Nucleocytoplasmic transport in C9orf72-mediated ALS/FTD

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\textbf{ABSTRACT}

A GGGGCC hexanucleotide repeat expansion in \textit{C9orf72} is the most common genetic cause of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Recent studies indicate that disruption of nucleocytoplasmic transport pathways play a critical role in the pathogenesis of \textit{C9orf72}-mediated ALS/FTD (C9-ALS). Here, we discuss mechanisms by which \textit{C9orf72} mutations cause nucleocytoplasmic transport deficits and contribute to disease pathogenesis. We review the current literature regarding nucleocytoplasmic transport disruption in C9-ALS, and discuss implications and directions for future research.

\textbf{KEYWORDS}

Amyotrophic Lateral Sclerosis (ALS); \textit{C9orf72}; Frontotemporal Dementia (FTD); hexanucleotide repeat expansion (HRE); Nucleocytoplasmic transport; Nuclear Pore Complex (NPC); Ran GTPase

\textbf{C9orf72-mediated ALS/FTD}

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects motor neurons of the brain and spinal cord causing progressive muscle weakness and atrophy.\textsuperscript{1} ALS shares clinical, neuropathological, and genetic features with frontotemporal dementia (FTD), a neurodegenerative disease affecting the frontal and/or temporal lobes of the brain that leads to progressive cognitive and behavioral impairments. Sporadic cases (sALS and/or sFTD) comprise ~90% of ALS or FTD, whereas ~10% are familial (fALS and/or fFTD). Increasingly, ALS and FTD appear to lie along a pathogenic spectrum, with ~15% of ALS patients developing FTD during the course of disease and ~15% of FTD patients developing ALS. Mutations in more than 10 genes have been shown to cause fALS, including \textit{SOD1} (superoxide dismutase 1),\textsuperscript{2} \textit{TARDBP} (TAR DNA-binding protein),\textsuperscript{3} \textit{FUS} (fused in sarcoma),\textsuperscript{4} \textit{hnRNPA1}/\textit{A2} (heterogeneous nuclear ribonuleoprotein),\textsuperscript{5} and \textit{C9orf72} (chromosome 9, open reading frame 72). A GGGGCC hexanucleotide repeat expansion (HRE) in the first intron of \textit{C9orf72} has recently been found to account for nearly half of fALS and a quarter of fFTD cases, and thus has become the focus of intense research.\textsuperscript{6,7} In addition, the \textit{C9orf72} HRE can cause both ALS and FTD in the same patient and/or family, providing compelling evidence that they share similar molecular mechanisms of pathogenesis.\textsuperscript{1}

The precise mechanism through which GGGGCC HRE causes ALS/FTD remains unclear despite intense investigation. Three hypotheses have been proposed: 1) loss of function of \textit{C9ORF72} due to disruption of transcription; 2) gain of toxicity caused by RNA repeats; 3) gain of toxicity caused by dipeptide protein repeats (DPRs) derived from repeat-associated, non-ATG translation (RAN translation) of the RNA.\textsuperscript{8-11} To date, the vast majority of studies support gain of toxicity as the major contributor to the pathogenesis, although whether such toxicity is caused by RNA and/or DPR remains unclear. Studies in \textit{Drosophila} demonstrate that arginine-containing DPRs are neurotoxic, and phenotypes correlate with level of DPR production.\textsuperscript{12,13} However, DPRs are easily detected within tissues generally unaffected in c9-ALS (e.g. cerebellum) and extremely difficult to detect in the spinal
cord, suggesting that the DPR aggregates are unlikely to be the primary cause of disease. Despite the uncertain mechanism, recent studies by several independent research groups, using multiple different model systems, all converge on a central role for nucleocytoplasmic transport disruption in C9-ALS pathogenesis.\(^{15-17}\)

**Cytoplasmic mislocalization and aggregation of nuclear proteins in ALS/FTD**

Protein aggregation is a pathological hallmark of neurodegenerative diseases including ALS/FTD, Alzheimer’s, Parkinson’s, and Huntington’s diseases.\(^8\) Cytoplasmic mislocalization and aggregation of TDP-43 (TAR DNA-binding protein of 43 kD) is observed in >95% of ALS cases and ~45% of FTD.\(^3\) Less commonly, aggregation of FUS\(^4\) and other hnRNPs\(^5\) are observed in ALS or FTD. These RNA-binding proteins normally regulate RNA metabolism within the nucleus; whereas in ALS/FTD, they are mislocalized and accumulate in the cytoplasm.\(^3-5\) Such cytoplasmic mislocalization and accumulation leads to at least two deleterious effects: 1) loss of function in the nucleus and 2) gain of toxicity in the cytoplasm.\(^1\)

One potential explanation for cytoplasmic mislocalization and aggregation of TDP-43 or other hnRNPs is that these proteins all contain both nuclear localization (NLS) and nuclear export (NES) signals, allowing them to normally cycle in and out of the nucleus. With aging and/or environmental stress, these proteins may aggregate within the cytoplasm and are therefore unable to be imported into the nucleus to perform their normal function.\(^1\) At least 3 lines of evidence support this hypothesis.\(^5,19,20\) First, these proteins all contain low complexity prion-like domains that are sensitive to phosphorylation and/or environmental changes that make them prone to aggregate. Second, mutations in their prion-like domains that make the proteins more likely to aggregate can cause familial ALS and/or FTD. Third, mutations that disrupt the nuclear import signal (NLS) of these proteins (especially FUS) leads to their cytoplasmic mislocalization and aggregation and causes familial ALS. As with other prion-like proteins such as \(\beta\)-amyloid, \(\alpha\) synuclein, and poly-glutamine proteins implicated in neurodegenerative diseases, TDP-43 and FUS have been proposed to cause progressive neurodegeneration by spreading from cell to cell.\(^21,22\) However, the machinery that drives and regulates the nucleocytoplasmic trafficking of these proteins is less understood. In new studies of \(C9ORF72\)-mediated ALS/FTD (C9-ALS or FTD), defects in nucleocytoplasmic trafficking may be a primary cause of the mislocalization/aggregation of nuclear proteins, including TDP-43.

**RanGAP and nucleoporins in C9-ALS**

A forward genetic screen of putative GGGGCC HRE RNA-binding proteins in *Drosophila*\(^8\) identified RanGAP (RanGTPase activitating protein, the *Drosophila* homolog of mammalian RanGAP1) as a potent modifier of GGGGCC-mediated neurodegeneration.\(^15\) Overexpression of RanGAP or a single copy of a gain-of-function allele of RanGAP (RanGAP[SD]) markedly suppressed GGGGCC-mediated neurodegeneration whereas knockdown of RanGAP enhanced it. Given its essential role in regulating nucleocytoplasmic transport, these findings suggested that the nucleocytoplasmic transport pathway is an important modulator of C9-ALS pathology. Indeed, other proteins in the same pathway, including RanGEF (Ran guanine nucleotide exchange factor), importin \(\alpha\), and exportin 1 (see Fig. 1), also genetically modify HRE-mediated neurodegeneration. Consistent with these findings, unbiased genetic screens in *Drosophila* and yeast independently identified components of the

![Figure 1. HRE suppresses nucleocytoplasmic transport. (A) Importin proteins are associated with RanGTP that are converted to RanGDP in the presence of RanGAP. Importin proteins are dissociated from RanGDP and are able to import cargos including TDP-43. GGGGCC repeats sequester RanGAP and inhibit the import process. (B) RanGEF converts RanGDP to RanGTP in the nucleus. (C) Exportin exports cargos in the presence of RanGTP. Proteins such as TDP-43 contains both nuclear localization sequences (NLS) and nuclear export sequences (NES) and are cycled between both import and export processes. Cytoplasmic mislocalization of TDP-43, due to insufficient import, causes neuronal toxicity.](image-url)
nuclear pore complex (NPC) as additional genetic modifiers of HRE-mediated neurodegeneration.\textsuperscript{16,17}

Macromolecules enter and exit the nucleus through the nuclear pore complex (NPC), a large protein complex consisting of \( \sim 30 \) subunits called nucleoporins (Nups).\textsuperscript{23} Molecules smaller than \( \sim 40 \) kilo Daltons (kD) can passively cross the NPC, whereas those larger than \( \sim 40 \) kD need active transport powered by GTP hydrolysis of Ran GTPase (Ran, Fig. 1). In the presence of RanGAP in the cytoplasm, Ran[GTP] is converted to Ran[GDP], thereby activating import carriers, e.g., importins, to bind the NLS of cargos and import them from the cytoplasm into the nucleus.\textsuperscript{24} In the nucleus, RanGEF catalyzes Ran to exchange GDP for GTP, activating export carriers, e.g. exportins, to bind the NES of cargos and export them from the nucleus to the cytoplasm. Therefore, the localization, abundance, and nucleotide-binding state of Ran GTPase provides the driving force for nucleocytoplasmic transport. Given an abundance of HRE RNA within cells of C9-ALS patients and our observation that the HRE consisting of 6 or more GGGGCC repeats can directly interact with RanGAP\textsubscript{1}, we hypothesize that the HRE directly sequesters RanGAP\textsubscript{1} (Fig. 1A). Indeed, RanGAP\textsubscript{1} forms accumulations that co-localize with some RNA foci in C9-ALS patient postmortem brain tissue, cultured neurons differentiated from iPS (induced pluripotent stem) cells derived from C9-ALS patients, and \textit{Drosophila} cells expressing GGGGCC repeats.\textsuperscript{15} These observations, together with the genetic interaction analyses, suggest that mislocalized/accumulated RanGAP is unable to activate Ran GTPase to hydrolyze GTP, and therefore fails to activate nuclear import.

As RanGAP is normally tethered to the cytoplasmic surface of the NPC via its interaction with a nucleoporin (Nup), RanBP2 (also called Nup358), RanGAP mislocalization in C9-ALS suggests that there may be additional defects in the NPC. Indeed, several nucleoporins also accumulate in C9-ALS patient postmortem brain tissue and iPS neurons,\textsuperscript{15} including NPC scaffolding proteins Nup205 and Nup107.\textsuperscript{25} The NPC can be divided into 5 main structural/functional compartments: cytoplasmic ring and filaments, central channel, scaffold, nuclear ring and basket, and transmembrane. One important future question is which nucleoporins and NPC functions are affected in C9-ALS. In addition, it is important to understand the sequential events in the interplay of the HRE and NPC: is nucleoporin pathology caused by the HRE-mediated sequestration and loss-of-function of RanGAP\textsubscript{1}? Is RanGAP\textsubscript{1} mislocalization a consequence of the HRE being stuck within the NPC during export? Or is nucleoporin pathology a result of dipeptide repeat disruption of the pore? Indeed, a recent study does suggest that GA dipeptide repeat can itself cause mis-localization of RanGAP\textsubscript{1} and Pom121.\textsuperscript{26} One way that we are seeking to elucidate the molecular mechanism is to determine the structural changes of the NPCs in C9-ALS, using super-resolution\textsuperscript{27} and electron microscopy.\textsuperscript{28}

### Nucleocytoplasmic transport deficits precede TDP-43 proteinopathy in C9-ALS

The substrate of RanGAP\textsubscript{1}, Ran GTPase, is primarily a nuclear protein, and its nuclear localization is required for proper nucleocytoplasmic transport. Since only Ran[GDP] is imported into the nucleus, if RanGAP\textsubscript{1} is dysfunctional, the Ran gradient is disrupted, inhibiting both nuclear import and export. Indeed, in both fly and iPS models of C9-ALS, Ran is mislocalized to the cytoplasm.\textsuperscript{15} In addition, live imaging of nucleocytoplasmic transport dynamics using FRAP of an NLS-tagged fluorescent protein shows a \( \sim 50\% \) decrease in protein import in C9-ALS iPS neurons, providing direct evidence that protein import is impaired.

The mislocalization of RanGAP\textsubscript{1} and nucleoporins\textsuperscript{15} seen in C9-ALS iPS neurons, together with the ability of RanGAP\textsubscript{1} to physically interact with the HRE,\textsuperscript{15} raises the possibility that defects in nucleocytoplasmic transport machinery are an early event in C9-ALS pathogenesis. These cells exhibit cytoplasmic mislocalization of TDP-43 without forming cytoplasmic punctae, suggesting that deficits in nucleocytoplasmic transport occur prior to formation of TDP-43 aggregates. In addition, the phenotypic severity of TDP-43 mislocalization correlates with the severity of Ran GTPase mislocalization, consistent with TDP-43 requiring Ran for its nucleocytoplasmic transport.\textsuperscript{29} Hence, defects in nucleocytoplasmic transport may be the primary cause of TDP-43 cytoplasmic aggregation in C9-ALS (Fig. 1C).

One question is whether a disruption of nucleocytoplasmic transport underlies the mislocalization of TDP-43 in other familial and sporadic forms of ALS. Indeed, we have observed RanGAP\textsubscript{1} mislocalization in postmortem brain tissue from some sALS patients
(unpublished data), suggesting that nucleocytoplasmic transport deficits may be a primary cause of TDP-43 proteinopathy in these cases. Interestingly, many nucleoporins are among the longest-lived proteins in post-mitotic cells due to the lack of turnover, which makes NPCs more likely to accumulate damage with aging. As patients age, NPCs deteriorate, potentially because of environmental exposures such as oxidative stress. In this way, once impaired NPC function reaches a certain threshold with aging, TDP-43 may begin to aggregate within the cytoplasm, leading to cytotoxicity and spread to adjacent cells.

Another question is whether nucleocytoplasmic transport of RNA is also disrupted. RNA metabolism plays an important role in ALS pathogenesis, and mutations of the RNA export factor Gle1 cause juvenile motor neuron diseases. Indeed, many nuclear RNAs are retained in the nucleus in C9-ALS fly models, in HEK293 cells overexpressing GGGGCC repeats, and in iPS neurons derived from C9-ALS patients. This raises several questions. First, how does mRNA mislocalization affect protein synthesis, and which proteins are most affected? Second, is RNA accumulation in the nucleus toxic? Does it contribute to nuclear stress that has been implicated in C9-ALS? Third, are the RNA deficits up- or down-stream of or in parallel with TDP-43 mislocalization? Future studies are required to address these questions.

DPRs have also been shown to affect nucleocytoplasmic transport via an unknown mechanism, possibly due to an interaction between DPRs and the NPC. Although it remains unclear whether such defects caused by DPRs precede TDP-43 pathology, it has been suggested that DPR accumulation may precede TDP-43 accumulation in some C9-FTD patient brain. Together with the fact that genes in the nucleocytoplasmic transport pathway are genetic modifiers of DPR toxicity in yeast, these findings collectively raise the intriguing possibility that DPRs may disrupt nucleocytoplasmic transport independent of the HRE RNA.

**Concluding remarks and perspective**

Despite the uncertainty as to whether RNA, DPR, or both cause neurodegeneration in C9-ALS, several research groups have independently identified nucleocytoplasmic transport deficits in different C9-ALS animal models and patients. In addition, C9orf72 has been recently shown to encode 2 protein isoforms, one of which was identified to be perinuclear, suggesting the intriguing possibility that C9orf72 may play a normal function in nucleocytoplasmic transport. Hence, defects in nucleocytoplasmic transport may be the underlying cause of neurodegeneration, preceding and initiating the cytoplasmic aggregation of prion-like proteins including TDP-43 in both C9-ALS and some sporadic ALS cases. In addition, given that both HRE RNA and DPR repeats can disrupt nucleocytoplasmic transport, we aim to study nucleocytoplasmic transport in diseases mediated by other types of repeats, e.g., polyglutamine (polyQ) repeats. Indeed, a recent study has reported that cytoplasmic aggregation of amyloid β, Huntingtin-polyQ, or TDP-43 severely affect nucleocytoplasmic transport and cause mRNA retention in the nucleus, indicating a more general mechanism through which accumulations of macromolecules disrupt nucleocytoplasmic transport.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This work was supported by grants from NIH (R01 NS085207 and NS091046 to J.D.R. and R01 NS082563 to T.E.L.), Brain Science Institute, Robert Packard Center for ALS Research at Johns Hopkins, Muscular Dystrophy Association (J.D.R.), Alzheimer Drug Discovery Foundation (J.D.R.), and ALS Association (T.E.L., K.Z. and J.D.R.). K.Z. is a Milton Safenowitz fellow in the ALS Association. J.C.G. is a recipient of a National Science Foundation Graduate Research Fellowship Award and the Thomas Shortman Training Fund Graduate Scholarship.

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