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## ***FMR1*: a gene with three faces**

**Ben A Oostra<sup>\*</sup>** and **Rob Willemsen**

*Dept of Clinical Genetics, Erasmus MC Rotterdam, The Netherlands*

### **Abstract**

The *FMR1* gene is involved in three different syndromes, the Fragile X syndrome (FXS), premature ovarian insufficiency (POI) and the Fragile X-associated tremor/ataxia syndrome (FXTAS) at older age. Fragile X syndrome is caused by an expansion of a CGG repeat above 200 units in the *FMR1* gene resulting in the absence of the *FMR1* mRNA and protein. The FMR1 protein is proposed to act as a regulator of mRNA transport and of translation of target mRNAs at the synapse. FXS is seen as a loss of function disorder. POI and FXTAS are found in individuals with an expanded repeat between 50–200 CGGs and are associated with increased *FMR1* mRNA levels. The presence of elevated *FMR1* mRNA in FXTAS suggests that FXTAS may represent a toxic RNA gain-of-function effect. The molecular basis of POI is yet unknown. The role of the *FMR1* gene in these disorders is discussed.

### **Introduction**

Fragile X syndrome (FXS) is the most prevalent cause of inheritable mental retardation often presenting as an autism spectrum disorder with a frequency of 1:4000 males and 1:6000 females (for review [1]). FXS is an X-linked disorder and is notable for its unusual inheritance pattern, showing increased penetrance as the mutant gene passes to subsequent generations (the Sherman paradox) [2]. In 1991 the responsible gene was identified by positional cloning and named the fragile X mental retardation-1 gene (*FMR1*) [3] FXS is almost exclusively caused by an expansion of a CGG repeat in the 5' untranslated region of the *FMR1* gene and was the first example of a trinucleotide repeat expansion mutation (Figure 1). In the normal population, the CGG repeat is polymorphic and ranges from 5–55 CGGs with an average length of 30 CGG units [4]. In Fragile X patients, however, the CGG repeat is found to be expanded beyond 200 repeats known as the full mutation (FM), that is usually hypermethylated and the methylation extends to the adjacent promoter region of the *FMR1* gene [3,5,6]. As a consequence the gene is transcriptionally silenced and the gene product, the fragile X mental retardation protein (FMRP), is absent.

Unmethylated expansions of 55–200 CGG units, called premutations (PM), are unstable in meiosis and are found in both males and females and may expand to a full mutation only upon maternal transmission to the next generation. The risk of transition is dependent on the size of the premutation, which accounts for the Sherman paradox [4]. The smallest CGG repeat number known to expand to a full mutation is 59 repeats to date [7].

<sup>\*</sup>Correspondence to Oostra BA, PhD, Dept. of Clinical Genetics, ErasmusMC, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands, +31 10 7043382, +31 10 7044736, E-mail: b.oostra@erasmusmc.nl.

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## FMR1 protein

The cognitive impairment in FXS is caused by the absence of the fragile X mental retardation protein (FMRP) in neurons. FMRP expression is widespread with abundant expression in neurons and with testicular expression in spermatogonia [8–13]. The subcellular distribution of FMRP is largely cytoplasmic, with high concentrations of FMRP found associated with (poly)ribosomes attached to the endoplasmic reticulum and with free ribosomes in the cytoplasm, at the bases of dendrites and within dendritic spines [10,12,14,15]. Interestingly, both *in vitro* and *in vivo* studies illustrated the presence of FMRP in the nucleus [12,14,16–19]. The association of FMRP with ribosomes is mRNA dependent via large ribonucleoprotein (RNP) particles, which contain several other proteins including FXR1P and FXR2P, nucleolin, YB-1, NUFIP1, CYFIP1 and CYFIP2 [20–23].

## Expansion of the fragile X mutation

Many models have been proposed to explain the expansion of trinucleotide repeats. One of the first proposed mechanisms involved in repeat instability at the molecular level was slippage of the replication fork during replication. Unpaired bases form loops, which result in expansions or contractions in a next round of replication, depending on whether the looped repeats are located in the newly synthesized or template strand [24]. However, slippage alone cannot explain all aspects of repeat expansions, especially large expansions and contractions. Strong experimental support came from studies in a yeast model deficient in *RAD27*. *RAD27*, like its mammalian homologue *FEN1*, is involved in removing DNA loops, such as those arising during displacement synthesis of the Okazaki fragments. As predicted by the above model, propagation of a CGG repeat in the *RAD27* null background results in a highly significant increase of repeat expansions [25].

Recognition of the unusual structural properties of trinucleotide repeats yielded new insights. Disease-causing repeats are almost exclusively formed by  $(CNG)_n$ -triplets. Single-stranded  $(CNG)_n$  can form hairpin-like structures that can include both Watson-Crick and mismatched base pairs. Due to their different sequences, the leading and lagging strand have different tendencies to form hairpins. The secondary structures are likely to affect recognition and subsequent repair or recombination of this structure [26,27]. Unusual DNA structures may stall DNA polymerases. Studies in yeast replication mutants showed a marked increase in frequency of repeat instability. A complex model based on replication fork stalling and restarting is described in detail by Mirkin [28].

Unlike other trinucleotide repeat disorders such as DM1 there is absence of repeat instability in somatic cells. For full mutations it has been proposed that methylation stabilizes the CGG repeat and prevents expansion or contraction. However, males with an unmethylated full mutation do not show somatic instability of the repeat [29]. Recently human embryonic stem cells containing a full mutation have been described [30]. The original embryonic cells show clear instability of the repeat, but after subcloning the unmethylated repeat becomes stable while the cells are still displaying all the characteristics typical of embryonic stem cells. Premutation alleles (that are not methylated) are also stable. What is causing this different behavior of the repeat in different repeat disorders is still unknown.

As observed for other dynamic mutations, the degree of expansion in humans depends on the gender of the transmitting parent. Repeat expansion from the premutation to the full mutation only occurs during maternal transmission. The maternal transmission bias could result from sex-specific differences in gametogenesis leading to the production of full expansion oocytes in females and premutation size repeats in sperm in carrier males. This is compatible with the observation that in sperm from full-mutation male patients only a premutation is found [31]. If the zygote starts development with a fully expanded *FMR1* repeat, then somatic mosaicism

must result from reductional repeat instability limited to a window in early development. Such reductional instability may be even more pronounced in the fetal testes. This contraction hypothesis is supported by evidence that in a 17-week old male fetus carrying a FM, germ cells harbor a premutation, while in the other cells a full mutation is present [32]. However, the timing of repeat expansion during development remains uncertain. A prezygotic model predicts that an expansion of PM to FM could occur during maternal meiosis, with the FM contracting to a PM in gametes of male offspring. PM gametes might have some selection advantage either due to the presence of FMRP, or against the presence of an expanded CGG-repeat [32]. Furthermore, FMs are responsible for a delay in replication of the *FMR1* gene during the cell cycle [33]. Thus, primordial germ cells with a PM might have a proliferative advantage, thereby overgrowing FM cells. A second model considers a postzygotic expansion, direct after separation of the germ line. It assumes that the FM allele has never been present in male or female gametes [34]. In contrast to this hypothesis, oocytes of a foetal female FM carrier show only FM alleles [32]. A final conclusion can only be drawn after analysis of oocytes from a PM carrier. This material is not available for obvious reasons. Expanded (CGG)<sub>n</sub> knock-in mouse models have been developed [35,36], that show intergenerational repeat instability [36,37]. When the CGG repeat in the mouse expands into a full mutation they might be used to study the timing of repeat instability.

### Epigenetic changes in the *FMR1* gene

Methylation of the expanded *FMR1* CGG repeat occurs early in embryonic development and is a dynamic process. In early germ cells from female FM fetuses, the *FMR1* repeat is fully expanded and unmethylated [32], whereas in chorionic villus samples from FM fetuses, the expanded repeat in this extraembryonic tissue is methylated to an increasing degree as development progresses [38]. The difficulty in studying this biological phenomenon is the absence of suitable material for study. Transgenic mouse models bearing more than 200 CGG repeats (human origin) within the murine *Fmr1* gene have been generated but they do not show epigenetic changes up till now [36,37]. Recently, Eiges et al. developed an embryonic stem cell line that was shown to contain the FM of *FMR1* with repeats up to 1000 triplets [30]. In this pluripotent undifferentiated stem line, the *FMR1* gene is unmethylated and transcriptionally active. When differentiated as embryoid bodies, transcription of *FMR1* is nearly eliminated. The embryoid bodies show a substantial increase in *FMR1* DNA methylation, similar to cells derived from fragile X patients. Chromatin immunoprecipitation studies show, the epigenetic switch of histone H3 deacetylation and histone H3K9 methylation which is also seen in fragile X patients [39,40]. The molecular mechanism behind these changes is not clear and the question remains how the repeat length itself is responsible for DNA methylation and histone modifications in the *FMR1* gene. These embryonic stem cells might help to unravel the mechanism and timing of gene silencing and to identify the proteins involved.

Almost all fragile X patients carry an expanded FM and show methylation of the promoter region of the *FMR1* gene and the coding region is intact. Rare individuals with expanded, but unmethylated repeats have been described. Such individuals are not showing the full spectrum of the phenotype of fragile X syndrome, demonstrating that methylation and not repeat elongation per se causes the typical features of FXS [29,41]. This observation prompted to investigate the possibility of epigenetic reactivation of the *FMR1* gene. It was shown that treatment of fragile X cell lines with 5-azadeoxycytidine (5-azadC) leads to reactivation of the *FMR1* gene (figure 2) [42] and DNA demethylation [43]. As normal methylation in the cell is followed by deacetylation it was hypothesized that a combination of demethylating and acetylating drugs might enhance demethylation of the CGG repeat. Indeed, a synergistic effect of the two types of drugs was demonstrated to enhance the reactivation of the FMRP production approximately five-fold. Histone acetylating drugs, such as butyrate, can potentiate the

reactivation induced by 5-azadC [39,40]. Unfortunately, 5-azadC cannot be administered to fragile X patients because of its toxicity and histone acetylating drugs alone do not reactivate the *FMR1* gene, as DNA methylation appears to be the dominant epigenetic modification responsible for gene silencing.

### Structural domains of FMRP

Two types of RNA binding domains have been identified in FMRP, including two KH domains and an RGG box containing a conserved Arg-Gly-Gly triplet [44,45]. The biological significance of the RNA-binding capacities and (poly)ribosomal association of FMRP-associated RNP particles is demonstrated in cells from a severely affected fragile X patient, who has a missense mutation (I304N) in the second KH domain within the *FMR1* gene [46]. The mutation disrupts the normal folding of the KH domain and the mutant I304N protein no longer associates with active polyribosomes [47–49].

In addition, a nuclear localization signal (NLS), a nuclear export signal (NES), two coiled coils and a G-quartet binding structure have been identified. The presence of both a NES and NLS suggests that FMRP may shuttle into and out of the nucleus. The nuclear export mediated by the NES of FMRP is exportin1-dependent [50]. In accordance with the shuttling hypothesis, the protein has been observed in the nuclear pore during transfer between the nucleus and cytoplasm [10]. Nuclear FMRP has been shown to exit the nucleus through its bound mRNA [19].

In human cell lines, FMRP co-localizes primarily with polyribosomes and ribosomes at/in the endoplasmic reticulum membrane. There is strong evidence to support an important role for FMRP in regulation of translation of specific target mRNAs. Evidence was presented that FMRP *in vitro* may function as a repressor of translation of its own mRNA [51]. It is interesting to note that the Ile304Asn mutant FMRP still is able to interact with polyA-mRNA but loses its function *in vitro* as a translational repressor due to a loss of homo-oligomerization. FMRP-associated target mRNAs contain a sequence that can form an intramolecular G quartet structure. FMRP binds also to its own mRNA via a G quartet that is found at the C terminal end of the part coding for the open reading frame [52,53]. A subset of mRNAs containing a G-quartet has been identified that are potential targets for FMRP, including important neuronal proteins like microtubule associated protein 1B (MAP1B) and semaphorin [53–55]. A validated list of targeted FMRP mRNAs can be found in Bassell and Warren [56]. But FMRP also can bind to mRNAs that do not contain a G quartet. It is still not fully clear what the role of the different binding regions is and whether they all are important for FMRP functioning.

### FMRP and mRNP transport

In 1987 Steward demonstrated that mRNAs were transported into dendrites of cultured hippocampal neurons [57]. Since then a large number of dendritic localized mRNAs have been identified and it is suggested that the translation of those mRNAs can be regulated in a spatially restricted manner in response to stimulation (for review [58]).

The dynamics of the transport of mRNP particles in neurons has been studied by different experimental approaches and a supramolecular complex was identified containing mRNAs, translational factors and ribosomal subunits [59–61]. FMRP containing RNP particles have been observed as RNA granules traveling in the dendritic branching [61,62] (figure 3). The migration of mRNP particles over long distances within processes towards the growth cone is established by movement along microtubules [59,63,64]. A similar model has been proposed in which FMRP binds specific mRNAs and mediates the targeting/transport of these transcripts into the dendrite using intact microtubules. During transport they remain translationally inactive until appropriate synaptic input allows translation. Recently, Dictenberg *et al* have

shown that FMRP is involved in a stimulus-induced dendritic mRNA transport [65]. FMRP-positive mRNP granules were associated with KIF5, which is a neurospecific motor protein. In the absence of FMRP, a number of mRNAs are diminished in their association with KIF5 supporting the role of FMRP as a molecular adaptor to bind mRNA targets during kinesin-mediated transport along microtubules to synaptic sites.

It is not known whether certain mRNAs can not be transported into the dendrite in the absence of FMRP. However, it is evident that FMRP plays a role at the synapse in controlling the translation of certain mRNAs. A role of FMRP in translationally silencing during transport into the dendrites have been proposed [66,67].

## FMRP and spines

Transport and regulated translation of mRNAs in dendrites is important for neuronal function, including modulation of synaptic plasticity. This is essential in memory consolidation and learning [68,69]. Altered spine morphology (long and thin dendritic spines) has been observed in post-mortem brains of fragile X patients [70–72] and in *Fmr1* KO mice [73–75]. The presence of the protein machinery near synaptic connections allows neurons to rapidly respond to signals at particular synapses through local translation of (specific) mRNAs. Weiler *et al* showed that FMRP is synthesized in response to mGluR activation using glutamate [15]. Controlled efficient translation of mRNAs in the vicinity of the synapse is important and herein FMRP plays a crucial role. It was proposed that FMRP located at the synapse is repressing translation of mRNAs encoding proteins that regulate endocytic events of AMPA. Upon synaptic stimulation FMRP may dissociate from these mRNA targets to allow translation and facilitation of AMPA receptor internalization. The model predicts that in the absence of FMRP the upregulated translation of a subset of mRNAs would result in the perturbation of AMPA receptor internalization dynamics.

A model was proposed in which FMRP is regulating mRNA translation upon mGluR stimulation [76] (figure 4). This mGluR theory states that AMPA receptor internalization triggered by mGluR5 stimulation [77], is exaggerated in *Fmr1* KO mice [78]. Recently it was shown that FMRP deficient dendrites indeed show aberrant AMPA receptor internalization resulting in a significantly reduced number of AMPA receptors at the postsynaptic membrane [79]. Moreover, *Fmr1* KO mice that are crossbred with mice that have a 50% reduction in mGluR5 expression were shown to be rescued in several phenotypic aspects [80]. This illustrates that reducing the mGluR5 signaling pathway potential can rescue some phenotypic features such as Inhibitory Avoidance Extinction test and the occurrence of audiogenic seizures while the macroorchidism was not rescued [80]. Since the amount of AMPA receptors at the postsynaptic membrane is correlated with protrusion shape, this might also explain the immature protrusion morphology that has been found in different brain areas of both fragile X patients and *Fmr1* KO mice [70,73–75,81–83]. An antagonist of mGluR5 receptors might reduce the mGluR5 signaling pathway potential and would theoretically counteract the increased internalization of AMPA receptor internalization in *Fmr1* KO neurons. Behavioral studies have shown that *Fmr1* KO mice treated with the mGluR5 antagonist MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochloride) clearly display less sensitivity to audiogenic seizures and show more wild type-like behavior in an open field test compared with untreated mice [84]. A similar rescue of the phenotype of the Prepulse Inhibition (PPI) paradigm in *Fmr1* KO mice was seen upon treatment with different mGluR antagonists including MPEP and fenobam [75] (figure 4). Treatment with these two mGluR antagonists also rescued the immature spine morphology in cultured hippocampal neurons [75]. Since spine shape is correlated with the number of AMPA receptors at the postsynaptic membrane [85], these data are in line with the rescue effect of MPEP on AMPA receptor trafficking as shown by Nakamoto *et al* [79]. Recently, Phase I clinical studies in fragile X patients have been started

with different mGluR antagonists, including fenobam and AFQ056. [86]. Thus far, no site-effects have been described both in mice and men.

## Translation of FMRP target mRNAs at the synapse

Protein kinases are crucial for the regulation of neuronal development and synaptic transmission upon response to extracellular or intracellular signals. The mGluR theory is in line with the translation control pathways within the dendritic spines: a simplified version is depicted in figure 5. Compelling evidence supports the postsynaptic FMRP signaling model. Ceman *et al* [19] showed that unphosphorylated FMRP associated with actively translating polyribosomes while a fraction of phosphorylated FMRP is associated with apparently stalled polyribosomes. The data suggest that dephosphorylation of FMRP may regulate FMRP and that the release of FMRP-induced translational suppression may involve a dephosphorylation signal. Rapid dephosphorylation of FMRP allows target mRNAs to be translated, whereas rephosphorylation represses translation. Subsequent experiments identified protein phosphatase 2A (PP2A) as an FMRP phosphatase. Rapid FMRP dephosphorylation has been reported after mGluR stimulation (<1 min) in neurons caused by enhanced PP2A enzymatic activity. On the other hand, extended mGluR activation (1–5 min) resulted in mammalian target of rapamycin (mTOR)-mediated PP2A suppression and FMRP rephosphorylation [66,67]. Ribosomal protein S6 kinase (S6K1) was identified as an important kinase involved in FMRP phosphorylation in the mouse hippocampus. It was shown that activity-dependent phosphorylation of FMRP by S6K1 requires signaling inputs from mammalian target of rapamycin (mTOR), ERK1/2, and PP2A. FMRP rephosphorylation is known to partly involve mTOR-dependent PP2A suppression (and thus suppression of dephosphorylation) and phosphorylation by S6K1. TSC1 and TSC2 proteins have been shown to negatively regulate cell growth through inhibition of the mTOR pathway.

Deficiency of one of these proteins results in Tuberous Sclerosis, a syndrome which may include intellectual disabilities. In cells lacking either TSC1 or TSC2 protein, the downstream targets of mTOR, including p70 S6 kinase (S6K) and ribosomal protein S6, are constitutively phosphorylated [87] resulting in activation of protein synthesis. Hu *et al* [88] showed that MEK and ERK signaling appears normal, and phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB; or Akt) signaling is compromised in *Fmr1* knock-out mice. Enhancing Ras-PI3K-PKB signaling restores synaptic delivery of GluR1-containing AMPA-receptors and normal LTP in *Fmr1* knock-out mice

Further research is needed to characterize the cascade of signaling upon mGluR activation and the mechanism whereby FMRP phosphorylation regulates translation of target mRNAs.

## Primary ovarian insufficiency

Furthermore, it became apparent that 20% of female PM carriers manifest premature ovarian failure (POF: cessation of menstruation at or before 40 years of age)[89]. It has been proposed that primary ovarian insufficiency (FXPOI) is a more accurate term for the disorder, to describe the broad range of clinical manifestations associated with what used to be classified as POF [90]. Hundscheid *et al.* [91] reported evidence for a paternal-parent-of-origin effect on FXPOI (fragile X associated primary ovarian insufficiency) in female PM carriers; however, subsequent studies by others did not support this observation [92,93]. Differences between the different data sets may be related to the observed discrepancy. Women with the PM, even if they are still cycling, have higher levels of follicle stimulation hormone (FSH) than do healthy women [94]. It has recently been suggested that Anti-Müllerian Hormone (AMH) may be a better marker of ovarian decline. AMH is expressed in granulosa cells of growing follicles, thus serves as a marker for the size of the primordial follicle pool in women. Indeed, female PM carriers, with (CGG) repeat lengths beyond 70, had lower AMH levels than did female

PM carriers with (CGG) repeat shorter than 70 repeats. Thus, lower AMH levels are suggestive of early ovarian decline in women with (CGG)<sub>>70</sub> [95]. Penetrance and age of onset of FXPOI, as well as the increase of FSH levels, correlate with (CGG) repeat length [96]. However, a non-linear relationship has been described for age at menopause and premenopausal size, in which premenopausal women in the mid-size range are at greatest risk for POF, while larger repeat tracts are associated with a lower risk [97].

## Fragile X-associated tremor/ataxia syndrome

Over the past few years, it has become apparent that PM carriers are also at risk of developing a progressive neurodegenerative disorder, which is clinically and neuropathologically entirely distinct from FXS [98–100]. This syndrome is called Fragile X-associated tremor/ataxia syndrome (FXTAS). Although both disorders involve repeat expansions in the *FMR1* gene, the clinical presentation and molecular mechanisms underlying each disease are completely distinct. The most common clinical features of FXTAS are a progressive action tremor and ataxia. More advanced or severe cases may show a progressive cognitive decline that ranges from executive and memory deficits to dementia (for review of clinical symptoms see [101]). The major neuropathological hallmark and postmortem criterion for definitive FXTAS is eosinophilic, ubiquitin-positive intranuclear inclusions located in broad distribution throughout the brain in neurons and astrocytes [102,103]. A study of the penetrance of the tremor and ataxia among PM carriers, ascertained through families with known probands with FXS, revealed more than one third of carriers, aged 50 years and older, show symptoms of FXTAS. The penetrance of this disorder exceeds 50% for men over 70 years of age [104]. Since the prevalence of the PM alleles in the general population is approximately 1/800 for males and 1/250 for females. It has been estimated that 1 in 3000 men older than 50 years in the general population will develop symptoms of FXTAS [105,106]. It has been suggested that penetrance increases with CGG repeat length.

An RNA gain of function mechanism has been suggested for FXTAS [98,99,107] based on the observation of elevated levels of CGG containing *FMR1* mRNA [108], along with either no detectable change in FMRP or slightly reduced FMRP levels, observed in peripheral blood leukocytes [108,109] and brain regions [110,111] of PM carriers. The mechanism underlying the elevated *FMR1* mRNA levels is unknown. The observation that increased levels of transcripts were also measured when constructs bearing the *FMR1* 5' UTR with PM-sized (CGG)<sub>n</sub>, fused to a luciferase reporter were transfected into two different cell lines [112], suggests that the expanded (CGG)<sub>n</sub> itself, rather than the reduced FMRP levels, is responsible for increased transcription [109,113,114] (figure 6).

## Mouse models for FXTAS

Knock-in mouse models have been generated in which the murine (CGG)<sub>8</sub> repeat within the endogenous *Fmr1* gene was replaced by a human (CGG)<sub>98</sub> or (CGG)<sub>118</sub> repeat using a homologous recombination technique in embryonic stem (ES) cells [35,36]. An expansion bias was observed, with the largest expansion being 43 CGGs [37]. Although it was expected that these longer CGG repeat expansions would eventually lead to methylation of the *Fmr1* gene, to date, and despite CGG repeat tracts well over 200 CGGs long, no methylation of the promoter region has been detected. Biochemical analysis of the brains of these mice revealed elevated *Fmr1* mRNA levels and reduced *Fmrp* expression. Importantly, elevated *Fmr1* mRNA levels were detected throughout development from 1–72 weeks of age [115]. Since *Fmr1* mRNA levels were already elevated by 1 week of age, the potential exists for as yet undocumented developmental consequences of excess transcript production in human FXTAS. On average, a two-fold increase of *Fmr1* mRNA levels in brain tissue was reported.

Both CGG repeat mouse strains showed an inverse correlation between CGG repeat length and *Fmrp* expression in brain using quantitative Western blot analysis [36,116]. The degree of change in *Fmrp* expression in the brain appears to be brain region-specific with significant reduced expression throughout the brain and relatively high expression in the hippocampus [36,37]. Thus, in spite of the elevated levels of *Fmr1* transcripts reduced *Fmrp* expression was found. This apparent paradox was explained by a hypothetical model in which the CGG expansion in the 5' UTR of the transcript hampers the initiation of translation (figure 6).

The knock-in model shows ubiquitin positive intranuclear inclusion formation in several neurons [115]. A comprehensive study for the presence of these intranuclear neuronal inclusions in different brain regions at different ages demonstrated the occurrence of intranuclear neuronal inclusions throughout the brain with high percentages of inclusions in specific brain structures, including olfactory nucleus, parafascicular thalamic nucleus, medial mammillary nucleus, colliculus inferior, cerebellum, amygdala and pontine nucleus at 72 weeks of age [115,116]. Furthermore, the average size of the inclusions within one specific brain area correlated significantly with the age of CGG repeat mice. In younger mice, smaller sized inclusions were found compared to older mice. Interestingly, the gradual increase in the size of the inclusions and the percent of ubiquitin-positive neurons over lifetime probably parallels the progressive development of the neurological phenotype of FXTAS in humans [100]. In an attempt to further characterize the constituents of the inclusions, a systematic analysis was performed to localize a panel of protein candidates (related to FMRP or other disorders with inclusions) using double-labeling immunohistochemistry. Next to ubiquitin, molecular chaperone Hsp40, 20S proteasome complex and DNA repair-ubiquitin-associated HR23B accumulated in the inclusions [115,117]. Importantly, *Fmrp* could not be detected in the inclusions, although *FMR1* mRNA is detectable.

Further evidence of the role of rCGG came from fly models and from transgenic mice [107, 118]. An rCGG transgenic fly study in which a DNA fragment containing 90 CGG repeats with a downstream EGFP coding sequence used as a reporter system, reported the first evidence that rCGG outside of the context of *FMR1* could induce neurodegeneration and inclusion formation [107]. Given that the fragile X PM rCGG repeat itself is sufficient to cause neurodegeneration in *Drosophila* it has been hypothesized that specific rCGG binding may sequester rCGG-repeat-binding proteins from their normal function [119]. Using biochemical and genetic approaches, three proteins, Pur  $\alpha$ , hnRNP A2/B1 (two protein isoforms from one gene), and CUGBP1 were found to bind rCGG repeats either directly (Pur  $\alpha$  and hnRNP A2/B1) or indirectly (CUGBP1, through the interaction with hnRNP A2/B1) [120,121]. These proteins are RNA-binding proteins, and have been shown to play a role in transcription, mRNA trafficking, splicing and translation. Furthermore, *in vivo* Pura was found to be present in the inclusions of both human and fly tissues, suggesting that expression of the fragile X PM rCGG repeats can sequester the rCGG-binding proteins from their normal cellular function(s) and cause neurodegeneration [120] (figure 6). This idea is further supported by the fact that over-expression of either Pur  $\alpha$  or hnRNP A2/B1 ameliorated neurodegeneration in the fly model of FXTAS. For Myotonic Dystrophy (DM1) it is proposed that CUG-binding proteins (CUG-BP) are sequestered by expanded *DMPK*-(CUG)<sub>n</sub> mRNAs and this is causing aberrant expression of other transcripts that are normally regulated by these proteins [122]. MBNL1 is a specific CUG-BP, homologous to the *Drosophila* muscleblind protein, which is essential in terminal differentiation of muscle and photoreceptor cells. It accumulates in the nuclear foci in DM1 cells, such that MBNL1 cannot exert its normal function during a critical period of cell differentiation and mRNA splicing [123]. In a similar way proteins can be sequestered by the expanded CGG repeat in *FMR1* mRNA and the lack of these proteins can contribute to the FXTAS phenotype.



PM carriers (with or without FXTAS) may develop a variety of neuropsychological symptoms, including mood and anxiety disorders [100,124–127]. Elevated stress hormone levels have been suggested as a possible explanation. Additional evidence for an elevated stress response and thus aberrant hypothalamo-pituitary-adrenal (HPA) axis function in PM carriers comes from reports demonstrating ubiquitin-positive intranuclear inclusions in the pituitary gland of autopsy brain material from patients with FXTAS [128,129]. Similarly, studies in expanded CGG mice at 72 weeks of age point to an increased anxiety phenotype in the open field behavior test [130]. Further characterization of the HPA axis physiology in CGG repeat mice revealed dramatically elevated corticosterone levels in serum in response to a mild stressor, as well as intranuclear ubiquitin-positive inclusions in both the pituitary and adrenal gland of 100-week-old mice [116].

## Future directions

For Fragile X syndrome research will focus on the cellular function of FMRP, especially in spines of neurons. Based on the current knowledge the first attempts have been started to treat Fragile X patients with drugs that are counteracting the lack of FMRP and the control of protein synthesis at the synapse. The first signs in animal studies are very promising. A next question is the repeat instability leading to FM. The availability of human embryonic stem cells opens the door for studies about the timing and the mechanism underlying repeat instability. This knowledge can form the basis for intervention in repeat instability from generation to generation.

For FXTAS the main question remains however; namely how the  $(CGG)_n$  RNA leads to formation of inclusions and how this relates to cellular dysfunction originates, ultimately leading to neurodegeneration and clinical problems. Future studies should focus on the identification and role of  $(CGG)_n$ -BP, and the cellular consequences of their depletion. If these proteins are indeed sequestered away from their normal cellular function, as the toxic RNA gain-of-function model predicts, their function might provide important links between the mutant RNA and clinical symptoms.

The mouse model will remain valuable since the onset as well as the course of disease can be studied. Furthermore, the  $(CGG)_n$  mice will furthermore be valuable in attempts at reversing the neurodegenerative phenotype. Therapeutic strategies will likely focus on diminishing  $(CGG)_n$  mRNA levels. The initial studies conducted in *Drosophila* are promising in this light, as reversing the negative effect of the expanded  $(CGG)_n$  RNA by co-expressing an anti-sense repeat or by overexpressing the proteins sequestered by the repeat RNA, rescued the neurodegenerative phenotype. Hence, strategies involving the siRNA pathway might prove beneficial in the future. In addition, when more  $(CGG)_n$ -BPs will be identified, this might provide new therapeutic targets.

## Conclusion

*FMR1* gene expression is involved in three important disorders with distinct entities. The fragile X syndrome, a neurodevelopmental disorder and the most prevalent cause of heritable mental retardation is caused by the total lack of the *FMR1* gene product, FMRP. Thus, in FM carriers the disease is caused by a loss-of-function mechanism. In contrast, PM carriers show increased transcription of the *FMR1* gene that results in elevated levels of *FMR1* mRNAs and causes a new progressive neurological syndrome, called FXTAS. Although most data is reported about male FM and PM carriers, but female FM and PM carriers can be affected as well. The molecular basis of FXTAS is unknown; however, a toxic RNA gain-of-function mechanism has been proposed and is currently under study. The third disorder is Premature Ovarian Insufficiency which is seen in female PM carrier. The molecular basis of POI is yet unknown.

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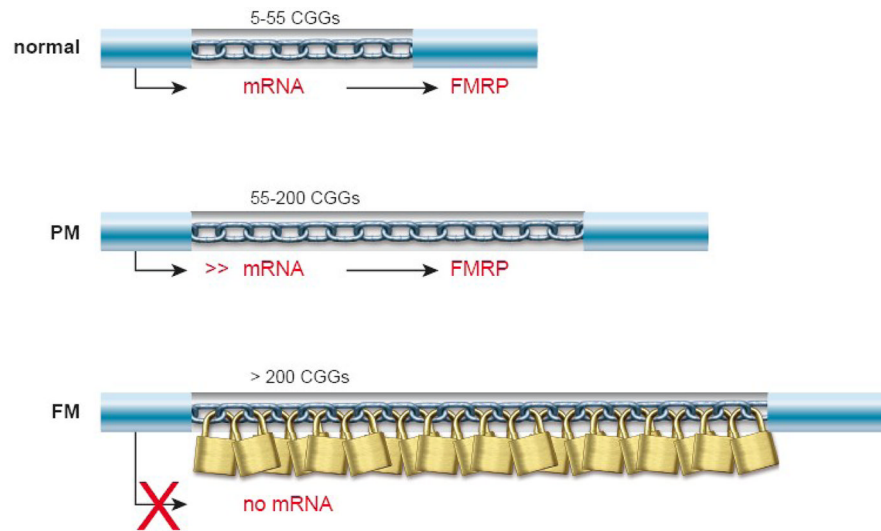
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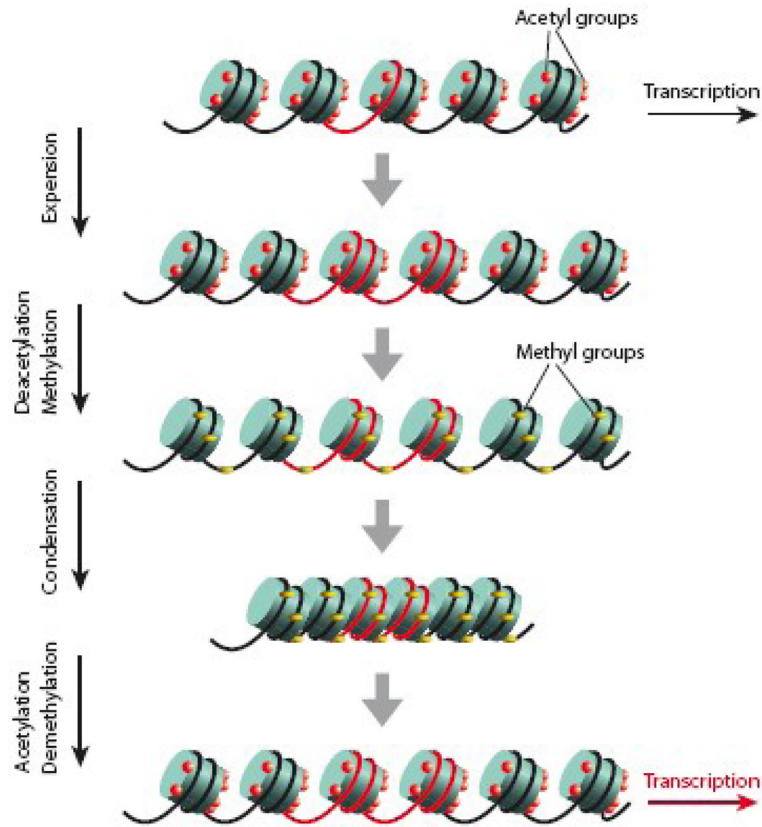


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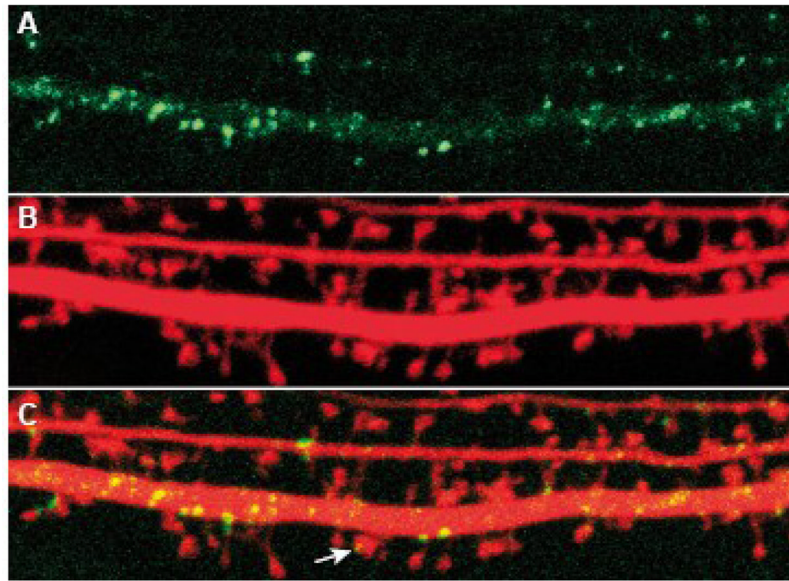


**Figure 1. The CGG repeat in the *FMR1* gene**

Schematic representation of normal, PM (premutation) and FM (full mutation) alleles of the *FMR1* gene and the effect of the expansion on transcription and translation. Methylation due to extensive elongation of the CGG repeat in the 5'-UTR of the *FMR1* gene is depicted as a lock.

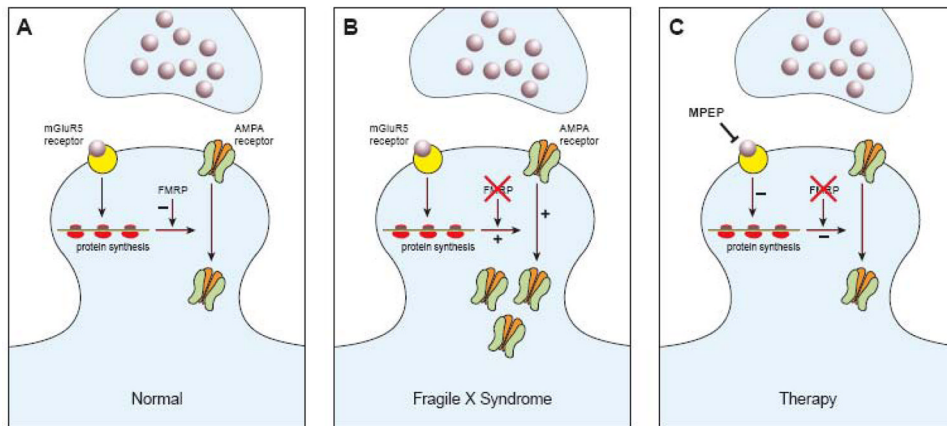


**Figure 2. Schematic representation of the chromatin structure of the *FMR1* gene**  
In the normal situation the active gene has an open chromatin structure. When the CGG repeat (red line) is expanded, deacetylation and methylation of the promoter and CGG region takes place leading to a packaged and less accessible chromatin structure causing inactivation of the *FMR1* gene. Treatment with 5-azadC results in demethylation and acetylation leading to an open chromatin structure and transcription will be (partly) restored.



**Fig. 3. Localization of EGFP-FMRP in dendritic spines**

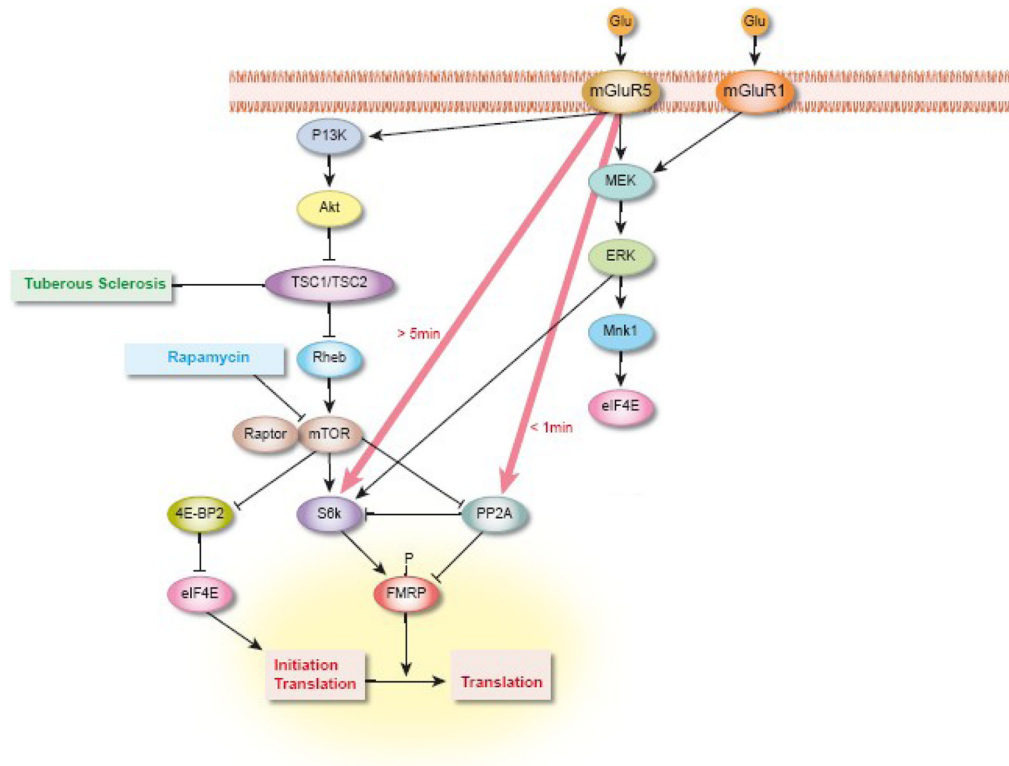
Primary hippocampal mouse neurons of E18 *Fmr1* KO mice were co-transfected with  $\beta$ -actin-EGFP-Fmr1 (A) and  $\beta$ -actin-mCherry (B). The dendrite, including many spines, of one neuron is depicted. Note the presence of EGFP-FMRP in a spinehead in the overlay (arrow in C). Images were acquired using a Zeiss LSM510 confocal microscope (scalebar= 5  $\mu$ m). Courtesy by Femke de Vrij.



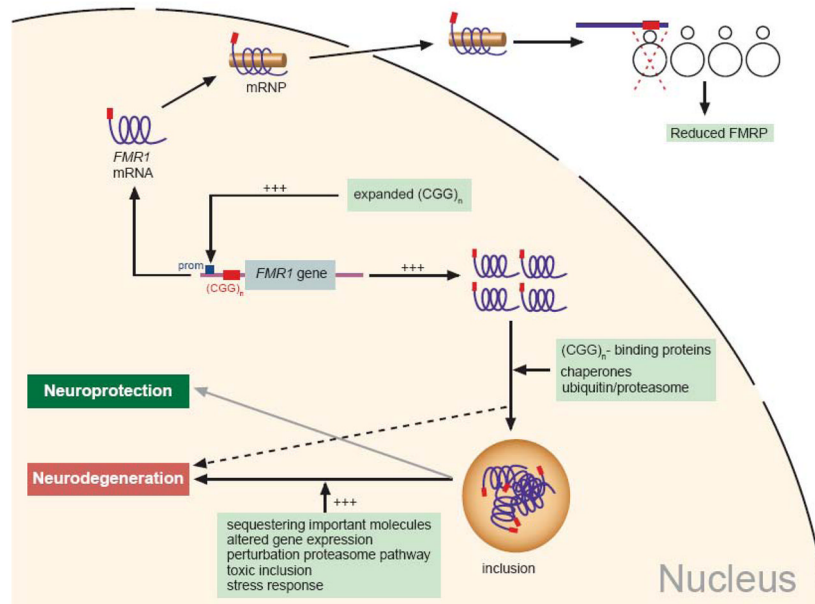
**Figure 4. The mGluR theory**

Hypothetical model for the action of FMRP at the synapse, adapted from reference [76].

Treatment with MPEP, an mGluR5 antagonist, results in the rescue of some phenotypic features because mGluR5 stimulation is reduced and subsequently local translation at the synapse is no longer exaggerated. Ultimately, the number of internalized AMPA receptors is reduced and restored to normal levels. **A.** Stimulation of mGluR5, a metabotropic glutamate receptor, induces local mRNA translation. This results in novel protein synthesis that on its turn stimulates the internalization of the ionotropic AMPA receptor, essential for in long-term plasticity. FMRP acts as a negative regulator of transcription [51,76], reducing the internalization of the ionotropic glutamate receptor. **B.** In neurons from fragile X patients the absence of FMRP leads to an increase internalization of the ionotropic glutamate receptors which results in enhanced LTD. **C.** Rescue of normal translation due to the mGluR antagonist MPEP, slowing down the internalization of the ionotropic glutamate receptors.



**Figure 5. Simplified model of the translation control pathways at the dendritic spines**  
 Stimulation of mGluR leads to active PP2A (<1min) which dephosphorylates FMRP, and this results in rapid translation of FMRP-associated mRNAs. Within 5 min, mTOR is activated, inhibiting PP2A and activating S6K1, leading to FMRP phosphorylation and translational inhibition of FMRP target messages. Based on [66,67].



**Figure 6. A schematic representation of the RNA gain of function mechanism proposed for the pathogenesis of FXTAS**

The *FMR1* gene is transcribed in the nucleus and transported to the ribosomes. The expanded CGG repeat present in the 5' UTR of the *FMR1* mRNA hampers translation, leading to lower levels of FMRP. The presence of the expanded CGG repeat results in enhanced transcription via a thusfar unknown mechanism and leads to elevated *FMR1* mRNA levels. In an attempt to get rid of the excess of *FMR1* mRNAs, the cell might attract chaperones or elements of the ubiquitin/proteasome system. Also CGG-binding proteins might be recruited. These processes could lead to the formation of intranuclear inclusions. Sequestration of proteins into the inclusion might prevent them from exerting their function, thereby disturbing normal cellular function, which in the end might cause neurodegeneration. However, it cannot be excluded that the formation of inclusions has a neuroprotective effect, such that neurons that are capable of capturing the toxic transcripts in the inclusions are the cells that survive.