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REVIEW

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# The multiple molecular facets of fragile X-associated tremor/ataxia syndrome

Chantal Sellier<sup>1</sup>, Karen Usdin<sup>2</sup>, Chiara Pastori<sup>3</sup>, Veronica J Peschansky<sup>3</sup>, Flora Tassone<sup>4,5</sup> and Nicolas Charlet-Berguerand<sup>1,6\*</sup>

## Abstract

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset inherited neurodegenerative disorder characterized by intentional tremor, gait ataxia, autonomic dysfunction, and cognitive decline. FXTAS is caused by the presence of a long CGG repeat tract in the 5' UTR of the *FMR1* gene. In contrast to Fragile X syndrome, in which the *FMR1* gene harbors over 200 CGG repeats but is transcriptionally silent, the clinical features of FXTAS arise from a toxic gain of function of the elevated levels of *FMR1* transcript containing the long CGG tract. However, how this RNA leads to neuronal cell dysfunction is unknown. Here, we discuss the latest advances in the current understanding of the possible molecular basis of FXTAS.

## Review

### Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder that affects older adults who have a large CGG-repeat tract in the 5'-untranslated region (UTR) of the *Fragile X Mental Retardation 1 (FMR1)* gene [1]. Historically, carriers of Fragile X (FX) premutation alleles with 55 to 200 CGG repeats are considered at risk for FXTAS. The prevalence of premutation alleles is approximately 1 in 260 to 1 in 800 for males and 1 in 130 to 1 in 250 for females in the general population [2,3]. Given reduced penetrance of FXTAS, it is estimated that 1 in 2,000 men over the age of 50 years in the general population will show symptoms of FXTAS [2,4-6]. Clinical features of FXTAS include progressive intention tremor and gait ataxia, which is frequently accompanied by progressive cognitive decline, parkinsonism, peripheral neuropathy, and autonomic dysfunction [7]. The neuropathology of FXTAS consists of mild brain atrophy and degeneration of the cerebellum, including hyperintensity of the middle cerebellar peduncle (MCP), loss of Purkinje neuronal cells, spongiosis of the deep cerebellar white matter, Bergman gliosis, and swollen axons [8-10]. Immunocytochemical staining of

post-mortem brain tissue from FXTAS reveals the presence of eosinophilic and ubiquitin-positive intranuclear inclusions that are broadly distributed throughout the brain, including in neurons and astrocytes [9,10], the spinal column, and several non-nervous tissues including thyroid, heart, and the Leydig cells in the testes [11,12].

In contrast to the absence of *FMR1* mRNA and protein expression seen in carriers of a full mutation (over 200 CGG repeats), individuals with premutation alleles have markedly increased expression of *FMR1* mRNA, but only moderately decreased FMRP levels [13-16]. FXTAS is not seen in carriers of fully silenced *FMR1* alleles, suggesting that a novel mechanism, involving increased expression of the long CGG repeat tract in the *FMR1* mRNA, is responsible for FXTAS. In support of this hypothesis, multiple studies have demonstrated adverse consequences of expressing CGG repeats in fly, mouse, and cell models [17-22]. Consistent with an RNA-based pathological mechanism, FXTAS has also been reported in individual carriers of intermediate alleles (45 to 55 CGG repeats) [23,24], and in full mutation allele carriers who are mosaics, both for repeat size and methylation, and who still express some *FMR1* mRNA [25-27]. In addition, there has been a report documenting the presence of intranuclear inclusions in the brains of three older adult males with Fragile X syndrome (FXS) [12]. These results have implications for the spectrum of FX-associated disorders, and suggest that the definition of FXTAS may need to be

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broadened to include individuals whose *FMRI* allele, irrespective of its size, makes sufficient RNA for its deleterious effects to be apparent.

How the RNA containing expanded CGG repeats leads to FXTAS pathogenesis is not yet fully known. This review will cover the recent advances in the understanding of the molecular mechanisms that may contribute to the pathogenesis of FXTAS, including the data presented at the First International Conference on *FMRI* Premutation (23 to 26 June, Perugia, Italy). For other aspects of FXTAS, there are a number of excellent reviews available [28-31], as well the additional articles published in this special issue of *JND*.

#### **CGG repeats are unstable, and tend to expand over time or with successive generations**

Increased CGG repeat numbers are associated with an increased risk of FXTAS and with an increased severity and reduced age of onset of FXTAS symptoms [5,6]. The CGG repeat tract responsible for FXTAS is polymorphic in the human population. Normal alleles have between 6 and 45 repeats, and are relatively stable on intergenerational transmission. However, as the repeat number increases, so too does the likelihood that the repeat tract will expand or gain additional repeat units on intergenerational transfer. AGG interruptions, which are commonly seen within *FMRI* alleles, typically at 10 to 11 and 20 to 21 triplets from the 5' end [32], are associated with a reduced risk of expansion [33,34]. Contractions do occur [35], but much less frequently. One of the consequences of the expansion bias is that alleles tend to increase in repeat number with successive generations. In addition to intergenerational expansion, somatic expansion is also seen in certain organs, including the brain in mice, and both lymphocytes and brain tissue in humans [27,36]. Somatic expansion may contribute to the repeat-length mosaicism that is seen in some human premutation carriers [27,37-40]. This somatic expansion has the potential to exacerbate FXTAS symptoms, particularly in carriers of alleles with more than 100 CGG repeats, where the repeat may be particularly prone to expansion.

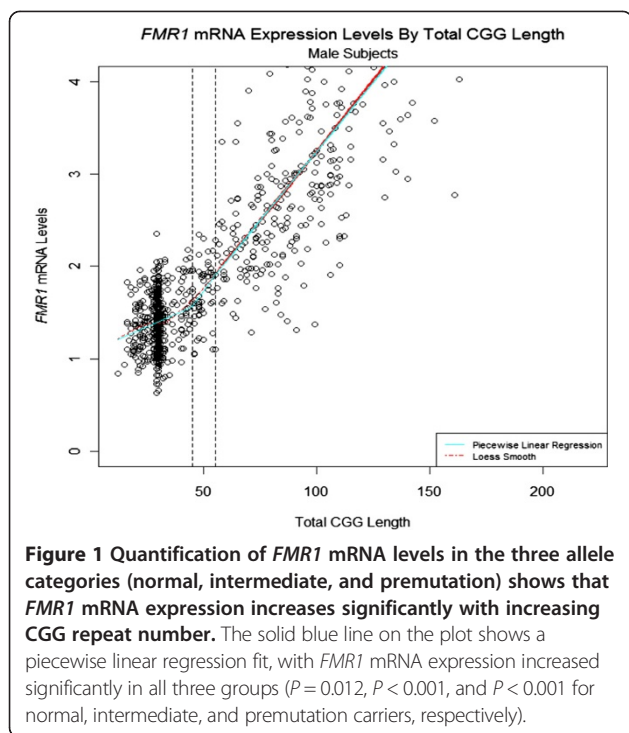
The mechanism responsible for these expansions is unknown. A number of other diseases are known to result from expansions of tracts containing these repeats or other short repeat units. Whether or not these diseases, which are referred to collectively as the repeat expansion diseases [41], share a common expansion mechanism is unknown. However, the unusual nature of these mutations suggests that they might. The expansion bias clearly differentiates the instability in these diseases from the classic microsatellite instability seen in certain cancers, where the repeat is as likely to lose repeats as it is to gain them.

The individual strands of expanded CGG repeats, like other repeats that cause repeat expansion diseases, form

secondary structures, including hairpins and quadruplexes [42]. These structures affect DNA processing enzymes such as DNA polymerase, both in vitro [42] and in vivo ([43,44]. It is generally thought that these structures are the trigger or substrate for expansion [41]. However, expansion in brain and liver, which are organs with a low proliferative capacity, along with expansion in mouse oocytes [45,46], suggest that the expansion mechanism may not involve aberrant DNA replication. Rather, given that oxidative stress exacerbates expansion in mice [47], it may be that expansion results from the aberrant repair of oxidized DNA or of DNA that is damaged in other ways. In contrast to generalized microsatellite instability, expansion in premutation mice actually requires *Msh2* [48]. However, whether expansion involves disruption of classic mismatch repair or involves another MSH2-dependent process is unknown. Although *Msh2* is required for expansion, it is not required for contractions [45,46,48]. Thus, it seems likely that expansions and contractions occur by different mechanisms, and the expansion bias seen in FX pedigrees may reflect a more efficient operation of the expansion process relative to the process that generates contractions.

#### **Expression of *FMRI* mRNA is increased in premutation carriers**

Several studies have shown that premutation alleles are characterized by high mRNA expression levels [13,15,16,49]. In our recent analysis, *FMRI* gene expression levels were measured in peripheral blood leukocytes from a total of 806 males across the whole range of CGG repeats, including normal individuals ( $n = 463$ ), and individuals carrying intermediate ( $n = 60$ ) and premutation ( $n = 283$ ) alleles [27]. The results showed that *FMRI* mRNA levels increased with increased CGG repeat number, and that a significant increase ( $P < 0.001$ ) was detectable for allele lengths as short as 35 CGG repeats (Figure 1). In addition, nuclear run-on experiments indicated that this elevated level of *FMRI* mRNA in premutation carriers is caused by increased transcription efficiency rather than increased mRNA stability [14,15]. Despite higher levels of *FMRI* transcripts, mild deficits of FMRP have been found in premutation carriers, and are probably due to a deficit in translational efficiency, particularly in the upper premutation range [13,16,49]. Thus, an FMRP deficiency is probably not the principal cause of FXTAS. Instead, the crucial observation that RNAs containing expanded CGG repeats accumulate in nuclear RNA aggregates in brain sections of patients with FXTAS [50] supports the notion that elevated levels of *FMRI* mRNA trigger neuronal toxicity. In support of this hypothesis, heterologous expression of 90 CGG repeats in *Drosophila melanogaster* was shown to cause neurodegeneration and formation of ubiquitin inclusions [18]. Similarly, a knock-in (KI) mouse model, in which the endogenous eight CGG repeats of the murine *Fmr1* were



replaced with an expansion containing around 100 CGG repeats of human origin, showed ubiquitin-positive nuclear inclusions, and mild neuromotor and behavioral disturbances [17,51,52]. Finally, expression of transcripts containing 90 CGG repeats in a transgenic mouse model recapitulated some of the neuropathological and molecular features of FXTAS, despite the presence of a normal *Fmr1* allele [19] (see also review on animal models for FXTAS in this issue). These animal models show that the expression of *FMR1* mRNA containing expanded CGG repeats is both necessary and sufficient to cause pathological features characteristic of human FXTAS. Several mechanisms have been proposed to explain how increased expression of a RNA containing expanded CGG repeats could be pathogenic.

#### Is pathology the result of an RNA gain-of-function mechanism?

The first recognized examples of RNA gain-of-function diseases were two other repeat expansion diseases, myotonic dystrophy type 1 and 2 (DM1 and DM2) [53]. DM is the most common muscular dystrophy in adults, and in this condition, RNAs containing hundreds to thousands of CUG (DM1) or CCUG (DM2) repeats accumulate in nuclear RNA aggregates that sequester the Muscleblind-like (MBNL) splicing factors. Depletion of the free pool of MBNL1 leads to specific alternative splicing changes, which ultimately result in the symptoms of DM [53]. Extending this RNA gain-of-function model to FXTAS, the expanded CGG repeats are predicted to

sequester specific proteins, resulting in loss of their normal functions, which would ultimately cause the symptoms of FXTAS [54,55]. Consistent with this idea, Iwahashi and collaborators [56] identified more than 20 proteins from inclusions purified from brains of patients with FXTAS. Of these, two RNA binding proteins were of special interest. The first, hnRNP A2/B1 is mutated in families with inherited degeneration affecting muscle, brain, bone, and motor neurons [57], while the second, MBNL1, is the splicing factor that is involved in DM [58]. However, a role for MBNL1 in FXTAS has been excluded, because no genetic interaction between MBNL1 and CGG-mediated neurodegeneration was observed in the fly model of FXTAS [59], and no misregulation of splicing events regulated by MBNL1 was observed in brain samples from patients with FXTAS [60]. By contrast, binding of hnRNP A2/B1 to RNA containing expanded CGG repeats was confirmed by independent proteomic and in vitro analyses [60,61]. Furthermore, overexpression of hnRNP A2/B1 rescued the neurodegeneration in transgenic *Drosophila* expressing 90 CGG repeats [59,61]. Interaction of hnRNP A2/B1 with RNA containing expanded CGG repeats was evident in cytoplasmic cerebellar lysates. By contrast, nuclear hnRNP A2/B1 presented little binding to CGG RNA, suggesting that some modifications of hnRNP A2/B1, either in the nucleus or in the cytoplasm, may alter the ability of hnRNP A2/B1 to bind to CGG RNA repeats [59]. The importance of titration of the cytoplasmic pool of hnRNP A2/B1 was further demonstrated by expression of expanded CGG repeats in primary cultures of rat sympathetic neurons [62]. RNA containing CGG repeats competed for binding of hnRNP A2/B1 to BC1 RNA, a dendritic regulatory RNA, resulting in impaired dendritic delivery of the BC1 RNA [62]. However, no misregulation of splicing events regulated by nuclear hnRNP A2/B1 was observed in brain samples of patients with FXTAS [60]. Overall, these data suggest that expanded CGG repeats recruit hnRNP A2/B1, resulting in depletion of the cytoplasmic but not the nuclear pool of hnRNP A2/B1. In addition, the ability of hnRNP A proteins to unfold tetraplex RNA structures, formed by expanded CGG repeats [63,64], raises the possibility that hnRNP A2/B1 may also act as a RNA chaperone that destabilizes these RNA structures. Finally, Sofola and collaborators [59] demonstrated that hnRNP A2/B1 recruits, *in trans* and through protein-protein interactions, other proteins such as CUGBP1, an RNA binding protein, whose expression is increased in heart samples of patients with DM [65]. These data indicated that proteins binding to CGG RNA may recruit other proteins, resulting in dynamic aggregates that expand over time, a model later confirmed in COS7 cells expressing 60 CGG repeats [60]. Overexpression of either hnRNP A2/B1 or CUGBP1 rescued neurodegeneration in a *Drosophila* model of FXTAS,

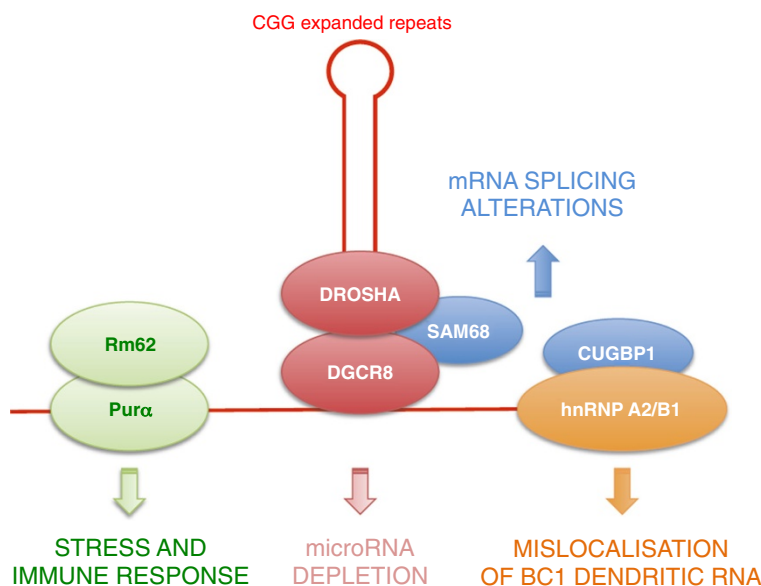
highlighting the potential importance of hnRNP A2/B1 and CUGBP1 to FXTAS pathology [59].

In addition to hnRNP A2/B1, proteomic analyses performed by Jin and collaborators [61] also showed that purine-rich binding protein  $\alpha$  (Pur $\alpha$ ) binds robustly 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 [61]. However, presence of Pur $\alpha$  within nuclear aggregates in FXTAS brain samples is inconsistently observed. Jin et al. found Pur $\alpha$  in cytoplasmic inclusions in *Drosophila* expressing 90 CGG repeats, and in inclusions in superior-mid temporal cortex neurons from human FXTAS brain sections [61]. By contrast, Iwashashi et al. did not detect Pur $\alpha$  in purified inclusions from cerebral cortex of patients with FXTAS [56]. Furthermore, Pur $\alpha$ -positive inclusions have not been observed in mouse models of FXTAS [66], or in hippocampal and cortical brain section of patients with FXTAS [67]. These results suggest that the composition of the inclusions varies from one brain region to the next and from one model organism to the other. Analogous to the recruitment of CUGBP1 by hnRNP A2/B1 to RNA containing expanded CGG repeats, Pur $\alpha$  was shown to recruit Rm62, the *Drosophila* ortholog of the RNA helicase P68/DDX5 [68]. Expression of expanded CGG repeats resulted in the post-transcriptional downregulation of Rm62, ultimately resulting in nuclear accumulation of *Hsp70* mRNA and of other mRNAs involved in stress and immune responses [68]. Overexpression of Rm62 rescued neurodegeneration in flies expressing 90 CGG repeats, highlighting the potential importance of P68/DDX5 to FXTAS pathology [68].

SAM68, a splicing regulator encoded by the *KHDRBS1* gene, was also found in CGG RNA aggregates [60]. However, overexpression of SAM68 was not sufficient to rescue neuronal cell death induced by expression of expanded CGG repeats [67]. As with CUGBP1 and Rm62, SAM68 did not bind directly to CGG repeats, and recruitment of SAM68 within CGG RNA aggregates occurred in *trans* through protein-protein interactions [59]. Sellier and collaborators [67] also showed that DROSHA-DGCR8, the enzymatic complex that processes pri-miRNAs into pre-miRNAs, associated specifically with CGG repeats of pathogenic size. Sequestration of DROSHA-DGCR8 within CGG RNA aggregates resulted in reduced processing of pri-miRNAs in cells expressing expanded CGG repeats, and in brain samples from patients with FXTAS. Overexpression of DGCR8 rescued neuronal cell death induced by expression of expanded CGG repeats [67]. These results suggest that titration of DGCR8 by expanded CGG repeats is a leading event

to CGG-induced neuronal cell death. However, recent analyses of miRNA expression in blood samples of patients with FXTAS and in *Drosophila* expressing CGG repeats did not show a global downregulation of miRNA, but rather, the expression of some specific miRNAs was misregulated [69,70]. Whether depletion of DROSHA-DGCR8 varies in blood and brain of patients with FXTAS, and whether the Drosha-Pasha complex is sequestered in cytoplasmic aggregates in *Drosophila* expressing expanded CGG repeats, remains to be determined. Similarly, whether overexpression of hnRNP A2/B1, P68/DDX5, DROSHA-DGCR8, or CUGBP1 rescues any phenotype in mouse models expressing expanded CGG repeats, would be necessary to determine the importance of these candidate proteins to FXTAS pathology.

These caveats aside, the observations described above suggest that CGG repeats could be pathogenic by sequestering specific RNA binding proteins, resulting in loss of their normal functions, and thus lead to neuronal cell dysfunction (Figure 2) [56,58,61,67,68]. However, this attractive model has some weaknesses. First, the inclusions observed in FXTAS brain sections differ from those seen in DM, a typical RNA gain-of-function disorder. In FXTAS, inclusions are larger and ubiquitinated, and contain various chaperone proteins such as Hsp27, Hsp70, and  $\alpha$ B-crystallin [9,56]. In short, these large inclusions resemble the aggregates seen in protein-mediated disorders, although they are negative for the typical proteins found in tauopathies, synucleinopathies, or polyQ disorders (for example, Huntington's disease). Second, and most disconcerting, although inclusions in brain samples of patients with FXTAS contain the mutant *FMR1* RNA with expanded CGG repeats [50], a mouse model, in which the endogenous eight CGG repeats of *Fmr1* is replaced with an expansion containing around 100 CGG repeats, shows numerous ubiquitin inclusions but only rare aggregates of SAM68 or DROSHA-DGCR8, associated with rare RNA aggregates of expanded CGG repeats [60,67]. Similarly, overexpression of expanded CGG repeats leads to formation of nuclear RNA aggregate in some cell types, including primary cultures of hippocampal embryonic mouse neurons and PC12, COS7, and SKOV3 immortalized cell lines, but no RNA aggregates have been observed in A172, U-937, THP1, HeLa, HEK293, NG108-15, IMR-32, Neuro-2a, SH-SY5Y, SK-N-MC, or SK-N-SH cells [22,60]. In short, not all cell lines can support CGG repeat aggregate formation, whereas in DM, expression of expanded CUG or CCUG repeats consistently results in formation of RNA foci. Thirdly, a recent and provocative study demonstrates that the toxic effect of CGG repeats depends on their location [71]. Moving expanded CGG repeats from a 5' UTR to a 3' UTR position reduced their toxic effect in *Drosophila*, whereas expanded CUG or CCUG repeats were found



**Figure 2** Various RNA binding proteins have been found to associate with RNAs containing expanded CGG repeats. Pura, DGCR8, and hnRNP A2/B1 bind directly to the CGG-containing RNA, whereas Rm62, SAM68, and CUGBP1 are recruited *in trans* through protein-protein interactions.

to be pathogenic in whatever location tested, provided they were expressed in sufficient amounts to deplete MBNL proteins. These data led Todd and collaborators to reconsider the model of RNA binding protein sequestration, and to explore further the molecular mechanisms of FXTAS.

#### Non-canonical AUG translation produces a polyglycine-containing protein in FXTAS

An unexpected observation, made by Todd and colleagues in flies transgenic for a construct containing 90 expanded CGG repeats cloned upstream of the green fluorescent protein (GFP) cDNA, was that some of the GFP signal was found in cytoplasmic inclusions. Western blotting analysis showed a band of the expected size for GFP, but also detected a protein 12 kDa larger [71]. Because translation of expanded CAG repeats in the absence of an ATG initiation codon (repeat-associated non-ATG translation or RAN translation) had been previously reported [72], Todd and collaborators tested whether expanded CGG repeats could be translated despite the fact that no ATG codon is present upstream of the repeats. Their analysis revealed that, indeed, translation of CGG repeats occurs in two out of the three frames, giving rise to short proteins containing either a polyalanine or a polyglycine stretch. Expression of the polyglycine protein resulted in the formation of protein inclusions, which were toxic both in neuronal transfected cells and in *Drosophila*. Further analyses of the polyglycine protein revealed that its translation was probably initiated at non-canonical AUG codons, such as CUG and GUG, which were located upstream of

the CGG repeats. A role for non-canonical translation initiation in inclusion formation is consistent with data from two different KI mice mouse models. In a mouse model that showed numerous ubiquitin inclusions, the expanded CGG repeat from a human premutation allele was cloned, along with sequences upstream of the CGG repeats in humans that contained the non-AUG initiations codons [17]. By contrast, in a second mouse model, in which the mouse 5' flanking sequence was retained, a stop codon was found to be located just upstream of the expanded CGG repeats [21]. These latter mice showed relatively few ubiquitinated aggregates, thus supporting the notion that non-ATG-initiated translation of the CGG tract is required to generate most of the inclusions [71]. That this unusual mode of translation may play a role in FXTAS is evidenced by the fact that, with the aid of specific antibodies, polyglycine protein can be seen in brain sections of patients with FXTAS [71]. Overall, these observations suggest that a protein gain of function may also occur in cells of patients with FXTAS. However, what contribution the polyglycine-containing or polyalanine-containing proteins make to the etiology of FXTAS is an exciting open question.

#### Non-coding transcription of the FMR1 locus: a role in FMR1 mRNA toxicity?

The majority of the human genome is transcribed but not translated. Such RNAs are classified as long non-coding RNAs (lncRNAs) when longer than 200 nucleotides [73-75]. To date, relatively few lncRNAs have been functionally characterized, but increasing evidence suggests that many may have important functions, including

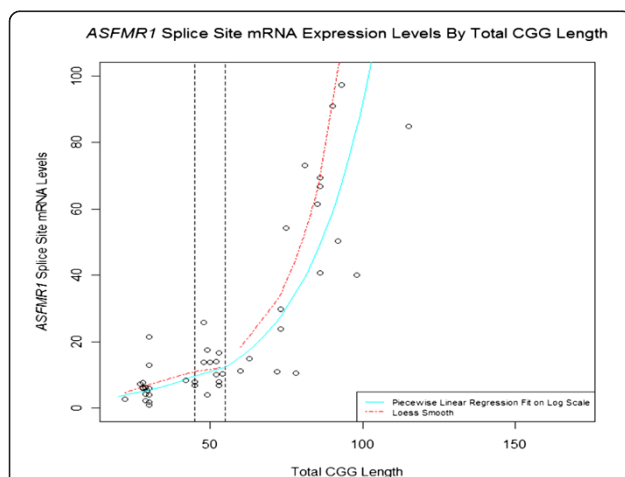
the regulation of transcription, RNA processing and translation, DNA methylation, and chromatin architecture, both locally (*cis*-acting) and across some genomic distance (*trans*-acting) [76-78].

In addition to the *FMR1* transcript, a variety of RNAs are produced from the *FMR1* locus. Therefore, it is possible that these lncRNAs produced from the *FMR1* locus may modulate certain aspects of FXS/FXTAS, as has been shown in other human diseases [79]. For example, Kumari and Usdin described an abundant antisense transcript of about 5 kb that spans the region upstream of the *FMR1* promoter, and whose expression does not change in response to repeat expansion [80]. By contrast Ladd and coworkers described a transcript, Antisense *FMR1* (*ASFMR1*), that spans the expanded CGG repeats and whose expression is elevated in lymphoblastoid cells and peripheral blood leukocytes of individuals with premutation alleles, while it is not expressed in those with full mutation alleles [81]. Multiple splice forms of *ASFMR1* have been identified, which show differential expression in carriers of premutation and normal alleles [81]. One of these *ASFMR1* splice variants contains a small intron that uses a non-consensus CT-AC splice site that is transcribed in a premutation cell line, but is absent in a normal cell line [81]. We compared the expression levels of this *ASFMR1* isoform in blood from individuals with alleles ranging from normal to premutation, and found a significant increase with CGG repeat number ( $P < 0.001$ ) (Figure 3) [27]. Of interest, both unspliced and spliced *ASFMR1* transcripts contain putative open-reading frames encoding polyproline peptides, resulting from antisense-oriented translation of

the expanded CGG repeats [81]. Whether *ASFMR1* containing expanded CCG repeats is translated and participates in the formation of the pathogenic nuclear inclusions observed in patients with FXTAS remain to be tested.

Another antisense transcript, *FMR4*, originates upstream of the *FMR1* start site, and covers 2.4 kb of sequence [82]. *FMR4* is widely expressed in fetal and adult human tissues, and throughout human and macaque brain regions. Expression of *FMR4*, like that of *ASFMR1* and *FMR1*, is increased in brain tissue of premutation individuals and is silenced in individuals with the full mutation [82]. Importantly, *FMR4* overexpression was shown to increase cell proliferation, whereas *FMR4* downregulation induced apoptosis in vitro [82]. Additionally, no *cis*-acting effect was observed upon expression of the *FMR1* gene. Therefore, it was hypothesized that *FMR4* influences proliferation pathways *in trans*, by targeting distal genomic loci. Current work is focused on defining a role for this transcript, as it has been found to affect the chromatin state and transcription of several genes involved in neuronal differentiation, axon guidance, and synaptic signaling, as well as cell cycle regulators (Peschansky and Pastori, unpublished data).

Two new transcripts arising from the *FMR1* locus, *FMR5* and *FMR6*, were recently identified [83]. *FMR5* is a sense-oriented lncRNA transcribed from approximately 1 kb upstream of the *FMR1* transcription start site (TSS). *FMR5* is not differentially expressed in human brain from unaffected individuals compared with full mutation and premutation patients, suggesting that its transcription is independent of CGG repeat expansion. Furthermore, the TSS of *FMR5* appears not to be affected by the chromatin silencing that occurs within full mutation alleles, or by the open chromatin hypothesized to increase transcription of *FMR1* premutation alleles. *FMR6* is a spliced long antisense transcript, 600 nucleotides in length, whose sequence is entirely complementary to the 3' region of *FMR1* [83]. It begins in the 3' UTR, ends in exon 15 of *FMR1*, and uses the same splice junctions as *FMR1*. An unexpected finding was that *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 *FMR1* in premutation carriers have yet to be described. The function of *FMR6* remains to be identified, but its complementarity to the 3' region of *FMR1* presents several interesting possibilities. *FMR6* may bind to *FMR1* mRNA, thereby regulating the stability, splicing, subcellular localization, or translational efficiency of *FMR1*, as has been described for other lncRNAs [77]. Notably, *FMR6* overlaps miR-19a and miR-19b binding sites in the *FMR1* 3' UTR [84], suggesting that *FMR6* may modulate the stability or translational efficiency of *FMR1* by interfering with



**Figure 3 Expression of the minor splice isoform in the *ASFMR1* transcript (131 bp), located near the *ASFMR1* promoter.**

Expression of this isoform increases in premutation carriers ( $P < 0.001$ ), and shows a similar trend in subjects with intermediate alleles ( $P = 0.0528$ ) compared with normal alleles. The solid blue line on the plot shows a piecewise linear regression fit (fitted on the log scale then exponentiated for plotting), with separate slopes in the normal, intermediate, and premutation alleles.

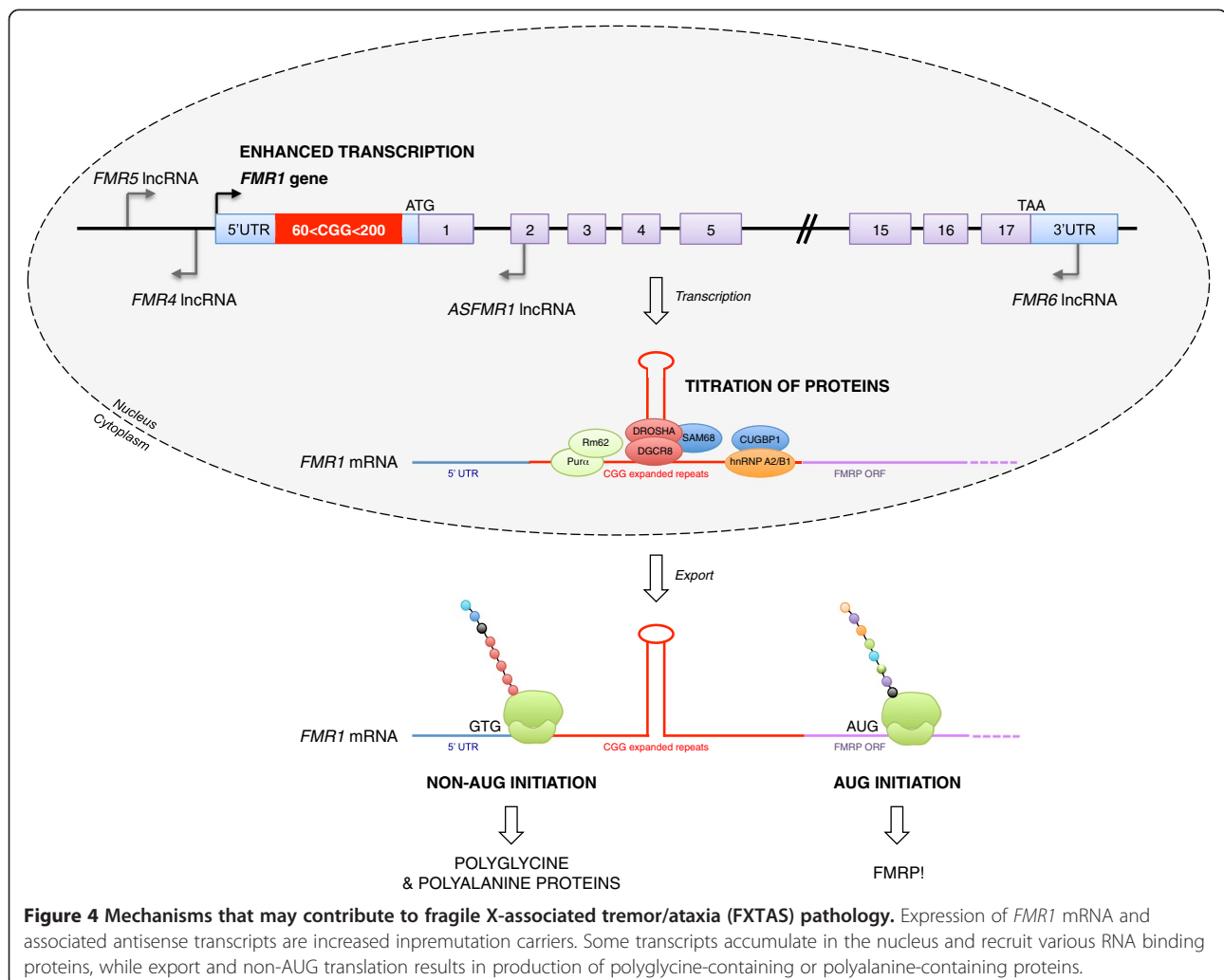
microRNA binding. Overall, these results highlight the importance of non-coding transcription of the *FMR1* locus. However, much work remains to fully understand the relevance of these transcripts to the pathology observed in premutation carriers.

### Conclusion

The restriction of FXTAS clinical features to unmethylated, transcriptionally active alleles with large CGG repeat numbers suggests that the expression of a mutant RNA is pathogenic to neuronal cells [55]. This hypothesis is supported by data from cell, fly, and mouse models [17-22]. However, how these RNAs cause neuronal cell dysfunction and FXTAS symptoms remains unclear. One model proposes that the RNA containing expanded CGG repeats is pathogenic via its sequestration of specific RNA binding proteins. Various proteins, including Pur $\alpha$ , Rm62, CUGBP1, hnRNP A2/B1, SAM68, and DROSHA-DGCR8, have been shown to bind directly or through a protein partner to expanded CGG repeats [56,59,61,67,68]. However, it remains

to be tested whether overexpression of these candidate proteins rescues any phenotype in mouse models expressing expanded CGG repeats. A second mechanism involves non-canonical translation initiation of expanded CGG repeats, resulting in expression of toxic polyglycine-containing and polyalanine-containing proteins [71]; however, how these proteins promote neuronal cell dysfunction is an open question. A third model is associated with the expression of antisense *FMR1* transcripts. Further investigation is needed to evaluate the pathological consequences of expression of *ASFMR1* or other long non-coding RNA mapping within the *FMR1* gene. Finally, although decreased expression of FMRP is probably not the principal cause of FXTAS, it cannot be excluded that a reduction in FMRP plays a role in modulating some of FXTAS features. In that context, the level of FMRP depletion in brain samples from a larger cohort of patients with FXTAS needs to be measured.

In conclusion, in addition to increased *FMR1* mRNA production, protein titration, non-AUG translation,



**Figure 4 Mechanisms that may contribute to fragile X-associated tremor/ataxia (FXTAS) pathology.** Expression of *FMR1* mRNA and associated antisense transcripts are increased in premutation carriers. Some transcripts accumulate in the nucleus and recruit various RNA binding proteins, while export and non-AUG translation results in production of polyglycine-containing or polyalanine-containing proteins.



antisense transcription, and decreased expression of FMRP are a number of non-exclusive mechanisms that may all contribute to FXTAS pathology. It is possible that contributions to pathology from more than one mechanism may help to explain the great variability in clinical presentation of premutation individuals, aspects of which have heretofore not been accounted for by CGG expansion size, mosaicism, methylation, alternative spliced isoforms, additional genomic changes, or other known factors. Thus, more work is needed to determine the relative contribution of these processes to disease pathology in this multifaceted disorder (Figure 4).

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

CS, KU, CP, VJP, FT and NCB wrote the paper. All authors read and approved the final manuscript.

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