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Xinmei Wen
Thomas Jefferson University, xinmei.wen@jefferson.edu

Thomas Westergard
Thomas Jefferson University, Thomas.Westergard@jefferson.edu

Piera Pasinelli
Thomas Jefferson University, Piera.Pasinelli@jefferson.edu

Davide Trotti
Thomas Jefferson University, Davide.Trotti@jefferson.edu

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Pathogenic determinants and mechanisms of ALS/FTD linked to hexanucleotide repeat expansions in the *C9orf72* gene

Xinmei Wen[1,*], **Thomas Westergard**[1], **Piera Pasinelli**[1], and **Davide Trotti**[1]

[1]Jefferson Weinberg ALS Center, Department of Neuroscience, Vickie and Jack Farber Institute for Neuroscience, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abstract

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two apparently distinct neurodegenerative diseases, the former characterized by selective loss of motor neurons in the brain and spinal cord and the latter characterized by selective atrophy of frontal and temporal lobes. Over the years, however, growing evidence from clinical, pathological and genetic findings has suggested that ALS and FTD belong to the same clinic-pathological spectrum disorder. This concept has been further supported by the identification of the most common genetic cause for both diseases, an aberrantly expanded hexanucleotide repeat GGGGCC sequence located in a non-coding region of the gene *C9orf72*. Three hypotheses have been proposed to explain how this repeats expansion causes diseases: 1) *C9orf72* haploinsufficiency-expanded repeats interfere with transcription or translation of the gene, leading to decreased expression of C9orf72 protein; 2) RNA gain of function-RNA foci formed by sense and antisense transcripts of expanded repeats interact and sequester essential RNA binding proteins, causing neurotoxicity; 3) Repeat associated non-ATG initiated (RAN) translation of GGGGCC repeat expansion-RAN translation of expanded sense and antisense repeats produces potential toxic dipeptide repeat protein (DPR). In this review, we assess current evidence supporting or arguing against each proposed mechanism in C9 ALS/FTD disease pathogenesis. Additionally, controversial findings are also discussed. Lastly, we discuss the possibility that the three pathogenic mechanisms are not mutually exclusive and all three might be involved in disease.

Keywords

C9orf72; ALS; FTD; repeat expansion disease; RNA foci; dipeptide repeat protein

[*]Correspondence: Xinmei Wen, MD, PhD, Jefferson Weinberg ALS Center, Department of Neuroscience, Vickie and Jack Farber Institute for Neuroscience, Thomas Jefferson University, 900 Walnut Street, Philadelphia, PA 19107, USA, Tel. 215-955-4325, Xinmei.Wen@jefferson.edu.

1. ALS and FTD: One Clinicopathological Spectrum Disorder

Amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease, causes the degeneration of upper motor neurons in the cortex and lower motor neurons in the medulla and spinal cord. Although few juvenile cases have been reported, ALS has generally an adult onset and progresses rather rapidly in most patients (few years from onset to end-stage). There are two forms of ALS: sporadic (sALS) and familiar (fALS). About 5% of ALS cases are inherited, in which there is a clear family history of disease [1]. In familial ALS cases, an autosomal dominant hereditary pattern is usually observed. Since the identification of the first causative mutation in *SOD1* in 1993, more than 30 genes have been identified to cause familial ALS worldwide [2]. These genetic mutations have been found to induce disturbances in protein homeostasis, RNA metabolism and/or cause abnormalities in axonal structures and functions [2, 3]. ALS involves also non-cell autonomous components, meaning cell types other than motor neurons are also affected by the disease pathogenic mechanisms and contribute to the demise of the motor neurons.

Frontal temporal dementia (FTD) is second most common form of dementia after Alzheimer's disease in the under 65-age group. As its name implies, FTD is characterized by atrophy of the frontal and temporal lobes. Symptoms include changes in personality and behaviors, disturbances in language, and cognitive deficits, which worsen progressively [4]. Like ALS, there are two types of FTD: familial (fFTD) and sporadic (sFTD). FTD has a strong genetic component with up to 50% of the cases being hereditary. Clinical subtypes include behavioral variant FTD (bvFTD), progressive non-fluent aphasia (PNFA) and progressive aphasia semantic dementia (SD) [5]. The average survival is 8–10 years after disease onset [6, 7]. Selective serotonin reuptake inhibitors and serotonin norepinephrine reuptake inhibitors have been shown to have positive effects only in some bvFTD patients [5]. Over the past twenty years, our understanding of the genetic causes and molecular pathogenic mechanisms has been growing at a surprisingly fast pace. Neuropathological classification of FTD has also been transformed. Mutations in *MAPT* (microtubule-associated protein tau), *GRN* (progranulin), *VCP* (AAA-type ATPase valosin-containing protein), *CHMP2B* (charged multiversicular protein 2B), the FET family (DNA/RNA binding proteins), including *FUS*, *EWS*, and *TAF15*, have been identified to cause FTD [8]. A variety of mechanisms have been implicated in FTD including impairments in neurotrophic support, autophagy and proteasome pathways, RNA metabolisms and neurofilament structures [7].

Although ALS and FTD present with distinct clinical features, strong associations have been found between them. ALS and FTD often occur in the same family and same patients sometimes. Indeed, there are overlaps in clinical presentations, genetic causes, pathological findings and pathogenic mechanisms. Both diseases are age dependent, and symptoms usually start in fifth decade of life. Both diseases relentlessly progress over time, and eventually lead to death. More than 50% of sALS patients and 60% of fALS patients have cognitive deficits; and around 15% of sALS patients are actually diagnosed with concomitant FTD [9, 10]. Similarly, 12.5–14% of FTD patients have concomitant motor neuron disease (MND), and another 27–36% of FTD patients have clinical evidence of motor system dysfunction [11, 12]. Disease progression seems to be more rapid in FTD

patients with concomitant motor neuron disease, as these patients were reported to have an earlier onset age and a shorter mean survival [13]. ALS and FTD are not only heterogeneous in clinical presentations; they are highly heterogeneous in genetic causes. A number of genetic mutations have been found to cause both ALS and FTD, namely mutations in *FUS*, *TDP-43*, *SQSTM1*, *UBQLN2*, *CHCHD10*, *VCP* and *C9orf72* [14–18]

2. Discovery of GGGGCC/CCCCGG Hexanucleotide Repeat Expansions in *C9orf72*

Multiple groups conducted extensive sequencing in families with hereditary ALS/FTD with unknown mutations, and linked the diseases to a 3.7 Mb region in the short arm of chromosome 9. Later the region was narrowed down to an 80kb area by genome wide association studies (GWAS) [4]. Deep sequencing of exons and exon-intron boundaries failed to identify the mutation. However, two independent groups pinpointed the nature and location of the mutation by carefully examining non-coding regions and manually aligning individual sequences [14, 16].

The mutation turned out to be a vastly expanded GGGGCC repeat (CCCCGG in the antisense direction) located in a non-coding region of the *C9orf72* gene. Since C9orf72 has three different transcript variants, the mutation resides in different regions in different variants: Promoter region for variant 2 and first intron for variant 1 and 3.

C9orf72 is located on chromosome 9, in the 72nd open reading frame and consists of 12 exons. *C9orf72* (also referred here as C9) is a highly conserved gene with three transcript variants, encoding two protein isoforms. Variants 2 and 3 encode C9orf72 isoform *a,* the longer version with 481 amino acids, and variant 1 encodes C9orf72 isoform *b*, the shorter version containing 222 amino acids. Both isoforms are still of unknown biological function (Figure 1).

The most common GGGGCC repeat size in *C9orf72* was reported to be 2 in normal, non-diseased individuals [19]. However, variable ranges of repeat number have been reported for controls and patients: fewer than 14 repeats in controls as 20–22 repeats were associated with FTD [20]; fewer than 23 repeats in controls [15]; 800–4400 repeats in patients [19]; 0–30 repeats in controls and more than 500 repeats in patients [21]. And finally a more recent study employing a southern blot based semi-automated quantification showed that most control carry fewer than 10 repeats, while patients carry 300–3800 repeats [22].

Similar to what was observed in other repeat expansion diseases, the repeat size was also found to be associated with sample collection time [22, 23]. *C9orf72* repeat expansion sizes have been widely reported to vary in different tissue types and cell types [19, 22–24], indicating the expansion of repeats might be caused by some cell division-independent mechanism in addition to somatic instability. The repeat size variation among different tissues within a same carrier was larger than that between different carriers, suggesting that intrinsic properties of specific tissue might affect the stability of the expanded repeats. In some cases, the difference in size between peripheral blood and CNS tissues was several thousand [21, 25]. It was also shown that repeat sizes smaller than 16 were somatically

stable, implying higher repeats were prone to expansion [25]. In addition to the repeat size variation within the same patient, there are also technical challenges and limited DNA available, thus it remains to be answered what is the minimal size for pathogenesis and what is the normal range.

3. Disease Mechanisms

Discovery of the expanded GGGGCC repeat in a non-coding region of the *C9orf72* gene, the most common genetic cause for both ALS and FTD, brought researchers' attention to a group of diseases called repeat expansion disease. Repeat expansions were known to cause a number of hereditary diseases, such as fragile X syndrome (FXS), fragile X tremor ataxia syndrome (FXTAS), myotonic dystrophy (DM), Huntington's disease (HD), and spinocerebellar ataxia 1 (SCA1) [26]. Three common pathogenic mechanisms underly repeat expansion diseases:

1. Loss-of-function, as seen in FXS, where expanded CGG repeats in *FMR1* causes hypermethylation of the promoter region and subsequent loss of FMRP protein, which is an essential RNA binding protein that regulates local protein synthesis leading to abnormal synaptic function [27];
2. RNA mediated gain-of-function, as seen in DM1, where expanded CTG repeats in the gene *DMPK* form nuclear RNA foci in muscle cells, sequester essential RNA binding proteins and causes misregulated RNA splicing [28];
3. Protein mediated gain-of-function, as seen in HD, where expanded CAG repeats in the coding region of the *huntingtin* gene are translated into poly-glutamine (poly-Q) tract, which leads to proteins aggregation and disruption of the normal functions of huntingtin [29, 30]. Protein mediated gain-of-function can also arise from repeats located in a non-coding region, (e.g. intron, 3′UTR, 5′ UTR) where expanded repeats are translated into toxic protein through a novel translational mechanism called Repeat-Associated Non-ATG initiated (RAN) translation, which circumvents the requirement for a canonical start codon [31]. RAN translation was first reported in myotonic dystrophy type 1 (DM1) and in spinocerebellar ataxia type 8 (SCA8), where expanded CAG•CTG repeats in non-coding regions were translated into poly-Gln and poly-Ala [31].

The characteristics of *C9orf72* mutation suggest all three potential mechanisms. Here, findings in favor of or against each potential mechanism are discussed.

3.1 *C9orf72* Haploinsufficiency

C9orf72 expression levels were thoroughly assessed in repeat expansion carriers and control population. *C9orf72* transcripts were found to be significantly reduced in lymphobast cells, frontal cortex, spinal cord, cerebellum and induced pluripotent stem cells (iPSCs) derived neurons from patients carrying the repeat expansion, and variant 2 was found to be mostly affected [16, 17, 32–36]. In a separate study, variant 2 was identified to be the primary

transcript for *C9orf72* [37], indicating loss of variant 2 would contribute greatly to loss of overall *C9orf72* protein levels. Furthermore, higher variant 1 expression levels in frontal cortex and cerebellum were found to be survival favorable [32].

Using a customized antibody recognizing both C9orf72 protein isoforms, decreased levels of the long isoform were found in patients frontal cortex samples but not in cerebellum samples compared to control samples [38]. In another publication, two customized antibodies against C9orf72 short and long isoforms respectively were developed, and researchers found that C9orf72 long isoform was down regulated in temporal and frontal lobes and no changes were detected in cerebellum, motor cortex and lumber spinal cord (low signal) while the short isoform was up regulated in C9-ALS patients [39].

However, a case report on a homozygous repeats expansion patient argued against the C9 loss-of-function (LOF) hypothesis. If C9 LOF were the major disease mechanism, more severe clinical course and neuropathological abnormality would be expected in a homozygous mutation carrier. In this case, homozygosity of C9 repeat expansion did cause a further reduction of *C9orf72* transcript levels, but clinical phenotype and neuropathological features were not unusual when compared to heterozygous carrier [40].

Do decreased *C9orf72* expression levels contribute to disease pathogenesis? To answer this question, C9 knockdown or C9 knockout models have been developed. Deletion of *alf-1,* the *c. elegans* ortholog of *C9orf72,* led to age-dependent progressive motor deficits similar to those seen in TDP-43 and FUS models and increased sensitivity to stress-induced paralysis, and GABAergic motor neuron was primarily affected [41]. Similarly, knockdown of *C9orf72* in zebrafish caused motor deficits and impaired axonal projection [35]. Although *c. elegans* and zebrafish C9 knockdown models suggested loss of C9orf72 was sufficient to cause disease phenotype, this hypothesis remained to be further tested since neither *C9orf72* homologs of c. elegans nor zebrafish shared high homology with human *C9orf72*. Later, a series of C9 knockdown and knockout mouse models were generated, where mouse ortholog of *C9orf72* is highly similar to human *C9orf72*. Mouse *C9orf72* was either conditionally removed in neuronal and glial cells using Nestin-Cre system or constitutively removed in all cell types using gene targeting or Crispr/Cas9 systems. Heterozygous and homozygous mice were analyzed to understand the functional consequences of decreased C9 protein level (resembling most of the patients) vs. full knockout of C9 protein. All together, mice with decreased or abrogated *C9orf72* level failed to show ALS/FTD-like features including motor deficits, behavioral abnormality, shortened life span, pathological hallmarks of ALS/FTD (TDP-43-positive inclusions, and p62-positive inclusions) [42–47]. To be noted, two C9 knockout mouse models showed mild motor deficits after 40 weeks [45] and decreased survival [47], but these phenotypes were suggested to be caused by other underlying conditions rather than ALS/FTD. Using a different approach, C9 level was also successfully reduced when antisense oligonucleotides (ASOs) against *C9orf72* were injected into adult (8 week old) mouse intracerebral ventricles, yet no ALS/FTD phenotypes were observed [43]. Taken together, absence of ALS/FTD-like phenotypes in C9orf72 knockdown or knockout mouse models indicates *C9orf72* haploinsufficiency is not the primary cause of ALS/FTD.

The above C9 knockout mouse models also provide insights into physiological functions of C9orf72, albeit they failed to display ALS/FTD related disease phenotypes. Upon close examination, these mice showed enlarged spleens and lymph nodes and they also showed microglia activation with abnormal lysosomal activity and altered cytokine production, suggesting that C9orf72 protein might be involved in regulating immune homeostasis, and autophagy/lysosome pathway [44–48]. Surprisingly, neoplastic events were also reported seen in one C9 knockout mouse model, which might be triggered by defects in immune systems [47].

Understanding the physiological function of C9orf72 is not only important for defining the role of C9 haploinsufficiency but also crucial for developing therapeutic strategies to mitigate the repeat expansion toxcity. In the hope that knowing where C9orf72 protein is located would provide some insight into its functionality, several groups made attempts to characterize C9orf72 protein localization in cells, with variable localization profiles reported: nuclear [14], primarily cytosolic [49], nuclear and cytoplasmic vesicles [50], nuclear, cytosolic and synaptic vesicles [51], and nuclear, P-bodies and stress granules [52]. Different cells and tissues were evaluated in these studies. Additionally, various commercial antibodies were used, which are still poorly characterized. These differences most likely led to different observations. Thus, a more specific antibody is crucial for an accurate characterization. Recent studies using customized antibodies provided more information on this matter. Using two newly developed antibodies against C9 short or long isoforms, researchers demonstrated that C9 long isoform showed diffuse cytoplasmic staining pattern and C9 short isoform was found to localize to nuclear membrane [39]. The long isoform of the C9orf72 protein also showed distinct speckle-pattern staining only in cytoplasm of cerebellar Purkinje neurons-the less affected neuronal cell type in diseases, not in spinal motor neurons-the primarily affected cell type [39]. The implications of these interesting findings remain to be explored.

Computational algorithms based on sequence and structural similarity predicts that C9orf72 is a guanine exchange factor with a DENN (Differentially Expressed in Normal and Neoplastic) like domain [53]. Consistent with the computational prediction of its function, C9orf72 protein was found to colocalize and interact with Rab proteins and was involved in endosomal trafficking in cells [50]. The role of C9orf72 protein in autophagy regulation was further established in a series of follow up studies: C9orf72 protein was implied in regulation of autophagy initiation by functioning as Rab1a effector and interacting with ULK1 initiation complex [54]; C9orf72 protein interacted with SMCR8 and WDR41 to participate in autophagy initiation [46, 55, 56].

Taken together, *C9orf72* haploinsufficiency might not be driving disease pathogenesis, but it is highly likely involved in it. It would be of great interest to further characterize the function of C9orf72 protein and investigate whether *C9orf72* is beneficial in stabilizing expanded repeats.

3.2 RNA Gain of Function

Nuclear and cytoplasmic RNA foci formed by sense or anti-sense transcript of the GGGGCC repeat expansion were found in C9 patient lymphoblast cells and fibroblast cells.

In the CNS, RNA foci were found in glia cells and neurons (more frequently than glia cells) of frontal cortex, cerebellum, hippocampus and spinal cord; the RNA foci most likely consisted of expanded GGGGCC sequence without flanking regions upstream or downstream of the repeat [14, 16, 33, 43, 57–59]. Besides forming RNA foci, the GGGGCC repeat expansion was also revealed to form highly stable G-quadruplex structure and R-loops *in vitro* [34, 60–62]. G-quadruplex consists of planar stacks formed by four guanine residues that are stabilized by Hoogsteen hydrogen bonding (Figure 2A). R-loops are hybrids of DNA and RNA formed by nascent RNA strand and template DNA strand during transcription [62] (Figure 2B).

Characterization of functional consequences of expanded repeats in C9 patients and their iPSC-derived neurons (iPSNs) supported the toxic role of RNA foci. Sequestration of ADARB2 protein by RNA foci and altered gene expression profiles were detected in C9 patient iPSNs and motor cortex; C9-iPSNs' susceptibility to glutamate toxicity can be alleviated by ASOs against *C9orf72* accompanied by decreased RNA foci number and continuing presence of RAN-DPR (RAN translation product poly-GlyPro level was evaluated), suggesting that RNA foci were the primary toxic species [33]. So far, a list of RNA binding proteins (RBPs) was identified to co-localize with sense and anti-sense RNA foci in patient tissues, including ALYREF, ADARB2, hnRNP-A1, hnRNP-H/F, hnRNP-K, hnRNP-U, nucleolin, Pur-α, SC35, SRSF2 [33, 34, 63–66] (summarized in Figure 2C). Another group examined the role of G-quadruplex formed by GGGGCC repeats in disease pathogenesis. They found that GGGGCC repeat expansion formed G-quadruplex and R-loops *in vitro*, which led to aberrant interaction with nucleolar proteins, resulting in nucleolar stress, reminiscent of traits seen in C9 patients and their cells, as well as loss of full length transcripts and the accumulation of abortive transcripts due to disrupted RNA polymerase processivity [34]. To be noted, a different group reported contrary findings that the intron containing expanded repeats were transcribed and spliced out correctly in C9 patients [36]. Overt cellular toxicity, altered RNA processing and nucleolar stress caused by expressing GGGGCC repeats *in vitro* and *in vivo* lend support to the notion that C9 repeat expansion causes disease phenotype through a RNA gain-of-function pathway. A higher number of RNA foci in the frontal cortex positively correlated with a younger disease onset age in C9 FTD patients, adding further support to RNA toxicity in disease pathogenesis [59].

To explore whether these abundant RNA foci seen in patients would cause neurodegeneration, transgenic *Drosophila* and cell models were developed. When 30 GGGGCC repeats were cloned into a 5′ UTR, toxicity was observed *in vitro* in N2a cells, as well as decreased locomotor activity and disrupted eye morphology when expressed in motor neurons and retinal cells, respectively, in aged flies [67]. In the same study, the purine-rich element binding protein A, Pur-α was found to interact with GGGGCC repeats and overexpression of Pur-α rescued the degenerative phenotypes. Later, using the same fly model, a different group showed expanded GGGGCC transcripts and RNA foci interacted with and sequestered the Ran GTPase-activating protein RanGAP, leading to reduced nuclear import *in vitro* and *in vivo* [68]. Moreover, expression of 38 or longer GGGGCC repeats caused apoptosis in neuronal cell lines and fish embryos via sequestration of hnRNP-H by RNA foci [64]. Besides hnRNP-H, several other translation regulators like eIF2a, FUS and ILF3 were also shown to be sequestered into RNA foci leading to translation arrest in

cells expressing 31 repeats of GGGGCC [69]. Most of the RBPs sequestered in RNA foci would fall into two categories: RNA processing regulators and translation regulators. Gene profiling of C9 patient cells and tissues revealed that splicing error rate was associated with disease severity, providing support for RBP sequestration by RNA foci as a potential disease mechanism [70]. Nevertheless, direct evidence showing functional consequences resulted from sequestration of these critical proteins are somewhat lacking. Interestingly, GGGGCC repeat RNA particles were also found in neurites of iPSNs from C9 patients, and these repeat RNA particles correlated with neuritic arborization defect seen in primary neurons expressing expanded repeats and *Drosophila* carrying 48 GGGGCC repeats (no RAN translation product was detected) [71].

And it was suggested that the toxicity might be due to dysfunctional granule transport caused by GGGGCC repeat expansion [71]. Contradictory results, however, were also reported in several studies where *in vitro* and *in vivo* models were used, suggesting RNA foci alone were not sufficient to cause neurodegeneration. In a transgenic fly model carrying up to 288 pure GGGGCC repeats (RNA only without production of RAN translated proteins), neither neurodegeneration nor decreased viability was observed [72]. More recently, in another fly model expressing up to 160 GGGGCC repeats with intronic and exonic flanking sequences from human *C9orf72*, which closely resembled the mutation in C9 patients, displayed no toxicity when expressed in a variety of tissues. More importantly, the average number of sense RNA foci per cell was a lot higher than that in C9 patient iPSCs derived neurons [36], suggesting that it was unlikely for RNA foci to be toxic. Similar claims have been made from several mouse models. In a mouse model expressing 80 repeats of GGGGCC in a 5′-UTR region, the repeat size was proven to remain stable through multiple generations and no RAN translation product was detected, however, researchers did not find motor/behavior phenotypes, or neuronal cell loss [73]. Moreover, two independently generated BAC transgenic mouse models carrying either the full human *C9orf72* gene with expanded GGGGCC repeats or first six exons of the human *C9orf72* gene with expanded GGGGCC repeats. These two mouse models both showed formation of abundant sense and anti-sense RNA foci as well as Gly-Pro dipeptides similar to pathological findings seen in C9 patients, but the mice lived a normal life span without developing any cognitive, behavioral or motor defects [74, 75]. In contrast, another two BAC transgenic mouse models carrying human *C9orf72* gene together with the expanded GGGGCC repeats showed ALS/FTD like phenotypes, including cognitive deficits, motor deficits and decreased survival [48, 76]. Similarly, these mice also showed pathological and molecular signatures of C9 ALS/FTD, including sense and anti-sense RNA foci, and RNA translation products (poly-GP, poly-GA and poly-GR inclusions). Moreover, RAN translation was dependent on length of repeats, expression levels, age, and disease course [48, 76]. However, it remains possible that the observed toxicity could come from RAN translation product.

3.3 RAN Translated Dipeptide Repeat Protein Toxicity

RAN translation of expanded repeats has become an emerging mechanism underlying nucleotide repeat disorders since the initial discovery in DM1 and SCA8. Efforts were also made to determine whether these unexpected protein products resulted from RAN translation would contribute to disease. In fragile X-associated tremor ataxia syndrome

(FXTAS), CGG repeats in the 5′ UTR of the fragile X mental retardation gene (*FMR1*) were shown to undergo RAN translation and produce poly-Ala protein and poly-Gly protein from different reading frames, the latter of which caused neural toxicity in *Drosophila* model [77] and was shown to trigger impairment in ubiquitin proteasome system (UPS) [78], supporting the role of novel protein products from RAN translation in disease pathogenesis. Recent progress on how RAN translation occurred in FXTAS provided mechanistic support. It was shown that RAN translation of CGG repeats was 30–40% efficient compared to canonical translation, and it utilized the canonical translation machinery and was dependent on repeat length and surrounding sequence [79].

If both sense and anti-sense transcripts of expanded GGGGCC repeats underwent RAN translation, five different dipeptide proteins (DPRs), namely poly-Gly-Ala(GA), poly-Gly-Pro(GP), poly-Gly-Arg(GR), poly-Pro-Ala(PA), and poly-Pro-Arg(PR), would have been produced from different reading frames (Figure 3). In C9 ALS/FTD patient tissues, DPRs were initially observed in p62 positive neuronal cytoplasmic inclusions (NCI), which stained negatively for the nuclear transcription factor TDP-43 [14, 16, 80–82]. Since their discovery, DPR pathology has been a highly consistent and definitive feature for *C9orf72* cases. Later, the presence of each potential DPR has been described in C9 ALS/FTD patient brain tissues and less frequently in spinal cord tissues, using a spectrum of antibodies, targeting either specific C-terminal regions or dipeptide-repeat regions of the individual DPRs [57, 58, 83–87]. DPR inclusions were not limited to neurons, and they were also shown in glial cells [83, 85, 88, 89]. Most studies reported that individual DPRs shared similar localization patterns but displayed different abundances: from highest to lowest are poly-GA, poly-GP, poly-GR, and poly-PA/poly-PR [89, 90]. Similar findings were also reported in C9 patient iPSCs derived neurons and C9 patient CSF (poly-GP was detected) [33, 49, 91–94]. Fundamental questions regarding the role of these C9-DPRs remain: Is the presence of DPRs a mere pathological hallmark? Are these DPRs actually causing neurodegeneration?

To clearly dissect the pathogenic mechanisms, researchers used a “random-alternative codon” strategy to design DNA constructs encoding each specific DPR or synthetic dipeptides, so they could study DPRs independent of one another and from potential interference from pure GC sequences at transcript level. Using this approach, GR and PR, the two arginine rich DPRs, have been shown to be strongly toxic by multiple groups when overexpressed in yeast, cultured neurons and *Drosophila*, while other DPRs were not or were only mildly toxic [49, 72, 95–99]. Interestingly, GR and PR showed distinct localization pattern: predominantly nucleolar accompanied by occasional cytoplasmic localization. Further analysis revealed that these arginine rich DPRs interact with a number of nucleolar/nuclear proteins and cause global translational dysregulations, including induction of nucleolar stress, suppression of rRNA synthesis, abnormal stress granule formation, splicing aberration, and inhibition of protein translation [49, 95, 96, 100]. Several cellular pathways have also been reported as potential disease mechanisms: Dysfunction of UPS [101]; Disturbance in nucleocytoplasmic transport [99, 102]; Suppression of Notch signaling pathway [98]. Surprisingly, co-expression of GA was found to partially mitigate toxicity caused by GR in *Drosophila* cells and human iPSNs [98]. In addition, *Drosophila* carrying 160 intronic GGGGCC repeats showed abundant RNA foci but no neurodegeneration when grown at 25°C, while *Drosophila* expressing 36 GGGGCC repeats with a poly(A) tail, which

readily produced GR and GP, displayed neurodegeneration phenotype [36]. More interestingly, when grown at a higher temperature, decreased survival was observed in *Drosophila* carrying 160 intronic GGGGCC repeats, which correlated with the production of GR, lending further support to the theory that R-rich DPRs induce neurotoxicity [36].

Tandem repeats of Gly-Ala is characteristic of EBNA1 protein of Ebstein-Barr virus and has been shown to impair proteasome functions [103, 104]. Likewise, studies showed that overexpression of poly-GA in cultured cells induced sequestration of Unc119, inhibition of proteasome activity, endoplasmic reticulum (ER) stress, reduced dendritic branching complexity, and apoptosis; Overexpression of Unc119 or inhibitors of ER stress ameliorated toxicity caused by GA expression [105, 106]. Another study showed that overexpression of GA or GP caused cellular toxicity via disrupting the UPS [101]. More intriguingly, 15 repeats of GA dipeptides were shown to form toxic amyloid fibrils when incubated in test tubes and could be transmitted from cell to cell [107]. However, it remains to be tested whether GA would spontaneously form amyloid fibrils when expressed in cells. Recently, mice expressing GA dipeptides were generated using a viral delivery system by injecting AAV1 encoding GFP tagged 50 repeats of GA into intracerebral ventricles [108]. These mice showed brain atrophy, motor deficits, and behavioral abnormality indicative of ALS/FTD like symptoms, confirming that expression of GA causes neurotoxicity *in vivo*. Consistent with the previous study, 50 repeats of GA dipeptides were found to form toxic fibrils, and the toxicity was conformation dependent since disruption of fibril formation would abolish toxicity. Furthermore, sequestration of HR23 proteins and nuclear pore proteins by GA aggregates were implied as potential mechanisms. It is of notice that each research group employed a unique sequence with different combination of alternative codons, different G/C content and different levels of repetitive sequences to encode the same DPR. These differences might lead to varied expression levels of the same DPR species and other unknown effects, which might explain the inconsistency in DPR toxicity seen by different groups.

In summary, overexpression of arginine rich DPRs cause neurotoxicity *in vitro* and *in vivo* by disturbing global translational regulation, nucleocytoplasmic transport, UPS, and by suppressing Notch signaling pathway; whether other DPRs cause direct neurotoxicity remains conflicting. Nevertheless, expression of C9-RAN DPR (GA) alone is sufficient to cause neurodegenerative phenotypes *in vivo*, strengthening the role of DPR toxicity in disease pathogenesis (summarized in Figure 3).

How relevant is DPR toxicity to human diseases remains debatable. If DPR toxicity causes direct neurodegeneration in disease, one would intuitively expect to see an overall heavier DPR burden or more toxic DPR species in regions or cell types that are affected most in the disease, or correlation between DPR pathology load and disease severity. The main argument is that there is low abundance of DPRs in key regions, and DPR distribution patterns and abundances do not differ between disease types (ALS vs. FTLD), nor do they correlate with disease onset, neurodegeneration, or phenotype [85, 89, 90, 109]. Exceptions were also reported in a few studies. In one study, the poly-GA positive dystrophic neurites were found associated with frontal cortex degeneration in FTLD and total poly-GA loads were associated with disease onset [90]. Cerebellar poly-GP levels were significantly lower

in ALS patient samples compared to FTLD and FTLD-MND patient samples and was also associated with cognitive deficits [110]. Although poly-PR inclusions were rare, the total burden was shown to be significantly higher in CA3/4 regions in FTLD cases than MND cases [89]. Moreover, the lack of abundant DPRs pathology, especially toxic arginine rich DPRs, in patient post-mortem tissues could also be explained by the possibility that toxic DPRs caused neuronal death, thus neurons "survived" and showed up in pathology tissue sections were those who did not have abundant DPRs. So, non-invasive or minimally invasive assays that could detect R-rich DPRs in patients throughout disease course would provide invaluable information. Interestingly, pathological findings from three *C9orf72*-FTLD patients who prematurely died shortly after disease onset suggested that DPRs were abundant throughout the cerebral cortical regions, hippocampus, and cerebellum, while TDP-43 pathological changes were sparse [111].

Additionally, some studies state that TDP-43 cytoplasmic inclusions were more abundant than DPRs, and that the occurrence of TDP-43 correlates to degeneration in specific areas in *C9ORF72*-positive patients [85, 109]. However, a recent systematic review of a large cohort of pathological studies looking at TDP-43, p62, and DPR inclusions in the brains and spinal cords of *C9ORF72*-positive patients reported different findings [112]. A total of 261 *C9orf72*-positive patients were analyzed after excluding studies that only looked at one type of inclusion or one specific CNS region, and it was shown that there was greatly lower prevalence of TDP-43 inclusions in the cerebellum and hippocampus than DPRs and p62. Additionally, there was little to no difference between the prevalence of TDP-43, DPRs, and p62 inclusions in the frontal lobe, temporal lobe, spinal cord, and brainstem [112].

4. Concluding Remarks

Despite the excitement of identifying *C9orf72* mutation as the most common genetic cause for ALS and FTD, we need to acknowledge that it is critical and challenging to decipher how the enormously expanded GGGGCC repeats in a non-coding region of *C9orf72* gene cause neurodegeneration. Three pathogenic mechanisms were proposed. The research field of *C9orf72* has been moving at a fast pace to determine which mechanism induce neurotoxicity and benefited greatly from lessons learned from other repeat expansion diseases. Abundant pathological and experimental findings have emerged. It remains to be investigated which pathogenic mechanism strikes neurons first and if there is a sequential cascade of neurotoxic events. Successful development of targeted therapeutics also relies on defining the contribution of each pathogenic mechanism.

Recent development of several lines of BAC transgenic mice carrying partial or full human *C9orf72* gene and expanded GGGGCC repeats has greatly advanced modeling of the diseases in rodents. What is more intriguing is the discrepancy of phenotypes of different lines-some of the lines showed ALS/FTD like phenotypes [48, 76] while others did not show any phenotypes [74, 75]. For the line that showed more robust phenotypes, one obvious difference is the different strain background-FVB/NJ was used in this line as opposed to C57BL/6 or SJL/B6 in other lines. The mutation specific for one genetic strain has led to the discovery of novel functions of GTPBP2 and potentials of mutations in tRNAs in causing neurodegeneration [113]. Although the reasons for the discordant results are complicated

and still unknown, there is a possibility that the different genetic background could be one underlying cause like in the case. A thorough comparison of different lines would provide more mechanistic insights.

Keeping in mind that these pathogenic mechanisms are not mutually exclusive, one should also explore the consequences of two or more mechanisms co-existing in the same model, e.g. crossing the BAC mouse with the C9 knockout mouse. It is possible that one mechanism might protect neurons against the toxicity caused by other mechanism(s). It is also possible that one mechanism might enhance the toxicity induced by other mechanism(s). There might also be convergence of mechanisms. Indeed, loss of C9orf72 protein sensitized cells to toxicity induced by expression of 30 repeats and 60 repeats of GGGGCC [52]. Moreover, decreased level of C9orf72 short isoform nuclear membrane staining has been found in C9-ALS patients, and the short isoform of the C9orf72 protein has also been found to physically interact with importin-b and RAN-GTPase [39]. Disruption of nucleocytoplasmic transport was associated with RNA toxicity as well as protein toxicity from R-rich DPRs [68, 97, 99]. Although it is premature to say C9 loss-of-function induce neurodegeneration together with toxic RNA and DPRs via disruption of nuclear transport, it is definitely worth further exploration.

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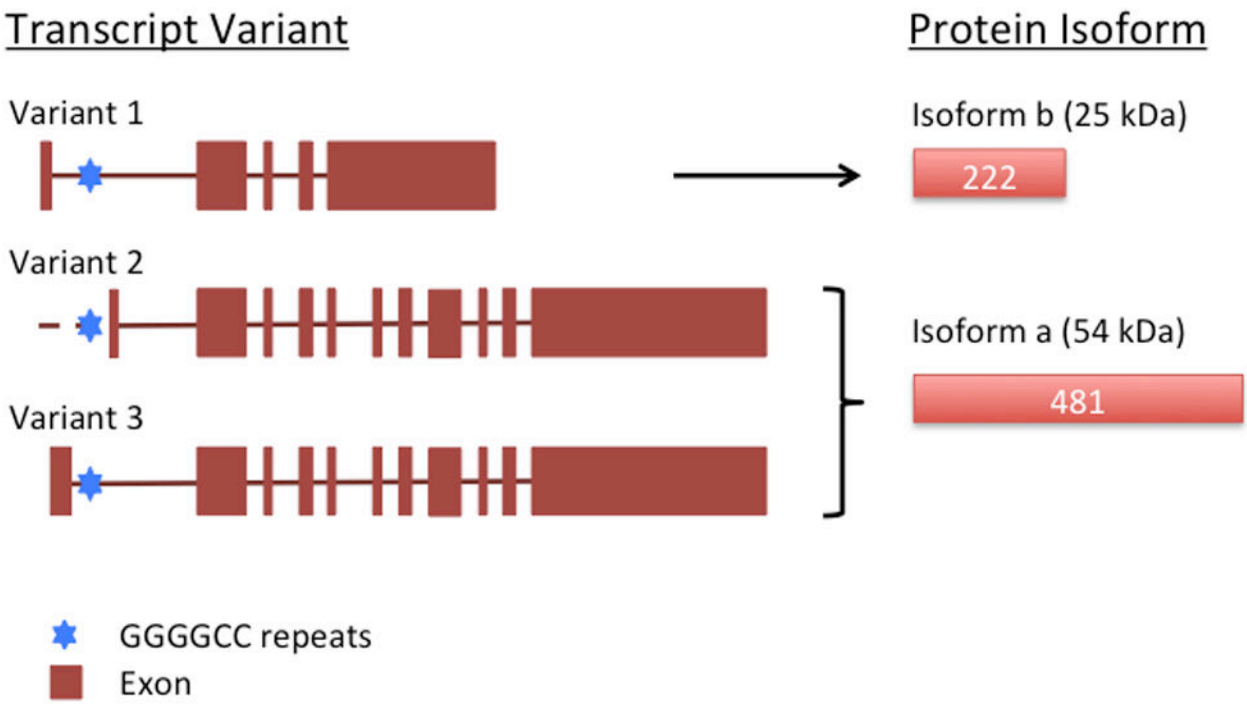
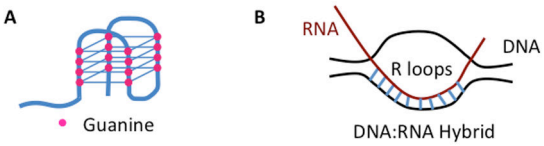


Figure 1. The three *C9orf72* transcripts and GGGGCC hexanuleotide repeat expansion

Three C9 transcript variants and two C9 protein isoforms are listed and named as in PubMed. GGGGCC hexanucleotide repeat (blue hexagon) and exons (brown block) are indicated. Predicted molecular weight for each protein isoform is listed.



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Protein	Function	Assays	Probe
ADARB2	adenosine deaminase: RNA editing	proteome arrays; RNA- colocalize with RNA foci in patient iPSNs and brain tissue; RNA pull down [33]	rGGGGCC
ALYREF	mRNA export adaptor	colocalize with RNA foci in patient cerebellum tissues and motor neurons; RNA pull down [63, 66]	rGGGGCC & rCCCCGG
ASF/SF2	pre-mRNA splicing	*in vitro* electrophoretic mobility shift assay [61]	rGGGGCC
eIF2a	translation initiation	colocalize with RNA foci in mammalian cell model [69]	rGGGGCC
FUS	RNA binding	colocalize with RNA foci in mammalian cell model [69]	rGGGGCC
hnRNPs (hnRNP-A1,-A3, -F, -H, -K, -U)	a group of heterogeneous nuclear proteins, involved in RNA processing	-A1: colocalize with RNA foci in patient iPSC-MNs [65]	rGGGGCC
		-A1: colocalize with RNA foci in patient cerebellum tissues; RNA pull down [63, 66]	rGGGGCC & rCCCCGG
		-A3: RNA pull down [114]	rGGGGCC
		-F: RNA pull down [34]	rGGGGCC quadruplex
		-H: colocalize with RNA foci in cell model and patient brain tissues; RNA pull down [64]	rGGGGCC
		-H/F: colocalize with RNA foci in patient cerebellum tissues, motor neurons; RNA pull down [63, 66]	rGGGGCC & rCCCCGG
		-K: colocaliza with RNA foci in patient cerebellum tissues [66]	rCCCCGG
		-U: SILAC; RNA pull down [34]	rGGGGCC quadruplex
ILF3	RNA binding protein	colocalize with RNA foci in mammalian cell models [69]	rGGGGCC
NCL	constituent protein of nucleolus	SILAC; RNA pull down; colocalize with RNA foci in patient brain tissue [34]	rGGGGCC
Pur α, β, γ	transcriptional activator	RNA pull down in vitro (cell model) and in vivo (Drosophila model) [67]	rGGGGCC
		colocalize with RNA foci in patient iPSC-MNs [65]	rGGGGCC
Ran-GAP1	nucleocytoplasmic trafficking protien	RNA pull down [68]	rGGGGCC
SRSF2 (SC35)	pre-mRAN splicing	colocalize with RNA foci in mammalian cell model [64]	rGGGGCC
		colocalize with RNA foci in patient cerebellum tissues, motor neurons; RNA pull down [63, 66]	rGGGGCC & rCCCCGG

Figure 2. GGGGCC/CCCCGG repeat expansions form G quadruplex and R-loops and interact with RBPs

(A) Four guanine residues (pink ball) form a G-quartet through eight pairs of hoogsteen hydrogen bonding. G-quartets stack on top of each other, forming helical G-quadruplex structure. (B) R-loops are formed newly transcribed RNA transcript and unwound DNA template during transcription. (C) A summary of RBPs reported to be sequestered in RNA foci or to interact with GGGGCC or CCCCGG RNA repeats.

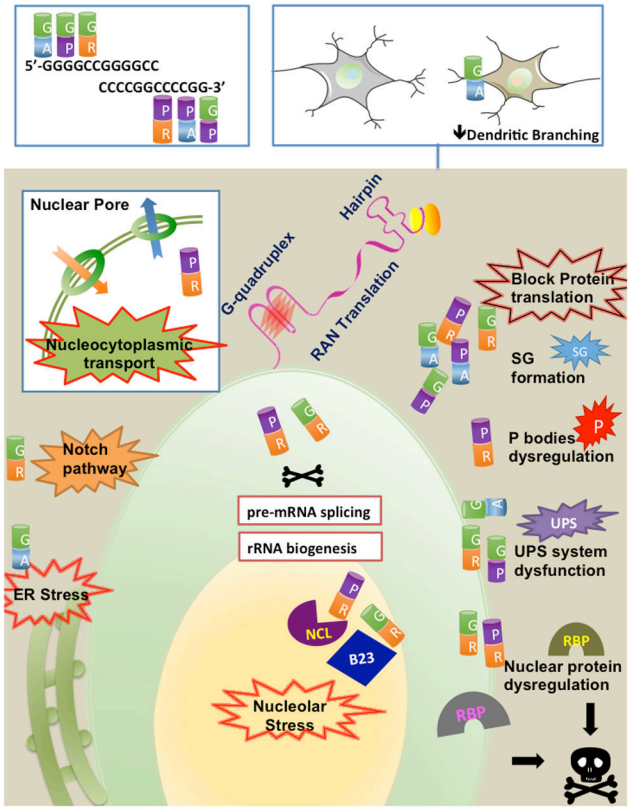

Figure 3. RAN translated DPRs impair cellular functions

RAN translation of sense and anti-sense transcripts of expanded GGGGCC repeats in all possible reading frames gives rise to five DPRs. Sense strand: poly-GA, poly-GP and poly-GR. Anti-sense strand: poly-PA, poly-PR and poly-GP (same as sense strand). Impairments in multiple cellular functions are found caused by C9-DPRs.