A fragile balance: FMR1 expression levels

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The FMR1 gene is involved in three different syndromes, the Fragile X syndrome, premature ovarian failure (POF) and the Fragile X-associated tremor/ataxia syndrome (FXTAS) at older age. Fragile X syndrome is caused by an expanded 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/or translation that plays a role in synaptic maturation and function. POF and FXTAS are found in individuals with an expanded repeat between 50 and 200 CGGs and are associated with increased FMR1 mRNA levels. The presence of elevated FMR1 mRNA in all patients suggests that these syndromes may represent a gain-of-function effect from the elevated message levels. The level of FMR1 mRNA is in fragile balance and is therefore critical for normal functioning.

Fragile X syndrome is the most prevalent cause of heritable mental retardation with a frequency of 1:4000 males and 1:6000 females (reviewed in 1). This X-linked disorder is usually caused by the absence of the fragile X mental retardation protein (FMRP). In addition, a rare atypical case of Fragile X syndrome has been reported that is associated with a single point mutation (2). The subcellular distribution of FMRP is largely cytoplasmic and FMRP expression is widespread with abundant expression in neurons, in dendrites in particular, and with testicular expression in spermatogonia (3,4). The association of FMRP with ribosomes is mRNA-dependent via ribonucleoprotein (RNP) particles, which contain several other proteins like nucleolin, YB-1, NUFIP1, CYFIP1 and CYFIP2 and the fragile X (structurally) related proteins FXR1P and FXR2P (5–7). FMRP contains RNA-binding sequence motifs, including two KH domains and an RGG box (8,9). The precise physiological function of FMRP is still not defined; however, a role in transport and/or translational efficiency of mRNAs, including its own mRNA, has been suggested (10).

PREVALENCES AND CGG-REPEAT INSTABILITY

In fragile X syndrome, mental retardation is almost exclusively caused by an expansion of a polymorphic CGG repeat in the 5′ untranslated region of the FMR1 gene (11,12). Fragile X patients have a number of more than 200 units. As a result of the repeat expansion, the CGG repeat and the surrounding promoter region of the FMR1 gene is methylated, inhibiting FMR1 transcription and causing absence of the protein product.

Although the number of CGG repeats in the general population is highly variable, the majority of FMR1 alleles have 29–30 repeats and are stable upon transmission to the next generation (13). These normal CGG repeats in the FMR1 gene are usually interrupted by two AGG triplets. Individuals with the fragile X premutation (PM) have expanded repeat lengths varying from 50 to 200 CGG repeats. PM alleles may become unstable, however, only through maternal transmission, and lengths usually increase in subsequent generations. Previous estimates of the prevalence of PMs in the general population in Canada yielded a prevalence of 1:259 females and 1:813 males with more than 54 repeats (14). Interestingly, this population-based study in males identified a number of alleles of intermediate size (between 40 and 54 CGG repeats) that, next to the identified PM alleles, showed AGG interruptions, suggesting that loss of AGG interruptions is a late event in the expansion of normal CGG repeats to intermediate sized alleles. Other studies in both Israel and Italy showed actually higher prevalences of PM alleles with prevalences approaching 1:100 females (15,16); however, further population-based screening studies in different populations are necessary to determine accurate estimates of PM alleles in the general population.

A recent collaborative study was established to examine several issues regarding FMR1 CGG-repeat instability among females with PM and intermediate alleles (17). Nolin and co-workers (17) found that the smallest alleles to undergo expansion to full mutation in one generation contained 59 CGG repeats with no AGG interruptions, and accurate risk estimates of full-mutation expansions in offspring of females with PM-size alleles were lower than previously supposed. Furthermore, no significant sex bias was observed in the
proportion of full mutation offspring born to mothers with PM alleles, and in contrast to PM alleles the intermediate alleles from females with no family history of fragile X, as a group, exhibited a stable transmission pattern. Notably, in contrast to PM-size alleles, Sullivan et al. (18) observed a higher degree of instability in paternal transmission of intermediate alleles. Altogether, these epidemiological studies are not only important to provide knowledge about the mutational pathway of repeat instability and understanding of FMR1 PM penetrance but also allow clinicians to improve risk estimates for genetic counselling of females with PM or intermediate-size alleles in making decisions about prenatal diagnosis.

**FEMALE PREMUTATION CARRIERS**

Cognitive functioning in female PM carriers is generally considered normal; however, a small subset of female PM carriers develops mild learning disabilities and emotional problems such as mood lability and anxiety (1). More importantly, ~20% of female PM carriers manifest premature ovarian failure (POF), defined by menopause before 40 years. POF represents the final stage of a variety of diseases that result in the loss of ovarian follicles. Hundscheid et al. (19) reported evidence for a paternal-parent-of-origin effect on POF in female PM carriers; however, subsequent studies by others did not support this observation (20,21). Differences between the different data sets may be related to the observed discrepancy. Perhaps the variation in FMR1 transcript levels in female PM carriers contributes to the development of POF (22). Further investigations are needed to understand the molecular pathways underlying POF among PM carriers, including the role of increased FMR1 transcripts on follicular development (23).

**MALE PREMUTATION CARRIERS**

Male PM carriers have generally been thought not to develop mental disabilities because they produce normal levels of the FMR1 gene product, FMRP. Recent studies of the expression of the FMR1 gene show compelling evidence that in cells of males with alleles in the PM range significantly increased FMR1 mRNA levels can be detected. The increased transcriptional activity of the FMR1 gene seems to be positively correlated with the size of the CGG repeat. That is, CGG repeats in the upper range (100–200 CGGs) result in an average 5-fold elevation, whereas CGGs in the lower range (50–100 CGGs) result in an average 2-fold elevation (22,24,25). Paradoxically, FMRP levels in cells from male PM carriers were mildly reduced (24,25). This observation has led to the hypothesis that expanded CGG repeats lead to the translational impediment of the FMR1 transcripts by conformational changes in the FMR1 transcript that influence the initiation of translation and stalled 40S ribosomal subunits and consequent FMRP reduction (22,24,25). Such a mechanism has been proposed for unmethylated alleles in the fragile X full mutation range too (26). In cells with alleles in the PM range the increased transcriptional activity of the FMR1 gene could be caused by a feedback mechanism for the diminished translational efficiency of the FMR1 transcript (Fig. 1). However, direct proof for such a mechanism is lacking and several other possible explanations for the enhanced transcriptional activity of the FMR1 gene have been proposed, including more open promoter conformation due to the expanded CGG repeat (25), and up-regulation of transcription by CGG binding proteins such as CGGBP1, a protein known to be able to regulate FMR1 expression (27). Increased stability of the FMR1 transcripts due to expanded CGG repeats has been excluded as an alternative explanation (24). In addition, the normal FMR1 transcript levels in the mutant I304N cell line argues against a role for the lack of functional FMRP as regulator of transcriptional activity of the FMR1 gene (22,25). The reduced FMRP production despite elevated FMR1 transcript levels suggests that transcriptional reactivation of the PM alleles as therapeutic intervention of fragile X syndrome will not be an effective therapy and thus should also include strategies to circumvent the block at the level of translation initiation.

**UNEXPECTED PHENOTYPE IN MALE PM CARRIERS**

The recent description of older males carrying a PM (ranging between 71 and 135 CGGs, to date), who exhibit an unique neurodegenerative syndrome characterized by progressive intention tremor and ataxia (FXTAS; fragile X-associated tremor/ataxia syndrome) suggests that a new neurodegenerative disorder has been linked to the PM state (28). More advanced cases may be accompanied by memory and executive function deficits, anxiety, parkinsonism, peripheral neuropathy, essential tremor and autonomic dysfunction (28–32). Significant dementia has been observed in a limited number of patients (28,33). MR imaging studies (T2 signal) of the brain of symptomatic adult male premutation carriers showed a characteristic imaging, including hyperintensities of the middle cerebellar peduncle, cerebellar white matter lateral, superior and inferior to the dentate nuclei and volume loss involving the pons, mesencephalon, cerebellar cortex, cerebral cortex, white matter of the cerebral hemispheres, and corpus callosum (34). Neurohistological studies on the brains of four symptomatic elderly premutation carriers demonstrated neuronal degeneration in the cerebellum and the presence of eosinophilic intranuclear inclusions in both neurons and astroglia. Furthermore, the inclusions showed a positive reaction with anti-ubiquitin antibodies, which suggests a link with the proteasome degradation pathway (33).

To better understand the timing and mechanism involved in FMR1 CGG repeat instability and methylation, a mouse model has been generated in which the endogenous mouse CGG repeat was replaced by a human CGG repeat carrying 98 CGG units (35). This ‘knock-in’ CGG triplet mouse shows moderate CGG repeat instability upon both maternal and paternal transmission and the aging ‘knock-in’ CGG triplet mouse was used to study the pathogenesis of FXTAS in these mice. Neurohistological, biochemical and molecular studies of the brains of these expanded-repeat mice (20–72 weeks) were undertaken and elevated Fmr1 mRNA levels and intranuclear inclusions with ubiquitin, Hsp40 and the 20S catalytic core complex of the proteasome as constituents were reported (Fig. 2) (36). An increase was observed in both the number and
the size of the inclusions in specific brain regions during the course of life, which correlates with the progressive character of FXTAS. These observations in expanded-repeat mice support a direct role of the \( Fmr1 \) gene, by either CGG expansion \emph{per se} or by elevated \( Fmr1 \) mRNA levels, in the formation of the inclusions and suggest a correlation between the presence of intranuclear inclusions in distinct regions of the brain and the clinical features in symptomatic premutation carriers. This mouse model will facilitate molecular studies to further analyse the pathogenesis of FXTAS from onset of symptoms till the final stage of the disease.

The origin and constitution of the inclusions in FXTAS is poorly understood; however, the presence of elevated \( Fmr1 \) mRNA levels in all patients has been proposed to be important for the formation of the inclusions and may represent a gain-of-function effect from the elevated message levels (29,33). A toxic RNA gain-of-function effect by non-coding portions of mRNAs, containing expanded repeats, has been proposed for several triplet repeat-related ataxias, such as SCA8, SCA10 and SCA12 and both genetically characterized forms of myotonic dystrophy (DM1 and DM2) (37). Extensive studies in both forms of DM1 and DM2 have shown that expanded tracts of CUG and CCUG repeats, respectively, within the untranslated part of the mRNA sequestered nuclear CUG-binding proteins which disrupts either mRNA processing (splicing) of other genes or transport of other mRNAs, eventually leading to abnormal muscle differentiation in both DM1 and DM2 and insulin resistance in DM1 (38–43). Whether the phenotype in male PM carriers arises from a similar mechanism is unknown; however, the expanded CGG tract in the \( Fmr1 \) transcript may attract high quantities of CGG-binding proteins with a consequent cumulative cytotoxic effect that may lead to intranuclear inclusion formation and ultimately neuronal cell death (27,44).

The presence of components of the ubiquitin–proteasome pathway and molecular chaperones is shared with several hereditary ataxias and other trinucleotide repeat disorders, including Huntington's disease (45,46), SCA type 1 (47), SCA type 3 (48), SCA type 7 (49) and OPMD (50). For the polyglutamine disorders a model has been proposed in which the polyglutamine expansion has a toxic gain-of-function property on the protein. However, a direct cause-and-effect relation between nuclear inclusions and the disease mechanisms is still

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**Figure 1.** Proposed model for molecular neuropathology in fragile X associated tremor/ataxia syndrome. (1) \( Fmr1 \) transcripts containing an expanded CGG repeat are normally incorporated in a mRNP particle and translocated out of the nucleus. (1a) The long CGG repeat in the \( Fmr1 \) transcript impede 40S ribosomal subunit migration resulting in hampered translation. Consequently, the nerve cell produces reduced levels of FMRP, the gene product of the \( Fmr1 \) gene. (2) In response to lowered FMRP levels an up to now unidentified feedback mechanism may increase the level of specific transcription factors that results in increased transcription of the \( Fmr1 \) gene. Enhanced transcription leads to elevated \( Fmr1 \) mRNA levels. Alternatively, long CGG tracts in the \( Fmr1 \) transcript may sequester high quantities of CGG-binding proteins and lowered CGG-binding protein levels result in an increased \( Fmr1 \) transcription. (3) The nerve cell attempts to clear itself from elevated \( Fmr1 \) transcript levels by employing molecular chaperones, and components of the ubiquitin–proteasome degradation pathway. If elevated \( Fmr1 \) transcript levels resists refolding/degradation intranuclear inclusions will be formed. Ultimately, the formation of inclusions will trigger neurodegeneration by activation of neurotoxic signalling pathways. Here, several mechanistic pathways can influence this process.
under debate (51–53). Recent studies of cellular models of polyglutamine diseases point to impaired proteasome function in the presence of polyglutamine fragments; however, it has been suggested that the impaired protein clearance is only at the start of the pathogenesis. The formation of aggregates seems to be a healthy response to misfolded proteins. For instance, the knock-in SCA1 mice (154Q) show exactly the abundant number of inclusions in neurons that are spared neurodegeneration, whereas Purkinje cells are the last to form inclusions (54). The cellular consequences of perturbation of the ubiquitin–proteasome degradation pathway in polyglutamine disorders may include transcriptional dysregulation of specific genes or sequestering of important proteins such as transcription factors and molecular chaperones, potentially leading to neuronal cell death (55–57). In addition, a recent study from Cowan et al. (58) suggest that abnormal stress response may contribute to the enhanced cell vulnerability. Cells expressing a truncated form of the androgen receptor containing an expanded polyglutamine tract show a reduction in the available levels of a specific heat shock protein involved in stress response (Hsp72). It was hypothesized that polyglutamine protein aggregates perturb normal stress response to routine metabolic insults and increase cell vulnerability. Whether the formation of aggregates underlies the clinical symptoms in male PM carriers remains unsolved.

It should be noted that mechanistically the FXTAS appears to fall into a different class of disorders than the polyglutamine diseases. That is, the expanded CGG repeat in the FMR1 gene is located in a non-coding region of the gene, whereas the expanded CAG repeat is located in each gene’s coding region, resulting in a polyglutamine tract in the disease protein. Thus, FXTAS seems to be caused by a pathogenic mechanism at the RNA level (dominant RNA-gain-of-function), wherein CGG repeat expansions in the FMR1 transcript disturb cellular function, finally leading to cell death.

Finally, the recognition of FXTAS as a new neurological disorder associated with older male PM carriers has implications for genetic counselling of fragile X families. Preliminary studies suggest that FXTAS occurs in ~20–30% of male PM carriers. With a population frequency of the PM carrier state of 1:813 males, FXTAS also represents a significant group in idiopathic ataxia. An initial analysis for the presence of the fragile X premutation in a cohort of 59 patients referred for genetic analysis of SCA genes showed three patients to carry the premutation (59). Further research should be focused on the true incidence of FXTAS among male PM carriers, the constituents of the inclusions and whether the elevated FMR1 transcripts are related etiologically to the formation of the inclusions.

**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 (8,9). The importance of the KH domain for a proper FMRP function is illustrated by a rare, unique patient with an Ile304Asn mutation, located in the second KH domain (2). The mutation disrupts the normal folding of the KH domain.

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 the fragile X protein shuttles through the cell into and out of the nucleus (Fig. 3). The nuclear export mediated by the NES of FMRP is exportin1-dependent (60). In accordance with the shuttling hypothesis, the protein has been observed in the nuclear pore during transfer between the nucleus and cytoplasm (61).

In human cell lines, FMRP co-localizes primarily with polyribosomes and rough endoplasmic reticulum. There is strong evidence to support that FMRP is important in regulating mRNA translation. Evidence was presented that FMRP in *vitro* may function as a repressor of translation of its own mRNA (10). 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. Two recent papers describe FMRP-associated mRNAs as containing a sequence that can form an intramolecular G quartet structure. Schaefler reported that FMRP binds to its own mRNA via a purine quartet that is found at the C terminal end of the part coding for the open reading frame (62). Darnell identified FMRP-bound RNA sequences out of a random RNA pool using the SELEX methodology (63). These RNA sequences had in common that they were able to form a G quartet structure. The presence of a G quartet structure in the FMRP associated mRNAs may indicate a high level of selectivity as only 4% of the mRNAs contain a G quartet structure.

**mRNA TARGETS OF FMRP**

As FMRP has RNA binding capacities, identifying the mRNAs bound by FMRP in the cell is a pre-requisite for understanding...
its function. An interaction of FMRP with the 3'-UTR of the myelin basic protein mRNA was found using purified recombinant FMRP. Sung et al. (64) identified nine mRNAs from adult brain that are able to bind FMRP, including a neuronal NT2 EST and Tip60a, a tat interactive protein. Also the *Xenopus* elongation factor 1A, xEF-1A, binds strongly to human FMRP. FMRP was demonstrated to inhibit mRNA translation of this gene and, in the absence of FMRP, the translation of human EF-1A is derepressed (65).

Brown et al. (66) examined FMRP-associated mRNAs from mouse brain and human lymphocytes. Using microarrays they identified 432 mouse mRNAs that were selectively immuno-precipitated in FMRP ribonucleoprotein particles and 251 human mRNAs that appeared differentially present in poly-somes of lymphoblast cells compared to cells from fragile X patients. Of 12 overlapping mRNAs identified in both data sets, eight contained a G quartet structure. It was shown that those mRNAs were either overexpressed or underexpressed in brains of individuals with fragile X syndrome. Among the identified mRNAs are the important neuronal proteins semaphorin, the microtubule-associated protein MAP1B and NAP22, which is present in axon terminals and dendritic spines.

Miyashiro et al. (67) have developed an approach to identify the mRNA cargoes of FMRP-associated RNP particles in situ using antibody-positioned RNA amplification, called APRA. Using APRA as a primary screen discrete changes in abundance and/or subcellular distribution of a subset of mRNAs (some previously proposed FMRP targets containing G-quartet motifs and other novel FMRP target mRNAs) was observed in brain tissue from *Fmr1* knockout mice. The identified mRNAs include the glucocorticoid receptor α (GRα). The receptor showed a change in dendritical distribution in the hippocampus of the knockout mouse. Diminished responsiveness of the receptor is compatible with learning problems.
observed in fragile X patient. Many of the mRNA targets were confirmed to bind to FMRP by gel shift assay and changes in protein expression levels were observed in total and synaptosomal brain extracts. However, there is little overlap with the other published mRNA targets.

Using the Fmr1 knockout mouse model, Zalfa et al. (68) revealed that FMRP regulates translation of specific dendritic mRNAs. Interestingly, FMRP associates directly with the dendritic, non-messenger RNA BC1, and BC1 is able to form an RNA duplex with a number of mRNAs that are potential targets for FMRP, via base-pairing to this mRNA. This suggests that FMRP acts through BC1, thereby determining the specificity of FMRP function via a novel mechanism of translational repression. The precise mechanism of the proposed BC1-facilitated FMRP repression is still not defined; however, two binding modes for the target mRNAs of FMRP have been identified now either defined by BC1 base-pairing or G-quartet recognition. Perhaps both modes occur in the nerve cell and are linked to specific functions, e.g. transport to different postsynaptic target sites.

**ROLE OF FMRP IN THE DENDRITES**

Pathological examination of brains of Fmr1 knock-out mice revealed the presence of long thin and tortuous spines along the apical dendrites (69). The presence of immature synaptic connections correlates with similar findings in human fragile X patients (70). It is postulated that FMRP by modulating mRNA translation is directly involved in synapse maturation during development and for synaptic activity in the adult.

Originally it was thought that only a few mRNAs were specifically targeted into dendrites (71). However, a recent review from Eberwine and colleagues shows the presence of many mRNAs, encoding proteins that fall into multiple functional classes within the dendrites using a very sensitive linear amplification protocol called aRNA on isolated live dendrites (72). There is compelling evidence that for dendrites an active sorting mechanism is involved.

In the synapse, FMRP might regulate the translation of certain mRNAs. Interestingly, local protein synthesis plays an important role in neuronal processes, including learning and memory. FMRP can be detected in polyosomes of synaptoneurosomes, neuronal preparations highly enriched in synapses. Synaptoneurosomes respond to stimulation by metabotropic glutamate agonists with fast increasing polyribosome formation and accelerated protein synthesis.

The synthesis of FMRP in the synaptoneurosomes increases after stimulation with the neurotransmitter glutamate, suggesting that synthesis of this protein in synaptoneurosomes is triggered by a class I glutamate receptor (73). FMRP synthesis can also be affected by brain-derived neurotrophic factor, BDNF, a known regulator of synaptic plasticity (74). BDNF downregulates the FMRP expression in cultured hippocampal neurons as well as in mouse brains. This downregulation by BDNF is a response to increased tyrosine kinase receptor signalling. The decreased FMRI mRNA amounts could be correlated to a decreased amount of FMRP in the cell.

The in vivo trigger of FMRP synthesis is not known, but FMRP increases in the barrel cortex after whisker stimulation in rats, a model of experience-dependent plasticity (75). This increase was notably observed in subcellular fractions enriched for synaptoneurosomes and polyribosomes suggesting a site-specific production of the protein. The altered level of FMRP most likely influences the translation of specific mRNAs in the synapse. Inhibition of the synthesis of its own mRNA, MAP1B, Arc and α-CaMKII has been demonstrated, but it remains unclear whether all cellular RNAs that are bound by FMRP are translationally repressed.

**FMRP AND mRNP TRANSPORT**

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 (76–78). The migration of mRNP particles over long distances within processes towards the growth cone is established by movement along microtubules (76,79,80).

The role of FMRP in dendritic mRNA transport in vivo was studied in a PC12 (neuroendocrine) cell line stably transfected with human FMR1-GFP fusion gene with an inducible expression system (78). After induction, FMRP-GFP appeared first in the cell soma and later as large granules in the neurites. Using time-lapse microscopy the movement of FMRP-GFP-positive granules was demonstrated from the cell soma into the neurites of living PC12 cells. The movement of the granules was microtubule dependent and the average velocity of the granules was 0.2 μm/s, which is in line with granular mRNA transport kinetics (81–84). Co-localization studies showed the presence of RNA, ribosomal subunits, FXR1P and kinesin heavy chain as components of the granules (78). In Figure 3 the trafficking of FMRP from the cell soma into the dendrites is shown schematically.

**FMRP AND LTD**

Since it is known that FMRP is synthesized in response to mGluR activation by glutamate (73), the involvement of FMRP in hippocampal long-term depression (LTD; long-lasting decrease in synaptic connectivity) has recently been investigated (85). In Fmr1 knockout mice, hippocampal LTD was found to be selectively enhanced compared with wild-type mice, which is consistent with a role of FMRP as repressor of translation (10,86). Stimulation of metabotropic glutamate receptors mediates internalization of AMPA and NMDA receptors and this form of LTD requires protein synthesis (87). This finding implicates FMRP in repressing translation of proteins that regulate endocytotic events, and upon synaptic stimulation FMRP may dissociate from these mRNA targets to allow translation and facilitation of 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 receptor internalisation dynamics and consequently enhanced hippocampal LTD (Fig. 3).

Thus, FMRP plays an important role in expression of proteins in the dendrites after specific triggering. Abnormal synaptic protein synthesis in absence of FMRP could underlie variable symptoms of the fragile X syndrome, including the presence of immature spines and impaired synaptic maturation.


