RNA-mediated pathogenesis in fragile X-associated disorders

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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 (∼30Mb) 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.

Fragile X syndrome (FXS) is the most common form of inherited mental retardation, with an estimated prevalence of 1 in 4000 males and 1 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) (Fig. 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 (Fig. 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.

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 auto-
somal paralogs, the fragile X-related proteins FXR1P and FXR2P,
constitutes a small, well-conserved family of RNA-binding pro-
teins (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 clus-
ter of arginine and glycine residues (RGG box) [51,65]. Both the
KH domains and RGG box of FMRP have been found to medi-
ate FMRP-RNA interactions both in vitro and in vivo [2,16,41,43].
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
target mRNA through miRNA interaction. The ability of FMRP to bind
RNA and suppress translation has definite clinical relevance, as evi-
denced by a severely affected individual with an I304N missense
mutation in the KH2 domain of FMRP [17]. As an RNA-binding pro-
tein, FMRP is found to form a messenger ribonucleoprotein (mRNP)
complex that associates with translating polyribosomes [16]. Fur-
thermore, 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 knock-
out (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 endoge-
nous 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 path-
ways 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 silenc-
ing 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 over-
expression 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]. Recombi-
nant 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 elf2c2 (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 [8,10]. 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 trans-
lational 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 exam-
ined 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 extrin-
sic factor for germline stem cell maintenance [64]. Furthermore,
we showed that bantam genetically interacts with dFmr1 to regu-
late 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 ban-
tam 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 miRNA 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 gen-
erally remain to be explored.
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 liability, 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 are 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 appears to remain at or slightly below normal levels, as I304N mutant cells with 30 CGG repeats demonstrate 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 1304N 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 or slightly reduced FMRP levels in premutation carriers.
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 man 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 postulated 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). 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.

regulated by these proteins that are altered by the expression of rCGG repeats and contribute to the pathogenesis of FXTAS (Fig. 3).

Interestingly an antisense transcript, ASFMR1, has recently been identified to overlap the CGG repeat region of the FMR1 gene [38,40]. Similar to FMR1, 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.

It is remarkable that the same type of mutation in the FMR1 gene is involved in three distinct disorders that affect patients in an age-dependent manner (Fig. 1). Interestingly, two of these syndromes, FXS and FXTAS, are known to be caused, at least in part, by altered noncoding RNA-mediated gene regulation. Understanding the role of noncoding RNAs in these diseases will not only help unravel the molecular pathogenesis of fragile X-related disorders, but will also tell us much about the role of noncoding RNAs in human diseases in general.

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References


containing neuronal RNPs are structurally and functionally related to somatic P bodies, Nature 52 (2006) 997–1009.


P. Kapranov, J. Cheng, S. Dike, D.A. Nix, R. Duttagupta, A.T. Williamson, P.F. Stadler, J. Hertel, J. Hackm Functions den...
L.P. Ranum, T.A. Cooper, RNA-mediated neuromuscular disorders, Annu. Rev.
G. Stefani, C.E. Fraser, J.C. Darnell, R.B. Darnell, Fragile X mental retardation pro-
O.A. Sofola, P. Jin, Y. Qin, R. Duan, H. Liu, M. de Haro, D.L. Nelson, J. Botas, RNA-
G. Lugli, J. Larson, M.E. Martone, Y. Jones, N.R. Smallheiser, Dicer and eIF2c are
Z. Li, Y. Zhang, L. Ku, K.D. Wilkinson, S.T. Warren, Y. Feng, The fragile X mental
B. Laggerbauer, D. Ostareck, E.M. Keidel, A. Ostareck-Lederer, U. Fischer, Evi-
M.A. Leehey, E. Berry-Kravis, C.G. Goetz, L. Zhang, D.A. Hall, L. Li, C.D. Rice, R.
I. Oberle, F. Rousseau, D. Heitz, C. Kretz, D. Devys, A. Hanauer, J. Boue,
B. Laggerbauer, D. Ostareck, E.M. Keidel, A. Ostareck-Lederer, U. Fischer, Evi-
M.C. Siomi, H. Siomi, W.H. Sauer, S. Srinivasan, R.L. Nussbaum, G. Dreyfuss, FXR1,
Y. Yang, S. Xu, L. Xia, J. Wang, P. Jin, D. Chen, MicroRNA Bantam is associated
C. Zuhlke, A. Budnik, U. Gehlken, A. Dalski, S. Purmann, M. Naumann, M.
K. Xu, B.A. Bogert, W. Li, K. Su, A. Lee, F.B. Gao, The fragile X-related gene affects
F. Tassone, R.J. Hagerman, A.K. Taylor, L.W. Gane, T.E. Godfrey, P.J. Hagerman, Ele-
vated levels of FMR1 mRNA in carrier males: a new mechanism of involvement
F. Tassone, C. Iwashashi, P.J. Hagerman, FMR1 RNA within the intranuclear inclu-
103–105.
A.J. Verkerk, M. Pieretti, J.S. Sutcliffe, YH. Fu, D.P. Kohl, A. Pizzuti, O. Reiner, S.
Richards, M.F. Victoria, F.P. Zhang, B.E. Eussen, G.B. Van Ommeren, L.A. Blonden,
Oostra, S.T. Warren, Identification of a gene (FMR-1) containing a CGG repeat coinci-
dent with a breakpoint cluster region exhibiting length variation in fragile X
B. Laggerbauer, D. Ostareck, E.M. Keidel, A. Ostareck-Lederer, U. Fischer, Evi-
M.A. Leehey, E. Berry-Kravis, C.G. Goetz, L. Zhang, D.A. Hall, L. Li, C.D. Rice, R.
I. Oberle, F. Rousseau, D. Heitz, C. Kretz, D. Devys, A. Hanauer, J. Boue,
F. Tassone, C. Iwashashi, P.J. Hagerman, FMR1 RNA within the intranuclear inclu-
103–105.
A.J. Verkerk, M. Pieretti, J.S. Sutcliffe, YH. Fu, D.P. Kohl, A. Pizzuti, O. Reiner, S.
Richards, M.F. Victoria, F.P. Zhang, B.E. Eussen, G.B. Van Ommeren, L.A. Blonden,
Oostra, S.T. Warren, Identification of a gene (FMR-1) containing a CGG repeat coinci-
dent with a breakpoint cluster region exhibiting length variation in fragile X
F. Tassone, C. Iwashashi, P.J. Hagerman, FMR1 RNA within the intranuclear inclu-
103–105.