

# Chapter 6

## The Molecular Biology of Premutation Expanded Alleles

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**Abstract** Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late adult onset neurodegenerative disorder that mainly affects male carriers of an allele of 55–200 CGG repeats in the *FMR1* gene (premutation). FXTAS symptoms include progressive intention tremor, gait ataxia, neuropathy, psychiatric symptoms, cognitive decline, and autonomic dysfunctions. Neuropathological features of FXTAS include global cerebral and cerebellar atrophy, spongiform changes of white matter, marked Purkinje cell dropout and presence of ubiquitin-positive intranuclear inclusions throughout the brain. In contrast to fragile X Syndrome (FXS), FXTAS is associated with elevated expression of repeat containing *FMR1* mRNA, which binds to and sequesters specific RNA binding proteins and impedes their normal functions. In addition, the CGG repeat expansion triggers a non-canonical translational initiation event to produce a polyglycine containing protein (FMRpolyG) that accumulates in patients brains. Here we discuss these and other putative molecular mechanisms for FXTAS pathogenesis with a focus on recent findings.

**Keywords** RAN translation • RNA disease • Transcription • Antisense  
• Neurodegenerative disease • Disease mechanisms

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## Introduction

Although the developmental disorder, fragile X syndrome (FXS), is almost always caused by CGG repeat expansions exceeding 200 CGG repeats (full mutation range), with hypermethylation of the promoter region and consequent transcriptional silencing (Fu et al. 1991; Oberlé et al. 1991; Pieretti et al. 1991; Verkerk et al. 1991; Yu et al. 1991), the adult onset neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome (FXTAS), almost exclusively affects carriers of premutation alleles (55–200 CGG repeats) with nearly all cases having alleles that exceed ~65–70 CGG repeats (Jacquemont et al. 2006). Premutation alleles are generally unmethylated but several studies have reported on the presence of premutation alleles partial methylated even in the lower premutation size range and in some case the methylation status correlated with the severity of the observed clinical involvement (Allingham-Hawkins et al. 1996; Pretto et al. 2014c; Tassone et al. 1999). Furthermore, several studies have reported FXTAS in individuals with a partially methylated full mutation (Loesch et al. 2012; Pretto et al. 2014a; Santa Maria et al. 2013) and also in individuals with smaller alleles in the “gray zone” or intermediate range (45–54 CGG repeats; normal <45 CGG repeats) (Hall et al. 2012; Liu et al. 2013).

Concerning instability of the CGG repeat, alleles in the normal, gray, and premutation size ranges generally have one or more AGG trinucleotides within the CGG repeat tract, typically spaced with intervals of 8–11 intervening CGG repeats (e.g., 9-10-10 pattern). However, these intervening repeats are generally lost with larger CGG repeat sizes within the premutation size range (Kunst and Warren 1994; Zhong et al. 1996), which may result in the loss of repeat length stability (Zhong et al. 1995). Interestingly, expansion from a premutation allele to a full mutation allele occurs only during maternal transmission; that is, transmission of a full mutation allele never occurs from the father, even if the father is a carrier of a full mutation allele (Nolin et al. 2003). Although the presence of AGG interruptions within the CGG repeat does not affect either the expression of the *FRM1* mRNA (Yrigollen et al. 2012, 2014) or its translation (Ludwig et al. 2009), it has been demonstrated to play a key role in the stability of the repeat (Nolin et al. 2013; Yrigollen et al. 2012, 2014). Importantly, Yrigollen et al. demonstrated that the risk of expansion from premutation to full mutation alleles during maternal transmission decreases by increasing number of AGG interruptions. In addition, the risk of instability of an intermediate or premutation allele during maternal or paternal transmission is reduced with the presence of AGG interruptions, as is the magnitude of size change that occurs during transmission (Nolin et al. 2013; Yrigollen et al. 2014). Using logistic regression of 710 maternal transmissions and considering the total length of the CGG repeat allele, the number of AGG interruptions, and the age of the mother at childbirth, a model for measuring the risk of expansion to a full mutation during maternal transmission was calculated. This model was determined to be more suitable than that which considered pure CGG stretch instead of total length, confirming the previous data on a smaller sample (Yrigollen et al. 2012).

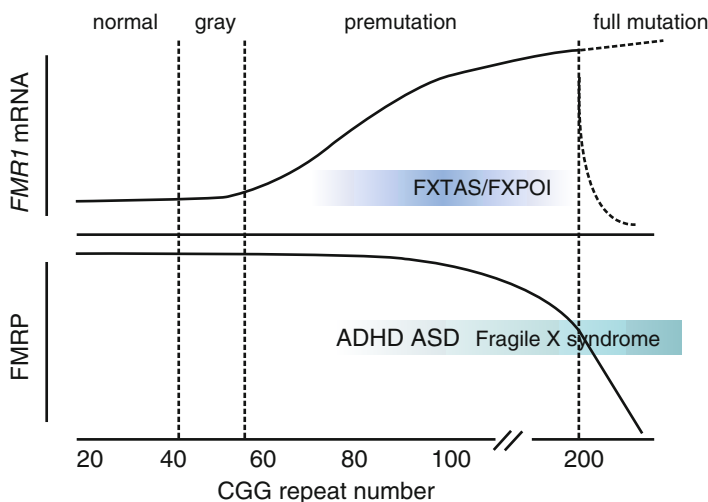
The prevalence of full mutation is approximately 1 in 5000 males and 1 in 2500–8000 females (reviewed in (Tassone et al. 2012a)). However, based on a recent systematic review and meta-analysis, approximately 1/7000 males and 1/11,000 females carry the full mutation (Hunter et al. 2014).

Reported frequencies of the premutation allele vary among studies of various populations. Canadian studies reported a premutation frequency of approximately 1 in 250 for females and 1 in 800 for males (Dombrowski et al. 2002; Rousseau et al. 1995). However, the frequency of the premutation in females in Israel is substantially higher, at approximately 1 in 113 (Toledano-Alhadeef et al. 2001), and lower in an Asian population with a rate of 1 in 1674 premutation males in Taiwan (Tzeng et al. 2005). In a recent report, using the known frequency for premutation females (using the average of 1 in 126 from (Pesso et al. 2000; Toledano-Alhadeef et al. 2001)) to calculate the expected number of full mutations, Hagerman (2008) determined that the expected frequency for premutation males would be 1 in 282 and for full mutation (both males and females) would be 1 in 2355. Interestingly, the former value is in agreement with what was observed in recent larger screening studies in which the prevalence of premutation alleles ranged between 150 and 210 in females and 290 and 430 in males (Hunter et al. 2014; Maenner et al. 2013; Tassone et al. 2012a).

## Clinical Involvement in *FMRI* Premutation Carriers

Over the last several years, there has been increasing awareness of the spectrum of the phenotypes associated with premutation alleles of the *FMRI* gene (Hagerman and Hagerman 2013). While the presence of a full mutation allele (>200 CGG repeats) generally result in FXS, with autism spectrum disorders in over 60% of subjects (Harris et al. 2008), smaller repeat expansions in the premutation range give rise to several distinct forms of clinical involvement: (a) fragile X-associated tremor/ataxia syndrome (FXTAS) mainly in older males; (b) fragile X-associated primary ovarian insufficiency FXPOI in approximately 20% of all carrier females; and may give rise to (c) behavioral, physical, emotional, and cognitive problems in some children who are premutation carriers, particularly at repeat sizes in the upper end of the premutation range.

FXTAS is mainly observed in males as demonstrated by the absence of significant differences between carrier females and controls when rated by CRST, ICARS, or UPDRS (Berry-Kravis et al. 2003; Jacquemont et al. 2004), or FXTAS Rating Scale (Leehey et al. 2007)—for details of use and findings with these rating scales in FXTAS the reader can see Chaps. 1 and 7. However, a percentage of female carriers clearly present clinical and neuropathological signs of FXTAS (Berry-Kravis et al. 2005; Jacquemont 2005; Peters et al. 2006; Zuhlke et al. 2004). In contrast to fragile X syndrome, both FXPOI and FXTAS appear to be generally confined to the premutation range with some exceptions (Liu et al. 2013; Loesch et al. 2012; Pretto et al. 2014c; Santa Maria et al. 2013). Moreover, while full mutations are



**Fig. 6.1** FXTAS and FXPOI are largely confined to the premutation range and are thought to occur through an RNA toxic gain of function due to excess *FMR1* mRNA; however, patients with partially methylated full mutation alleles continue to express mRNA and are potentially at risk of developing FXTAS. By contrast, fragile X syndrome is caused by reduced/absent FMRP, due to silencing of the *FMR1* gene in the full mutation range. Features of the fragile X syndrome spectrum may also occur in the upper premutation range due to reduced protein production. *Dashed lines* for *FMR1* mRNA levels in the full mutation range reflect variations in degree of methylation; FMRP levels are reduced due to both *lower* mRNA levels and reduction in translation efficiency from the *upper* premutation range

characterized by the absence of *FMR1* mRNA, and consequently by the absence of FMRP, premutation carriers possess elevated levels of *FMR1* mRNA, with the extent of the increase depending upon the size of the premutation repeat expansion (Sellier et al. 2014; Tassone et al. 2000a). For the largest premutation alleles, FMRP levels are moderately reduced (up to twofold) due to a relative impediment to translation, demonstrated in both human and mouse (Ludwig et al. 2014; Primerano et al. 2002). A diagram of the relative levels of *FMR1* mRNA and proteins (FMRP) as a function of the number of CGG repeats and the association of clinical phenotypes is shown in Fig. 6.1.

The expanded CGG repeat, located in the 5' UTR region of the *FMR1* gene, is now believed to cause the cellular toxicity in FXTAS, whereas FMRP is not thought to play a role in the neurodegenerative syndrome, since full mutation alleles (where FMRP is absent) are not associated with FXTAS. It is, however, possible that the decreased levels of FMRP could somehow act in concert with the effects of the toxic mRNA, particularly in the upper end of the premutation range, where lower FMRP levels are detected; however, many cases of FXTAS are observed for CGG repeats in the 80–100 range, where FMRP levels are not substantially reduced. Thus, it appears clear that FXS and FXTAS are caused by a CGG expansion in the *FMR1* gene but with completely different molecular mechanisms (RNA Gain of Function for FXTAS; Loss of FMRP function for FXS).

As noted above, FXTAS is more frequent in males, as would be expected for an X-linked disorder (Adams et al. 2007; Berry-Kravis et al. 2007; Coffey et al. 2008; Greco et al. 2008; Hagerman and Hagerman 2004b; Hagerman et al. 2004, 2007). In particular, female carriers are less likely to develop the disorder due to the protective effect of the expression of the normal allele in a portion of cells. Moreover, for those females who do experience features of FXTAS, expression of the normal *FMR1* allele is also likely to be responsible for the less severe clinical outcome (Hagerman and Hagerman 2004b; Zuhlke et al. 2004). X-inactivation effects are therefore predicted to have a protective effect toward the neurological features of FXTAS. Several reports, in which a correlation between the severity of clinical signs and the X-inactivation ratio has been observed, seem to be supportive of such theory (Berry-Kravis et al. 2005; Coffey et al. 2008; Jacquemont 2005). However, females can present with significant higher rates of muscle pain, thyroid problems, hypertension, and fibromyalgia (Coffey et al. 2008; Hagerman and Hagerman 2013; Hundscheid et al. 2003; Rodriguez-Revenga et al. 2009).

Although details of the medical, cognitive, psychiatric, and neurological phenotypes of FXTAS and in premutation carriers in general, are discussed more extensively in other chapters of this book, a few additional comments regarding phenotype are pertinent here. Borderline to mild cognitive deficits generally are observed when FMRP levels are moderately reduced, as is often the case for individuals with alleles in the high premutation range (>150 CGG repeats) or for individuals with alleles in the low full mutation range that remain unmethylated and therefore retain transcriptional and somewhat translational activity (Tassone et al. 2000b). Numerous phenotypic problems have been reported in premutation carriers that seem to begin in many cases in childhood. The most consistent deficits seen in such carriers of a large premutation include shyness, anxiety, social deficits, ADHD, and executive function deficits (Aziz et al. 2003; Chonchaiya et al. 2012; Cornish et al. 2005; Farzin et al. 2006). Thus, for the larger premutation alleles, cognitive involvement may reflect the combined effects of both RNA toxicity and lowered FMRP levels (Farzin et al. 2006; Goodlin-Jones et al. 2004; Tassone et al. 2000a). The occurrence of seizures in about 8–13% of those with the premutation (Bailey et al. 2008; Chonchaiya et al. 2012; Hagerman and Stafstrom 2009), appears to be associated with the development of autism spectrum disorder (ASD) in males with the premutation (Chonchaiya et al. 2012). Further, immune mediate disorders, including hypothyroidism and fibromyalgia, sleep apnea, hypertension, and migraine are more common in premutation carriers than controls (Hagerman and Hagerman 2013). Finally, very recent studies of early development in infant premutation carriers has evidenced the presence of processing deficits similar to infants with FXS. Specifically, a visual discrimination deficit in babies with the premutation that is similar to what seen in babies with the full mutation and that is significantly different from visual discrimination in age- and sex-matched control babies has been recently reported. These observations have suggested that spatiotemporal processing impairment may constitute an endophenotype in infant premutation carriers (Gallego et al. 2014). In addition, examination of cognitive, communication, and social-behavioral profiles has demonstrated subtle developmental differences in premutation infants as young as 12 months of age (Wheeler et al. 2015).

A number of studies have reported on the correlation between the length of the CGG repeat within the premutation range and the severity of clinical involvement in premutation carriers. These correlations are particularly strong with the neurological (FXTAS) phenotypes including the age of onset of tremor and ataxia (Tassone et al. 2007a), overall motor impairment (Leehey et al. 2007), severity of white matter disease and degree of brain atrophy (Cohen et al. 2006; Loesch et al. 2005), severity of neuropathic signs (Berry-Kravis et al. 2007), degree of neuropathy as measured by nerve conduction studies (Soontarapornchai et al. 2008), reduced cerebellar volume (Adams et al. 2007), and the percent of inclusions and age at death (Greco et al. 2006). These associations are discussed further in Chap. 5.

## ***FMRI* Gene Structure**

The *FMRI* gene consists of 17 exons spanning 38 kb of Xq27.3 (Eichler et al. 1993). The gene is expressed in many tissues; however, the highest expression of the 4.4 kb transcript is observed in brain, placenta, testis, lung, and kidney (Hinds et al. 1993). In both human, including fetal brain neurons, and mouse, extensive alternative splicing of the *FMRI* gene has been demonstrated by RT-PCR analysis, and several FMRP isoforms have been observed on Westerns (Ashley et al. 1993; Huang et al. 1996; Sittler et al. 1996; Verheij et al. 1993; Verkerk et al. 1993) due to alternative splicing in the carboxy-terminal half of the *FMRI* gene. Published studies have suggested that alternative splicing in the *FMRI* gene does not seem to be tissue specific, as similar ratios of transcripts were found in several fetal tissues, including brain and testis (Verkerk et al. 1993); however, quantitative approaches were not taken. The main splice variants observed in the *FMRI* gene involve the use of alternative splice acceptors in exons 12, 14, 15, and 17 (Ashley et al. 1993; Verkerk et al. 1993). The existence of 16 different mRNA isoforms has been recently described in different tissues (Pretto et al. 2015). Although the relative abundance of these isoforms was reported to be significantly increased in premutations, interestingly the abundance of isoforms spliced at both exons 12 and 14, specifically *Iso10* and *Iso10b*, containing the complete exon 15 and differing only in splicing in exon 17 was four to sixfold increased compared to controls. Based on their findings, the authors suggested that RNA toxicity is likely to arise from an increased level of all *FMRI* mRNA isoforms and such increase may have a functional relevance in the pathology of *FMRI*-associated disorders. Although the specific function of the splice variants is not known, four or five different FMRP isoforms have been described using FMRP-specific antibodies (Verheij et al. 1995) some of which act as shuttling proteins that transport their mRNA targets from the nucleus to the cytoplasm (Dury et al. 2013).

In addition to the presence of alternative splicing of the *FMRI* gene, a detailed analysis of the CG-rich, TATA-less, promoter region of the *FMRI* gene has revealed an influence of the CGG repeat with respect to initiation or the start of initiation. The existence of three distinct groups of transcriptional initiation sites, and the

distribution of these start sites, which is modulated by the number of CGG repeats in the downstream (5' UTR) region, has been demonstrated in different tissues including lymphocytes and neuronal cells (Beilina et al. 2004; Tassone et al. 2011). While premutation alleles appear to preferentially express the longer *FMRI* mRNA (50 bp longer compared to the most frequent transcription initiation site in the normal alleles) (site II or site III), normal alleles appear to preferentially use the shorter transcripts initiating at site I (Beilina et al. 2004; Tassone et al. 2011). Interestingly, the nucleotide sequence of all three transcriptional initiation sites was found to be highly similar to the consensus sequence of pyrimidine-rich initiator (Inr) elements (consensus sequence YYAN(T/A)YY) (Javahery et al. 1994), which are usually located near the start site and have been implicated in transcription initiation in TATA-less genes (Chow et al. 1995). In addition, a fourth transcription initiation site, located between the previously identified sites I and II, was identified in brain tissues, suggesting the presence of a brain-specific transcription start site (Tassone et al. 2011). The dependency of alternative transcription initiation site usage on the CGG repeats length has also been observed in mice (Tassone et al. 2011).

Alternative polyadenylation site usage in the 3'UTR, which is implicated in the regulation of gene expression of many genes, has also been observed in the *FMRI* mRNA. Several different mRNA transcripts through the usage of different polyadenylation signals were identified in both human and mouse brain tissue (Tassone et al. 2011). Thus, the combination of both alternative 5' and 3' UTRs in addition the complexity of expression of the *FMRI* isoforms suggest their potential role in neuronal physiology, as well as in *FMRI*-associated disorders; however, it needs to be investigated.

Importantly, the differential usage of initiators in normal and premutation alleles may imply a possible variation in translational efficiency (post-transcriptional regulation) due to variation in 5' structure and sequence. Indeed, the FMRP deficit observed, especially in the upper premutation range, is CGG dependent and is due to decreased translational efficiency. Reduced translational efficiency was observed both in cell lines and in transient transfection experiments using expanded alleles spanning the entire premutation range (Chen et al. 2003; Primerano et al. 2002). Similar findings are observed in two independently generated knock-in mouse models of FXTAS (Brouwer et al. 2008; Entezam et al. 2007; Iliff et al. 2013; Ludwig et al. 2014; Willemsen et al. 2003). To date, the precise mechanism by which the expanded CGG repeat impedes translation is not clear. Translation from the larger premutation alleles is expected to be severely inhibited by the predicted free energies of stabilization of the CGG repeat element. One interesting feature of the transition from normal to the premutation range is the gradual loss of AGG interruptions within the CGG element located in the 5' UTR region of the *FMRI* mRNA.

As mentioned earlier, a few studies have shown that both transcription and translation expression levels (Ludwig et al. 2009; Tassone et al. 2007b; Yrigollen et al. 2012) of the *FMRI* mRNA are not influenced by AGG interruptions. Therefore, even if the presence of AGG repeats may modify the secondary/tertiary structure of the UTR region of the *FMRI* gene, such alterations do not seem to have an effect on transcription or represent a rate-limiting step in translational initiation.

## Higher *FMRI* Transcription Rate in Premutation Alleles

While hypermethylation of the *FMRI* gene in full mutation alleles leads to transcriptional silencing, premutation alleles are generally unmethylated and, therefore, transcriptionally active. Moreover, *FMRI* message levels are two to tenfold higher than normal, particularly in the upper end of the premutation range (~100–200 CGG repeats), despite their reduced protein (FMRP) levels (Sellier et al. 2014). Both elevated mRNA levels (up to fivefold) and FMRP deficit were also observed in some fragile X males with a partially methylated full mutation, even for alleles greater than 300 CGG repeats (Tassone et al. 2000b).

Elevated *FMRI* mRNA levels observed in premutation carriers appear to result not from increased mRNA stability (Tassone et al. 2000a) but rather from increased transcriptional activity of the *FMRI* gene. This was best demonstrated by increased levels of run-on transcription in premutation alleles compared to normal (Tassone et al. 2007a). Nuclear retention of expanded repeat *FMRI* mRNA was not observed by RNA in situ hybridization experiments in patient derived lymphocytes (Tassone et al. 2007a) although this has not yet been evaluated in neurons. Elevated levels of *FMRI* messenger RNA and a deficit in translation efficiency in expanded alleles has been evaluated using in vitro translation experiments with a luciferase reporter mRNA containing the 5'UTR of the *FMRI* gene containing varying numbers of CGG repeats. Specifically, the translation efficiency gradually decreases with increasing CGG repeat number (Chen et al. 2003).

The augmented transcription of *FMRI* mRNA is associated with epigenetic alterations induced by the CGG repeat expansion itself (Todd et al. 2010). Specifically, ectopically expressed CGG repeat expansions are sufficient to elicit chromatin changes in a *Drosophila* model of the disease and similar changes are observed in patient derived cell lines. Interestingly, these alterations in local chromatin structure are dynamic and modifiable by genetic and pharmacologic means, suggesting that agents aimed at chromatin remodeling might have therapeutic potential in FXTAS (Todd et al. 2010). How exactly the repeat leads to these epigenetic alterations is unclear. Long tracts of CGG repeats are known to exclude nucleosomes in vitro (Wang et al. 1996). Should this also occur in vivo it could lead to enhanced transcription by enhancing access of transcription factors to the promoter. Alternatively, recent reports demonstrate that transcribed CGG repeats form R-loop structures that could also potentially trigger increased *FMRI* expression (Groh et al. 2014; Loomis et al. 2014; Usdin et al. 2014). Future work will be required to define the sequential pathway and proximal events that lead to transcriptional up-regulation.

## *ASFMRI*: The Antisense Transcript at the *FMRI* Locus

Using RACE analysis and strand-specific RT-PCR, Ladd and collaborators (Ladd et al. 2007) identified an antisense transcript of the *FMRI* gene (*ASFMRI*) overlapping with the CGG repeat region. The *ASFMRI* is widely expressed in human



tissues, with a higher expression in brain and similarly to the *FMRI* gene, the expression appears to be influenced by the CGG repeat number as it is upregulated in peripheral blood leukocytes of premutation carriers and silenced in full mutation alleles. Moreover, *ASFMRI* transcript is alternatively spliced, polyadenylated, exported to the cytoplasm, and contains an open reading frame encompassing the CCG repeat that potentially encodes a polyproline peptide (Ladd et al. 2007). Shortly after this report, Khalil and collaborators described a second antisense transcript to *FMRI*, *FMR4*, which originates upstream of the *FMRI* start site and covers 2.4 kb of sequence (Khalil et al. 2008). Expression of *FMR4*, like that of *ASFMRI* and *FMRI*, is increased in brain from premutation individuals and silenced in individuals with the full mutation (Khalil et al. 2008). Importantly, *FMR4* overexpression increases cell proliferation while *FMR4* down-regulation induces apoptosis in vitro (Khalil et al. 2008). More recently, two novel transcripts arising from the *FMRI* locus, *FMR5* and *FMR6*, were identified (Pastori et al. 2013). *FMR5* is a sense-oriented long non-coding RNA (lncRNA) transcribed from approximately 1 kb upstream of the *FMRI* transcription start site. In contrast to *FMRI*, the *FMR5* transcript is not differentially expressed in human brain from unaffected individuals compared to full and premutation patients, suggesting that its transcription is independent of CGG repeat expansion (Pastori et al. 2013). *FMR6* is a spliced 600 nt long antisense transcript whose sequence is complementary to the 3' region of *FMRI*. *FMR6* begins in the 3'UTR, ends in exon 15 of *FMRI*, and uses the same splice junctions as *FMRI*. Unexpectedly, the expression of *FMR6* is reduced in premutation carriers suggesting that abnormal transcription and/or chromatin remodeling occurs toward the distal end of the locus. However, the chromatin marks associated with the 3' end of *FMRI* in premutation carriers are yet to be described and further studies are needed to determine the potential contribution of these long non-coding RNA to the variable clinical phenotypes associated with FXTAS and to the *FMRI*-associated disorders.

## RNA Toxic Gain-of-Function Model

As stated above, FXTAS has been observed, with rare exceptions, only in premutation carriers (Loesch et al. 2012; Pretto et al. 2014c; Santa Maria et al. 2013). The absence of FXTAS in carriers of fully silenced *FMRI* alleles has led to the hypothesis that FXTAS is the result of a toxic gain-of-function of the *FMRI* mRNA itself (Hagerman and Hagerman 2004a; Hagerman et al. 2001). This hypothesis is based on a precedent set by the myotonic dystrophies, DM1 and DM2, in which expression of mutant RNAs containing hundreds to thousands of CUG (DM1) or CCUG (DM2) repeats accumulate in nuclear RNA aggregates that sequester specific RNA-binding proteins (the Muscleblind-like proteins). Depletion of the free pool of MBNL proteins leads to specific RNA metabolisms changes, which ultimately results in the symptoms of myotonic dystrophy (Nelson et al. 2013; Ranum and Cooper 2006).

Extending this RNA gain-of-function model to FXTAS predicts that the mutant *FMRI* RNA containing expanded CGG repeats may sequester specific proteins

resulting in loss of their normal functions, which would ultimately cause the symptoms of FXTAS (Hagerman and Hagerman 2004a). Consistent with this hypothesis, Iwahashi and collaborators identified more than 20 proteins from inclusions purified from brains of FXTAS patients (Iwahashi et al. 2006). Among these, two RNA-binding proteins were of special interest, hnRNP A2/B1, that is mutated in families with inherited degeneration affecting muscle, brain, bone and motor neurons (Kim et al. 2013), and MBNL1, the RNA-binding protein that is sequestered in myotonic dystrophy (Kanadia et al. 2003; Miller et al. 2000). However, a role for MBNL1 in FXTAS appears unlikely, since no genetic interaction between MBNL1 and CGG-mediated neurodegeneration was observed in fly model of FXTAS (Sofola et al. 2007), and no misregulation of splicing events regulated by MBNL1 were observed in brain samples from FXTAS patients (Sellier et al. 2010).

Association of hnRNP A2/B1 to RNA containing expanded CGG repeats was confirmed by several independent analyses (Jin et al. 2007; Sellier et al. 2010; Sofola et al. 2007). Specifically, the interaction of hnRNP A2/B1 with RNA containing expanded CGG repeats was observed in cytoplasmic cerebellar lysates (Sofola et al. 2007), and the importance of the titration of the cytoplasmic pool of hnRNP A2/B1 by expanded CGG repeats was demonstrated by the impaired dendritic delivery of the BC1 RNA, a known target of hnRNP A2/B1 (Muslimov et al. 2011). In contrast, nuclear hnRNP A2/B1 exhibited little binding to CGG RNA (Sofola et al. 2007), and alternative splicing regulated by nuclear hnRNP A2/B1 were not altered in brain samples of FXTAS patients (Sellier et al. 2010). These data suggest that some modifications of hnRNP A2/B1, either in the nucleus or in the cytoplasm, may modify the ability of hnRNP A2/B1 to bind to CGG RNA, so that expanded CGG repeats may recruit and deplete the cytoplasmic pool of hnRNP A2/B1, but not the nuclear pool of hnRNP A2/B1. In addition, the ability of hnRNP A proteins to unfold RNA structures formed by expanded CGG repeats (Khateb et al. 2004; Ofer et al. 2009) raises the interesting hypothesis that hnRNP A2/B1 may also act as an RNA chaperone that destabilizes the RNA structures formed by expanded CGG repeats.

hnRNP A2/B1 recruits other proteins, such as the CUGBP1 RNA-binding protein, to RNA containing expanded CGG repeats (Sofola et al. 2007). Overexpression of either hnRNP A2/B1 or CUGBP1 rescues the neurodegeneration in a *Drosophila* expressing 90 CGG repeats, highlighting the potential importance of hnRNP A2/B1 and CUGBP1 to FXTAS pathology (Jin et al. 2007; Sofola et al. 2007). Similarly, hnRNP A2/B1 interacts with the heterochromatin protein 1 (HP1) to silence the expression of specific *Drosophila* retrotransposons, such as *gypsy* or *copia*, and the titration of hnRNP A2/B1 by expanded CGG repeats results in increased expression of these retrotransposons in fly model of FXTAS (Tan et al. 2012). Knockdown of *gypsy* RNA expression, but not of *copia*, suppress the toxicity induced by expanded CGG repeats in *Drosophila* (Tan et al. 2012). Whether the expression of retrotransposons is altered in FXTAS patients remains to be determined, but it is interesting to note that increased levels of 5-hydroxymethylcytosine (5hmc) have been identified in repetitive elements DNA in a mouse model of FXTAS (Yao et al. 2014).

In addition to hnRNP A2/B1, proteomic analyses revealed that SAM68, a splicing regulator encoded by the *KHDRBS1* gene, was also found in CGG RNA aggregates (Sellier et al. 2010). However, overexpression of SAM68 was not suf-

ficient to rescue neuronal cell death induced by expression of expanded CGG repeats. Similarly to hnRNP A2/B1 that recruits CUGBP1 through protein–protein interactions, SAM68 did not bind directly to CGG repeats and recruitment of SAM68 within CGG RNA aggregates occurred *in trans*.

Sellier and collaborators (2013) found that DROSHA-DGCR8, the enzymatic complex that processes pri-microRNAs into pre-miRNAs, associates directly with expanded CGG repeats. Sequestration of DROSHA-DGCR8 within CGG RNA aggregates resulted in reduced processing of pri-miRNAs in neuronal cells expressing expanded CGG repeats, and overexpression of DGCR8 rescued neuronal cell death induced by expression of CGG repeats (Sellier et al. 2013). DROSHA-DGCR8 also recruits SAM68 to CGG repeats. Other studies also demonstrated alteration of the expression of specific miRNAs in blood samples of FXTAS patients and in *Drosophila* expressing CGG repeat (Alvarez-Mora et al. 2013; Tan et al. 2012). These data suggest that misregulation of the processing and/or of the expression of miRNA may be of importance for the pathogenicity of FXTAS.

Proteomic analysis performed by Jin and collaborators (2007) showed that Pur $\alpha$  (purine-rich binding protein  $\alpha$ ) binds to RNA containing expanded CGG repeats. Pur $\alpha$  is a single-stranded cytoplasmic DNA- and RNA-binding protein that has been implicated in many biological processes, including RNA transport and translation. Importantly, overexpression of Pur $\alpha$  rescued neurodegeneration in a *Drosophila* model of FXTAS (Jin et al. 2007). However, the presence of Pur $\alpha$  within nuclear aggregates in FXTAS brain samples was not consistently observed. Jin et al. (2007) found Pur $\alpha$  in cytoplasmic inclusions in *Drosophila* expressing 90 CGG repeats. In contrast, Pur $\alpha$  was not identified within the intranuclear inclusions in brain sections of mouse premutation models, or in hippocampal or cortical brain sections derived from patients with FXTAS (Galloway et al. 2014; Iwahashi et al. 2006; Sellier et al. 2013). These results indicate that composition of the inclusions may vary from one model organism to the other.

As with hnRNP A2/B1 that recruits CUGBP1, Pur $\alpha$  recruits Rm62, the *Drosophila* ortholog of the RNA helicase P68/DDX5 (Qurashi et al. 2011). Overexpression of Rm62 rescued neurodegeneration in flies expressing 90 CGG repeats, highlighting the potential importance of P68/DDX5 to FXTAS pathology (Qurashi et al. 2011). Of interest, the RNA helicases P68/DDX5 and DDX6 have been recently reported to be involved in the unfolding of expanded CUG repeats in myotonic dystrophy (Laurent et al. 2012; Pettersson et al. 2014). These data raise the hypothesis that regulating the RNA structures of CUG expanded repeats in DM, or of CGG repeats in FXTAS may be of therapeutic interest (Disney et al. 2012; Tran et al. 2014; Yang et al. 2015).

Finally, simultaneous studies by Peter Todd and David Nelson's groups demonstrated that overexpression of TDP-43, encoded by the *TARDBP* gene, suppresses the neurodegeneration induced by expanded CGG repeats in *Drosophila* (Galloway et al. 2014; He et al. 2014). Interestingly, the expression of *Tardbp* is down-regulated in Purkinje neurons of a mouse model of FXTAS expressing the 5'UTR of *FMR1* with 90 CGG repeats under the *Pcp2* promoter (Galloway and Nelson 2009). TDP-43 is of special interest for neurodegenerative diseases since protein inclusions of TDP-43 in neurons is a histopathological hallmark of amyotrophic lateral sclerosis (ALS) (Neumann et al. 2006), and mutation of *TARDBP* leads to amyotrophic lateral sclero-

sis and frontotemporal dementia (ALS-FTD) (Kunst and Warren 1994). Interestingly, TDP-43 is not found within the RNA aggregates of CGG repeats, but requires the hnRNP A2/B1 homologues Hrb87F and Hrb98DE to modulate the CGG repeat triggered toxicity (He et al. 2014). Overall, these observations suggest that mutant *FMR1* RNA containing expanded CGG repeats could be pathogenic by sequestering specific RNA-binding proteins, resulting in loss of their normal functions, ultimately leading to neuronal cell dysfunction and death. In that aspect, it remains to be determined whether expression of Pur $\alpha$ , hnRNP A2/B1, P68/DDX5, DROSHA-DGCR8, CUGBP1, or TDP-43 rescues any phenotype in mouse models expressing expanded CGG repeats. Such functional rescue experiments will be instrumental to determine the importance of these candidate RNA-binding proteins to FXTAS pathology.

## Inclusion Formation

The presence of eosinophilic intranuclear inclusions, broadly distributed throughout the brain and brainstem of affected individuals (Greco et al. 2002, 2006; Tassone et al. 2004) represent the neuropathological hallmark of FXTAS. These inclusions are present as single, spherical (~2–5  $\mu\text{m}$  of diameter) particles that are found exclusively within the nuclei. Inclusions have been identified in both neurons and astrocytes throughout the cerebrum and the brainstem, including cells of the ependymal and sub-ependymal layers (Tassone et al. 2004). They are most numerous in the hippocampus and are rarely seen in Purkinje cells of the cerebellum (Greco et al. 2002, 2006). Outside of the CNS, rare inclusions have been observed in both the pineal and the posterior and anterior pituitary glands but also in the pancreas, in the heart, in the thyroid and adrenal glands, in the Leydig cells of the testis, in ganglia and in periadrenal fat tissue (Greco et al. 2007; Hunsaker et al. 2011). Inclusions have also been observed in postmortem brain tissue of females with FXTAS (Hagerman et al. 2004; Tassone et al. 2012b).

Pioneering work from Hagerman and Tassone's laboratories demonstrated that these inclusions are ubiquitin-positive and contain various chaperone proteins such as Hsp27, Hsp70, and  $\alpha\text{B}$ -crystallin, but are negative for tau proteins,  $\alpha$ -synuclein, or polyglutamine (Greco et al. 2002; Iwahashi et al. 2006). Importantly, these inclusions also stain positive for the *FMR1* RNA messenger as well as for its polyglycine RAN-translated product (Buijsen et al. 2014; Tassone et al. 2004; Todd et al. 2010) suggesting that expression of mutant *FMR1* mRNA may trigger inclusion formation. In support of this hypothesis, the formation of inclusions can be recapitulated in neuronal cell cultures using a minimal construct expressing an expansion of 99 CGG repeats embedded within the 5'UTR of *FMR1* (Arocena et al. 2005; Sellier et al. 2010; Todd et al. 2010). Of interest, plasmids harboring either no expanded CGG repeats or expanded CGG repeat but under an inactive promoter (no RNA) did not lead to inclusion formation. Also, intranuclear inclusions formed even when the portion encoding FMRP was deleted (Arocena et al. 2005; Todd et al. 2010). Thus, expression of the 5'UTR of *FMR1* containing a CGG repeat expansion is necessary and sufficient for the formation of these inclusions.

These observations in neuronal cell cultures are consistent with the work of Jin et al. (2003), who showed that expression in *Drosophila* of a construct containing the 5'UTR of *FMRI* with 90 CGG repeat induces the formation of cytoplasmic ubiquitin-positive inclusions associated with neurodegeneration and eye pathology (Jin et al. 2003). Similarly, a knock-in (KI) mouse model, in which the endogenous eight CGG repeats of the murine *Fmr1* were replaced with an expansion containing ~100 CGG repeats of human origin, demonstrated ubiquitin-positive nuclear inclusions and mild neuromotor and behavioral disturbances (Brouwer et al. 2008; Van Dam et al. 2005; Willemsen et al. 2003). A result confirmed by recent mouse models where heterologous expression of the sole 5'UTR of *FMRI* containing expanded CGG repeats under strong chimeric promoters is sufficient to cause cellular toxicity, despite the presence of normal *Fmr1* alleles and unaltered expression of *Fmrp* (Hashem et al. 2009; Hukema et al. 2014). These animal models demonstrate that the expression of *FMRI* mRNA containing expanded CGG repeats is both necessary and sufficient to cause the pathological features of human FXTAS (Berman et al. 2014).

## RAN Translation Produces a Polyglycine-Containing Protein in FXTAS

What drives inclusion formation in FXTAS was unclear until recently. The working hypothesis in the field was that the CGG RNA repeats were serving as a nidus for inclusion formation by nucleating together a set of RNA-binding proteins. However, while some inclusions in brain samples of FXTAS patients contain mutant *FMRI* RNA with expanded CGG repeats (Tassone et al. 2004), mouse models in which express expanded CGG repeats show numerous ubiquitin-positive inclusions but only rarely RNA aggregates, suggesting that other factors might trigger inclusion formation (Sellier et al. 2013). Moreover, the inclusions observed in FXTAS are ubiquitin positive, which is a characteristic not commonly observed in other RNA-dominant disorders such as Myotonic Dystrophy.

A hint for how nucleotide repeats in the 5'UTR of a messenger RNA might trigger inclusion formation came with the discovery of repeat associated non-AUG (RAN) translation by Laura Ranum and colleagues (Zu et al. 2011). They found that expression of either CAG in cells outside the context of an AUG-initiated open reading frame were capable of supporting translational initiation and production of homopolymeric proteins. Surprisingly, this RAN translation occurred in all three possible reading frames to produce polyglutamine, polyalanine, and polyserine containing proteins. Initiation was largely independent of the surrounding sequence context but was influenced by the cell type in which the repeats were expressed and was somewhat different for different repeat frames. They then established that these alternative "RAN" products accumulated in tissues in animal models of Spinocerebellar Ataxia type 8 and in lymphoblasts from patients with DM1 (where a CAG repeat is expressed as an antisense transcript). Since these initial observations, RAN translation has been reported in association with CUG repeats (as seen in DM1) and with both GGGGCC and CCCCGG repeat expansions in C9 or f72

that cause ALS and Frontotemporal Dementia (Ash et al. 2013; Gendron et al. 2013; Mori et al. 2013a, b; Zu et al. 2011, 2013).

Todd and colleagues (2013) independently discovered that placement of CGG repeats upstream of a fluorescent reporter protein (GFP) in certain reading frames led to aggregation of GFP in transfected cells and in *Drosophila*. This aggregation was associated with production of a higher molecular weight species that resulted from translational initiation within the 5'UTR just above the CGG repeat. They were able to demonstrate by mass spectroscopy and biochemical analysis that this unconventional translational initiation (CGG RAN translation) occurred at one of several "near" AUG codons (one nucleotide different from AUG) to produce products in two reading frames, producing a polyglycine containing protein (FMRpolyG) and a poly-alanine containing protein (FMRpolyA) (Todd et al. 2013). Production of FMRpolyG was confirmed in patients by use of antibodies generated against the predicted C terminus of this novel protein. The FMRpolyG protein was found in a fraction of ubiquitinated inclusions in brain autopsy samples from patients (Todd et al. 2013). Recent studies with a second set of antibodies covering different N-terminal and C-terminal epitopes revealed that most of the ubiquitin-positive inclusions observed in FXTAS brain sections are also FMRpolyG positive (Buijsen et al. 2014).

A role for FMRpolyG in disease pathogenesis is supported by work in *Drosophila*. In both transfected cells and in flies, it was demonstrated that moving the repeat to the 3'UTR or placing a stop codon just above the repeat prevented FMRpolyG production and suppressed CGG repeat associated toxicity. In contrast, placing an AUG start codon above the repeat-enhanced FMRpolyG production and led to greater lethality in flies and cells. However, the mechanism by which FMRpolyG induces toxicity is still unclear. Impairment of the ubiquitin proteasome system (UPS) enhances CGG repeat associated toxicity (Handa et al. 2005; Oh et al. 2015; Todd et al. 2013) and expression of chaperone proteins such as HSP-70 suppresses CGG repeat associated toxicity in flies (Jin et al. 2003). This genetic interaction between UPS impairment and CGG toxicity is largely driven by RAN translation (Oh et al. 2015). Moreover, RAN translation is capable of triggering UPS impairment in transfected cells (Oh et al. 2015). Together these studies suggest that failures in protein quality control are likely one method by which RAN translation cause toxicity, but future work will be needed to define how this unusual process contributes to FXTAS pathogenesis.

## Disruption of Lamin A/C Architecture

Among the various proteins that Iwahashi and collaborators identified within the inclusions in brain samples of FXTAS patients (Iwahashi et al. 2006), the lamin A/C proteins are of special interest. Lamin proteins constitute a matrix of protein that is located next to the inner nuclear membrane and that is involved in nuclear stability, chromatin structure, and gene expression. Mutations in the *LMNA* gene, which encodes both lamin A and C splicing forms, are associated with several diseases

called laminopathies, including limb girdle and Emery–Dreifuss muscular dystrophies, dilated cardiomyopathy, lipodystrophy, Charcot–Marie–Tooth disease and Hutchinson–Gilford progeria syndrome. Interestingly, nuclear organization of lamin A/C is disrupted in MEF of knock-in mice expressing ~200 CGG repeats, as well as in skin fibroblasts of FXTAS patients with loss of the typical architecture of lamin A/C within the nuclear ring (Garcia-Arocena et al. 2010). These alterations of the lamin A/C structure can be recapitulated in cell cultures upon transfection of a minimal construct expressing expanded CGG repeats embedded within the 5'UTR of *FMRI* (Arocena et al. 2005; Hoem et al. 2011; Sellier et al. 2010). Also, the expression of *Lmna* mRNA is decreased in Purkinje neurons of a mouse model of FXTAS expressing 90 CGG repeats under the *Pcp2* promoter (Galloway et al. 2014). In contrast, the expression of *LMNA* mRNA is increased while the amounts of soluble protein of Lamin A and C are slightly reduced in brain samples of FXTAS patients (Garcia-Arocena et al. 2010). Overall, these results suggest that lamin A/C architecture and expression are altered in FXTAS; however, it remains to determine the molecular mechanism leading to these alterations. It is therefore intriguing that recent data on GGGGCC repeat expansions in *C9orf72* which cause ALS and FTD are associated with changes in nuclear envelope architecture and nuclear pore complex function (Freibaum et al. 2015; Jovicic et al. 2015; Zhang et al. 2015). Thus, the pathological consequences of lamin alterations in FXTAS are an exciting area of research in need of further exploration.

## Mitochondrial Dysfunction

Mitochondrial biogenesis plays a central role in neurogenesis and neuroplasticity and several studies have demonstrated significant mitochondrial dysfunction in premutation carriers (Kaplan et al. 2012; Napoli et al. 2011; Ross-Inta et al. 2010), both with and without fragile X-associated tremor ataxia syndrome (FXTAS). Specifically, altered biochemical characteristics pointing to a lower ATP production, including decreased NAD- and FAD-linked oxygen uptake rates, decreased mitochondrial protein expression, increased oxidative/nitrative stress, and altered zinc bioavailability were demonstrated in both cultured dermal fibroblasts and brain samples from premutation carriers with and without FXTAS, within a wide range of CGG repeats (Napoli et al. 2011; Ross-Inta et al. 2010). Kaplan et al. (2012) observed fewer mitochondria and greatly reduced mitochondria mobility in hippocampal neuronal culture from preCGG KI mice, expressing low FMRP levels and higher *Fmr1* mRNA than that measured in wild type during the early stages of development. In addition, preCGG neurons presented with abnormal metabolic function including higher rates of basal oxygen consumption, ATP production, and proton leak. The authors suggested that deficits in mitochondrial trafficking and metabolic function could play a role in the early pathophysiology observed in premutation carriers and also potentially contribute to the risk of developing FXTAS.

In addition, an altered  $\text{Ca}^{2+}$  regulation was reported in both neuronal cultures from the premutation mouse model (*Fmr1* preCGG mouse) (Cao et al. 2012) but also in iPSC derived from premutation carriers (Liu et al. 2012). The impaired  $\text{Ca}^{2+}$  regulation was associated with alteration of the most abundant excitatory neurotransmitter in the vertebrate CNS, the Glutamate, with a decrease in the expression of GLT-1 and GLAST Glutamate uptake and of their expression levels in astrocytes cultures from preCGG mice and in postmortem cerebellum of PM carriers with FXTAS compared with age-matched controls. Higher CGG repeat number had the greatest reductions in both proteins (Cao et al. 2013; Pretto et al. 2014b). Abnormal clustered burst (CB) firing electrical activity and abnormal patterns of synchronized calcium oscillations in the cytosol were also observed with the premutation mouse neurons, which, interestingly, were reversed with mGluR5 antagonists or the GABAA receptor positive modulator allopregnanolone (Cao et al. 2012) making it a potential candidate for a beneficial therapeutic approach in FXTAS.

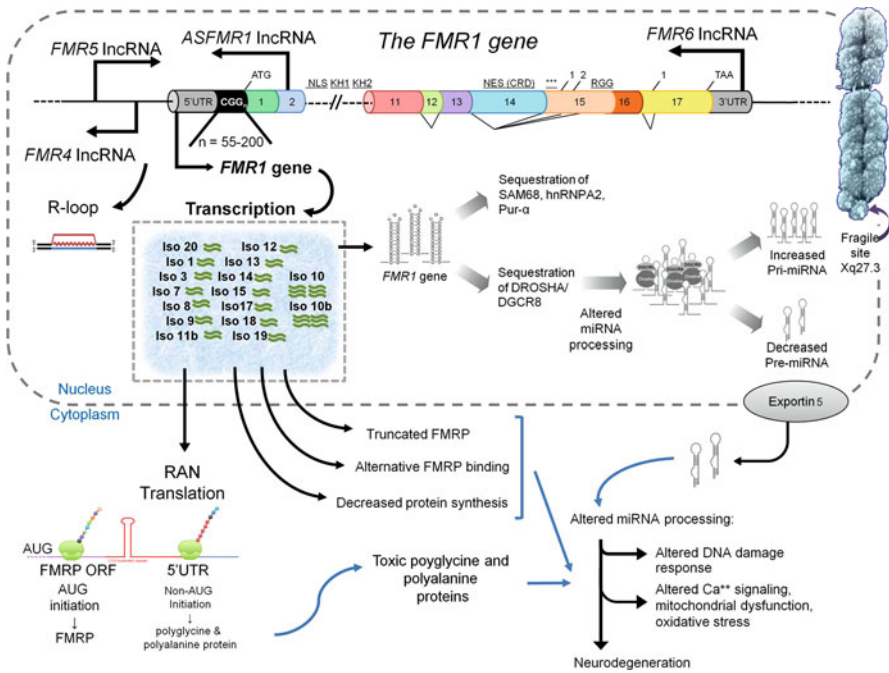
Finally, shorter telomeres have been observed in a number of neurodegenerative conditions including Alzheimer disease, cell senescence, and also in Down syndrome (Jenkins et al. 2006; Panossian et al. 2003). The role of telomeres includes protection against degeneration; they also appear to be essential for chromosomal stability and could lead to cell senescence. Telomere shortening has also been observed in a small sample of male carriers of the premutation (Jenkins et al. 2008) with FXTAS and with or without dementia. However, further studies are warranted to establish if telomere shortening could be considered a biomarker for the cellular dysfunction observed in FXTAS.

The above observations suggest that many triggering events can be associated with CGG expansion leading to cellular dysregulation and dysfunction and ultimately, potentially, to neurodegeneration and *FMRI*-associated disorders.

## Summary

Individuals with expanded repeat of 55–200 CGG repeats are at risk to develop the late onset disease FXTAS, which might be the most common progressing neurological disorder associated with a single gene defect in males. FXTAS is likely to be the consequence of the increased expression of mutant *FMRI* mRNA containing expanded (55–200) CGG repeats. Despite recent and important advances, the molecular mechanisms that lead to both inclusion formation and cell toxicity in FXTAS remain to be clarified. Several nonexclusive models for RNA-triggered pathogenesis in FXTAS can be hypothesized (summarized in Fig. 6.2). First, it is possible, as in the case of myotonic dystrophy, that expanded CGG repeats toxicity, derives from sequestration of CGG RNA-binding proteins, which would limit the availability of those proteins to carry out their normal cellular functions. Alternatively, interactions between yet to be defined proteins and expanded CGG repeats could lead to activation of downstream signaling cascades potentially harmful for the cell. A second mechanism involves noncanonical translation of the





**Fig. 6.2** Diagram showing the molecular alterations of premutation expanded alleles including increased FMR1 mRNA expression levels, R-Loop formation, sequestration of CGG binding proteins, miRNA dysregulation and RAN translation

expanded CGG repeats in a small protein composed of polyglycine, which is prone to aggregation. However, whether and how FMRpolyG alters neuronal function in mammalian systems requires further elucidation. Finally, mild decreased expression of the FMRP protein may contribute to FXTAS pathogenesis.

In conclusion, contributions to pathology from multiple pathogenic mechanisms may converge to explain the great variability in clinical presentations of FXTAS. Thus, more studies are warranted to improve our understanding of this multifaceted disorder and to design and develop appropriate targeted therapeutic approaches.

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