COMMENTARY

Toxic RNA as a driver of disease in a common form of ALS and dementia

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A recent and very stimulating finding in the field of neurodegenerative disease is the discovery that expansion of a hexanucleotide microsatellite DNA repeat (GGGGCC) in an intron of the *C90RF72* gene is a common cause of two devastating neurodegenerative diseases for which there are no cures: frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (1, 2). With this observation arose a fascinating question—how does expansion of this repeat induce a disease that can manifest as ALS, a motor neuron disease, as well as FTD, a leading cause of presenile dementia? Adding to the mystery is the almost complete absence of information on the *C9ORF72* gene or the function of the protein it encodes. However, initial characterization of *C9ORF72*-FTD/ALS combined with work on some other neurodegenerative diseases suggests three pathogenic mechanisms (Fig. 1). First is the evidence indicating that mRNA levels of *C9ORF72* transcripts are decreased in *C9ORF72*-FTD/ALS (1–3). Thus, a loss of *C9ORF72* function might contribute to disease. Second, accumulation of RNA transcripts containing the GGGGCC repeat within nuclear foci in frontal cortex and spinal cord material in *C9ORF72*-FTD/ALS patients suggests a toxic RNA gain of function (1). This pathogenic mechanism is patterned after other noncoding expansion disorders where RNA foci lead to the sequestration and altered activity of RNA-binding proteins (4). A third possible pathogenic mechanism is repeat-associated non-ATG translation (RAN translation). RAN translation, a mode of translation that occurs in the absence of an initiating ATG codon, was first reported to occur across expanded CAG repeats to produce potentially toxic homopolymeric peptides (5). It is among these competing possibilities that now enters the work of Xu et al. in PNAS (6), showing that expression of the expanded *C9ORF72* repeat is sufficient to cause neurodegeneration and that its ability to sequester the RNA-binding protein Pur α likely has a role in repeat-mediated neurodegeneration.

Using both mammalian neuronal cells and the fly *Drosophila melanogaster*, Xu et al. (6) first show that expression of an expanded GGGGCC repeat is sufficient to cause toxicity. This is a seminal observation because it directs one's attention to the GGGGCC repeat itself regarding pathogenesis (i.e., pathways II and III, Fig. 1). In the case of the neuronal cell study, either a control ($GGGGCC_3$) or an expanded ($GGGGCC_{30}$) repeat was inserted into an EGFP expression vector between the start site of transcription and the translational ATG start codon. Only the neuronal Neuro-2a cells expressing the vector containing the expanded repeat exhibited a significant reduction in viability. To examine toxicity in vivo, Xu et al. used a well-established *Drosophila* genetic approach whereby expression of either a control or expanded GGGGCC repeat was directed to a variety of neuronal tissues. Flies expressing an expanded repeat showed significant signs of neuronal death. In both a cellular model and an animal model, expression of an expanded GGGGCC repeat was sufficient to cause damage. Two additional experiments using the *Drosophila C9ORF72* disease model reveal that damage to retinal photoreceptors is more pronounced in older flies and that motor neurons, the primary disease target in ALS, undergo a progressive degeneration upon expression of an expanded GGGGCC repeat. These latter two results indicate that two seminal features of human *C9ORF72* disease, age dependence of disease and susceptibility of motor neurons, are replicated with expression of expanded GGGGCC repeat in flies.

Therefore, how might an expanded GGGGCC repeat alter cellular function to cause disease? Building on this group's experience with another DNA repeat-based disorder Fragile X tremor/ataxia syndrome (FXTAS), Xu et al. (6) looked at whether the capability of repeat containing RNA to bind normal RNA-binding proteins was enhanced; i.e., could *C9ORF72* disease be one of the disorders where RNA foci lead to the sequestration and altered activity of RNA-binding proteins?

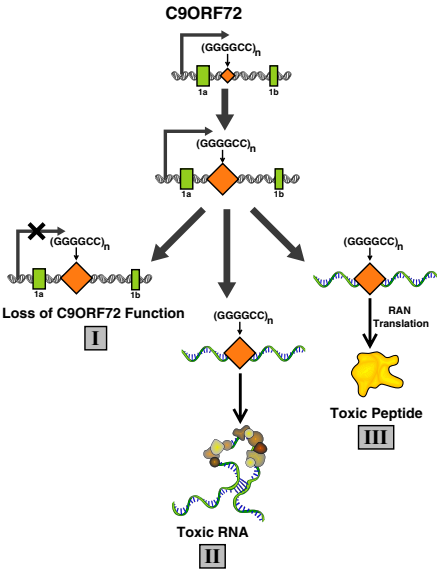

Fig. 1. Scheme depicting the three major putative mechanisms underlying expanded *C9ORF72* repeat ALS/FTD. Pathway I involves a loss of function of *C9ORF72* and/or other nearby genes. Both mechanisms II and III involve a toxic gain of function, with pathway II consisting of a toxic RNA and in III, toxicity being attributable to a protein or peptide.

Consistent with a RNA-mediated pathogenic mechanism, the authors show that synthesized GGGGCC repeat-containing RNAs are able to bind proteins in extracts prepared from mouse spinal cord. To identify proteins binding to GGGGCC repeat-containing RNA, they separated the bound proteins using polyacrylamide gel electrophoresis. Individual proteins were excised and identified using mass spectrometry. Intriguingly, the most prevalent *C9ORF72* GGGGCC-binding proteins found were several members of the Pur family of RNA-binding proteins, with Pur α being the most abundant. Intriguingly, Pur α was previously found to interact with expanded CGG repeat-containing *FXTAS* RNA and modulated disease in a *Drosophila* model of FXTAS (7).

Binding of Pur α to GGGGCC repeats was examined in vitro using GST-tagged Pur α

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and radiolabeled GGGGCC repeats (6). Both mouse and *Drosophila* Pur α bound with similar affinities, K_d of 14.8 and 5.1 nM, respectively. Xu et al. further show that a tagged version of *C9ORF* GGGGCC-repeat RNA could interact with endogenous Pur α in Neuro-2a cells and in mouse and human brain extracts. The brain extract assay found no evidence that TAR DNA-binding protein (TDP)-43, another RNA-binding protein implicated in ALS/FTD, was able to bind *C9ORF* GGGGCC-repeat RNA. The heterogeneous nuclear ribonucleoprotein RNA-binding protein hnRNP A2/B1, which does bind to FXTAS CGG repeat-containing RNA, failed to bind to *C9ORF72* GGGGCC-repeat RNA using either the in vitro or the in vivo assay. Xu et al. also failed to find evidence of *C9ORF72* GGGGCC-repeat RNA binding to another hnRNP, hnRNP A3. However, the significance of their failure to detect *C9ORF72* GGGGCC-repeat RNA to any of the hnRNPs needs to be taken with caution because another study found that *C9ORF72* GGGGCC-repeat RNA bound to all three of these hnRNPs, with hnRNP A3 being detectable in the p62-positive, TDP-43–negative inclusions seen in the brains of patients with *C9ORF* disease (8). Xu et al. did find Pur α-positive inclusions in flies expressing expanded *C9ORF72* GGGGCC repeat. Importantly, Pur α was found in inclusions in mutant *C9ORF72* repeat carriers and in noncarriers with FTD-TDP.

Lastly, and perhaps one of the more informative results, is the authors' demonstration that overexpression of Pur α is able to rescue expanded *C9ORF72* repeat-induced neurodegeneration in the mammalian Neuro-2a neuronal cell system and *Drosophila* model of disease (6). Decreasing expression of Pur α in Neuro-2a cells in itself caused cell death. Moreover, overexpression of hnRNP A2/B1 (that, in this study, did not bind to the *C9ORF72* repeat) failed to rescue expanded GGGGCC-induced toxicity. These results provide strong evidence that, at least in these model systems, manipulating the binding of Pur α levels impacts toxicity in a manner consistent with the hypothesis that sequestration of Pur α by mutant *C9ORF72* repeats contributes to pathogenesis.

Collectively, Xu et al. (6) provide two major insights into understanding how an expanded *C9ORF72* GGGGCC repeat alters neuronal function and induces disease. First, this study nicely demonstrates in two model systems that expression of the *C9ORF72* expanded repeat itself, in the absence of other components of the *C9ORF72* gene, is sufficient to be toxic. This finding strongly argues in favor of pathogenic models based on expression of the expanded repeat. Xu et al. further show that the sequestration of the RNA-binding protein Pur α by *C9ORF72* RNA containing an expanded repeat contributes to pathogenesis in both the mammalian neuronal cell and *Drosophila* models of *C9ORF72* disease, thus providing support for toxic RNA contributing to disease (pathway II, Fig. 1). This latter point is particularly noteworthy because it provides direct evidence linking a putative pathogenic pathway to *C9ORF72* repeat-induced disease.

The work of Xu et al. exemplifies what is becoming an effective strategy for identifying the events that have a substantive contribution to disease.

Although the work of Xu et al. (6) provides several important steps toward understanding the mechanism by which *C9ORF72* disease develops, there are several key points remaining to be clarified. It remains a mystery why expansion of the *C9ORF72* repeat leads to a neurodegenerative disease that presents as ALS and/or FTD. Another more specific issue that Xu et al. note is that this work used models of *C9ORF72* disease where the size of the expanded repeats, 30 GGGGCC repeats, was limited by what was readily isolated and manipulated experimentally. Although this length is above the two- to eight-repeat-length tracts typically seen in unaffected individuals, it is far less than the 100–1,000 of hexanucleotide repeats seen in ALS/FTD patients (1, 2). One needs to remain open to the idea that expanded repeats so much longer than 30 GGGGCCs either might not sequester RNA-binding proteins and/or trigger another mechanism such as gene silencing at the *C9ORF72* locus or translation of a toxic peptide. In this regard, it is worth noting that two groups recently reported immunostaining for GGGGCC repeat encoded insoluble peptides in the brains of patients who succumbed to *C9ORF72* disease (9, 10). Whether these peptides actually contribute to *C9ORF72* remains to be shown. As seen for many of the other unstable DNA repeat/microsatellite disorders (11), *C9ORF72* GGGGCC expansion very likely will have several molecular outcomes. Importantly, the work of Xu et al. exemplifies what is becoming an effective strategy for identifying the events that have a substantive contribution to disease (i.e., the application of a cross-species spectrum of model systems for testing pathways fundamental to pathogenesis). It is through the identification of such pathways that the likelihood of developing effective therapeutic approaches will be enhanced.

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