals of Clinical Translational Neurology*, vol. 5, no. 5, pp. 583–597, 2018.
- [171] T. Nonaka, M. Masuda-Suzukake, M. Hosokawa et al., "C9ORF72 dipeptide repeat poly-GA inclusions promote intracellular aggregation of phosphorylated TDP-43," *Human Molecular Genetics*, vol. 27, no. 15, pp. 2658–2670, 2018.