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Evidence for RNA-mediated toxicity in the fragile X-associated tremor/ataxia syndrome

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Abstract

Fragile X premutation carriers are at risk for developing a late-onset, progressive neurodegenerative disorder termed fragile X-associated tremor/ataxia syndrome (FXTAS). A growing body of evidence suggests the characteristic excess CGG repeat containing *FMR1* mRNA observed in premutation carriers is pathogenic and leads to clinical features of FXTAS. The current model suggests premutation mRNA transcripts can induce the formation of intranuclear inclusions by the sequestration of RNA-binding proteins and other proteins. The sequestered proteins are prevented from performing their normal functions, which is thought to lead to the neuropathology-observed FXTAS. This paper discusses the existing evidence that microsatellite expansions at the level of RNA play a role in the disease pathogenesis of FXTAS and some of the approaches that may uncover downstream effects of expanded riboCGG expression.

Keywords

FXTAS; intranuclear inclusion; neurodegeneration; premutation allele; riboCGG-mediated toxicity; RNA-binding protein

The X-linked *FMR1* gene has a polymorphic CGG repeat located in its 5′ untranslated region (UTR). The alleles of the *FMR1* gene can be grouped into three distinct classes depending on the size of the repeat. Among individuals in the general population, repeat length ranges between five and 54 triplets, with the majority of X chromosomes carrying either 29 or 30 repeats. A second allele class is known as the full mutation, wherein upon expansion of the CGG repeat tract to more than 200 triplets, the repeat sequence itself and an adjacent CpG island become hypermethylated and associated histones are modified. As a result, *FMR1* transcription is silenced [1–7]. Thus, fragile X syndrome (FXS), the most commonly inherited form of mental retardation, results from the loss of function of *FMR1*. The mechanism for CGG repeat expansion is still under investigation, however, evidence suggests the expansion to the full mutation occurs in female gametes [8,9]. Male and female carriers of FXS represent the

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third allele class, known as the premutation allele. Premutation carriers have an intermediate number of CGG triplets between 55 and 200 repeats, which show no unusual methylation. Although both males and females can transmit premutation alleles to their offspring, only alleles obtained via maternal transmission expand to a full mutation and cause FXS in a single generation. Younger premutation carriers have normal cognitive abilities and are not affected with FXS. However, reports of tremor and cognitive decline in grandfathers of FXS families with known probands sparked further study of older males carrying premutation alleles in families where FXS was present. Case studies of male premutation carriers led to the description of a new late-onset neurodegenerative disorder termed fragile X-associated tremor/ataxia syndrome (FXTAS) [10–12].

The most common clinical features of FXTAS include a progressive action tremor with ataxia. More advanced or severe cases may show a progressive decline in cognition that ranges from executive and memory deficits to dementia [13]. FXTAS patients may also exhibit psychological symptoms such as depression, increased anxiety and mood instability [14,15]. Approximately 30% of patients complain of muscle weakness, numbness and pain in the lower extremities [16], suggesting that the disorder is not confined to the CNS. A useful differential diagnostic criterion for FXTAS is increased T2 intensities of the middle cerebellar peduncle (MCP) and adjacent white matter observed by MRI [17]. These criteria distinguish the disorder from other late-onset movement disorders and dementias. FXTAS patients also demonstrate mild-to-moderate global brain atrophy, which is most common in the frontal and parietal regions, as well as in the pons and cerebellum. In addition, degeneration of the cerebellum including Purkinje cell loss, Bergman gliosis, spongiosis of the deep cerebellar white matter and swollen axons have been observed in nearly all case studies of autopsy brains of symptomatic premutation carriers [18,19].

A study of penetrance revealed that more than a third of all carriers aged 50 years and older will show symptoms of FXTAS and the penetrance of this disorder exceeds 50% for men over 70 years of age [20]. The prevalence of premutation alleles is approximately one in 800 for males and one in 250 for females in the general population [21,22]; however, it is estimated that approximately one in 3000 men of more than 50 years of age in the general population will show symptoms of FXTAS [23]. These estimates do not consider the influence of size on penetrance, which predicts a correlation between the age of onset of symptoms to the size of the repeat. Recent studies have correlated the age of onset of clinical symptoms with the length of expanded repeats, demonstrating that larger repeats represent an increased risk factor for the development of FXTAS [23,24]. The degree of brain atrophy [24] and severity of the tremor and ataxia [24], as well as the risk of developing cognitive impairments [25], have also been correlated with CGG repeat length [24].

Although few, there are cases of FXTAS clinical features in female premutation carriers [26–29]. The lower frequency of FXTAS in females is thought to be due to the partial protection offered by random X-inactivation, leading to approximately half of cells expressing only the normal allele in most female carriers. Recent studies have been aimed at defining differences between male and female FXTAS, as well as female penetrance. One such study, led by Adams *et al.*, demonstrated milder whole brain and cerebellar volumetric loss in FXTAS females than in FXTAS males; however, FXTAS females exhibit significantly reduced brain volume compared with unaffected premutation carrier females or control females without the premutation [30]. Similar patterns of brain atrophy and white matter disease were observed in both males and females affected by FXTAS. Unlike FXTAS males, FXTAS females do not show significant correlation between reduced cerebellar volume and both increased FXTAS severity and increased CGG repeat length [30]. A study of FXTAS penetrance in 85 women with premutation alleles predicted that approximately 16.5% of female carriers over 50 years of age will exhibit signs of FXTAS [31].

Female premutation carriers are at an increased risk for developing other medical and neurological problems. It has been established that between 16 and 24% of female premutation carriers will exhibit fragile X-associated primary ovarian insufficiency (FXPOI), which results in the cessation of menstruation before the age of 40 years [32,33]. Although the basis for FXPOI is not understood, the penetrance and age of onset appear to correlate with CGG repeat length [34]; however, a later study suggests a nonlinear relationship between age of menopause and premutation size, in which female carriers with premutations in the midsize range are at a greater risk for FXPOI, while larger repeat tracts are associated with lower risk [35]. Aside from FXTAS and FXPOI, increased prevalence of thyroid disease, fibromyalgia and hypertension have been observed in female premutation carriers [31,36].

Neuropathology: intranuclear inclusions

The neuropathological hallmark and post-mortem criterion for definitive FXTAS is the presence of eosinophilic, ubiquitin-positive intranuclear inclusions. FXTAS inclusions are negative for tau and α-synuclein, which rule out FXTAS as a tauopathy or synucleinopathy [37–39]. The inclusions are located in broad distributions throughout the brain – in neurons, astrocytes and the spinal column. FXTAS inclusions share the ubiquitin-positive hallmark with several other inclusion disorders, such as the polyglutamine disorders; however, FXTAS inclusions do not stain with antibodies that recognize polyglutamine. It is important to note that, unlike the polyglutamine disorders, there is no known structurally abnormal protein with FXTAS. The presence of ubiquitin and heat-shock proteins (HSP) in the FXTAS inclusions strongly suggests a defect in the proteasome degradation or protein-folding pathways; however, the link between these processes and RNA-mediated neurodegeneration is unknown.

Iwahashi *et al*. purified human FXTAS frontal cortex inclusions using fluorescence-activated flow-based methods [40]. Both mass spectroscopic analysis of purified inclusions and immunohistochemical analysis of isolated nuclei and tissue sections uncovered 19 proteins that constitute the protein complement of the inclusions (Table 1). The protein components of FXTAS inclusions fell into eight major functional categories, including: histone family; intermediate filament; microtubule; myelin-associated proteins; RNA-binding proteins (RBPs); stress-related proteins; chaperones and ubiquitin–proteasome-related proteins. Several proteins previously identified as components of FXTAS inclusions, such as ubiquitin, HSP70 and 20S subunit of the proteasome, were identified and purified by immunofluorescent staining of isolated nuclei of FXTAS frontal cortex. In addition, proteins such as tau and α-synuclein, which were not found in immunohistochemical analysis of FXTAS brains, were also not present in isolated nuclei of FXTAS frontal cortex in this study.

Molecular correlates of FXTAS

Before the discovery of FXTAS, premutation carriers were generally regarded as clinically unaffected [41–44]. No alterations in *FMR1* mRNA or FMRP levels could be detected using semiquantitative reverse transcription-PCR and immunohistochemistry [45–48]. Despite these findings, reports of learning disabilities [43,49,50] and psychiatric dysfunction [11,16,51–56] in both male and female premutation carriers raised suspicions that carriers may exhibit molecular abnormalities due to the premutation expansion in the *FMR1* mRNA [57]. The clinical observations warranted a more quantitative investigation of *FMR1* mRNA and FMRP levels in carriers. Tassone *et al*. used quantitative fluorescence PCR to demonstrate that relative *FMR1* mRNA levels in premutation carrier blood leukocyte samples were up to tenfold higher than that of control individuals [58]. In addition, immunocytochemical staining of premutation lymphocytes revealed a reduction in FMRP-positive staining, indicating a reduction in FMRP production. Other studies also revealed a reduction in FMRP levels in blood leukocytes of some premutation carriers with cognitive deficits [59]. Kenneson *et al.* developed a slot-blot-

based assay to measure FMRP levels in cell lysate and demonstrated significantly diminished FMRP levels in carriers that negatively correlated with repeat number; however, despite reduced FMRP levels, the carriers overexpressed *FMR1* mRNA, which resulted in a positive correlation between repeat number and *FMR1* message levels [60].

Several possible mechanisms for *FMR1* message overexpression have been put forward. One feedback mechanism suggests that the cell attempts to compensate for reduced levels of FMRP by increasing the amount of available *FMR1* transcript [61]. Alternatively, expansion of CGG repeats may change the distances between potential factor-binding sites, leading to a more open promoter that could favor increased access of transcription factors. Another suggested mechanism is that increased stability of the premutation *FMR1* transcript may be the cause of the message overexpression. After measuring primary transcription and stability of *FMR1* mRNA, Tassone *et al.* determined that the mechanism of elevated *FMR1* transcript levels results from increased transcription at the *FMR1* locus and not from increased stability [62]. Reduced translational efficiency of premutation alleles has been shown to result from a reduced association of the premutation mRNA with polysomes [63]. This decline in translation efficiency parallels increasing CGG repeat length. Other studies have considered the effect of strong secondary or higher order structures to impede ribosome entry or scanning of the 5′ UTR of FMR1 mRNA [64]. In addition, changes in FMRP may result from altered cellular localization of the transcripts carrying expanded repeats. It is possible that large fractions of the expanded transcripts are sequestered in the nuclear inclusions, and are therefore not accessible to the ribosomes; however, as of yet there is no evidence for this hypothesis.

RNA-mediated neurodegeneration model for FXTAS

A threshold in triplet repeat mutation length in *FMR1* defines two molecularly distinct diseases. The presence of elevated *FMR1* mRNA carrying repeats in premutation carriers along with the absence of FXTAS in older adults with FXS led to the proposal of RNA-mediated toxicity mechanism that describes the contribution of premutation RNA to FXTAS pathogeneisis [11,65,66]. Several clues regarding the molecular mechanisms involved in FXTAS come from the RNA gain-of-function model for myotonic dystrophy (DM), a typically adult-onset, dominant, muscle disorder caused by an abnormal expansion of a CUG triplet repeat present in the 3′ untranslated region of a protein kinase gene, *DMPK* [67]. In DM1 cells, mRNA transcripts with expanded CUG repeats are sequestered in nuclear RNA foci [68] along with decreased DMPK protein levels [69]. Expanded CUG repeats in *DMPK* mRNA can form hairpins that are thought to block the export of the RNA from the nucleus [70]. An RBP, MBNL1, can colocalize with the DM RNA foci [71]. Ultimately, disruption of MBNL1 leads to downstream misregulated splicing of MBNL1 targets that result in DM pathology [72]. The RNA toxicity model has been demonstrated in a DM mouse model expressed a transgene with expanded CTG repeats in the *DMPK* gene that resulted in myotonia [73,74]. A similar mechanism leading to FXTAS pathology has been recently tested (Figure 1). The proposed mechanism for RNA pathogenesis for FXTAS states that CGG-repeat-containing FMR1 transcripts recruit RNA binding and other proteins that accumulate as intranuclear inclusions. The sequestration of these proteins is thought to prevent them from performing their normal functions that would lead to downstream alterations in cellular function. The observation of expanded *FMR1* RNA transcripts in the FXTAS inclusions of a 70-year-old male who died with FXTAS helped to further support for the RNA toxicity hypothesis [75].

The major parallels between FXTAS and DM are the induction of intranuclear inclusions and nuclear foci as a result of expanded repeat RNA. If these aberrant structures are responsible for pathology, what are the proteins that might be altered in abundance by them? Attempts to define proteins in the inclusions in FXTAS have revealed a number of candidates (Table 1). Among these, is a mammalian ortholog of the *Drosophila* protein muscleblind, MBNL1.

Interestingly, MBNL1 is present in both FXTAS intranuclear inclusions [40] and DM nuclear foci suggesting at least a potentially parallel mechanism for FXTAS pathogenesis. A major caveat to the parallel with DM is demonstrated by considering different clinical features of FXTAS and DM. DM is a multisystemic disease that can present at any age from birth to old age. The major pathologies observed in DM are muscle wasting and myotonia, which are not observed in FXTAS. Although DM1 neuronal nuclear foci have been observed, it is uncertain whether the foci directly contribute to the neuronal dysfunction [76]. In DM muscle, there is evidence to support the direct involvement of RNA foci in disease pathogenesis through mechanisms that involve sequestration of MBNL1 and other proteins resulting in misregulation of genes that undergo alternative splicing [72,77]. MBNL1 is an RBP that functions as regulator of alternative splicing. In the case of DM1, human brain samples exhibited splicing misregulation with reversion to fetal isoforms of *NMDAR1*, *APP* and *Tau* genes [76]. The altered splicing that results from misregulation of *MBNL1* may lead to the mental retardation and other cognitive defects observed in some DM patients. Changes in alternative splicing have not been described for FXTAS; however, the cellular and murine models of FXTAS provide adequate tools for investigation of CGG-mediated splicing alterations.

Evidence for an RNA-mediated neurodegeneration mechanism for FXTAS pathology

Cellular models

Garcia-Arocena *et al.* demonstrated that expanded CGG repeats expressed in untranslated mRNA are toxic to cells and can induce inclusion formation [78]. In this study, human neural cells transfected with green fluorescent protein (GFP) reporters possessing *FMR1* 5′UTR with 88 CGG repeats, not only exhibited cytotoxicity and inclusions but also changes in nuclear morphology and nuclear lamin structure. Handa *et al.* performed microarray analysis on RNA from cells transfected with plasmids expressing transcribed but untranslated premutation length CGG repeats upstream of *FMR1* [79]. The microarray studies revealed upregulation of genes required for apoptosis, which were validated by quantitative-PCR, demonstrating that premutation CGG repeat RNA is toxic to cells.

Animal models

Fly models—Jin *et al.* developed a series of transgenic *Drosophila* to test the role of CGG in RNA as a toxic agent [66]. The authors made use of the Gal4-upstream activation sequence system to drive expression of expanded, untranslated riboCGG repeats upstream of the coding sequence for the *EGFP* reporter gene and demonstrated that ribo-CGG was sufficient to cause degeneration of neurons in the fly eye [66]. This model provided the first *in vivo* evidence for RNA toxicity in an animal model. The riboCGG induced neurodegeneration was neuron specific, dose sensitive and directly dependent on CGG repeat length. A repeat length of 90 CGGs was sufficient to cause neurodegeneration of photoreceptors. In addition, the flies developed inclusions that were positive for ubiquitin, Hsp70 chaperone and the proteasome. The finding that Hsp70 could suppress the riboCGG-mediated neurodegeneration of the fly eye [66] made this model particularly useful for the discovery of other genetic modifiers (see section ‘Genetic and chemical modifiers of CGG-mediated phenotypes in FXTAS models’).

Murine models

Knock-in mouse models of FXTAS: Oostra & Usdin mice—The Oostra group at Erasmus University, Rotterdam, The Netherlands, created a knock-in mouse model that replaced the eight endogenous murine CGG repeats with an expanded repeat of human origin (~100 repeats) [80]. This model exhibited up to 3.5-fold more *Fmr1* mRNA in the brain compared with controls. The mice developed intranuclear ubiquitin-positive inclusions in

several neuronal cell types [81], but not in Purkinje neurons of the cerebellum. An increase in both the number and size of the inclusions was correlated with the age of the animals. As in human post-mortem samples, the inclusions in this model were positive for Hsp40, Rad23B and the 20S catalytic core complex of the proteasome. These mice demonstrated cognitive decline, neuromotor and behavioral disturbances that were assessed by a battery of tests [82]. Possibly the most significant piece of evidence to support the RNA-mediated toxicity model for FXTAS was that these mice displayed no alterations in FMRP levels, as assessed by western blot, despite elevation in *Fmr1* mRNA levels [81]. Animals derived from the original knock-in with CGG repeat expansions greater than 200 triplets have now been developed after many generations of selecting offspring with longer repeats. Repeats of this length would result in FXS in humans. Interestingly, these mice do not develop molecular or phenotypic characteristics of FXS, and no methylation is observed at the *Fmr1* locus. Elevated *Fmr1* mRNA levels were only observed in the lower range for premutation alleles and Fmrp levels were reduced in the upper premutation range in these animals [83]. Loss of the intranuclear inclusions was observed in animals with greater than 200 triplets [83], which raises the question of the relevance of inclusions to disease pathology.

A second knock-in mouse model that has small serial ligated, short stable CGG•CGG repeat tracts knocked into exon 1 of the endogenous murine *Fmr1* gene was created by the Usdin group at the NIH [84]. This model exhibited genetic and pathophysiological changes that were similar to human FXTAS but not observed in the previous mouse model. For example, the Usdin knock-in repeats were found in rare cases to expand to a full mutation-sized repeat without evidence of methylation in a single generation. In addition, this model has Purkinje neuron pathology in the form of abnormal calbindin staining, swollen axonal torpedos and Purkinje neuron cell loss. A correlation between the repeat number and the *Fmr1* mRNA levels was also observed.

Transgenic mouse model of FXTAS

Purkinje neuron-specific trangenic model of FXTAS—Both knock-in mouse models suggest a direct involvement of riboCGG in neuropathology; however, it remained unclear which part of the expanded rCGG transcript was necessary or whether FMRP plays a role. Our group developed transgenic mouse models to explore the molecular requirement for CGG-mediated neurodegeneration observed in FXTAS. The mice expressed 90 CGG repeats in the 5′ untranslated region (UTR) of either the *Fmr1* gene or reporter gene EGFP under the regulation of the Purkinje neuron-specific promoter, L7/Pcp2 [85]. As controls L7*Fmr1* and L7EGFP lines were also created without expanded repeats. We found that mice with CGG-containing transcripts outside the context of *Fmr1* are sufficient to cause Purkinje neuron neurodegeneration and behavioral anomalies in our models. The presence of large and frequent Purkinje neuronal inclusions is unique to our models. This model has provided the first evidence in a mammalian system that rCGG outside the context of *Fmr1* was sufficient to cause neurodegeneration, as only CGG-containing repeat mice exhibited significant Purkinje neuron cell loss compared with wild-type or L7*Fmr1* mice. In additon, detailed examination of Purkinje neurons in transgenics revealed axonal swellings. Ubiquitin-positive inclusion formation was observed in mice expressing L7CGG$_{90}$*Fmr1* or L7CGG$_{90}$EGFP transgenes suggesting that inclusion formation is not directly correlated to *Fmr1* levels but rather, expression of expanded CGG repeats. Purkinje neuronal inclusions in our rCGG-containing transgenics also stained positive for antibodies against Rad23B, Hsp40 and the 20S core subunit of the proteasome, as assessed by immunohistochemical analysis. Our transgenics also exhibited a decline in motor-learning abilities as assessed by the accelerating rotarod. L7CGG$_{90}$*Fmr1* mice developed an age-dependent decline in rotarod walking since mice at 40 weeks performed worse than mice at 20 weeks, while L7*Fmr1* transgenics showed no age effect-related decline in rotarod performance. Each of the animal models has different

neurological phenotypes, which strongly suggests differences in CGG repeat length, RNA expression and proteins levels may play an essential role in FXTAS pathology (Table 2).

Yeast artificial chromosome transgenics—Our group also developed yeast artificial chromosome (YAC) transgenic mice carrying a premutation-sized CGG repeat within the entire human *FMR1* gene in order to study instability of the FXS repeat [86]. While they are not as well characterized as the models previously described, the YAC transgenics develop significant pathology but lack detectable intranuclear inclusions. In particular, Purkinje neurons show accelerated degeneration and neuropathology in the form of axonal torpedoes as animals age (Figure 2). These animals overexpress the human RNA and protein; whether differences result from human versus mouse overexpression is a factor remains unknown. Interestingly, *SCA1* transgenic mice expressing ataxin-1 with 82 glutamine repeats develop ataxia and Purkinje cell degeneration [87,88] in the absence of nuclear inclusions [89]. These studies suggest that inclusion formation may not be required for disease pathogenesis. However, conditional SCA1 transgenics [90], as well as a conditional mouse model of Huntington's disease (HD) expressing 94 CAG repeats upstream of the lacZ promoter under the regulation of a Tet inducible promoter [91] can recover from polyglutamine-induced inclusions. It would be of interest to determine whether similar inducible models of FXTAS pathogenesis show the ability to recover.

Genetic & chemical modifiers of CGG-mediated phenotypes in FXTAS models

Our group and others made use of the transgenic fly model by performing biased genetic screens that uncovered RBPs capable of modifying the neurodegenerative eye phenotype in the fly (Table 3). For example, overexpression of RBP, Purα, suppresses the riboCGG-mediated neurodegeneration in the *Drosophila* eye [92]. Jin *et al.* found Purα in both the riboCGG-induced fly inclusions as well as human FXTAS sup–mid temporal cortex inclusions [92]. However, Iwashashi *et al.* did not detect Purα in human FXTAS inclusions purified from the cerebral cortex [40]. In addition, Purα-positive inclusions have not been observed in either the Oostra knock-in [**Willemsen R, Erasmus University, Pers. Comm.**] nor our Purkinje neuron-specific transgenics. The reasons behind the differences in inclusion protein content from human to human or from human to mouse and fly are unclear; however, it is not difficult to imagine that inclusions from one brain region to the next will not mirror exactly in protein content, as gene expression varies from region to region; the same can be said for species to species gene expression differences. In addition, variations in technique to assess inclusion protein compositions may yield different results.

Although there is conflicting data regarding the presence of Purα in inclusions, the protein may still play a role in FXTAS pathology as Purα directly interacts with riboCGG repeats *in vitro* and *in vivo* [92]. Interestingly, Purα-null mice develop many of the hallmark features of human FXTAS. Similar to humans with FXTAS, Purα-null mice exhibit loss of neurons in the cerebellar granular layer as well as the Purkinje cell layer and develop tremor upon motion [93]. Even Purα-heterozygous mice develop neurological features, suggesting that haploinsufficiency is problematic, at least in the brain. Purα has been implicated in dendritic RNA transport and translation [94,95]. The RNA-toxicity model of FXTAS would suggest that sequestration of Purα into inclusions or even alterations in the Purα protein levels may lead to alterations in RNA transport or translation of Purα targets.

In another biased screen for RBPs that modify the transgenic fly neurodegenerative phenotype, Sofola *et al.* observed that overexpression of two additional RBPs, hnRNPA2/B1 and CUGBP1, could suppress the riboCGG-induced eye neurodegeneration and hnRNPA2/B1 could interact directly with the riboCGG repeats [96]. CUGBP1 has been implicated in the pathology of DM1. CUGBP1 steady-state levels are increased in DM1, which induces

downstream misregulated alternative splicing events. Sofola *et al.* demonstrated CUGBP1 could interact with rCGG repeats via hnRNPA2/B1 *in vitro* [96].

Several studies indicate that riboCGGs can fold into hairpin and tetraplex structures that could reduce the efficiency of translation of premutation *FMR1* mRNA. The reduced translational efficiency of premutation RNA results from its reduced association with polysomes [63]. Interestingly, hnRNPA2 can mediate the nonenzymatic destabilization of quadruplex forms of (CGG)n in both DNA and RNA [97]. Khateb *et al*. demonstrated, using an *in vitro* translation assay, that premutation repeats in the promoter region of a construct expressing the firefly reporter gene could reduce its translational efficiency [98]. In addtion, a similar effect was demonstrated *in vivo*; however, in the presence of overexpressed hnRNPA2, not only could the cell more efficiently translate the premutation RNA but also diminish the production of excess premutation RNA *in vivo*.

Ofer *et al.* recently demonstrated that TMPyP4, a quadruplex ribo-CGG destabilizing porphyrin, on its own and in cooperation with overexpressed hnRNPA2, can significantly increase the efficacy of translation of premutation RNA *in vivo* making it an attractive candidate for FXTAS therapeutics [99]. Although the mechanism that allows TMPyP4 to increase efficiency of premutation mRNA translation and diminish excess premutation RNA accumulation is unknown, it may be useful to decrease RNA toxicity in carriers affected by FXTAS. According to the RNA toxicity model, if hnRNPA2/B1 is sequestered to the cytoplasmic inclusions in flies, it may serve to enhance translation of premutation RNA, thereby maintaining the levels of FMRP in the cell. Since hnRNPA2/B1 can be found in both the nucleus and the cytoplasm, sequestration of hnRNPA2/B1 to human FXTAS nuclear inclusions may hamper its cytoplasmic functions and lead to reduced translation of *FMR1* mRNAs.

All of these observations taken together have led to the hypothesis that the neurodegeneration and inclusion formation observed in FXTAS are the result of a RNA-mediated toxicity caused by the increased expression of expanded ribo-CGG containing mRNA. A significant correlation between CGG repeat length and the frequency of intranuclear inclusions, in both neurons and astrocytes of human premutation carriers, further supports these models. In addition, intranuclear inclusions can be induced in primary progenitor cells and established cell lines when premutation-sized CGG repeats fused to *FMR1* are expressed in these cells [78]. Given the high prevalence of fragile X premutation carriers in the general population and their risk for developing clinical features of FXTAS, it is essential to understand the molecular mechanisms of FXTAS, and further analysis of the pathogenic effects of ribo-CGG in all of the models is essential for determining the mechanism of FXTAS.

Conclusion

Considerable data are now available implicating fragile X premutation mRNA in disease pathogenesis. While the cell, fly and mouse models have provided useful molecular and genetic tools that will aid in our understanding of FXTAS disease progression, the precise mechanisms involved remain obscure and need to be investigated further. Early events that lead to inclusion formation and neuronal death are not understood. The relevance of inclusion formation in neurodegeneration remains a mystery since FXTAS pathology is observed in animal models that express greater than 200 CGG repeats and YAC transgenics expressing 90 CGG repeat and human FMR1, but these rarely ever show inclusions. A reasonable suggestion for inclusion formation would be to sequester pathogenic RNA; however, the protective versus harmful effects of inclusion formation have been hotly debated in polyQ inclusions disorders [100]. The argument that inclusions have a role in neuronal dysfunction suggests that a key step in the pathogenesis of various polyQ disorders result from aberrant interactions of misfolded

protein with cellular proteins. The interaction would inhibit the sequestered proteins from performing their normal activities and trigger a cascade of events that lead to neuronal dysfunction and subsequently, inclusion formation. The argument for a neuroprotective role for inclusions suggests they function to sequester misfolded proteins from the pool of correctly folded proteins.

It is still unknown how the premutation and other pathogenic RNAs recruit RBPs and other proteins into inclusions or whether these proteins play some role in the inclusions. The downstream effects of sequestration of Purα, Hsp70, hnRNPA2/B1, MBNL1 and other as yet unidentified proteins, are essential to our understanding of the consequences of their depletion in cells. It is reasonable to suggest that, owing to the nature of the sequestered proteins, alterations in proteasome degradation and changes in neuronal function may be to blame for the clinical features of FXTAS. The variability of *Fmr1* transcript expression in knock-in mice with various repeat lengths may help us understand FXTAS penetrance and disease severity. Understanding how and why cells form inclusions in response to expanded RNA, as well as the nature of the inclusions will likely lead to new therapeutic approaches that will combat neurodegeneration and improve cognitive and motor performance. Therapy could take many approaches from gene delivery of genetic modifiers to prevent inclusion formation. One example is provided by a study aimed at the RNA interference knockdown of premutation CGG repeats in *Drosophila* that has already demonstrated that gene delivery can suppress ribo-CGG-mediated neurodegeneration [101].

Future perspective

The future of FXTAS should focus on genetic modifiers of neurodegeneration and behavioral anomalies. Additional studies that provide more insight into the roles of proteins such as Purα, hnRNPA2/B1 and MBNL1 in intranuclear inclusions are necessary to determine the alterations required for disease pathogenesis. In addition, the presence of these proteins in inclusions may provide useful information regarding the downstream effects of protein sequestration. Mouse models that employ a Tet ON/OFF or tissue-specific inducible system for the expression of premutation CGG repeats would prove invaluable for the study of the consequence of repeat expression on inclusion recovery, behavioral and cognitive rescue studies, cell morphology and function. The differences between inclusion content as well as an increased tolerance for expanded repeat length in mice must also be considered. As previously mentioned, Purα and MBNL1 have not been observed in murine inclusions, which may result in the use of different pathways for induction of inclusion formation between humans and mice. In addition, differences in phenotypes between CGG-repeat knock-in and Purkinje-specific CGG90 transgenic mice exist. The reasons for the variation in phenotypes between the animal models are unknown; however, differences such as the developmental timing of the expression of the CGG-containing transcript may account for the differences in phenotype. Both knock-in models of FXTAS use the endogenous mouse *Fmr1* regulatory sequences, while our transgenics use a robust Purkinje neuron-specific promoter active after birth, which may also account for the early inclusion phenotype observed in our transgenics. The differences in onset or severity of the pathogenic and behavioral phenotypes observed in our models may also be due to variations in the level of transgene expression due to positional effects. Better understanding of parallel pathways between FXTAS and other noncoding dominant repeat disorders may provide a useful insight into FXTAS pathogenesis.

The *Drosophila* model of FXTAS aids our understanding of the effect of varying levels of CGG-transcript expression and phenotype severity as we have observed a correlation between increased levels of CGG-transcript expression and RNA-mediated toxicity in the flies [**Sofola O, Baylor College of Medicine, Unpublished Data**]. The fly has helped to uncover modifiers of the neurodegenerative phenotype, and points to potential pathways involved in the disorder. The

fly offers important advantages to the study of RNA-mediated CGG repeat toxicity, such as the ability to perform rapid genetic screens for modifiers and to assess behavioral anomalies quickly. These flies can be used to investigate drug and gene therapies for FXTAS. Although the fly model of FXTAS may help us uncover additional modifiers of the neurodegenerative phenotype, it is important to consider that the *Drosophila* inclusions were almost always cytoplasmic, which is unlike those observed in patients or in mouse models. Therefore, the composition of the inclusions and pathways involved in neurodegeneration may differ significantly.

Studies that identify mechanisms for overexpression of CGG-containing FMR1 mRNA, as well as downstream molecular mechanisms for expanded CGG-repeat *FMR1* mRNA to trigger downstream events leading to pathology, are required to understand of FXTAS disease progression. A focus on defining the cell death mechanisms and markers of CNS toxicity will help us understand the events that precede inclusion formation. Identification of genetic and environmental risk and protective factors associated with the penetrance of FXTAS is required to develop treatments for the disorder. FXTAS is a relatively new disorder; therefore, there is clearly a need for additional animal models to increase our knowledge of disease pathology, molecular mechanisms and drug treatment. One thing is clear, expanded CGG-repeat containing mRNAs play an integral role in FXTAS pathology.

Executive summary

Background

- Premutation alleles of *FMR1* are linked to fragile X-associated tremor/ataxia syndrome (FXTAS) and fragile X-associated primary ovarian insufficiency (FXPOI).
- Approximately a third of premutation allele carriers will exhibit clinical features of FXTAS.
- The radiological criterion for definitive FXTAS is increased T2 intensities observed by MRI.
- This paper presents a number of observations/conclusions that provide evidence for a pathogenic RNA gain-of-function mechanism for FXTAS.

Neuropathology: intranuclear inclusions

- The presence of ubiquitin-positive eosinophilic intranuclear inclusions throughout the brain and spinal column are a major neuropathological hallmark of FXTAS.
- FXTAS is not associated with mutations or repeat expansion in *FMRP*.

Molecular correlates of FXTAS

- FXTAS patients display no signs of fragile X syndrome and vice versa.
- Premutation carriers have elevated levels of expanded CGG-containing *FMR1* mRNA.
- Premutation carriers have normal to slightly reduced FMRP levels.

Evidence for an RNA-mediated neurodegeneration mechanism for FXTAS pathology

- Premutation length riboCGGs as RNA are toxic to cells.
- A fly model of FXTAS provided the first *in vivo* evidence of an RNA-toxicity mechanism for FXTAS.

- Two premutation CGG knock-in mouse models exhibit ubiquitin-positive intranuclear inclusion formation and correlations between repeat length and *Fmr1* mRNA levels.
- CGG_{90}*Fmr1* and CGG_{90}*EGFP* Purkinje neuron-specific transgenic mice exhibit ubiquitin-positive intranuclear inclusions.
- FXTAS-like neuropathology observed in yeast artificial chromosome transgenics suggest neuropathology is independent of inclusion formation.

Genetic & chemical modifiers of CGG-mediated phenotypes in FXTAS models

- The transgenic fly model of FXTAS and cellular models are useful tools to uncover genetic and chemical modifiers.

Future perspective

- Modifiers of CGG-induced neurodegeneration may provide essential clues for downstream events leading to FXTAS pathology.
- Understanding the role of FXTAS inclusion formation and elucidating inclusion protein composition may help us understand the molecular mechanism of FXTAS.

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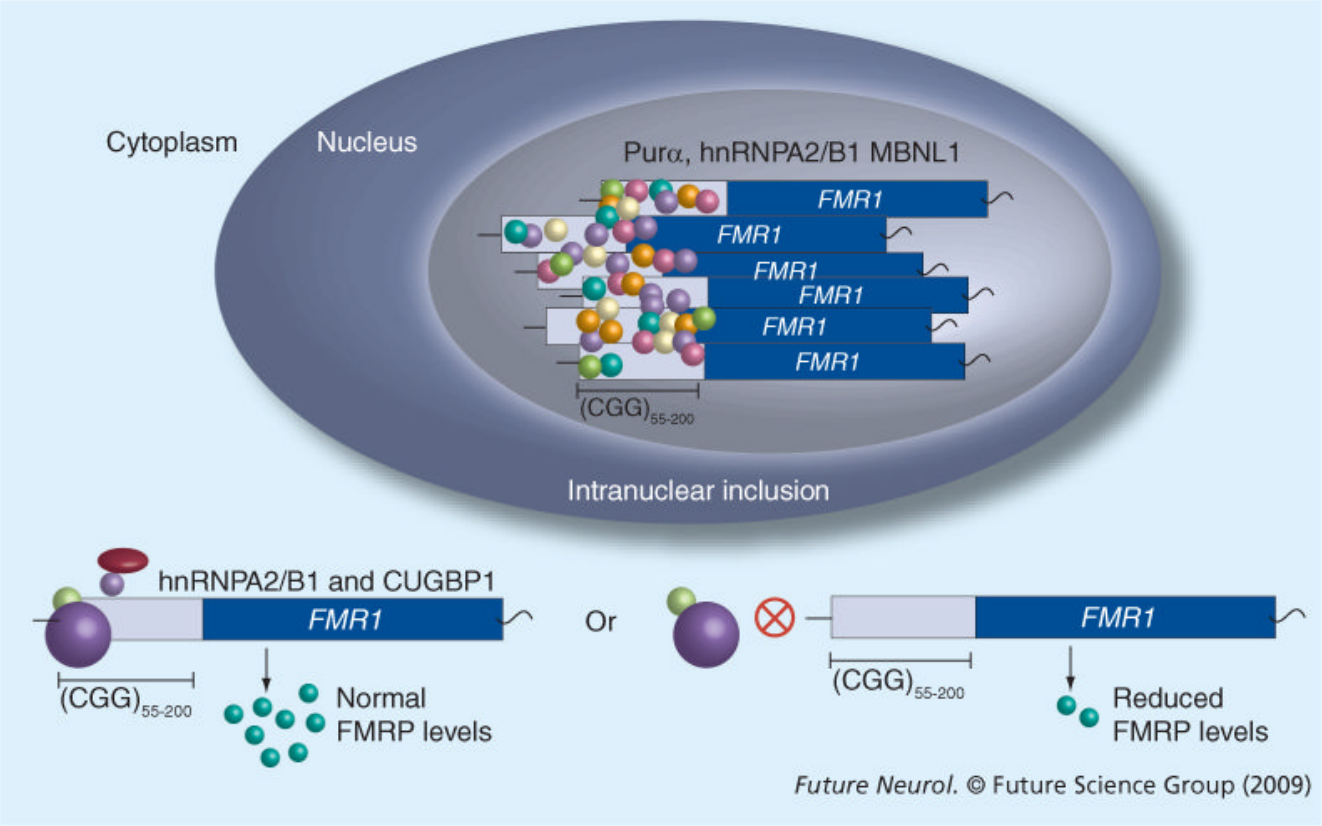

Figure 1. RNA toxicity model in a premutation cell

Via unknown mechanisms, increased transcription of premutation alleles of the *FMR1* gene leads to elevated levels of *FMR1* mRNA. The excess RNA is thought to exert its toxic effect on the cell via sequestration of RNA-binding proteins, ubiquitin, proteasome components and other unknown proteins, which would lead to abnormal regulation of cellular processes normally regulated by the sequestered proteins. Sequestration of proteins into intranuclear inclusions may lead to downstream neurodegeneration. In addition, sequestration of the toxic RNA into intranuclear inclusions could be a mechanism that the cell uses for survival. Premutation RNA exported to the cytoplasm may be translated normally. Reduced FMRP levels may result from, first, interactions of RNA-binding proteins in the cytoplasm with the expanded RNA, which could block ribosome entry; second, complex RNA structures due to expanded CGG repeats; third, reduced translation owing to nuclear sequestration of RNA-binding proteins required for translation; and/or last, reduced translation due to sequestration of premutation RNA in the inclusion.

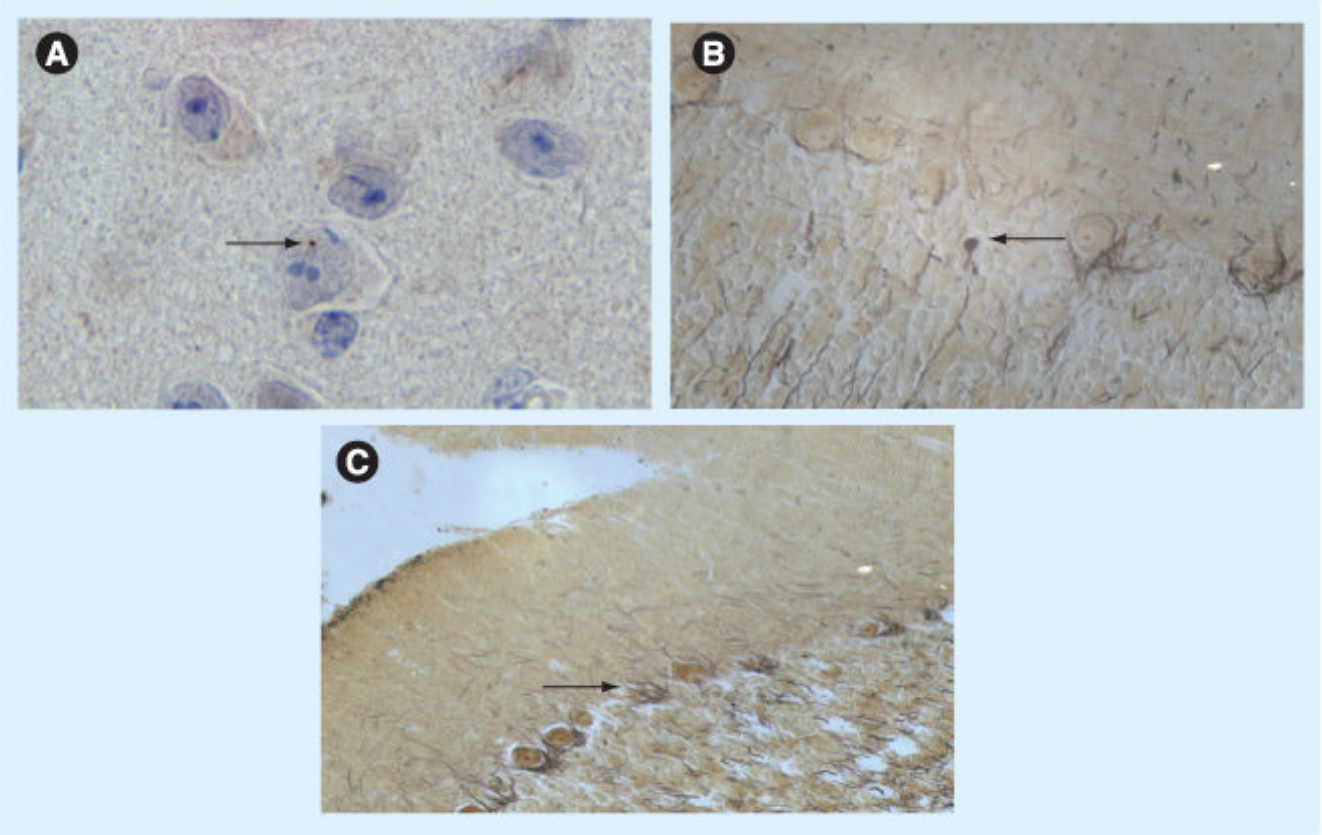

Figure 2. Expanded CGG repeats in a yeast artificial chromosome transgenic model using human *FMR1* lead to neuropathology with very few nuclear inclusions

(A) Infrequent ubiquitin-stained inclusions seen in hippocampus, CA3, mammilary nucleus and hypothalamus. **(B & C)** Cerebellar pathology including axonal torpedos (**B**, swellings) and empty baskets (**C**, Purkinje neuron loss).

Table 1
Protein components of CGG-induced inclusions identified by various methods

Model	Candidate protein	Category	Ref.
Humans, mice and flies	Ubiquitin	Ubiquitin–proteasome system	[66,81,84,102,103]
Humans	11S regulator to 20S proteasome	Ubiquitin–proteasome system	[40]
Mice and flies	20S proteasome	Ubiquitin–proteasome system	[40,66]
Mice	Homolog of yeast gene *Rad23A*	DNA repair and ubiquitin–proteasome system	[104]
Mice	Homolog of yeast gene *Rad23B*	DNA repair and ubiquitin–proteasome system	[102,104]
Humans	H2B histone family	Histone family	[40]
Humans	Similar to H2A histone family, member Z	Histone family	[40]
Humans	HIST I H4D protein	Histone family	[40]
Humans	H2A histone family A (L)	Histone family	[40]
Humans	H2A histone family, member Q (O)	Histone family	[40]
Humans	Neurofilament 3	Intermediate filament	[40]
Humans and mice	Lamin A/C	Intermediate filament	[40,78,84]
Humans	Vimentin	Intermediate filament	[40]
Humans	Internexin neuronal intermediate filament	Intermediate filament	[40]
Humans	NEFL protein	Intermediate filament	[40]
Humans	Glial fibrillary acidic protein (GFAP)	Intermediate filament	[40]
Humans	β5-tubulin	Microtubule	[40]
Humans	Tubulin, α6	Microtubule	[40]
Humans	Tubulin, alpha and ubiquitous	Microtubule	[40]
Humans	Myelin/oligodendrocyte glycoprotein β3	Myelin-associated protein	[40]
Humans	2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)	Myelin-associated protein	[40]
Humans	Myelin basic protein	Myelin-associated protein	[40]
Humans	Muscleblind-like 1	RNA-binding protein	[40]
Humans	Heterogenous nuclear ribonucleoprotein A2/B1	RNA-binding protein	[40]
Humans and flies	Purα	RNA-binding protein	[92]
Humans	αβ-crystallin	Stress-related protein	[40,78]
Humans and flies	Hsp70	Chaperone	[40,66]
Humans	Hsp27	Chaperone	[40]
Mice	Hsp40	Chaperone	[81,102]

Table 2

Phenotypic comparisons between mouse models of fragile X-associated tremor/ataxia syndrome

Mouse model	Brain *Fmr1* mRNA levels	Brain FMRP levels	Ubiquitin-positive inclusions	Purkinje neuronal phenotype	Onset of neuromotor behavioral symptoms (weeks)	Onset of inclusions (weeks)	Ref.
CGG_{98} knock-in	2–3.5-fold increase relative to wild-type	Normal	+	N/R	N/R	30	[81]
Expanded $CGG_{100–150}$	2–3.5-fold increase relative to wild-type	Normal	+	N/R	52	N/R	[105]
Expanded $CGG_{151–200}$	Normal	Decreased	+	N/R	N/R	N/R	[105]
Expanded $CGG_{>200}$	Normal	Decreased	Few/absent	N/R	N/R	N/R	[105]
Usdin CGG knock-in	Increased	Decreased (in some brain regions)	+	Swollen axons, cell death and abnormal calbindin staining	N/R	N/R	[84]
$L7CGG_{90}$ transgenics	N/A	N/A	+ (only in Purkinje neurons)	Inclusions, swollen axons and cell death	20	8	[102]

+ Positive;

N/A: Not applicable;

N/R: Not reported.

Table 3

Genes that modify the RNA-mediated neurodegeneration caused by the fragile-X premutation riboCGG repeat in *Drosophila melanogaster*

Gene	Protein present in fly inclusions	Ref.
Hsp70	Yes	[66]
Purα	Yes	[92]
Cugbp1	No	[96]
hnRNPA2/B1	No	[96]
riboGCC repeats	N/A	[101]

N/A: not applicable.