2.1 FXTAS: an overview of pathology

Fragile X-associated tremor/ataxia syndrome (FXTAS) belongs to family of trinucleotide repeats disorder that affects the carriers of 55 to 200 CGG repeats at the 5'-untranslated region (UTR) of the Fragile X Mental Retardation 1 (FMR1) gene [1]–[5]. The word "FXTAS" was first coined in 2001 by Hagerman and colleagues when they reported five elder males which were carriers of the PM and presented with intention tremor, difficulty in walking, cognitive decline and brain atrophy in combined with elevated FMR1 mRNA levels [6]. Initially, FXTAS was characterized narrowly as an adult-onset movement disorder, while now definition of FXTAS has broaden with the characterization and identification of clinical pleiotropy among children and adults carrying premutation alleles of the FMR1 gene [3], [7]–[10].

The core presenting features of FXTAS involves intention tremor, which is trembling or shaking of a limb during voluntary movement or resting condition (resting tremor), and problems with balance and coordination(ataxia) [11]. It is observed that intention tremors develops first followed by ataxia after few years. However, it is not mandatory to have both the features together in FXTAS. Many affected individuals develop other movement problems linked parkinsonism, neuropathy and executive dysfunctions [12]. People with FXTAS commonly show cognitive disabilities, short-term memory loss and loss of executive function, which is the ability to plan and implement actions and develop problem-solving strategies [13]. Due to this patients show impairment of skills such as impulse control, self-monitoring, focusing attention appropriately, and cognitive flexibility. Many people with FXTAS experience anxiety, depression, moodiness, or irritability [14]. Several other pathologies are also prevalent in PM carriers regardless of having FXTAS which include dysautonomia [15]–[17], sleep problems [18], migraine headaches [19], vestibular dysfunction [20], hearing deficit [10], olfactory deficit [21], chronic fatigue [22], and psychiatric problems [14], [23], [24]. Fibromyalgia [25], autoimmune disorders [26], and thyroid dysfunction [27] are common in females with FXTAS.

2.2 Radiological features of FXTAS

Individuals with FXTAS have different neuroimaging and neuropathological profiles. FXTAS patients show moderate to severe generalized brain atrophy along with specific cerebellar atrophy, ventricular enlargement, and white matter lesions [28],[29]. The magnetic resonance imaging(MRI) findings associated with FXTAS indicate diffuse cerebral, brainstem and

cerebellar volume loss, and increased T2 signal intensity in white matter of the middle cerebellar peduncles, termed as MCP sign which is major radiological feature of FXTAS (Fig. 2.1a). These white matter lesions are also found in underlying cerebellar white matter lateral to the dentate nuclei (Fig. 2.1c,d) [28]. Although, MCP sign is not specific to FXTAS, approximately 60% of males with FXTAS have white matter lesions or hyperintensities on T2-weighted signals in magnetic resonance imaging (MRI)[30].The radiological features of FXTAS also include patchy or confluent areas of increased signal intensity on T2 weighted or FLAIR images in periventricular and deep white matter of the cerebral hemispheres and corpus callosum (Fig. 2.1b). These cerebral alterations are more likely to be associated with older age[28],[31]. Volumetric studies also suggest significant volume loss in the cerebellum, cerebral cortex, amygdalohippocampal complex, thalamus, and brain stem in aging male premutation carriers with and without FXTAS [32],[33]. Loss in cerebellar volume, increased ventricular volume, and whole brain white matter hyperintensity correlate with CGG repeat length in premutation carriers [31]. The above-described constellation of MRI findings in an aging male should lead to consideration of FXTAS, particularly if the MCP sign is present.

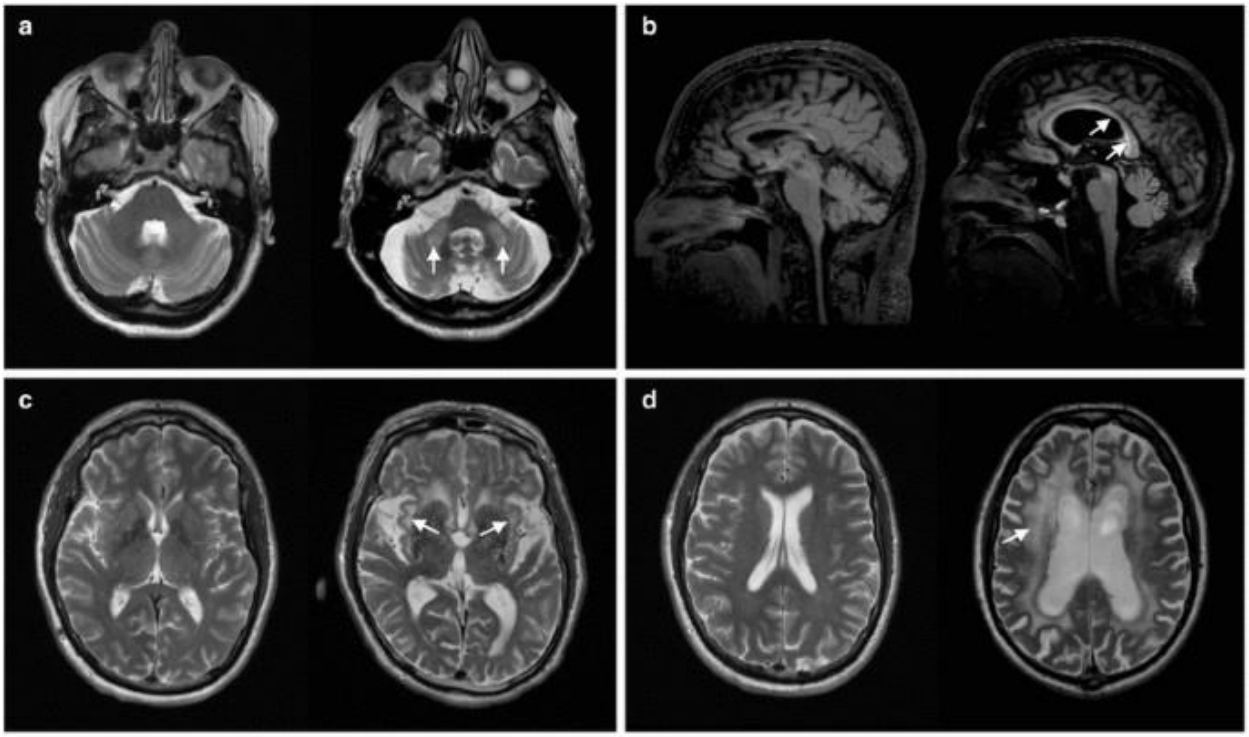

Figure 2.1. MRI images of white matter lesions and structural abnormalities associated with FXTAS. Image containing four panels where each panel represent pair of image, a normal control (left) and FXTAS case (right). The white arrows indicate increased signal intensity on T2 turbo spin-echo sequences in the middle cerebellar peduncle (MCP) (a), sub-insular white matter

(c), and cerebral white matter (d). Thinning and increased signal of the trunk and splenium of the corpus callosum (b; arrows) are from the T2 FLAIR sequence. Cases depicted in this figure are males, as follows: a. 70 years, FXTAS stage 3, 96 CGG repeats; b. same as a; c. 65 years, stage 3, 91 CGG repeats; d. 78 years, stage 4, 116 CGG repeats. The control used for all images and comparisons was a 68-year-old man with 32 CGG repeats [3].

The study of 26 male premutation carriers having symptoms of FXTAS suggested some provisional diagnostic criteria for FXTAS based on clinical and radiological criteria (Table 2.1).

Table 2.1. Diagnostic criteria and categories for FXTAS (Adapted from Hall et al., 2014 [155]).

Molecular	**Required**	**FMR1 Mutation ***
Clinical	Major	• Intention tremor • Cerebellar gait ataxia
	Minor	• Parkinsonism • Neuropathy • Memory and executive function deficits
Neuroradiological	Major	• White matter lesions in the middle cerebellar peduncles (MCP sign) or brainstem
	Minor	• White matter lesions in the splenium of the corpus callosum • Cerebral white matter lesions • Moderate–severe brain atrophy
Neuropathological	Major	• Ubiquitin-positive intranuclear inclusions Diagnostic categories
Diagnostic categories		
Definite	1 clinical major AND 1 neuroradiological major OR 1 neuropathological major	
Probable	2 clinical major OR 1 clinical minor + 1 radiological minor	
Possible	1 clinical major + 1 clinical minor	
* FMR1 mutation includes premutation, gray zone and FM with mosaicism.		

2.3 *FMR1* mutation: Genetics and molecular mechanisms

2.3.1. Range of expanded CGG repeats and associated pathologies

Fragile X syndrome was the first known trinucleotide repeat disorder and the gene responsible for fragile X associated pathologies, FMR1, was identified in 1991 [34]. Depending on number of CGG repeats at 5' UTR and the methylation status of the repeat region,FMR1 alleles are categorized into four types (mentioned below) (Fig. 2.2). Though, there is no clear distinction between all allele categories, some other important factors such as family history and repeat instability are also considered. Further, stability of alleles having less than 90 repeats is highly affected by the number of AGG interspersions within the CGG repeat sequence [35], [36]. Therefore, all this information should be considered while counseling patients and their family about expansion risk.

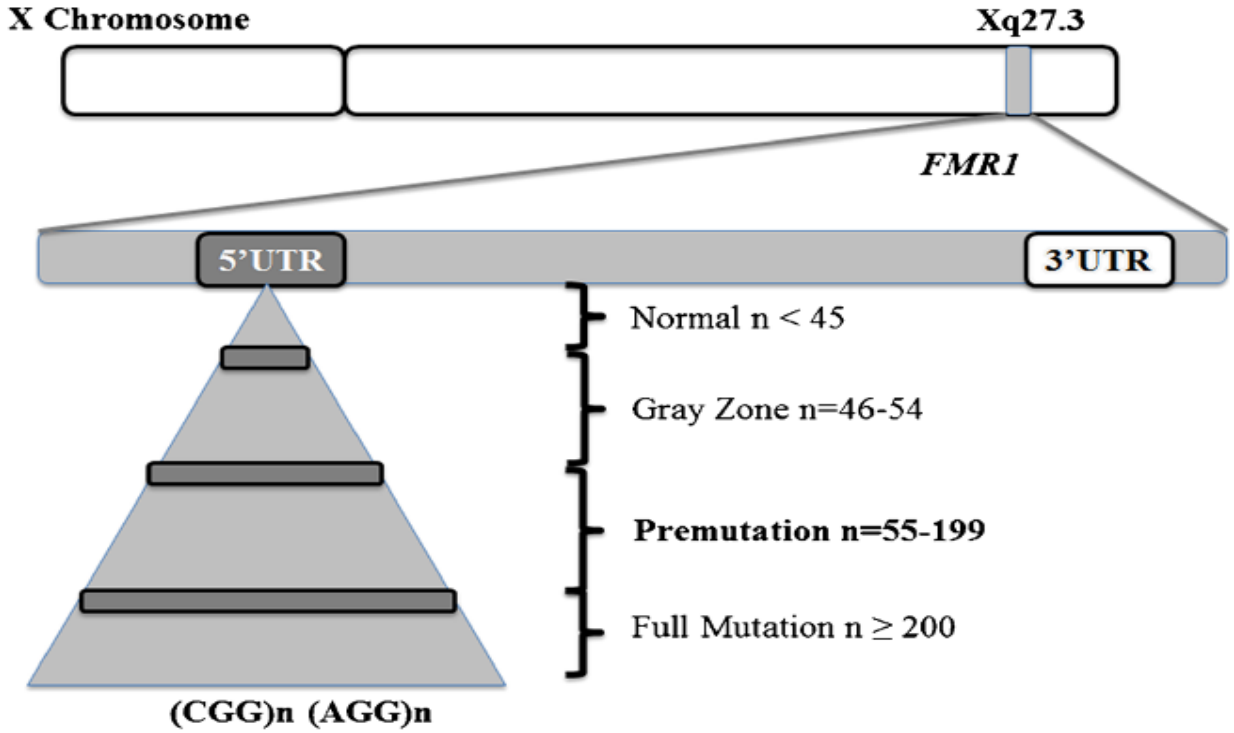

Figure 2.2. Classification of FMR1 allele based on number of expanded CGG repeats at 5UTR of FMR1. Schematic representation for position of FMR1 gene on q arm X chromosome where region spanning expanded CGG repeats at 5'UTR of FMR1 are classified in four types depending on number of CGG repeats (CGG)n.

2.3.1a Normal alleles. ~5-44 repeats

This allele is referred as "normal" because it is present in majority of the general population [37]. The population distribution indicates the highest percentage of individuals with approximately 29-31 repeats while smaller but significant percentages cluster have also been reported around 20

and 40 repeats. The low number of repeats in such range have less instability during mitosis or meiosis and are transmitted from generation to generation without any change in repeat number (Fig. 2.3). However, there are few cases where alleles having no AGG interspersions lead to a greater likelihood to be unstable [36].

2.3.1b Intermediate alleles. ~45-54 repeats(also known as gray zone or borderline)

Alleles of CGG repeats between 45 and 54 CGGs form an important category also termed as grey zone alleles [38]. The long and uninterrupted (CGG)n of grey zone determines its stability and susceptibility to the disease. More than 33 repeats greatly enhances the transmission of unstable repeats during mitosis or meiosis [39] while AGG interruptions provide stability to (CGG)n [40]. The presence of AGG between CGG expansions prevent the formation of stable secondary RNA structures such as hairpins which has been formed by (CGG)n reported in *in vitro* studies [41]. Nearly 14% of intermediate alleles are reported to be unstable and may expand to the range of premutation when transmitted by the mother [42]. However, there are no reports indicating conversion of intermediate to full mutations to the offspring.

2.3.1c Premutation alleles. ~55-200 repeats

Alleles of this size are associated with increased risk for clinical disorders including FXTAS, FXPOI and FXAND (Fig. 2.3). CGG repeats within this range are instable upon transmission, hence, female premutation carriers are considered to be at risk of having children with FXS. Though premutation allele shows mosaicism in terms of pattern of methylation of FMR1 promotor, overall methylation is less [44]. The risk of developing FXS from premutation is highly dependent on the number of AGG interspersions for small premutation alleles [35], [36]. It is important to consider that the upper limit of the premutation range is sometimes around 230.

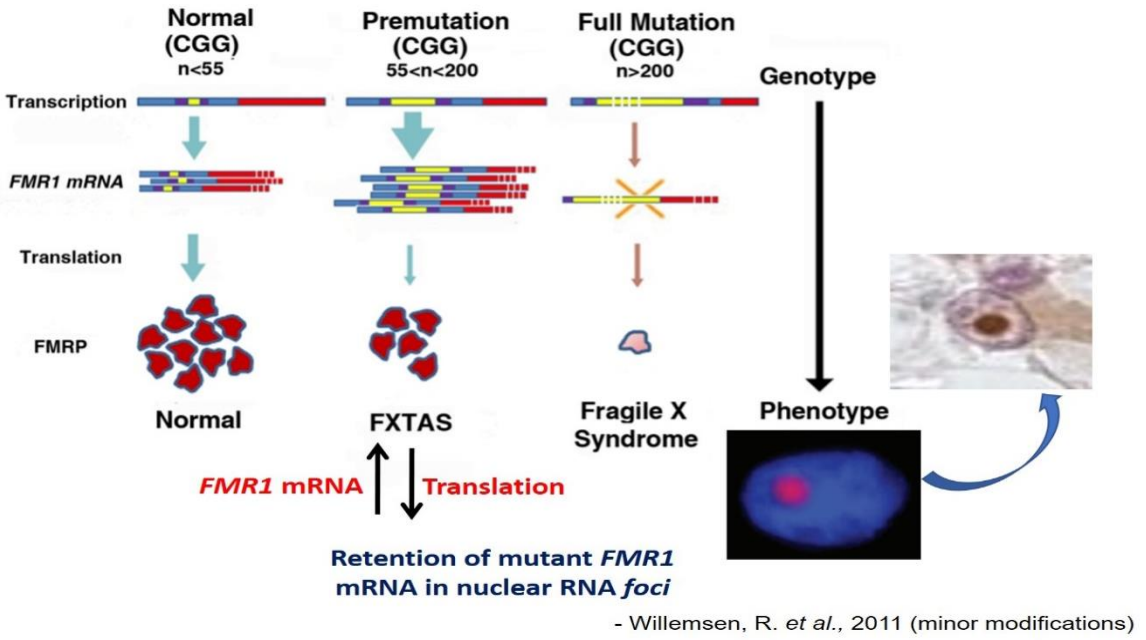

Figure 2.3. Correlation of CGG repeat length, FMR1 RNA levels, FMRP expression and clinical outcomes. Under normal condition, the CGG repeat at the 5'-UTR ranges between 5 and 55 including 45-54 (Gray zone), leading to normal FMR1 mRNA transcription, translation, and normal FMRP expression. While repeats of premutation range (55–200) results into increased FMR1 mRNA levels, but reduced FMRP expression. This causes increased risk of developing fragile X-associated tremor/ataxia syndrome (FXTAS) and/or fragile X-associated primary ovarian insufficiency (FXPOI) in females[43]. More than 200 repeats causes hyper methylation of FMR1 promoter and silencing of FMR1 gene. As a result, there is absence of FMRP which ultimately leads to fragile X syndrome.

2.3.1d Full-mutation alleles. > 200 repeats

The lower range of full mutation is roughly round 200 which can be extended to several thousand. Such high number of expanded CGG repeats are associated with aberrant hypermethylation of the FMR1 promoter. Thus, lead to transcriptionally silencing of FMR1 gene causing absence of FMRP which is responsible for fragile X syndrome [45],[46]. Mostly, all males who inherited a full mutation show FXS which usually causes intellectual disability, while only about 60% of the females with a full mutation show mild to severe clinical features. PBMCs from individual with a full mutation harbors extensive somatic variation of repeat number. Though, the full mutation occurs only when it is transmitted from the mother, there are rare observations where the unstable expanded allele is passed on by the mother [47].

2.3.2 Pathogenicity induced by expanded CGG repeats

Research of last two decades have suggested two key potential mechanisms to explain pathogenicity induced by expanded CGG repeats (Fig. 2.4). RNA gain-of-function was the first explained mechanism, where toxicity is mediated by sequestration of RNA binding proteins due to secondary RNA structures formed by FMR1-mRNA containing CGG repeats [48]. The RNA-protein aggregates lead formation of intranuclear ubiquitin positive inclusions that is responsible for RNA-mediated neurodegeneration in FXTAS [49]. Subsequent experimental evidence showed the sequestration of a various proteins (e.g., Purα, hnRNP A2/B1,Lamin A/C,DROSHA/DGCR8, CUGBP1 and Sam68) within the inclusions using *in vitro* and *in vivo* models [50]–[54]. Increased RNA toxic effects was shown to be associated with larger repeat sizes (100-200) and correlates positively with degree of neuropathology of central nervous system [32]. The other possible mechanism is translation of CGG repeat containing FMR1-mRNA to toxic, glycine rich protein called FMRpolyG [55]–[57]. FMRpolyG also sequesters several proteins and form intra nuclear inclusion. Moreover, recent study has demonstrated that FMRpolyG disrupts nuclear lamina architecture through binding to the nuclear envelope protein LAP2β and contributes to cytotoxic effects observed in FXTAS [57]. Although, sequestration of proteins due to RNA gain of function and protein toxicity due to FMRpolyG are currently the most widely accepted model (Fig. 2.4), other models including antisense transcript model, mitochondrial dysfunction model, altered DNA damage repair response and other stress response models have also shown significant impact in premutation pathology [58]–[60].

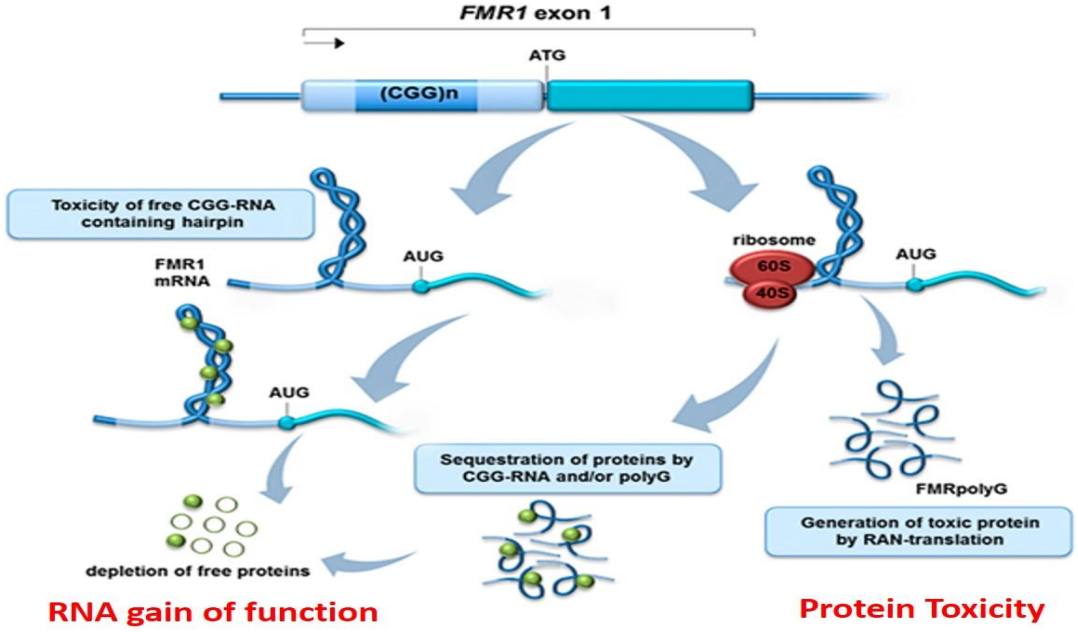

Figure 2.4. Two basic proposed mechanisms of CGG-repeat induced toxicity in PM carriers. (A) RNA gain of function model: expanded CGG-repeat FMR1 mRNA sequester several RNA binding proteins via forming secondary RNA structures. The sequestration of these crucial proteins causes their unavailability to carry out normal functions and critical cellular processes which ultimately lead to interruption and blockage in important cellular processes. (B) FMRpolyG, protein toxicity model: CGG repeat present at 5'UTR of FMR1 are translated via non cognate -AUG codon by phenomenon called repeat-associated non-AUG (RAN) translation. This results in production of the polyglycine-containing polypeptide (FMRpolyG) that is known to bind and sequester other proteins and affects normal cell function and proved to be toxic (Figure adapted from [61])

2.4 Fragile X mental retardation protein (FMRP)

FMR1 gene encodes fragile X mental retardation protein (FMRP), which is an RNA-binding protein known regulate the translation of its bound target mRNAs [62]. FMRP comprises of four different domains, including two RNA binding domains (KH1, KH2), RGG box, a nuclear localization (NLS) and a nuclear export signal (NES), which allows its shuttling between nucleus and cytoplasm (Fig. 2.5) [63]. Both RNA binding motifs can bind to noncoding RNA structures, including G-quartets and kissing complexes and help FMRP to suppress the translation of target mRNA [64],[65]. Though FMRP is ubiquitously expressed throughout the body, it is highly enriched in brain and can bind to approximately 400 mRNAs of brain [66],[67]. Within brain, FMRP is mainly found in the cell soma of neurons and is associated with other proteins and mRNAs in ribonucleoprotein (RNP) complexes. Significant quantities of these FMRP-positive RNP particles are transported into the dendrite, while a less quantity of the protein is localized in the axons during development [68].

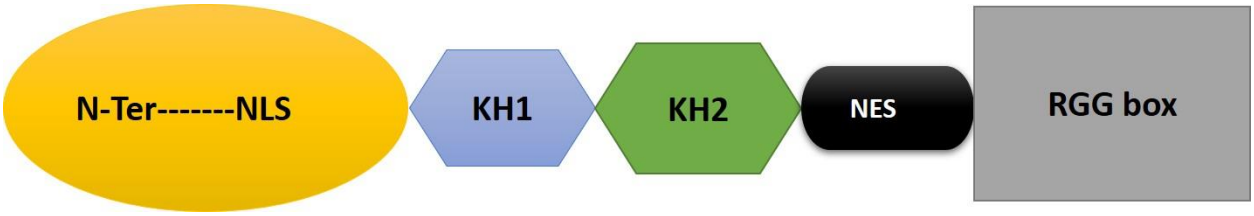

Figure 2.5. Structural and functional domains of FMRP. The FMRP is composed of four RNA binding domains including N-terminus, KH1, KH2, RGG box. It also contains functional nuclear localization (NLS) and export signals (NES). Each protein domain has also been shown to assign one or more functions like interaction with RNAs and other proteins.

In neurons, FMRP is part of different types of cytoplasmic granules including stress granules, P-bodies and RNA-granules (reviewed in [69]). Cytoplasmic granules are condensed areas of cellular material that may or may not be bounded by a membrane. Among this, stress granules are cytoplasmic foci where untranslated mRNAs and proteins accumulate when cells are

subjected to a variety of stress. Processing bodies (P-bodies), on the other hand, are cytoplasmic ribonucleoprotein(RNP) granules mainly composed of stalled mRNAs and proteins related to mRNA degradation, suggesting its roles in post-transcriptional regulation [70]. P-bodies are also hub for microRNA processing and contain the components of RNA-induced silencing complex (RISC). Lastly, RNA-granules comprise translationally silenced mRNAs, and in neurons RNA-granules are transported to dendritic synapses. These RNA-granules can be classified into two types on the basis presence and absence of ribosomal subunits [69]. Although the exact mechanism of action for FMRP in these different types of granules is still not clear, several reports suggest role of FMRP in inhibition of mRNA translation during dendritic transport and at the synapse in neuron. Corollary, FMRP is also developmentally expressed in astrocytes where it may be required for synaptogenesis [71]. In FXS, where there is total deficit of FMRP causes abrupt shaping of dendritic arbors of neurons suggesting a functional role of astrocytic FMRP in the pathogenesis of FXS [72].

2.5 FMRP and translational regulation of mRNAs by miRNA pathway

FMRP is RNA binding protein highly expressed in brain; however, the molecular mechanism underlying translational regulation of its bound mRNAs is elusive. Several studies using *Drosophila* model identified dFmrp(ortholog of FMRP) interaction with Dicer, miRNAs, Ago2 and other miRNA pathway components [73]–[76], which is also in complimentary with mammalian FMRP suggesting an important role of FMRP together with miRNA pathway in regulation of translation of target mRNAs (Fig. 2.6)[77]. Though, FMRP associates with components of the miRNA pathway, it is also controversial whether it plays an essential role in the function of miRNA pathway itself or it functions independently. This differences in opinion about whether FMRP is required for normal function of the miRNA pathway may be due to the use of different cell systems or the evaluation of different miRNAs which are developmentally timed and tissue specific [78]. Therefore, regardless of ambiguity in FMRP's role in RISC function, it is still possible that FMRP utilizes the miRNA pathway for translation regulation of its target mRNAs.

The interaction of FMRP with the miRNA pathway relies on phosphorylation of FMRP that provides a rapid and reversible way to modulate translation of its bound mRNAs. Using a phospho-specific antibody for three phosphorylated serine(496, 499 and 503), Cheever and

colleagues have observed that P-FMRP is associated with a high abundance of an 80 nt RNA species that is considered to be pre-miRNA. Further, FMRP probed with a Dicer antibody showed that Dicer associates only with FMRP and not P-FMRP [79]. Dicer complex is required for the processing of pre-miRNAs into mature miRNAs which is not bound to FMRP if it is phosphorylated [78]. Hence, this leads to the abundance of pre-miRNAs observed with P-FMRP [79]. While RNase treatment showed no effect on the FMRP- Dicer interaction further indicated protein interaction between them [78]. This result was further supported by the experiments where pull down of phosphorylated peptide sequence of FMRP did not capture Dicer while the same unphosphorylated peptide sequence could show pulldown of Dicer [79]. This suggests that that Dicer-FMRP association requires unphosphorylation of FMRP and phosphorylation of FMRP abolishes this interaction (Fig. 2.6).

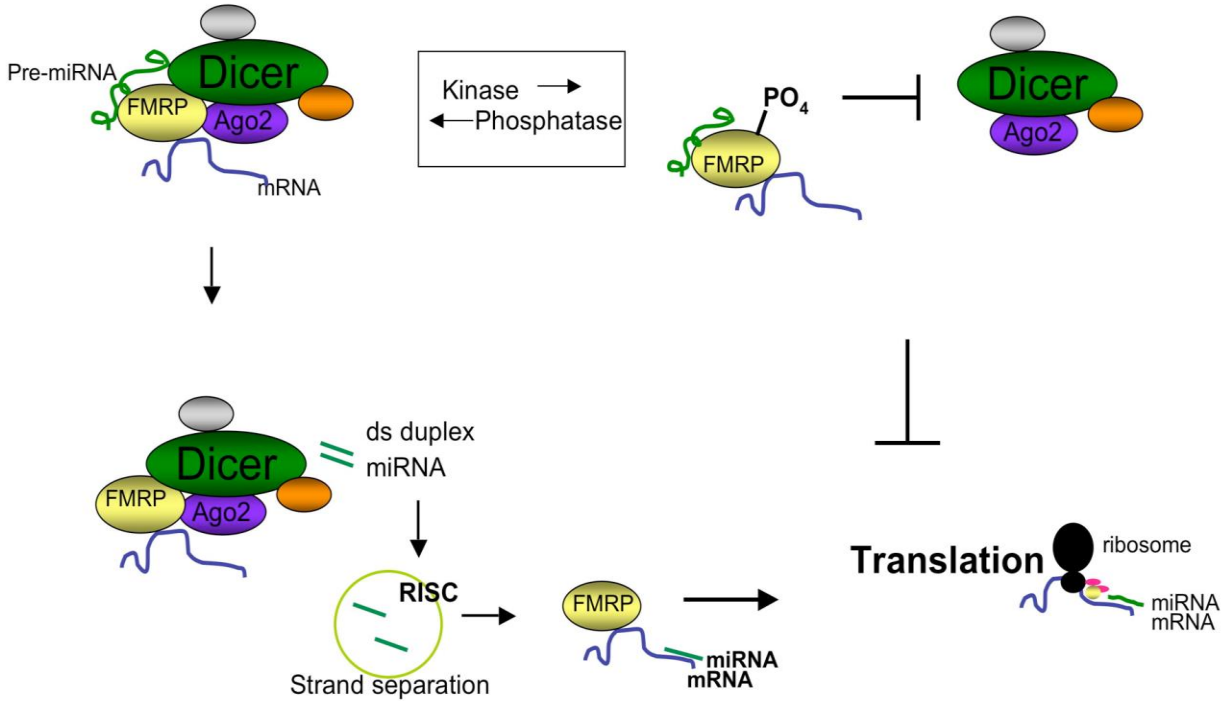

Figure 2.6. Role of Phosphorylated FMRP (P-FMRP) in regulation of translation. Phosphorylation of FMRP is crucial for its binding with Dicer and translational regulation of mRNAs along with other RISC components. Unphosphorylated FMRP associates with Dicer (top left) and Dicer containing complex then processes pre-miRNAs into mature miRNAs, containing double-stranded duplex (bottom left). After separation of double strand by RISC, the single stranded mature miRNA binds FMRP's target mRNA to activate its translation. Conversely, when FMRP is phosphorylated, dicer cannot bind, and pre-miRNAs are not

converted into mature miRNAs. Without activating the miRNAs, translation of target mRNA cannot occur, and as a result translation gets suppressed by FMRP due to loss of dicer binding.

2.6 miRNA biogenesis pathway dysregulation in FXTAS

miRNA belongs to family of small noncoding RNAs and have remined highly conserved across the species including plants, animals, fungi and viruses [80], [78]. Recent study have identified ~ 2300 true validated miRNAs which account for almost ~1% of the human genome [81]. The canonical pathway of microRNA biogenesis unidirectional and universal to all mammalian miRNAs [82]. This canonical pathway begins in the nucleus when transcription of primary miRNA (pri-miRNA) takes place by RNA polymerase II/III. This pri-miRNA are further processed by the microprocessor complex Drosha–DGCR8 (Pasha) into precursor, pre-miRNA, which is exported from the nucleus by Exportin-5–Ran-GTP [83]. Pre-miRNA are then cleaved by RNase, Dicer in complex with the double-stranded RNA-binding protein TRBP to its mature length (~ 20-25 bp). One strand of double stranded miRNAs, acts as guide strand which is the functional strand loaded together with Argonaute2 (Ago2) proteins in RNA-induced silencing complex (RISC) while the other strand known as 'passenger strand' is degraded [84].

The molecular consequence of the premutation suggests increased FMR1 mRNA levels and a slight or moderate reduction in FMRP [85]. As discussed in the previous section, FMRP as RNA binding protein known to be involved in translational control of mRNAs together with components of miRNA pathway *in vitro* and *in vivo* [73], [75]. Hence, decreased levels of FMRP observed in premutation condition can be linked to the altered expressions of its bound miRNAs. The study from Alvarez-Mora and group has first shown altered miRNA levels in FXTAS male patients where deep sequencing analysis evidenced 83 and miRNAs microarray analysis showed 31 miRNAs that were significantly deregulated. However, decreased level of FMRP could not fully justify the altered miRNA expression in FXTAS, a recent study has provided an important link of sequestration of proteins associated with miRNA processing with the disease process in FXTAS [54]. The proteomics data and subsequent experiments have revealed that the double-stranded RNA- binding protein DGCR8 binds preferentially to CGG repeats of premutation range. The DGCR8 plays central role in miRNA biogenesis via forming "micro- processor" with DROSHA which converts pri-miRNA to pre-miRNA. Hence, the sequestration of DGCR8 by expanded CGG repeats causes their less availability for miRNA processing and lead to globally

decreased miRNA biogenesis and accumulation of corresponding pri-miRNA [86], [87]. Further, *in vitro* overexpression of DGCR8 restored normal dendritic growth and branching, and alleviated cell death in cultured neurons expressing a toxic 60 CGG repeat [54]. Provided all the evidence, it can be concluded that decreased FMRP levels and sequestration of DROSHA/DGCR8 by expanded CGG repeats are main mechanisms responsible for abrupt miRNA expression in premutation condition.

2.7 Mitochondria and neurodegeneration

Mitochondria are cellular organelles involved in numerous physiological processes such as generation of ATP by oxidative phosphorylation, generation and detoxification of reactive oxygen species (ROS), calcium homeostasis and regulation of apoptosis [88]–[90]. Corollary, mitochondrial dysfunctions leads to oxidative stress, loss of calcium homeostasis and apoptosis, ultimately leading to numerous neuronal pathologies [91]–[93]. Mutations in mitochondrial proteins encoded by nuclear genes can also cause several mitochondrial associated diseases like Friedreich's ataxia and Wilson's disease [94]. Overall, it is estimated that 1 in 5000 people in general population has a genetic mitochondrial disease [95]. Furthermore, acute mitochondrial dysfunction have been associated with many pathologies, such as Parkinson's Disease, dementia and Alzheimer's Disease, epilepsy, stroke, cardiovascular disease, inflammation, cancer and diabetes mellitus [90], [94], [96], [97]. Hence, understanding the regulation of mitochondrial functions has been major focus and is important to understand not only FXTAS but also various mitochondrial associated developmental and aging disorders.

2.8 Mitochondrial dysfunctions in FXTAS

Various studies have shown mitochondrial dysfunctions in neuronal, non-neuronal cell and mouse models of FXTAS as well as in PBMCs, fibroblasts and brain tissue of premutation carriers (Fig. 2.7). A single case study on FXTAS patient using magnetic resonance spectroscopy (MRS) has first shown mitochondrial dysfunctions in FXTAS [98]. This study reported ventricular lactate accumulation and increased Pi to ATP ratio in the resting calf muscles of the patient. While proceeding study from Ross-Inta C et al., (2010) in fibroblasts from premutation carriers showed decreased NAD- and FAD-linked oxygen consumption rates and decreased ATP production compared to age matched controls[60]. These alteration of mitochondrial bioenergetics is associated with decreased mitochondrial proteins levels e.g.

ATPase β-subunit, MnSOD and CytC Oxidase subunit IV [60]. The import of nuclear encoded protein to different compartment of mitochondria is essential for mitochondrial functions. The mitochondrial disulfide relay system (MDRS) facilitates the import and folding of proteins in the intermembrane space (IMS) of the mitochondria. This system is generally composed of two proteins called Mia40 and Erv1. Mia40, interacts with newly imported proteins in its oxidized state and promote their correct folding by inducing formation of intramolecular disulfide bridges [99]. Decreased expression of Mia40 and altered MDRS have been observed in fibroblast derived from premutation carriers. This may lead to improper folding of protein in IMS, causing mitochondrial stress. The deficit of MDRS components may also lead to decrease levels of nuclear encoded proteins like cytochrome c and NDUFB7 as observed in fibroblasts from premutation carriers [100]. The knock in mice model of premutation (expressing an average of 170 CGG repeats) confirmed the pathogenic role of expanded CGG repeats on mitochondrial functions. The study depicted lower mitochondrial density and strong deficit in mitochondrial dynamics in neurites of hippocampal neurons in pre-CGG mice as compared to WT mice[101]. Notably, another study reported altered mitochondria trafficking, metabolic function and increased level of oxidative markers such as GPX1 and CytC in transgenic mice expressing 90XCGG repeats[102]. Furthermore, higher expression of *FMR1* RNA isolated from PBMCs from premutation carriers was associated with decreased transcript levels for various mitochondrial proteins (ND1, CytC) [103]. Similarly, a clinical studies involving plasma metabolic profiling of FXTAS patients showed altered bioenergetics and Warburg shift correlated with high lactate levels and altered Krebs' intermediates, neurotransmitters, and markers for neurodegeneration[104]. Importantly, altered bioenergetics have the potential to affect brain cognition and function leading to increase in susceptibility to the development of neurological or behavioral problems[13]. Mitochondrial number, which correlates with copy number of mitochondrial DNA, is variable in different cell types and depends upon the bioenergetic requirement of the cell. In consonance to this, recent study has shown reduced mtDNA copy number in the specific brain region such as cerebellar vermis, dentate nucleus, parietal and temporal cortex, thalamus, caudate nucleus and hippocampus is associated with disease progression in FXTAS patients, which further suggest an association of mitochondrial dysfunction with FXTAS [105].

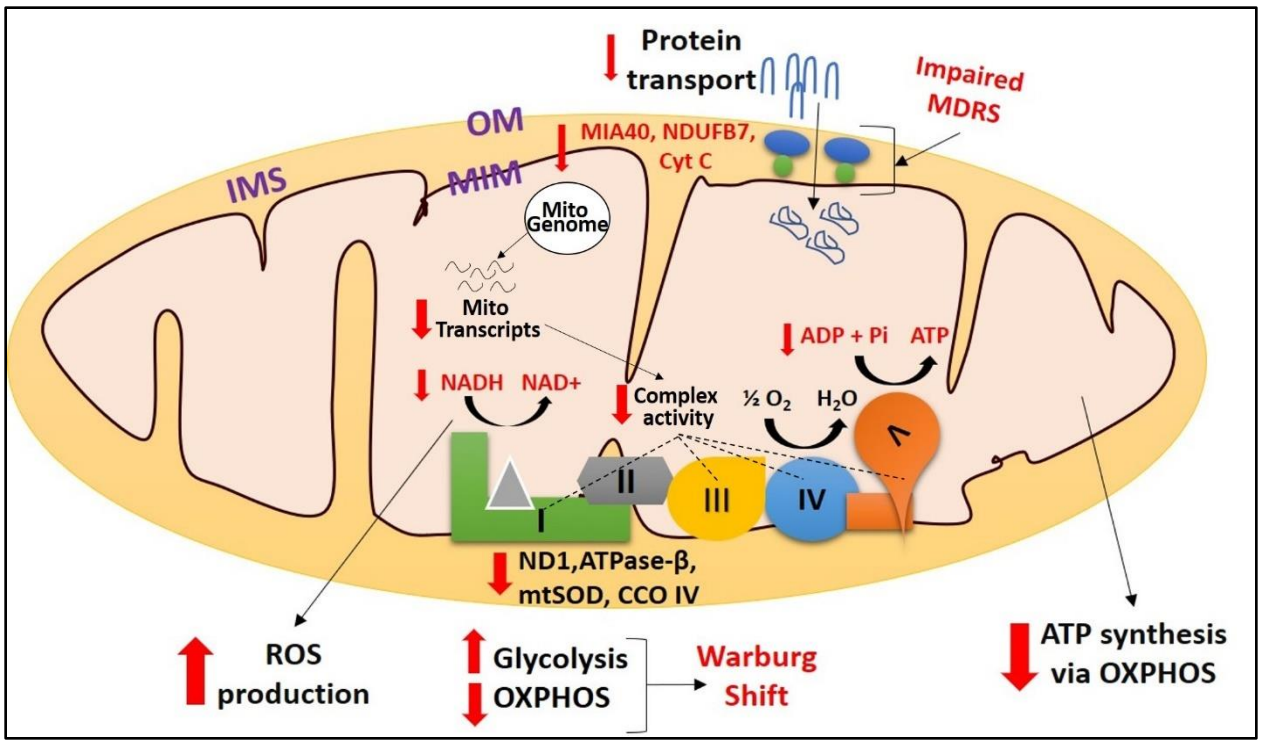

Figure 2.7. Schematic representation of mitochondrial dysfunction involved in premutation condition. (1) Decreased levels of ND1, ATPase β-subunit, MnSOD and CCO IV causes decreased OXPHOS activity and ATP levels. (2) Altered metabolic profile causes Warburg shift. (3) Impaired MDRS lead to decreased Cytochrome C and NDUFB7 which further manifest mitochondrial dysfunction. (4) Impaired OXPHOS activity and decreased mtSOD contribute to elevated ROS levels. (5) FMRpolyG small aggregates may associate with mitochondria and affects mitochondrial transcription leading to decreased mitochondrial transcripts levels.(Abbreviations: OM, outer membrane; MIM, mitochondrial inner membrane; IMS, inter membrane space; MDRS, mitochondrial disulfide relay system; Cyt C, Cytochrome C; mtSOD, mitochondrial superoxide dismutase; CCO IV, CytC Oxidase subunit IV; OXPHOS, oxidative phosphorylation).

Overall, these studies provide evidences that mitochondrial bioenergetics is altered in FXTAS, possibly through decreased mitochondrial respiratory chain complex levels and activity, resulting in increased superoxide levels and cellular damages (Fig. 2.7)[13], [100], [106]–[108] In the following section, we review some of the possible mechanisms leading to mitochondrial abnormalities.

2.9 Emerging mechanisms for mitochondrial dysfunction in FXTAS

2.9.1 Altered zinc transporter function and iron metabolism

The first possible molecular mechanism explaining mitochondrial dysfunctions in FXTAS was shown by Napoli and colleagues, who showed altered expression of Zinc transporter(ZnT) in fibroblasts from CGG premutation carriers[109]. Zn is a pivotal neuromodulator involved in brain development and its concentration changes with the age in the brain [110], [111]. This study indicated defective import/processing of nuclear encoded mitochondrial proteins lead to altered Zn homeostasis. Interestingly, treatment of premutation fibroblast with Zn showed rescue of mitochondrial dysfunctions in terms of increased OXPHOS activity and normal processing of mitochondrial proteins [109]. Further, iron is known to be an essential element playing crucial role in maintaining mitochondrial physiology [112] and a previous clinical finding has suggested altered transport of iron from general circulation into CSF. This leads to mild increase iron levels in neurons and oligodendrocytes in putamen region of the brain causing dysregulated iron metabolism in premutation carriers [113]. Same group has also reported overall decreased levels of the iron-binding proteins transferrin and ceruloplasmin, which further worsen the disease pathology [114]. However, it still remains to be clarified if altered ZnT protein expression, dysregulated iron metabolism or other pathological mechanisms lead to mitochondrial abnormalities in FXTAS, specifically through CGG RNA gain of function and/or FMRpolyG protein toxicity.

2.9.2 Transcription of CGG repeats and RNA gain of function

Historically, RNA toxicity was the first model proposed to explain pathogenesis of FXTAS [51], [53], [115]. This model is based on the myotonic dystrophy type 1, a neuromuscular disease caused by a large expansion of CTG repeats located in the 3'UTR of the *DMPK* gene. In this disease RNAs containing hundreds to thousands of CUG repeats sequester the Muscle blind-like (MBNL) RNA binding proteins into nuclear RNA foci, causing a partial loss of function of the MBNL proteins resulting in the symptoms of myotonic dystrophy [116]. Similarly, expanded CGG repeats at 5'UTR of *FMR1* mRNA may bind and titrate specific RNA binding proteins, resulting in loss of their normal functions [117]. Studies from the last decade have shown that mutant *FMR1* mRNA binds several RNA binding proteins such as hnRNP A2/B1, DROSHA/DGCR8, Purα etc. [50], [51], [53], [54], [115], [118]. Recently, using *in silico* approaches Cid-Samper and colleagues have identified some novel binding partners of CGG RNA scaffold embedded within 5' UTR of *FMR1*[119]. This computational analysis using catRAPID omics, a server for large-scale calculation of protein-RNA interactions and

confirmation using large scale *in vitro* experiments predicted novel partners of CGG repeats such as SRSF1,5,6 and 10, KHDRBS3 and MBNL1 [53], [57], [115]. Among high-confidence candidates predicted by catGRANULE [120], a pre-mRNA splicing factor TRA2A showed the highest score. As alternative splicing defects have been already observed in cell models and brain samples FXTAS patients [4], [53], Cid-Samper and colleagues focused their analysis on TRA2A. Interestingly, this protein was present in RNA aggregates as well as in ubiquitin positive inclusions in FXTAS human brain samples suggesting its involvement in FXTAS pathogenesis [121]. TRA2A has also been reported to be a component of ALS granules [122], but it does not appear in TAU inclusions [123], which indicates that its sequestration occurs only in specific neurodegenerative diseases. This strongly suggests that sequestration of TRA2A by CGG RNA aggregates can be a crucial mechanism for FXTAS pathogenesis.

The RNA gain of function model could be a potential contributor of mitochondrial dysfunction in FXTAS, as decreased RNA binding protein activities may impair normal mitochondrial function. Notably, the DROSHA/DGCR8 enzymatic complex plays a crucial role in miRNA biogenesis by cleaving pri-microRNAs into pre-miRNAs [83]. Partial sequestration of DROSHA-DGCR8 within CGG RNA foci reduces the processing and expression of various miRNAs in neuronal cells expressing expanded CGG repeats [86],[87]. Interestingly, the role of miRNAs in the regulation mitochondrial function is an emerging field and miRNAs may regulate nuclear encoded mitochondrial proteins (NEMPs) at post transcriptional level, and consequently affect mitochondrial homeostasis, OXPHOS, ATP synthesis, apoptosis, ROS generation and fatty acid metabolism in normal and pathological states[124], [125]. Therefore, impaired miRNA biogenesis due to titration of the DROSHA/DGCR8 enzymatic complex by expanded CGG repeats may ultimately affect mitochondrial functions.

2.9.3 FMRpolyG protein toxicity

The two models suggested above are based on post-transcriptional and co-transcriptional mechanism. Once *FMR1* mRNA gets transcribed, the next potential cause of CGG repeat pathogenicity is the recently described Repeats Associated Non-AUG (RAN) translation mechanism, where tri-, tetra-, penta- or hexa-nucleotide repeats promote their non-canonical translation in the absence of AUG start codon[126]–[128]. In FXTAS condition, *FMR1* mRNA containing expanded CGG repeats are RAN translated in glycine frame through initiation at an ACG near-cognate start codon located before the CGG repeats [57], [129], [130]. This leads to

expression of FMRpolyG, a toxic protein that forms sumo and ubiquitin-positive intranuclear inclusions. Expression of FMRpolyG in transformed human cells as well as in primary neuronal cell cultures reveals that it interacts with the Lamina-associated polypeptide 2 beta (LAP2β), a protein that anchors Lamin B1 and B2 to the inner nuclear membrane. This interaction leads to the disruption of the nuclear lamina architecture, ultimately resulting in nuclear pore alterations and nucleo-cytoplasmic transport defects [57]. This may have adverse consequence especially for mitochondria, as mitochondrial DNA encodes only 13 proteins and the vast majority of the mitochondrial proteins(~1500) are encoded by nuclear DNA which are transcribed into nucleus and transported to cytosol and translocate to mitochondria [131]. Hence, FMRpolyG may alter mitochondrial functions as a secondary hit by disrupting the nuclear lamina and the nucleo-cytoplasmic traffic. Beside this indirect pathogenic action, a recent report suggests that FMRpolyG also forms small cytosolic inclusions, which can interact with mitochondria and alter their functions, specifically, mitochondrial supercomplexes assembly and activity. Additionally, this study found decreased level of mitochondrial transcripts in samples of CGG premutation carriers [132]. However, the mechanisms of transient interaction of FMRpolyG with mitochondria and its implication in decreased level of mitochondrial transcripts need to be further investigated.

2.9.4 Decreased expression of FMRP

Expansion of over 200 CGG repeats (full mutation, responsible for Fragile X syndrome) lead to DNA methylation and consequently to promoter silencing of the *FMR1* gene, ultimately resulting in absence of the Fragile X Mental Retardation Protein (FMRP), a protein encoded by the *FMR1* mRNA and that is essential for synaptic plasticity and neuronal development[133]. In FXTAS, *FMR1* promoter is active and *FMR1* mRNA expressed, but there is moderate decrease expression of FMRP, particularly in the upper premutation range, as expanded CGG RNA repeats form secondary stable RNA structures (hairpin, duplexes) that impede ribosome translocation[134]. FMRP is an RNA binding protein that regulates transport and local translation of mRNAs in neurons[135], [136]. Recent reports indicate that FMRP could also be associated with Ago2 in the RNA Induced Silencing Complex (RISC) and may play a dual role as translation suppresser and activator[79], [137], [138]. Therefore, lower expression of FMRP in FXTAS may impair activity of RISC complex, which may lead to altered translation of nuclear-encoded mitochondrial proteins (NEMP). Furthermore, FMRP can bind to Superoxide Dismutase

1 (SOD1) mRNA and regulate its levels positively[139]. SOD1 is one of the three superoxide dismutase that reduces superoxide specie inside cells. Decreased levels of SOD1 may increase mitochondrial ROS levels in FXTAS conditions. In regard to this, accumulation of mutated SOD1 in mitochondrial intermembrane space impairs oxidative phosphorylation and mitochondrial metabolism [139]. In conclusion, it remains to be determined whether a mild decrease expression of FMRP may also contribute to mitochondrial alterations in FXTAS.

2.10 miRNAs translocation to mitochondria: implications in FXTAS

The noncoding RNAs, miRNAs, play an important role in posttranscriptional/translational regulation of mRNAs in several patho-physiological conditions and sparked great interest in disease research due to their capability of regulating a large number of target genes through either translational repression or mRNA degradation [140], [141]. The subcellular localization and association of miRNAs with various organelles and their site specific functional implication is now emerging (reviewed in [142]). Reports from our lab and others have confirmed the presence of nuclear encoded RNA species in the mitochondria including miRNAs, referred as mito-miRNAs [143]–[145]; however, their physiological relevance needs to be further explored. The transport of nuclear encoded miRNA to the mitochondria is required for optimal mitochondrial function [143], [146], [147]. In FXTAS condition, where secondary RNA structures (hairpin, duplexes) sequester several RNA binding proteins including proteins involved in miRNA biogenesis (e.g. DROSHA/DGCR8), which may lead to decreased pool of miRNAs and also affect translocation of miRNA to mitochondria. Thus, the role of mito-miRNAs in modulation of mitochondrial functions should be considered and needs to be investigated further to understand FXTAS pathogenicity.