Insights into *C9ORF72*-Related ALS/FTD from *Drosophila* and iPSC Models

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Abstract

GGGGCC (G_4C_2) repeat expansion in *C9ORF72* is the most common genetic cause of ALS and FTD. An important issue is how repeat RNAs and their translation products—various dipeptide repeat (DPR) proteins—cause neurodegeneration. *Drosophila* has been widely used to model G_4C_2 repeat RNA and DPR protein toxicity. Overexpression of disease molecules in flies has revealed important molecular insights. These have been validated and further explored in human neurons differentiated from induced pluripotent stem cells (iPSCs), a disease-relevant model in which expanded G_4C_2 repeats are expressed in their native molecular context. Approaches that combine the genetic power of *Drosophila* and the disease relevance of iPSC-derived patient neurons will continue to unravel the underlying pathogenic mechanisms and help identify potential therapeutic targets in *C9ORF72*-ALS/FTD.

Keywords

Autophagy; DNA Damage; DPR Protein; Nucleocytoplasmic Transport; RAN Translation; Repeat Expansion

C9ORF72 Repeat Expansion in ALS and FTD

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is a fatal, progressive neurodegenerative disorder caused by degeneration of upper and lower motor neurons in the brain and spinal cord [1]. Although ALS has long been considered a motor neuron disease, some ALS patients have symptoms of frontotemporal dementia (FTD), and the two disorders share many pathological and genetic features and several dysfunctional molecular pathways downstream of many common disease-causing genetic mutations [2]. FTD, the second most common form of presenile dementia after Alzheimer's disease, is accompanied by focal degeneration of the prefrontal and/or temporal cortex and changes in behavior, language, and executive functions [3]. FTD is a clinically heterogeneous disorder that

includes behavioral variant FTD, semantic dementia, progressive nonfluent aphasia, FTD with motor neuron disease, and other conditions [3].

The most common genetic cause of both ALS and FTD is a GGGGCC (G_4C_2) repeat expansion in the noncoding region of a previously uncharacterized gene, *C9ORF72* [4, 5]. How a single genetic mutation such as *C9ORF72* repeat expansion causes distinct yet overlapping diseases is unclear. It is possible that different genetic modifiers and environmental influences also play a role. The identification of this unusual ALS/FTD-causing mutation in 2011 galvanized the field, leading to breathtaking progress in recent years. Much of this progress reflects the intellectual challenge posed by the multifaceted nature of the underlying pathogenic mechanisms. First, *C9ORF72* expression is reduced by G_4C_2 repeat expansion, suggesting haploinsufficiency as a potential disease-causing mechanism. In cells and tissues of *C9ORF72* patients, expression of *C9ORF72* transcript variant 2 and total full-length C9ORF72 is reduced by expanded repeats in the promoter region [4, 6–11]. However, *C9orf72* knockout mice have no detectable neurodegeneration [12–18], arguing against haploinsufficiency as a primary mechanism of disease, although partial loss of *C9orf72* function in microglia may influence disease progression [16].

Additional pathogenic mechanisms of *C9ORF72*-ALS/FTD are suggested by other molecular hallmarks of the disease. Nuclear and cytoplasmic RNA foci formed by sense and antisense expanded G_4C_2 repeat transcripts imply that disease phenotypes are induced by RNA-mediated toxicity, since these foci might sequester RNA binding proteins and consequently alter RNA metabolism, as in other repeat expansion disorders [19]. Another distinctive feature of *C9ORF72*-ALS/FTD is the accumulation of dipeptide repeat (DPR) proteins, mostly in the cytoplasm of patient neurons [20–22]: poly(GR), poly(GA), and poly(GP) are synthesized from the G_4C_2 sense repeat transcripts, while poly(PR), poly(PA), and poly(PG) are produced from the antisense strand. Repeat-associated non-AUG (RAN) translation is proposed to be the mechanism responsible for DPR protein production [22]; however, other unconventional translational mechanisms may also be involved in neurons and other cell types of *C9ORF72* patients [23].

The presence of both RNA foci and DPR proteins in the brains of *C9ORF72*-ALS/FTD patients [4, 20–22], in human neurons derived from *C9ORF72*-induced pluripotent stem cells (iPSCs) [7, 8, 24], and in *C9ORF72* BAC transgenic mice [15, 25–27] raises important questions about their relative contributions to the disease pathogenesis: (1) Are G_4C_2 RNA foci toxic? If so, are they sufficient to cause disease? (2) Does unspliced *C9ORF72* pre-mRNA or an intron containing spliced G_4C_2 repeats serve as a template for DPR protein synthesis? (3) Are DPR proteins necessary or sufficient to induce neurotoxicity? (4) Which DPR proteins are more toxic and what is the mechanism of their toxicity? (5) What cellular defects do endogenously expressed repeat RNA and DPR proteins cause in human neurons? To address these important questions, both *Drosophila* and *C9ORF72*-ALS/FTD patient-specific iPSCs have been used productively in recent years. Insights from these studies are discussed below.

Drosophila as a Genetic Model for Neurodegenerative Diseases

During the last two decades, *Drosophila* has emerged as a highly valuable in vivo genetic organism for modeling various neurodegenerative diseases, such as Huntington's disease, spinocerebellar ataxia, and Parkinson's disease [28, 29], in part because of evolutionary conservation of many gene functions and related molecular mechanisms between human and fly. Basic research in *Drosophila* has generated many genetic tools for analyzing both loss- and gain-of-function mutants. Moreover, the reduced complexity of the fly brain and the brief life span of flies make it feasible to conduct unbiased large-scale genetic screens to discover novel modifier pathways and mechanisms. Fly models are also used to test drugs and therapeutic compounds [e.g., 30]. On the other hand, disease phenotypes associated with cognitive and behavioral defects may not be truly recapitulated in *Drosophila*. Another limitation is the anatomical difference between human and fly brain, which makes it impossible to address in *Drosophila* the selective vulnerability seen in most neurodegenerative diseases. Moreover, most fly models rely on the overexpression of disease molecules, and many cellular phenotypes under investigation are developmental rather than late onset in the adult brain.

Molecular Contexts of Expanded G_4C_2 Repeats in *Drosophila* Models

Since the discovery of *C9ORF72* repeat expansion in 2011 [4, 5], several *Drosophila* models expressing expanded G_4C_2 repeats have been established to analyze the gain of toxic function in a manner mostly similar to that used for other repeat expansion diseases [31–35]. These models have been useful for determining whether the RNA or DPR protein toxicity is the primary cause of disease and for discovering potential pathogenic mechanisms. However, each fly model has unique molecular features, and the length and molecular context of the G_4C_2 repeats in these models vary, which is critically important for the proper interpretation of the toxicity of repeats and the potential implications for our understanding of the pathogenic mechanisms of *C9ORF72*-ALS/FTD.

The first fly model of G_4C_2 repeat expansion was generated by cloning either 3 or 30 copies of repeats downstream of the upstream activating sequence (UAS) element in the *pUAST* vector, which allows spatial control of target gene expression in tissue-specific *Gal4* driver lines [31]. The 30 G_4C_2 repeats were interrupted in the middle by a 6-nucleotide *Xho*I restriction site in the 5′ untranslated region (5′UTR) of the gene encoding EGFP followed by the SV40 3′ UTR containing a polyadenylation signal [*(G₄C₂)₃₀-EGFP*, Figure 1A]. In this construct, it is predicted that poly(GP) is in frame with EGFP and that poly(GR) has an unnatural C-terminal polypeptide consisting of six amino acids. However, poly(GP) and poly(GR) were not detectable when the construct containing the interrupted 30 copies of G_4C_2 repeats was expressed with *GMR-Gal4* to induce retinal degeneration [36]. On the other hand, poly(GA) is predicted to be in frame with an unnatural protein of 317 amino acids (P. Jin, personal communication). It is not known whether this poly(GA)-containing artificial protein is actually produced in this fly model.

In another fly model of G_4C_2 repeat expansion, 3, 36, or 103 copies of uninterrupted G_4C_2 repeats were cloned into the *pUASTattB* vector between the UAS and an SV40 3′UTR

containing a polyadenylation signal (Figure 1) [32]. In this model, as in the $(G_4C_2)_{30}$-EGFP flies described above, G_4C_2 repeats are also expressed in the molecular context of poly(A)$^+$ mRNA; thus, as expected, repeat RNA is mainly localized in the cytoplasm [33]. Each salivary gland cell expressing $(G_4C_2)_{36}$ or $(G_4C_2)_{103}$ has one nuclear RNA focus [32,33], which seems to be the site of repeat RNA transcription, as the focus colocalizes with RNA polymerase II and flies homozygous for the repeat transgene have two foci in each cell [33]. In this model, poly(GP) can be detected by western blot and poly(GR) by dot blot [32]. Unnatural C-terminal fragments are likely also synthesized from the vector sequence adjacent to the repeat RNA, but this has not yet been demonstrated experimentally. To block DPR production, 6 nucleotides containing stop codons in both sense and antisense directions were inserted periodically after every 12 repeats. This construct was named "RNA only (RO)" [$(G_4C_2)_{36}$-RO, Figure 1) [32].

In human patients, expanded G_4C_2 repeats are located in the first intron of *C9ORF72,* and repeat RNA is mostly localized in the nucleus [4]. To recapitulate this genomic organization in a fly model, a mini *C9ORF72* gene was constructed in which 5, 20, 40, 80, or 160 copies of G_4C_2 repeats are flanked by human intronic sequences of *C9ORF72* and adjacent exons (Figure 1) [33]. This mini *C9ORF72* gene was cloned into the *pBID-UASC* vector, which contains the same SV40 3′UTR as in fly models described above. In this model, the intron containing 160 G_4C_2 repeats is efficiently transcribed and spliced out, as in human *C9ORF72* cells, and forms numerous nuclear RNA foci in neurons and glial cells [33]. Moreover, poly(GP) is detectable but expressed at roughly 1% of the level in $(G_4C_2)_{36}$ flies [33], probably because $(G_4C_2)_{36}$ in the context of poly(A)$^+$ mRNA is exported from the nucleus and translated in the cytoplasm much more efficiently than intronic repeat RNA.

In 2015, two additional fly models of G_4C_2 repeat expansion were reported. In the $(G_4C_2)_{58}$-GFP model, 58 copies of uninterrupted G_4C_2 repeats were placed 5′ to the coding region of GFP but without the AUG translation initiation site in the *pUASTattB* vector (Figure 1) [34]. In this model, G_4C_2 repeats are also part of poly(A)$^+$ mRNA. Poly(GP) is in frame with GFP, and indeed a single band on western blot could be detected by poly(GP) or GFP antibodies [34], although the translation initiation site for this chimeric protein has not been mapped yet. Moreover, poly(GR) with a predicted short C-terminus, but not poly(GA) or poly(PR), was detected by dot blot in $(G_4C_2)_{58}$-GFP flies [34]. Finally, in the $(G_4C_2)_{48}$ model, 6 stop codons were engineered into 3 different reading frames 5′ adjacent to 48 copies of uninterrupted G_4C_2 repeats in the *pUAST* vector containing the same 3′UTR as all the other fly models mentioned above (Figure 1) [35]. The production of DPR proteins in this fly model was not examined [35].

Drosophila Models of DPR Protein Toxicity

Are DPR proteins sufficient to cause toxicity in *C9ORF72*-ALS/FTD? To answer this important question, several groups created *Drosophila* models of DPR protein toxicity by optimizing codons to disrupt RNA G-quadruplex structure and to express individual DPR proteins of different lengths in *Drosophila* through conventional AUG-initiated translation. These models include 36 and 100 copies of GA, GR, PA, and PR [32]; GFP-tagged 50 copies of GA, PA, and PR [37]; Flag-tagged 80 copies of GA, GR and PR [38]; GFP-tagged

47 copies of GP, and 50 copies of GA and GR [34]; and Flag-tagged 25 or 50 copies of GA, GR, PA, and PR [39]. Unlike the expanded G_4C_2 repeat models described above, these DPR protein models differ only in their length and tag. It is worth noting that in *C9ORF72* patients, the exact length and composition of different DPR proteins remain unknown, and the expression level of each DPR protein may vary by anatomical region and in different neuronal cell types as a result of different rates of production and degradation. Moreover, sensitive ELISA assays to quantify the level of each DPR protein remain to be developed. Thus, *Drosophila* models overexpressing DPR proteins of different lengths have their limitations but are likely to be informative.

Insights from *Drosophila* Models of *C9ORF72* Repeat Expansion

RNA foci versus DPR toxicity

The relative contributions of repeat-containing RNA foci versus DPR proteins to disease pathogenesis were one of the first issues addressed in the *Drosophila* models of G_4C_2 repeat expansion described above. Expression of *$(G_4C_2)_{36}$* or *$(G_4C_2)_{103}$* in the eye caused neurotoxicity and rough eye phenotypes, and regulated expression of these constructs in adult postmitotic neurons reduced life span [32]. On the other hand, stop codons inserted into G_4C_2 repeat sequences to stop DPR protein abolished repeat toxicity [32]. Although repeat RNA toxicity cannot be completely ruled out since these stop codons may change the secondary structure of the repeat-containing mRNA, these results suggest that DPR proteins generated from these repeats are the dominant toxic agents in this fly model [32]. Consistent with this notion, expression of poly(GR) and poly(PR), but not other DPR proteins, in the fly eye causes retinal degeneration [32, 34, 37, 38]. Moreover, interrupted antisense RNA containing about 100 copies of G_2C_4 repeats that cannot be translated into DPR proteins does not elicit toxicity in *Drosophila* adult neurons [40].

In contrast to *$(G_4C_2)_{103}$* flies, flies with intronic *$(G_4C_2)_{160}$* expression that were grown at 25°C had no obvious toxicity in the eye or reductions in dendritic branching, locomotor activity, or life span despite the abundance of nuclear RNA foci, consistent with very low level expression of poly(GP) (and presumably other DPR proteins as well) [33]. Thus, the subcellular localization and toxicity of G_4C_2 repeats are heavily influenced by their molecular context (Figure 2). In intronic *$(G_4C_2)_{160}$* flies grown at 29°C, however, life span was modestly reduced and correlated with a 4-fold increase in poly(GP) production; the number of nuclear G_4C_2 RNA foci in neurons or glial cells remained the same [33]. Thus, DPR proteins appear to be primarily responsible for disease phenotypes caused by intronic *$(G_4C_2)_{160}$*.

Although results from *Drosophila* models cannot always be extrapolated to human patients, recent studies of postmortem tissues from *C9ORF72* patients indicate that RNA foci are not the determinant of the clinicopathological phenotypes [41]. Moreover, among all DPR proteins, only poly(GR) seems to be significantly abundant in clinically related brain areas and correlates well with neurodegeneration [42]. Thus, it is critically important to further understand how poly(GR) alone causes neurodegeneration. On the other hand, it is highly likely that multiple DPR proteins are expressed in the same patient neuron. Indeed, poly(GA) can recruit poly(GR) into p62-positive aggregates in *Drosophila* cells [38]. To

further investigate this issue, *Drosophila* will be an excellent model system for determining how different DPR proteins interact with each other.

Nucleocytoplasmic transport

Expression of $(G_4C_2)_{28}$-GFP and $(G_4C_2)_{58}$-GFP in a tissue-specific manner causes repeat-length- and dosage-dependent retinal degeneration in the fly eye and morphological and functional deterioration in motor neurons [34]. A collaborative large-scale genetic screen in this fly model showed that several components of the nuclear pore complex, including Nup50, Nup152, Nup107, and Nup160, enhance or suppress G_4C_2 repeat toxicity [34]. Moreover, other genes that function in RNA export and protein import were identified as genetic modifiers, highlighting the central role of nucleocytoplasmic transport in G_4C_2 repeat toxicity (Figure 3). Indeed, $(G_4C_2)_{58}$-GFP expression compromises the integrity of the nuclear envelope of *Drosophila* salivary gland cells [34]. Because poly(GR), but not poly(GA) or poly(GP), also causes retinal degeneration in the fly eye [32, 34, 37, 38], it is likely that poly(GR) detected in $(G_4C_2)_{58}$-GFP is at least partly responsible for the nucleocytoplasmic transport defects. Indeed, independent studies in yeast and fly by other groups also identified proteins involved in nucleocytoplasmic transport as strong genetic modifiers of toxicity induced by arginine-containing DPR proteins [39, 43] (Figure 3).

In a study of proteins that bind to G_4C_2 repeat RNA in vitro, overexpression of Ran GTPase-activating protein (RanGAP) suppressed the rough eye phenotype induced by $(G_4C_2)_{30}$-EGFP, and several other nucleocytoplasmic transport genes also genetically modified repeat toxicity [36]. RanGAP is a guanine exchange factor that catalyzes the hydrolysis of Ran-GTP to Ran-GDP, which mediates the import of proteins into the nucleus [44]. In *Drosophila* salivary gland cells expressing $(G_4C_2)_{30}$-EGFP, the nuclear:cytoplasmic ratio of shuttling proteins such as Ran and TDP-43 is decreased [36]. Since RanGAP directly binds to G_4C_2 quadruplex RNA in an in vitro assay, and poly(GP) and poly(GR) were not detected in $(G_4C_2)_{30}$-EGFP flies grown at ambient temperature, it was proposed that G_4C_2 repeat RNA is toxic in part because it sequesters RanGAP [36] (Figure 3). It is not known whether poly(GA)-containing protein is expressed in this fly model or why the RNA toxicity in this model, in which 30 copies of G_4C_2 repeats are interrupted by the *Xho*I restriction site, differs so drastically from that in flies expressing G_4C_2 repeat RNA interrupted by stop codons [$(G_4C_2)_{36}$-RO] [32].

RNP granules

Like RanGAP [36], Pur-alpha—a DNA/RNA binding protein that participates in stress granule formation and mRNA transport and translation in neuronal dendrites [45–47]—interacts with G_4C_2 repeat RNA in vitro [31]. In $(G_4C_2)_{30}$-EGFP flies, the rough eye phenotype was mitigated by overexpression of Pur-alpha, suggesting that it, too, modifies repeat toxicity [31]. A potential detrimental role for G_4C_2 repeats in dendrites was suggested by the finding that expression of $(G_4C_2)_{48}$ in *Drosophila* dendritic arborization neurons led to branching defects that were modified by RNA transport proteins such as FMR1 and Orb2 [35]. Since poly(GR) can itself decrease dendritic branching [38], poly(GR) produced through RAN translation might conceivably be at least partly responsible for dendritic defects, in addition to toxicity caused by G_4C_2 repeat RNA in neuronal transport granules.

Independent proteomics analyses show that arginine-containing DPR proteins bind preferentially to many RNA binding proteins, including components of stress granules (SGs) [48–52]. Genes encoding many of these DPR-interacting proteins enhance or suppress poly(GR) or poly(PR) toxicity in the *Drosophila* eye [48]. Similarly, a study using APEX proximity labeling and mass spectrometry identified ~150 previously unknown human SG proteins, some of which were stress- and cell type-dependent [53]. Moreover, fly homologues of some of these newly identified SG genes genetically modified poly(GR) toxicity in *Drosophila* cells [53]. Thus, dysregulation of SG dynamics and function may be a central pathogenic mechanism in *C9ORF72*-ALS/FTD.

Induced Pluripotent Stem Cell (iPSC) Models of *C9ORF72*-ALS/FTD

In addition to *Drosophila*, induced pluripotent stem cell (iPSC) lines derived from skin fibroblasts or other cell types is a complementary and powerful approach, since these iPSC lines can be differentiated into different cell types such as cortical or motor neurons or glial cells, and disease-causing mutations are expressed in their native genetic and molecular contexts [54, 55]. Human neurons differentiated from iPSCs can be used to reveal many disease-relevant molecular and cellular phenotypes, including some age-related phenotypes. On the other hand, the selective vulnerability of different brain regions is challenging to address in iPSC models of neurodegenerative diseases. Moreover, most if not all iPSC differentiation protocols lead to the production of relatively immature human neurons that may not fully recapiculate the aging process of mature neurons in vivo [56]. Nonetheless, shortly after *C9ORF72* repeat expansion was discovered, three laboratories independently generated iPSC lines from *C9ORF72*-ALS/FTD patients [7–9]. These and additional iPSC lines generated by other labs have been differentiated into cortical or motor neurons, and studies of these cells have provided important insights into disease mechanisms.

RNA Foci Toxicity in *C9ORF72* iPSC-Derived Human Neurons

Both nuclear RNA foci and DPR proteins are found in human cortical and motor neurons differentiated from multiple *C9ORF72* iPSC lines [7, 8, 24, 49, 57, 58]. Although RNase H-mediated degradation of repeat RNAs by antisense oligonucleotides appears to be beneficial in *C9ORF72* iPSC-derived neurons and *C9ORF72* BAC transgenic mice, treatment with antisense oligonucleotides reduces the levels of both repeat RNA and DPR protein production in neuronal and non-neuronal cells [8, 14, 24, 59, 60]. It is unclear therefore whether the beneficial effects are mediated by loss of either repeat RNA or DPR proteins. Some small molecules that bind to expanded G_4C_2 RNA and reduce the number of RNA foci also decrease DPR production in *C9ORF72* iPSC-derived neurons or neurons directly transdifferentiated from fibroblasts [58, 61]. Thus, the relative toxicities of RNA foci and DPR proteins in human neurons are still unknown.

In vitro biochemical studies have identified a large number of proteins that can bind to expanded G_4C_2 repeat RNA in cell lysates [7, 8, 31, 62–68]. *C9ORF72* iPSC-derived human neurons have been widely used to examine the extent of sequestration of some of these proteins in G_4C_2 RNA foci. For instance, proteins reported to partially colocalize with RNA foci in *C9ORF72* iPSC-derived human neurons and patient brain tissues include ADARB2

[8], hnRNP-A1 and Pur-α [9], nucleolin [67], RanGAP [36], and hnRNP-H [63, 65, 68]. It is not known whether any of these proteins play a key role in neurodegeneration mediated by repeat RNA toxicity as shown for Muscleblind in myotonic dystrophy [19].

Nucleocytoplasmic Transport Defects in *C9ORF72* iPSC-Derived Human Neurons

Nucleocytoplasmic transport defects reported in several *Drosophila* models of *C9ORF72*-ALS/FTD have been confirmed in *C9ORF72* iPSC-derived neurons and patient brain samples, further supporting the role of this gene in disease pathogenesis. Nuclear retention of RNAs has been observed in *C9ORF72* iPSC-derived cortical neurons, indicating the impairment of nuclear RNA export [34]. In *C9ORF72* iPSC-derived neurons or neurons trandifferentiated from *C9ORF72* fibroblasts, the nuclear:cytoplasmic ratio of Ran and RanGEF (RCC1) is decreased, leading to defects in nuclear import of proteins [36, 43]. Some defects related to nucleocytoplasmic transport were observed in *C9ORF72* iPSC-derived neurons but not in neurons generated from direct conversion [36, 43], probably because of differences in differentiation protocols and/or the age of the neurons. The exact mechanism of nuclear transport defects in *C9ORF72* neurons is unclear, although both G_4C_2 RNA-mediated RanGAP depletion and DPR-mediated disruption of nuclear pore complex have been suggested as potential mechanisms (Figure 3). Altered nucleocytoplasmic transport in iPSC-derived motor neurons from sporadic ALS cases and ALS patients with TDP-43 mutations has been attributed to TDP-43 pathology [69], providing further evidence that nucleocytoplasmic transport defects are a common pathogenic mechanism in ALS/FTD. In fact, nucleocytoplasmic transport is also impaired in iPSC-derived neurons from patients with Huntington disease and in mouse models of the disease [70, 71].

Other Insights from *C9ORF72* iPSC-Derived Human Neurons

Autophagy and Lysosomes

One of the first cellular defects revealed in *C9ORF72* iPSC-derived human neurons was compromise of the autophagy pathway [7]. Autophagy is a cytoplasmic bulk degradation pathway in which misfolded proteins and damaged organelles are transported to lysosomes for degradation, a process altered in many neurodegenerative diseases [72]. *C9ORF72* iPSC-derived cortical neurons are more prone to cell death induced by autophagy inhibitors, and the level of the autophagy adaptor protein p62 is elevated in these cells [7]. This cellular defect could in part reflect partial loss of C9ORF72 function, since C9ORF72 protein has been implicated in the regulation of autophagy through binding to SMCR8 and ULK1 [17, 73–76]. Under starvation conditions, C9ORF72 is also localizes to the surface of lysosomes [75], and *C9orf72* knockout mice have enlarged lysosomes, especially in microglia [17]. Although *C9orf72* knockout mice do not exhibit obvious neurodegeneration [12–18], partial loss of *C9orf72* function, such as in microglia, may contribute to disease progression [16]. Indeed, C9ORF72 is required for lysosomal biogenesis in iPSC-derived motor neurons, and partial loss of C9ORF72 impairs the clearance of endogenous DPR proteins [77]. More importantly, ectopic C9ORF72 expression in motor neurons derived from patient iPSCs

improves survival, indicating that C9ORF72 haploinsufficiency contributes to disease in the presence of toxic DPR proteins [77].

Neuronal excitability

Another cellular phenotype found in *C9ORF72* iPSC-derived neurons is abnormal neuronal excitability. *C9ORF72* motor neurons fire fewer action potentials after 2 months in culture, and the decrease correlates with altered expression of several genes including *KCNQ3*, a regulator of neuronal excitability [24, 78]. However, hyperexcitability is observed in 2-week-old iPSC-derived motor neurons with *C9ORF72* repeat expansion or other ALS mutations, in part because of reduced delayed-rectifier potassium current amplitudes [78, 79], suggesting an age-dependent transition from hyper- to hypoexcitability, at least in this cellular model of *C9ORF72*-ALS/FTD. Hyperexcitability was proposed to induce calcium imbalance and subsequent excitotoxic cell death. Indeed, *C9ORF72* iPSC-derived motor neurons are more susceptible to glutamate toxicity [8] and show increased Ca^{2+}-permeable AMPA receptor expression [80]. Because iPSC-derived motor neurons are more similar to human fetal than adult spinal tissues [81], additional studies are needed to further investigate the misregulation of neuronal excitability in adult neurons of *C9ORF72*-ALS/FTD patients [82].

Nucleolar stress

The nucleolus is the site for ribosome biogenesis and also serves as a sensor for different types of stress. As a central hub for coordinated stress response, the nucleolus shows significant changes in morphology and composition [83]. Nucleolar stress in *C9ORF72*-ALS/FTD was first suggested by the aberrant subcellular localization of nucleolin in *C9ORF72* iPSC-derived motor neurons and patient motor cortex [68]. Subsequently, nucleolin mislocalization was found in *C9ORF72* BAC transgenic mice but without significant changes in ribosomal RNA processing or splicing [25]. DPR toxicity is likely at least partly responsible for nucleolar stress in *C9ORF72*-ALS/FTD since overexpressed poly(GR) or poly(PR) causes nucleolar stress such as nucleolar enlargement in both *Drosophila* and mammalian neuronal or nonneuronal cells [37, 38, 84, 85]. However, poly(GR) or poly(PR) does not seem to localize within the nucleolus in *C9ORF72* patient brains [86], suggesting an indirect effect on nucleolar stress. Moreover, the extent of nucleolar stress in *C9ORF72* patient brains remains to be further clarified [85, 86].

DNA damage

In many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and ALS, DNA damage is increased and the DNA damage response is altered [87]. Indeed, increased DNA double-strand breaks are found in 4-month-old but not 2-week-old *C9ORF72* iPSC-derived motor neurons [50]. Similar observations have been made in spinal motor neurons from *C9ORF72* ALS patients [87]. Expression of poly(GR) in human control iPSC-derived motor neurons or poly(GR) and poly(PR) in human neuroblastoma cells can increase DNA damage [50, 88], in part because inhibiting ROS production partially decreases poly(GR)-induced DNA damage and increases oxidative stress in human motor neurons or poly(GR) toxicity in flies [50]. Moreover, increased DNA damage results in elevated P53 in *C9ORF72* motor neurons, and partial reduction of P53 activity suppresses

poly(GR) toxicity in flies [50]. The R-loop structure formed by G_4C_2 DNA-RNA hybrid and poly(GA) might also help increase DNA damage [89]. It remains to be determined how an altered DNA damage response (DDR) pathway contributes to eventual neurodegeneration in *C9ORF72*-ALS/FTD.

Concluding Remarks

Disease-relevant cellular phenotypes have been identified in *C9ORF72* iPSC-derived motor or cortical neurons, and these cells have been increasingly used to confirm many phenotypes first observed in other experimental systems (Figure 4). For instance, these neurons also show vesicle trafficking defects [90], mislocalization of poly(GR)-interacting splicing factors [91], and changes in expression levels of genes of interest [38, 92, 93]. *C9ORF72* iPSC-derived neurons have also been used to confirm cell-to-cell transmission of DPR proteins [94] and to test beneficial effects of therapeutic drugs [95]. However, iPSC-derived motor neurons are more similar to human fetal spinal tissues than adult spinal tissues [56], and *C9ORF72* promoter methylation status may change during reprogramming [96]. Thus, novel experimental strategies are needed to establish better iPSC models of *C9ORF72*-ALS/ FTD, such as use of three-dimensional organoid cultures [97]. Nonetheless, approaches combining the power of genetic analyses in *Drosophila* and the disease relevance of iPSC-derived human neurons will undoubtedly continue to be useful both for addressing many scientific challenges (see Outstanding Questions) and for revealing important insights into pathogenic mechanisms and therapeutic targets of *C9ORF72*-ALS/FTD and other neurodegenerative diseases.

Outstanding Questions

What are the major toxic molecules in each *Drosophila* or mouse model of G_4C_2 repeat expansion and iPSC-derived neurons? How does partial loss of C9ORF72 function contribute to disease pathogenesis?

What is the composition of the G_4C_2 repeats in different *C9ORF72* patients? Are they pure repeats or interrupted by other nucleotide sequences? How are repeats expanded or retracted in somatic cells?

What is the template for endogenous DPR production, spliced intron RNA containing repeats or unspliced mRNA? How are sense and antisense repeat RNAs translated in patient cells? Accordingly, what are the N-terminus, C-terminus, and size of different DPR proteins in patient cells?

Are all DPR proteins expressed in the same patient neuron or glial cell? What is the relative contribution of each DPR protein to disease pathogenesis? How do different DPR proteins interact with each other? What molecular events trigger TDP-43 pathology?

Several cellular pathways have been implicated in the pathogenesis of *C9ORF72*-ALS/FTD. What is the relative contribution of alterations in each pathway in disease initiation and progression? Which potential disease mechanism triggers

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the pathogenic events? How do different dysregulated molecular pathways interact with each other during disease progression?

How does the repeat expansion in *C9ORF72* cause ALS in some carriers and FTD in others? Do other genetic or environmental factors contribute to the different manifestations of the same mutation?

What are the most predictive biomarkers for *C9ORF72*-ALS/FTD? Which therapeutic approaches are the most effective?

Acknowledgments

References

1. Hardiman O, et al. Amyotrophic lateral sclerosis. Nat Rev Dis Primers. 2017; 3:17071. [PubMed: 28980624]

2. Gao FB, et al. Dysregulated molecular pathways in amyotrophic lateral sclerosis-frontotemporal dementia spectrum disorder. EMBO J. 2017; 36:2931–2950. [PubMed: 28916614]

3. Olney NT, et al. Frontotemporal dementia. Neurol Clin. 2017; 35:339–374. [PubMed: 28410663]

4. DeJesus-Hernandez M, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron. 2011; 72:245–256. [PubMed: 21944778]

5. Renton AE, et al. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron. 2011; 72:257–268. [PubMed: 21944779]

6. Gijselinck I, 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. Lancet Neurol. 2012; 11:54–65. [PubMed: 22154785]

7. Almeida S, et al. Modeling key pathological features of frontotemporal dementia with C9ORF72 repeat expansion in iPSC-derived human neurons. Acta Neuropathol. 2013; 126:385–399. [PubMed: 23836290]

8. Donnelly CJ, et al. RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. Neuron. 2013; 80:415–428. [PubMed: 24139042]

9. Belzil VV, et al. Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic event detectable in blood. Acta Neuropathol. 2013; 126:895–905. [PubMed: 24166615]

10. Waite AJ, et al. Reduced C9orf72 protein levels in frontal cortex of amyotrophic lateral sclerosis and frontotemporal degeneration brain with the C9ORF72 hexanucleotide repeat expansion. Neurobiol Aging. 2014; 3:1779e5–1779.e13.

11. Xiao S, et al. Isoform-specific antibodies reveal distinct subcellular localizations of C9orf72 in amyotrophic lateral sclerosis. Ann Neurol. 2015; 78:568–583. [PubMed: 26174152]

12. Atanasio A, et al. C9orf72 ablation causes immune dysregulation characterized by leukocyte expansion, autoantibody production, and glomerulonephropathy in mice. Sci Rep. 2016; 6:23204. [PubMed: 26979938]

13. Burberry A, et al. Loss-of-function mutations in the C9ORF72 mouse ortholog cause fatal autoimmune disease. Sci Transl Med. 2016; 8:347ra93.

14. Jiang J, et al. Gain of toxicity from ALS/FTD-linked repeat expansions in C9ORF72 Is alleviated by antisense oligonucleotides targeting GGGGCC-containing RNAs. Neuron. 2016; 90:535–550. [PubMed: 27112497]

15. Koppers M, et al. C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits. Ann Neurol. 2015; 78:426–438. [PubMed: 26044557]

16. O'Rourke JG, et al. C9orf72 is required for proper macrophage and microglial function in mice. Science. 2016; 351:1324–1329. [PubMed: 26989253]

17. Sullivan PM, et al. The ALS/FTLD associated protein C9orf72 associates with SMCR8 and WDR41 to regulate the autophagy-lysosome pathway. Acta Neuropathol Commun. 2016; 4:51. [PubMed: 27193190]

18. Ugolino J, et al. Loss of C9orf72 enhances autophagic activity via deregulated mTOR and TFEB signaling. PLoS Genet. 2016; 12:e1006443. [PubMed: 27875531]

19. Mohan A, et al. RNA-protein interactions in unstable microsatellite diseases. Brain Res. 2014; 1584:3–14. [PubMed: 24709120]

20. Mori K, et al. The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. Science. 2013; 339:1335–1338. [PubMed: 23393093]

21. Ash PE, et al. Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. Neuron. 2013; 77:639–646. [PubMed: 23415312]

22. Zu T, et al. RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. Proc Natl Acad Sci U S A. 2013; 110:E4968–E4977. [PubMed: 24248382]

23. Gao FB, et al. Rethinking unconventional translation in neurodegeneration. Cell. 2017; (171):994–1000. [PubMed: 29149615]

24. Sareen D, et al. Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. Sci Transl Med. 2013; 5:208ra149.

25. O'Rourke JG, et al. C9orf72 BAC transgenic mice display typical pathologic features of ALS/FTD. Neuron. 2015; 88:892–901. [PubMed: 26637796]

26. Peters OM, et al. Human C9ORF72 hexanucleotide expansion reproduces RNA foci and dipeptide repeat proteins but not neurodegeneration in BAC transgenic mice. Neuron. 2015; 88:902–909. [PubMed: 26637797]

27. Liu Y, et al. C9orf72 BAC mouse model with motor deficits and neurodegenerative features of ALS/FTD. Neuron. 2016; 90:521–534. [PubMed: 27112499]

28. McGurk L, et al. *Drosophila* as an in vivo model for human neurodegenerative disease. Genetics. 2015; 201:377–402. [PubMed: 26447127]

29. Senturk M, Bellen HJ. Genetic strategies to tackle neurological diseases in fruit flies. Curr Opin Neurobiol. 2017; 50:24–32. [PubMed: 29128849]

30. Costa MDC, et al. Unbiased screen identifies aripiprazole as a modulator of abundance of the polyglutamine disease protein, ataxin-3. Brain. 2016; 139:2891–2908. [PubMed: 27645800]

31. Xu Z, et al. Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. Proc Natl Acad Sci U S A. 2013; 110:7778–7783. [PubMed: 23553836]

32. Mizielinska S, et al. C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. Science. 2014; 345:1192–1194. [PubMed: 25103406]

33. Tran H, et al. Differential toxicity of nuclear RNA foci versus dipeptide repeat proteins in a *Drosophila* model of C9ORF72 FTD/ALS. Neuron. 2015; 87:1207–1214. [PubMed: 26402604]

34. Freibaum BD, et al. GGGGCC repeat expansion in C9orf72 compromises nucleocytoplasmic transport. Nature. 2015; 525:129–313. [PubMed: 26308899]

35. Burguete AS, et al. GGGGCC microsatellite RNA is neuritically localized, induces branching defects, and perturbs transport granule function. Elife. 2015; 4:e08881. [PubMed: 26650351]

36. Zhang K, et al. The C9orf72 repeat expansion disrupts nucleocytoplasmic transport. Nature. 2015; 525:56–61. [PubMed: 26308891]

37. Wen X, 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. 2014; 84:1213–1225. [PubMed: 25521377]

38. Yang D, et al. FTD/ALS-associated poly(GR) protein impairs the Notch pathway and is recruited by poly(GA) into cytoplasmic inclusions. Acta Neuropathol. 2015; 130:525–535. [PubMed: 26031661]

39. Boeynaems S, et al. *Drosophila* screen connects nuclear transport genes to DPR pathology in c9ALS/FTD. Sci Rep. 2016; 6:20877. [PubMed: 26869068]

40. Moens TG, et al. Sense and antisense RNA are not toxic in Drosophila models of C9orf72-associated ALS/FTD. Acta Neuropathol. 2018; 135:445–457. [PubMed: 29380049]

41. DeJesus-Hernandez M, et al. In-depth clinico-pathological examination of RNA foci in a large cohort of C9ORF72 expansion carriers. Acta Neuropathol. 2017; 134:255–269. [PubMed: 28508101]

42. Saberi S, 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 Neuropathol. 2017; Epub ahead of print. doi: 10.1007/s00401-017-1793-8

43. Jovicic A, et al. Modifiers of C9orf72 dipeptide repeat toxicity connect nucleocytoplasmic transport defects to FTD/ALS. Nat Neurosci. 2015; 18:1226–1229. [PubMed: 26308983]

44. Moore MS. Ran and nuclear transport. J Biol Chem. 1998; 273:22857–22860. [PubMed: 9722501]

45. Kanai Y, et al. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron. 2004; 43:513–525. [PubMed: 15312650]

46. Johnson EM, et al. Role of Pur alpha in targeting mRNA to sites of translation in hippocampal neuronal dendrites. J Neurosci Res. 2006; 83:929–943. [PubMed: 16511857]

47. Daigle JG, et al. Pur-alpha regulates cytoplasmic stress granule dynamics and ameliorates FUS toxicity. Acta Neuropathol. 2016; 131:605–620. [PubMed: 26728149]

48. Lin Y, et al. Toxic PR poly-dipeptides encoded by the C9orf72 repeat expansion target LC domain polymers. Cell. 2016; 167:789–802. e12. [PubMed: 27768897]

49. Lee KH, et al. C9orf72 dipeptide repeats impair the assembly, dynamics, and function of membrane-less organelles. Cell. 2016; 167:774–788. e17. [PubMed: 27768896]

50. Lopez-Gonzalez R, 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. 2016; 92:383–391. [PubMed: 27720481]

51. Boeynaems S, et al. Phase separation of C9orf72 dipeptide repeats perturbs stress granule dynamics. Mol Cell. 2017; 65:1044–1055. e5. [PubMed: 28306503]

52. Yin S, et al. Evidence that C9ORF72 dipeptide repeat proteins associate with U2 snRNP to cause mis-splicing in ALS/FTD patients. Cell Rep. 2017; 19:2244–2256. [PubMed: 28614712]

53. Markmiller S, et al. Context-dependent and disease-specific diversity in stress granules formed from pre-existing protein interactions. Cell. 2018; 164:487–498.

54. Takahashi K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131:861–872. [PubMed: 18035408]

55. Mertens J, et al. Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. Nat RevNeurosci. 2016; 17:424–437.

56. Ho R, et al. ALS disrupts spinal motor neuron maturation and aging pathways within gene co-expression networks. Nat Neurosci. 2016; 19:1256–1267. [PubMed: 27428653]

57. Dafinca R, 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 aclerosis and frontotemporal dementia. Stem Cells. 2016; 34:2063–2078. [PubMed: 27097283]

58. Simone R, et al. G-quadruplex-binding small molecules ameliorate C9orf72 FTD/ALS pathology in vitro and in vivo. EMBO Mol Med. 2017; 10:22–31.

59. Lagier-Tourenne C, et al. Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. Proc Natl Acad Sci U S A. 2013; 110:E4530–E4539. [PubMed: 24170860]

60. Gendron TF, et al. Poly(GP) proteins are a useful pharmacodynamic marker for C9ORF72-associated amyotrophic lateral sclerosis. Sci Transl Med. 2017; 9 pii: eaai7866.

61. Su Z, et al. Discovery of a biomarker and lead small molecules to target r(GGGGCC)-associated defects in c9FTD/ALS. Neuron. 2014; 83:1043–1050. [PubMed: 25132468]

62. Reddy K, et al. The disease-associated r(GGGGCC)n repeat from the C9orf72 gene forms tract length-dependent uni- and multimolecular RNA G-quadruplex structures. J Biol Chem. 2013; 288:9860–9866. [PubMed: 23423380]

63. Lee YB, et al. Hexanucleotide repeats in ALS/FTD form length-dependent RNA foci, sequester RNA binding proteins, and are neurotoxic. Cell Rep. 2013; 5:1178–1186. [PubMed: 24290757]

64. Mori K, et al. hnRNP A3 binds to GGGGCC repeats and is a constituent of p62-positive/TDP43-negative inclusions in the hippocampus of patients with C9orf72 mutations. Acta Neuropathol. 2013; 125:413–423. [PubMed: 23381195]

65. Cooper-Knock J, et al. Sequestration of multiple RNA recognition motif-containing proteins by C9orf72 repeat expansions. Brain. 2014; 137:2040–2051. [PubMed: 24866055]

66. Haeusler AR, et al. C9orf72 nucleotide repeat structures initiate molecular cascades of disease. Nature. 2014; 507:195–200. [PubMed: 24598541]

67. Rossi S, et al. Nuclear accumulation of mRNAs underlies G4C2-repeat-induced translational repression in a cellular model of C9orf72 ALS. J Cell Sci. 2015; 128:1787–1799. [PubMed: 25788698]

68. Conlon EG, et al. The C9ORF72 GGGGCC expansion forms RNA G-quadruplex inclusions and sequesters hnRNP H to disrupt splicing in ALS brains. Elife. 2016; 5:e317820.

69. Chou, et al. TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. Nat Neurosci. Epub ahead of print.

70. Grima JC, et al. Mutant huntingtin disrupts the nuclear pore complex. Neuron. 2017; 94:93–107. e6. [PubMed: 28384479]

71. Gasset-Rosa F, et al. Polyglutamine-expanded huntingtin exacerbates age-related disruption of nuclear integrity and nucleocytoplasmic transport. Neuron. 2017; 94:48–57. e4. [PubMed: 28384474]

72. Menzies FM, et al. Autophagy and neurodegeneration: Pathogenic mechanisms and therapeutic opportunities. Neuron. 2017; 93:1015–1034. [PubMed: 28279350]

73. Sellier C, et al. Loss of C9ORF72 impairs autophagy and synergizes with polyQ Ataxin-2 to induce motor neuron dysfunction and cell death. EMBO J. 2016; 35:1276–1297. [PubMed: 27103069]

74. Yang M, et al. A C9ORF72/SMCR8-containing complex regulates ULK1 and plays a dual role in autophagy. Sci Adv. 2016; 2:e1601167. [PubMed: 27617292]

75. Amick J, et al. C9orf72 binds SMCR8, localizes to lysosomes, and regulates mTORC1 signaling. Mol Biol Cell. 2016; 27:3040–3051. [PubMed: 27559131]

76. Jung J, et al. Multiplex image-based autophagy RNAi screening identifies SMCR8 as ULK1 kinase activity and gene expression regulator. Elife. 2017; 6:E23063. [PubMed: 28195531]

77. Shi Y, et al. Haploinsufficiency leads to neurodegeneration in C9ORF72 ALS/FTD human induced motor neurons. Nat Med. 2018; 24:313–325. [PubMed: 29400714]

78. Devlin AC, et al. Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability. Nat Commun. 2015; 6:5999. [PubMed: 25580746]

79. Wainger BJ, et al. Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. Cell Rep. 2014; 7:1–11. [PubMed: 24703839]

80. Selvaraj BT, et al. C9ORF72 repeat expansion causes vulnerability of motor neurons to Ca2+-permeable AMAP receptor-mediated excitotoxicity. Nat Commun. 2018; 9:347. [PubMed: 29367641]

81. Ho R, et al. ALS disrupts spinal motor neuron maturation and aging pathways within gene co-expression networks. Nat Neurosci. 2016; 19:1256–1267. [PubMed: 27428653]

82. Schanz O, et al. Cortical hyperexcitability in patients with *C9ORF72* mutations: Relationship to phenotype. Muscle Nerve. 2016; 54:264–269. [PubMed: 26799151]

83. Boulon S, et al. The nucleolus under stress. Mol Cell. 2010; 40:216–227. [PubMed: 20965417]

84. Kwon I, et al. Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. Science. 2014; 345:1139–1145. [PubMed: 25081482]

85. Mizielinska S, et al. Bidirectional nucleolar dysfunction in C9orf72 frontotemporal lobar degeneration. Acta Neuropathol Commun. 2017; 5:29. [PubMed: 28420437]

86. Schludi MH, et al. Distribution of dipeptide repeat proteins in cellular models and C9orf72 mutation cases suggests link to transcriptional silencing. Acta Neuropathol. 2015; 130:537–555. [PubMed: 26085200]

87. Madabhushi R, et al. DNA damage and its links to neurodegeneration. Neuron. 2014; 83:266–282. [PubMed: 25033177]

88. Farg MA, et al. The DNA damage response (DDR) is induced by the C9orf72 repeat expansion in amyotrophic lateral sclerosis. Hum Mol Genet. 2017; 26:2882–2896. [PubMed: 28481984]

89. Walker C, et al. C9orf72 expansion disrupts ATM-mediated chromosomal break repair. Nat Neurosci. 2017; 20:1225–1235. [PubMed: 28714954]

90. Aoki Y, et al. C9orf72 and RAB7L1 regulate vesicle trafficking in amyotrophic lateral sclerosis and frontotemporal dementia. Brain. 2017; 140:887–897. [PubMed: 28334866]

91. Yin S, et al. Evidence that C9ORF72 dipeptide repeat proteins associate with U2 snRNP to cause mis-splicing in ALS/FTD patients. Cell Rep. 2017; 19:2244–2256. [PubMed: 28614712]

92. Sivadasan R, et al. C9ORF72 interaction with cofilin modulates actin dynamics in motor neurons. Nat Neurosci. 2016; 19:1610–1618. [PubMed: 27723745]

93. Coyne AN, et al. Post-transcriptional inhibition of Hsc70-4/HSPA8 expression leads to synaptic vesicle cycling defects in multiple models of ALS. Cell Rep. 2017; 21:110–125. [PubMed: 28978466]

94. Westergard T, et al. Cell-to-cell transmission of dipeptide repeat proteins linked to C9orf72-ALS/FTD. Cell Rep. 2016; 17:645–652. [PubMed: 27732842]

95. Imamura K, et al. The Src/c-Abl pathway is a potential therapeutic target in amyotrophic lateral sclerosis. Sci Transl Med. 2016; 9 pii: eaaf3962.

96. Esanov R, et al. C9orf72 promoter hypermethylation is reduced while hydroxymethylation is acquired during reprogramming of ALS patient cells. Exp Neurol. 2016; 277:171–177. [PubMed: 26746986]

97. Kawada J, et al. Generation of a motor nerve organoid with human stem cell-derived neurons. Stem Cell Reports. 2017; 9:1441–1449. [PubMed: 29107592]

Highlights

- The toxicity of expanded G_4C_2 repeats in *Drosophila* models is influenced by their molecular context.

- DPR proteins but not RNA foci are major toxic molecules in *Drosophila* models of G_4C_2 repeat expansion.

- Nucleocytoplasmic transport defects are found in both *Drosophila* models of G_4C_2 repeat expansion and neurons derived from *C9ORF72* iPSCs.

- Multiple interconnected molecular pathways are dysregulated in *C9ORF72*-ALS/FTD, such as autophagy, stress granule dynamics, oxidative stress DNA damage, nucleolar stress, and neuronal excitability.

- Experimental approaches that combine the genetic power of *Drosophila* and the disease relevance of iPSC-derived patient neurons are excellent strategies to help uncover pathogenic mechanisms in *C9ORF72*-ALS/FTD.
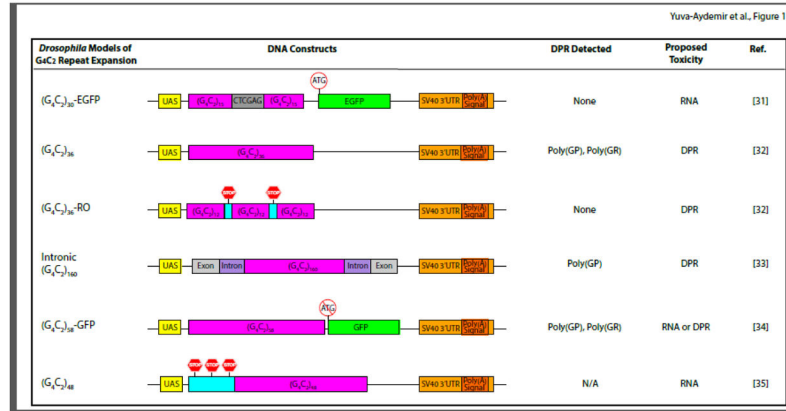
Figure 1. Schematic Representation of Different *Drosophila* Models of G$_4$C$_2$ Repeat Expansion
Major features of the DNA constructs used for each model are presented. In all *Drosophila* G$_4$C$_2$ models, the UAS-GAL4 system is used to overexpress G$_4$C$_2$ repeats with different lengthes, and these models share common SV40 3′UTR containing a polyadenylation signal. In the (G$_4$C$_2$)$_{30}$-EGFP model, repeats are interrupted in the middle by a six base pair sequence and followed by the *EGFP* coding region containing the ATG initiation codon. The (G$_4$C$_2$)$_{36}$ construct contains uninterrupted repeats, while the (G$_4$C$_2$)$_{36}$-RO construct harbors stop codons between every 12 repeats. The intronic (G$_4$C$_2$)$_{160}$ construct mimics the human *C9ORF72* locus, as 160 copies of repeat sequence are flanked by *C9ORF72* intronic sequences and adjacent exons. In the (G$_4$C$_2$)$_{58}$-GFP model, 58 copies of repeats are upstream to the GFP codon region but without the ATG start codon. The (G$_4$C$_2$)$_{48}$ construct contains stop codons in each frame 5′ to the repeat sequence. The three columns on the right list DPR proteins detected in these models when phenotypes are observed; the proposed mechanism of toxicity; and the respective references.
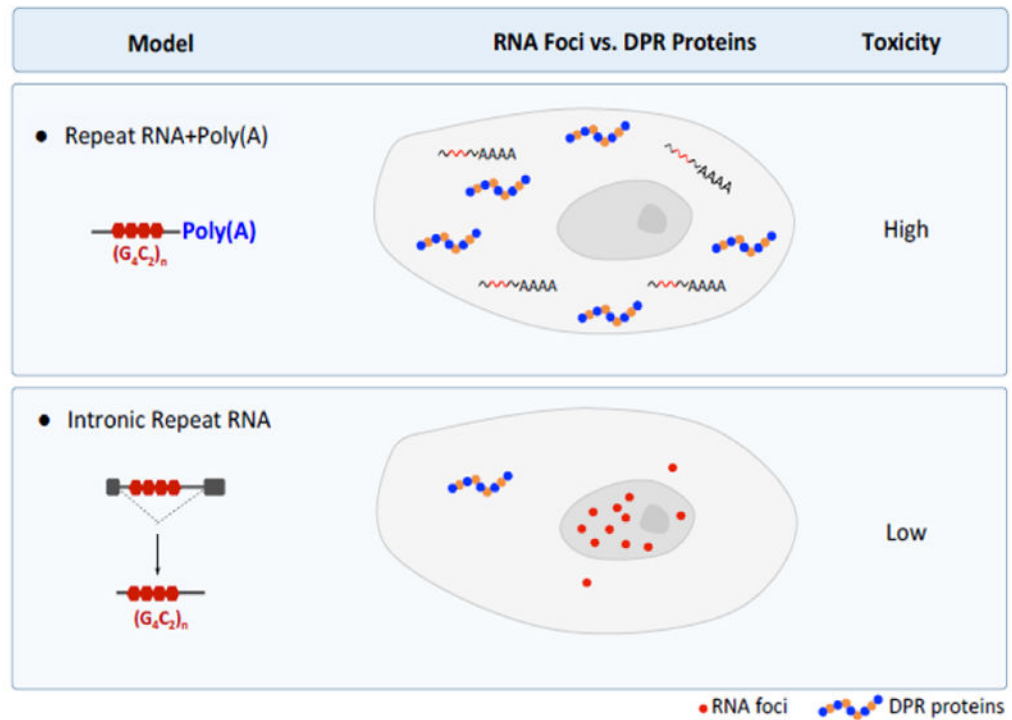
Figure 2. The Effect of Poly(A) Tail on the Subcellualr Localization and Toxicity of Expanded G_4C_2 Repeats

In $(G_4C_2)_n$-polyA models (top panel), repeat RNA is transported to the cytoplasm, where it is translated into DPR proteins. Diffuse G_4C_2 RNA is detected mostly in the cytoplasm, and higher toxicity correlate with high levels of DPR protein production. In intronic $(G_4C_2)_n$ models (bottom panel), repeat RNA accumulates in the nucleus as RNA foci and less DPR protein is produced. Thus, only low levels of toxicity are observed.
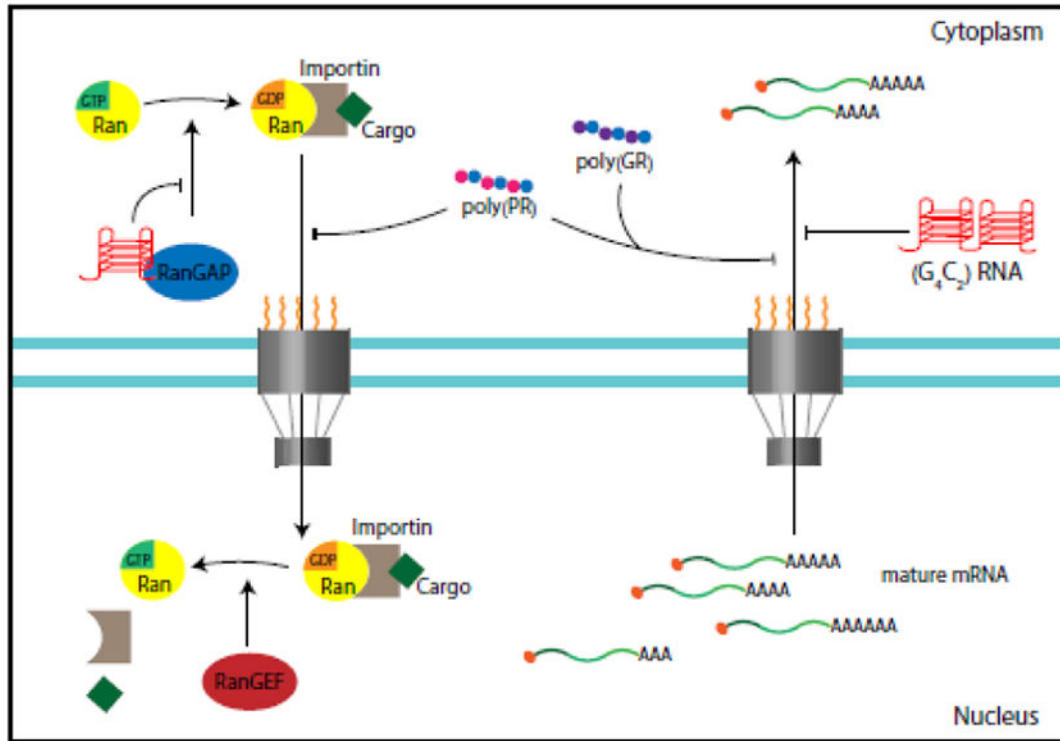
Figure 3. Overview of the Nucleocytoplasmic Transport Defects in *C9ORF72*-ALS/FTD
This cartoon summarizes the identified nuclear cytoplasmic transport defects in *Drosophila*, yeast and *C9ORF72* iPSC-derived neurons. G_4C_2 quadruplex RNA disrupts the RAN gradient and the nuclear import of proteins by binding to RanGAP, which catalyzes the conversion of Ran-GTP to Ran-GDP. Impairment of nuclear export caused by repeat RNA or DPR proteins leads to nuclear retention of RNA.
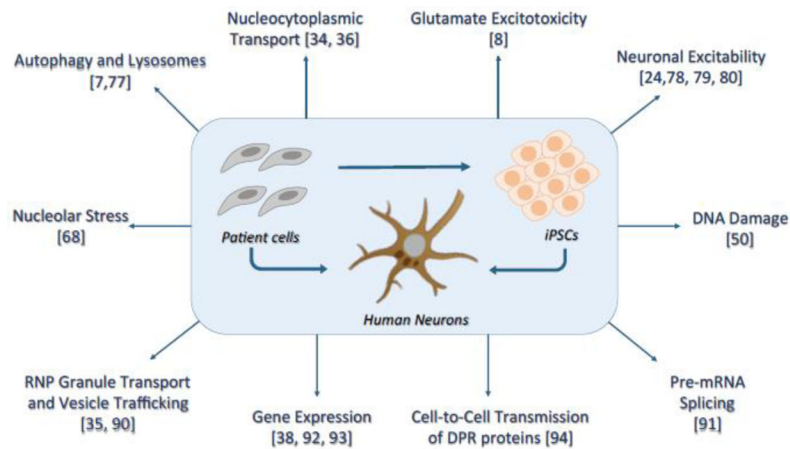
Figure 4. Insights from *C9ORF72* Patient-Derived Human Neurons

Currently, patient-derived human neurons can be obtained by direct conversion of patient cells to neurons or by reprograming of the patient cells into induced pluripotent stem cells (iPSCs) followed by differentiation into neurons (iPSC-derived neurons). Several disease-relevant cellular phenotypes have been identified in studies of neurons derived from *C9ORF72* patient. These include defects in autophagy and lysosome biogenesis, glutamate excitotoxicity, abnormalities in neuronal excitability, increased DNA damage, dysregulation of gene expression networks, vesicle trafficking defects, and nucleolar stress. In other instances, patient-derived neurons were used to confirm phenotypes observed first in other experimental systems. Examples include nucleocytoplasmic transport defects discovered in fly models, as well as pre-mRNA splicing and RNP granule transport defects and cell-to-cell transmission of di-peptide repeat (DPR) proteins discovered in other cell models.