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RNA-mediated pathogenesis in fragile X-associated disorders

Huiping Tan^{a,b}, He Li^b, and Peng Jin^{a,*}

^aDepartment of Human Genetics, Emory University School of Medicine, Atlanta, Georgia, USA

^bDivision of Histology and Embryology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China

Abstract

Noncoding RNAs play important and diverse regulatory roles throughout the genome and make major contributions to disease pathogenesis. The *FMR1* gene is involved in three different syndromes: fragile X syndrome (FXS), primary ovarian insufficiency (POI), and fragile X-associated tremor/ataxia syndrome (FXTAS) in older patients. Noncoding RNAs have been implicated in the molecular pathogenesis of both FXS and FXTAS. Here we will review our current knowledge on the role(s) of noncoding RNAs in FXS and FXTAS, particularly the role of the microRNA pathway in FXS and the role of noncoding riboCGG (rCGG) repeat in FXTAS.

Keywords

Noncoding RNAs; FMR1; Fragile X syndrome and FXTAS

Recent genome-wide interrogations of transcribed RNA have yielded compelling evidence for its pervasive and complex transcription throughout most of the human genome. Nevertheless, a significant portion of this transcribed RNA appears to be non-protein-coding and is currently uncharacterized. In-depth analysis of 1% of the human genome (~30 Mb) performed by the Encyclopedia of DNA Elements (ENCODE) project revealed that 92.6% of the interrogated bases could be detected as primary transcripts and that, among these, there were many novel non-protein-coding transcripts [7]. Similarly, another study found that the majority (64%) of polyadenylated (poly-A+) transcripts ≥ 200 nucleotides (nt) in length lay outside annotated protein-coding regions [35]. These and other genome-wide analyses have led to the identification of tens of thousands of noncoding RNA transcripts expressed from the human genome, most of which have yet to be functionally characterized. Along with the revelation that noncoding RNAs in the human genome are surprisingly abundant, there has been a surge in molecular and genetic data showing diverse and important regulatory roles for noncoding RNA. It has been shown that both the development and proper function of the nervous system require the intricate spatiotemporal expression of a wide repertoire of regulatory RNAs. Misregulation of these regulatory RNAs could contribute to the abnormalities in brain development that are associated with neurodevelopmental disorders.

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*To whom correspondence should be addressed: Peng Jin, Ph.D., Department of Human Genetics, Emory University School of Medicine, 615 Michael Street, Suite 301, Atlanta, Georgia 30322, USA. TEL: (404) 727-3729, FAX: (404) 727-5408, E-mail: peng.jin@emory.edu

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Fragile X syndrome (FXS) is the most common form of inherited mental retardation, with an estimated prevalence of one in 4000 males and one in 8000 females [58]. In addition to cognitive deficits, the phenotype of fragile X syndrome includes mild facial dysmorphism (prominent jaw, high forehead, and large ears), macroorchidism in postpubescent males, and subtle connective tissue abnormalities [58]. Many patients also manifest attention-deficit hyperactivity disorder and autistic-like behavior. As the first condition found to be caused by trinucleotide repeat expansion, fragile X syndrome is typically the result of a massive CGG trinucleotide repeat expansion within the 5' untranslated region (UTR) of the fragile X mental retardation 1 gene (*FMR1*), which results in transcriptional silencing of *FMR1* [19,39,47,48, 57]. The majority of affected individuals have expansions of over 200 CGG repeats, referred to as the full mutation. Most people in the general population carry between 5 and 54 repeats, while those individuals who carry CGG repeat expansions between 55 and 200 are referred to as premutation carriers. Over the last several years, male and to a lesser extent female premutation carriers are found to be at increased risk of developing an age-dependent progressive intention tremor and ataxia syndrome, which is uncoupled from fragile X syndrome [24] and known as fragile X-associated tremor/ataxia syndrome (FXTAS) (Figure 1). Besides a progressive action tremor with ataxia, patients with FXTAS also show a progressive cognitive decline that ranges from executive and memory deficits to dementia [24]. In addition, female premutation carriers are also found to have significantly higher rates of primary ovarian insufficiency (POI); however, the exact molecular basis of POI has yet to be determined (Figure 1).

Although both FXS and FXTAS involve CGG repeat expansions in the *FMR1* gene, the clinical presentations and molecular mechanisms underlying each syndrome are distinct. Interestingly, recent studies have implicated noncoding RNAs in the pathogenesis of both disorders. Here we will review our current knowledge about the involvement of noncoding RNAs in FXS and FXTAS, particularly the role of the microRNA (miRNA) pathway in FXS and the role of noncoding riboCGG (rCGG) repeat in FXTAS.

MiRNA pathway in fragile X syndrome

Identification of other mutations (e.g. deletions in patients with the typical phenotype) has confirmed that *FMR1* is the only gene involved in the pathogenesis of fragile X syndrome and that the loss of the *FMR1* product, fragile X mental retardation protein (FMRP), causes fragile X syndrome [14,44,60]. FMRP along with its autosomal paralogs, the fragile X-related proteins FXR1P and FXR2P, constitute a small, well-conserved family of RNA-binding proteins (fragile X-related gene family) that share over 60% amino acid identity and contain two types of RNA-binding motifs: two ribonucleoprotein K homology domains (KH domains) and a cluster of arginine and glycine residues (RGG box) [51,65]. Both the KH domains and RGG box of FMRP have been found to mediate FMRP-RNA interactions both *in vitro* and *in vivo* [2,16,17,41,43]. Both motifs contribute to the role of FMRP as a suppressor of target messenger RNA (mRNA) translation via binding of noncoding RNA structures, including G-quartets and "kissing complexes" (also known as loop-loop-pseudoknots), within the untranslated regions of target mRNAs [2,12,13,16,41,43,50,53]. The ability of FMRP to bind RNA and suppress translation has definite clinical relevance, as evidenced by a severely affected individual with an I304N missense mutation in the KH2 domain of FMRP [17]. As an RNA-binding protein, FMRP is found to form a messenger ribonucleoprotein (mRNP) complex that associates with translating polyribosomes [16]. Furthermore, FMRP is known to be involved in translational control and could suppress translation both *in vitro* and *in vivo* [41,43]. At the cellular level, abnormal dendritic spines are found in the brains of both human patients with fragile X syndrome and *Fmr1* knockout (KO) mice, implying that synaptic plasticity is affected in the absence of FMRP [11,46]. Based on these observations, it has been proposed that FMRP is involved in synaptic plasticity via regulation of mRNA transport and local protein synthesis

of specific mRNAs at synapses. Due to space limitations, we will focus here on the potential role of the miRNA pathway in fragile X syndrome. (For an in-depth discussion of the molecular pathogenesis of fragile X syndrome, please see the recent review by Bassell and Warren) [6].

MicroRNAs (miRNAs) are a new class of small noncoding RNAs that are ~22 nucleotides (nt) in length and generated from endogenous double-strand transcripts [5]. Typically, miRNAs bind to the 3' UTR of target mRNA, leading to translational repression or mRNA degradation. The roles miRNAs play in diverse biological pathways have been reviewed extensively. Endogenous miRNAs were found associated with FMRP, and FMRP interacts biochemically and genetically with known components of the miRNA pathway [8,28,61]. Experiments in *Drosophila* revealed specific biochemical interactions between *dFmrp* and functional RNA-induced silencing complex (RISC) proteins, including *dAGO1*, *dAGO2*, and *Dicer* [8,28,61]. *dFmr1* displays strong genetic interaction with *dAGO1*, and *dAGO1* dominantly interacts with *dFmr1* in both *dFmr1* overexpression and loss-of-function models [33]. Furthermore, *dFmr1* also interacts genetically with *AGO2*, as exemplified by their ability to coregulate *ppk1* mRNA levels [61]. Additional studies provide further evidence for the involvement of FMRP in miRNA-containing RISC and P body-like granules in *Drosophila* neurons [4]. Recombinant human FMRP can also act as an acceptor for Dicer-derived miRNAs, and importantly, endogenous miRNAs themselves are associated with FMRP in both flies and mammals [8, 28,33]. In the adult mouse brain, *Dicer* and *eIF2c2* (the mouse homolog of *AGO1*) interact with FMRP at postsynaptic densities [45]. Recently, the phosphorylation of FMRP was also shown to increase its association with miRNA precursors [9,10]. Presumably, this interaction works to regulate translation of target mRNAs in an activity-dependent manner. Based on these findings, it has been proposed that the RISC proteins, including Argonaute (Ago) and Dicer, could interact with FMRP and use the loaded guide miRNA(s) to interact with target sequences within the 3' UTR of RNA bound to FMRP and suppress its translation [31,33]. In this model, FMRP facilitates the interaction between miRNAs and their target mRNA sequence, ensuring proper targeting of guide miRNA-RISC within the 3' UTRs and proper translational suppression.

The fact that FMRP is associated with Dicer, miRNAs, and specific mRNA targets raises the question of whether FMRP is associated with specific miRNAs and modulates their processing. To address this question, the expression and processing of miRNAs were examined in *Drosophila dFmr1* mutants; in the fly brain, *dFmrp* was specifically associated with miR-124a, a nervous-system-specific miRNA [62]. *dFmrp* is required for the proper processing of pre-miR-124a, whereas the loss of *dFmr1* leads to a reduced level of mature miR-124a and an increased level of pre-miR-124a. These results suggest a modulatory role for *dFmrp* to maintain proper levels of miRNAs during neuronal development [62]. In our own studies, we have shown that *dFmr1*, the *Drosophila* ortholog of the *FMR1* gene, plays a role in the proper maintenance of germline stem cells in *Drosophila* ovary, potentially through the miRNA pathway [63]. To test this, we recently used an immunoprecipitation assay to reveal that specific microRNAs (miRNAs), particularly the bantam miRNA (*bantam*), are physically associated with *dFmrp* in ovary [64]. We found that, like *dFmr1*, *bantam* is not only required for repressing primordial germ cell differentiation, it also functions as an extrinsic factor for germline stem cell maintenance [64]. Furthermore, we showed that *bantam* genetically interacts with *dFmr1* to regulate the fate of germline stem cells [64]. Collectively, our results support the notion that the FMRP-mediated translational pathway functions through specific miRNAs to control stem cell regulation; however, we saw no effect of *dFmrp* on the biogenesis of the bantam miRNA. Whether FMRP is associated with specific miRNAs in mammalian cells remains to be determined.

All the current findings support the idea that FMRP could regulate the translation of its mRNA through miRNA interaction. However, the exact mechanism of its action together with the

RISC and miRNAs is not yet clear. Thus the relevance of these observations to FMRP-mediated translational regulation and the possible involvement of miRNAs in mental retardation more generally remain to be explored.

RNA-mediated neurodegeneration in FXTAS

Fragile X-associated tremor/ataxia syndrome (FXTAS) has been recognized in older males of FXS families and is uncoupled from the neurodevelopmental disorder, FXS [23]. Although both disorders involve repeat expansions in the *FMR1* gene, the clinical presentation and molecular mechanisms underlying each are completely distinct. The most common clinical feature of FXTAS is a progressive action tremor with ataxia. More advanced or severe cases may show a worsening cognitive decline that ranges from executive and memory deficits to dementia [22]. Patients may also present with common psychiatric symptoms, such as increased anxiety, mood lability, and depression [3,27]. Nearly all case studies from autopsies on the brains of symptomatic premutation carriers demonstrate degeneration in the cerebellum, which includes Purkinje neuronal cell loss, Bergman gliosis, spongiosis of the deep cerebellar white matter, and swollen axons [20,21]. The major neuropathological hallmark and postmortem criterion for definitive FXTAS is eosinophilic, ubiquitin-positive intranuclear inclusions located in broad distribution throughout the brain in neurons, astrocytes, and in the spinal column [20]. The inclusions are both tau and α -synuclein negative, which indicates that FXTAS is not a tauopathy or synucleinopathy. Notably, the FXTAS inclusions share the same ubiquitin-positive hallmark as several other inclusion diseases, such as polyglutamine disorders, yet the inclusions do not stain with antibodies that recognize polyglutamine, which suggests a defect in the proteasomal degradation pathway [21,24,54]. Furthermore, unlike the polyglutamine disorders, there is no known structurally abnormal protein associated with FXTAS.

One logical explanation for the variability in the FXTAS clinical phenotype might be variability in the size of the premutation alleles of individual patients. Interestingly, all these patients are carriers of a premutation-size CGG repeat (55-200 triplets) in the 5' UTR of the *FMR1* gene. The repeat is expressed in the mature *FMR1* mRNA in premutation carriers, as well as in individuals with normal CGG repeat lengths. A study of families with known FXS probands into the penetrance of tremor and ataxia among premutation carriers revealed that more than a third of carriers 50 years or older show symptoms of FXTAS, and that the penetrance of this disorder exceeds 50% for men over 70 years of age [29]. The prevalence of premutation alleles is approximately 1 in 800 for males and 1 in 250 for females in the general population; however, it is estimated that 1 in 3000 men over 50 in the general population will show symptoms of FXTAS [15]. These estimates do not consider a size bias, which takes into account the correlation between the age of onset of symptoms and the size of the repeat. Recent studies have correlated the age of onset of clinical FXTAS symptoms with the length of expanded repeats and shown that larger CGG repeats represent an increased risk factor for the development of FXTAS [30,42]. Moreover, the degree of brain atrophy and severity of the tremor and ataxia are associated with the CGG repeat length [42]. Some female carriers also develop clinical features of FXTAS, but at a much lower frequency than males, which may be due to partial protection conferred by random X-inactivation of the premutation allele [25, 29,66].

At the molecular level, the premutation is different from either the normal or full mutation alleles. Besides the obvious difference of the CGG repeat length itself, there are a number of other distinctions. In cells from premutation carriers over a wide range of repeat lengths, the level of *FMR1* mRNA is elevated some two to eight times above normal levels, while the stability of *FMR1* mRNA is unchanged (Figure 2). Indeed, even in the high-end normal range (~55 repeats), the *FMR1* message level was near double that found in normal alleles (~30

repeats) [36,55]. In addition, some hypomethylated full mutation alleles produce substantial amounts of *FMR1* message (~4.5-fold normal) [55]. Although the precise mechanism for this overexpression is unknown, it is likely that the increasing length of the CGG repeat near the *FMR1* promoter proportionally opens the chromatin, allowing more ready access to transcription factors. Despite this elevation in *FMR1* levels, the amount of *FMR1* protein is modestly reduced in the premutation range (~80% of normal) and may be largely absent in cells expressing *FMR1* message with very long repeats (> 300 repeats) [18,36]. This paradoxical reduction of translation despite elevated mRNA levels can be explained by the fact that long CGG repeats in the *FMR1* mRNA impede 40S ribosomal subunit migration from the 5' cap to the initiating codon. It is unlikely that overexpression of *FMR1* mRNA is a response to the reduction of FMRP levels, as I304N mutant cells with 30 CGG repeats, which produce nonfunctional protein, do not show an elevated *FMR1* mRNA [16,36].

An RNA gain-of-function mechanism has been suggested for FXTAS based on the observation of increased levels of CGG-containing *FMR1* mRNA, along with either no detectable change in FMRP or slightly reduced FMRP levels in premutation carriers [21,29,34,55,59]. The absence of FXS, which results from the loss of function of the *FMR1* gene product, in patients with FXTAS, along with the absence of FXTAS symptoms in older individuals with FXS also suggests a role for the expanded rCGG repeat in FXTAS pathology. This type of RNA gain-of-function mechanism may also account for some triplet repeat-related ataxias, such as spinocerebellar ataxia type 8 (SCA8), SCA10, and SCA12, and in myotonic dystrophy (DM) [49]. The untranslated repeat expansion in DM has in fact given us major insight into the underlying molecular mechanisms of FXTAS. DM1 is caused by a CTG repeat expansion in a region of the 3' UTR of the *DMPK* gene that is transcribed into RNA but not translated into protein. The mutant transcripts sequester MBNL and other proteins, which form ribonuclear foci or inclusions. Indeed, using *in situ* hybridization, Tassone et al. demonstrated the presence of expanded *FMR1* RNA transcripts in the FXTAS inclusions of a 70-year-old male who died with FXTAS [56].

Beyond the observation of increased levels of CGG-containing *FMR1* mRNA in fragile X premutation carriers, several other lines of evidence further support an RNA-mediated gain-of-function toxicity model for FXTAS. First, in a “knock-in” mouse model, in which the endogenous CGG repeats (five CGG repeats in the wild-type mouse *Fmr1* gene) were replaced with a ~100-CGG repeat fragment, intranuclear inclusions were found to be present throughout the brain, with the exception of cerebellar Purkinje cells [59]. There was an increase in both the number and size of the inclusions over the life course, which correlates with the progressive character of the phenotype observed in humans [20]. Second, neuropathological studies in humans have revealed a highly significant association between length of the CGG tract and frequency of intranuclear inclusions in both neurons and astrocytes, indicating that the CGG repeat length is a powerful predictor of neurological involvement clinically (age of death) as well as neuropathologically (number of inclusions) [20]. Third, intranuclear inclusions can be formed in both primary neural progenitor cells and established neural cell lines, as was revealed using a reporter construct with an *FMR1* 5' UTR harboring expanded (premutation) CGG repeats [1]. Fourth, we described a model of FXTAS using *Drosophila* expressing the *FMR1* untranslated CGG repeats 5' to the EGFP coding sequence and demonstrated that premutation-length rCGG repeats are both toxic and sufficient to cause neurodegeneration [34]. And finally, it has been found recently that rCGG expressed in Purkinje neurons outside the context of *Fmr1* mRNA can result in neuronal pathology in a mammalian system [26]. These observations led us and others to propose that transcription of the CGG₉₀ repeats leads to an RNA-mediated neurodegenerative disease. We further posited a mechanism by which rCGG repeat-binding proteins (RBPs) may become functionally limited by their sequestration to lengthy rCGG repeats, mechanistically similar to the pathophysiology of DM1.

To test this model using both biochemical and genetic approaches, we and others have identified three proteins, Pur α , hnRNP A2/B1 (two protein isoforms from one gene), and CUGBP1, which bind rCGG repeats either directly (Pur α and hnRNP A2/B1) or indirectly (CUGBP1, through the interaction with hnRNP A2/B1) [32,52]. Thus, there are protein complexes that physically interact with rCGG repeats, and all of these are RNA-binding proteins that have been shown to function in transcription, mRNA trafficking, splicing, and translation. Interestingly, Pur α knock-out mice appeared normal at birth, but developed severe tremor and spontaneous seizures at two weeks of age due to drastically lower numbers of neurons in regions of the hippocampus and cerebellum [38]. The hypothesis is that overproduced rCGG repeats in FXTAS sequester these proteins from their normal cellular functions, contributing significantly to the pathology of this disorder. This idea is given strong support by the fact that overexpressing either Pur α or hnRNP A2/B1 alleviated neurodegeneration in a fly model of FXTAS [32,52]. The next important step will now be to further identify the RNAs regulated by these proteins that are altered by the expression of rCGG repeats and contribute to the pathogenesis of FXTAS.

Interestingly an antisense transcript, *ASFMR1*, has recently been identified to overlap the CGG repeat region of the *FMRI* gene [37,40]. Similar to *FMRI*, the *ASFMR1* transcript is silenced in full mutation individuals and up-regulated in premutation carriers. However, whether *ASFMR1* contributes to the pathogenesis of either FXTAS or FXS remains to be determined.

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Figure 1. Mutations in the *FMR1* gene can lead to three distinct disorders

The normal human *FMR1* gene possesses a CGG trinucleotide repeat size of between 5 and 54 in the 5' UTR. A large expansion of over 200 CGG repeats triggers the transcriptional silencing of *FMR1*, which results in fragile X syndrome (FXS). For those individuals with CGG repeat expansions between 55 and 200, an age-dependent progressive neurodegenerative disorder, called fragile X-associated tremor/ataxia syndrome (FXTAS), has been recognized among many male carriers in or beyond their fifth decade of life. Female carriers, on the other hand, may suffer from primary ovarian insufficiency (POI).

Figure 2. Genotype-phenotype correlation at the *FMR1* locus

In fragile X syndrome, large expansions of the CGG trinucleotide repeat (> 200 repeats) in the 5' UTR of the *FMR1* gene causes hypermethylation of the *FMR1* promoter, which leads to the transcriptional silencing of *FMR1* and the loss of the *FMR1* product, FMRP. However, the level of *FMR1* mRNA is elevated some two to eight times above normal levels in premutation carriers (55-200 CGG repeats), whereas the amount of FMRP appeared to remain at or slightly below normal levels. The observation of increased *FMR1* mRNA levels in premutation carriers supports the idea that FXTAS is an RNA-mediated neurodegenerative disorder.

Figure 3. RNA-binding proteins (RBPs) are involved in FXTAS pathogenesis

Three RNA-binding proteins, Pur α , hnRNP A2/B1, and CUGBP1, were found to bind rCGG repeats either directly (Pur α and hnRNP A2/B1) or indirectly (CUGBP1, through the interaction with hnRNP A2/B1). hnRNP A2/B1 forms a complex with CUGBP1 to bind to rCGG repeats, which is independent of Pur α . Overexpression of these proteins can suppress neuronal cell death caused by fragile X premutation rCGG repeats, supporting the model that sequestration of these proteins by the overproduced rCGG repeats in FXTAS prevents them from carrying out their normal functions, leading to abnormal RNA metabolism and neurodegeneration.