Earlier *in vitro* studies have reported that RAN translation of CGG repeats of premutation range drives the expression of FMRpolyG[1] and FMRpolyG inclusions were also detected in brain tissue from mice model and FXTAS patients [2],[3]. However, dynamics and sub cellular localization of this mutated FMRpolyG in premutation condition have never been explored. Therefore, we first analysed the cellular trafficking of FMRpolyG by expressing CGG repeats constructs in cell lines and mice model. Our results were in consonance with earlier findings where FMRpolyG was known to form intra nuclear inclusion. Strikingly, we also observed some small cytoplasmic aggregates which were in close contact with mitochondria. We further checked implication of this interaction on mitochondrial function. Interestingly, expression of expanded CGG repeat also affects various mitochondrial functions including altered mitochondrial supercomplexes assembly and activity, and global decreased levels of mitochondrial genome encoded transcripts.

6.1 Expanded CGG repeats translated into toxic protein FMRpolyG and forms intranuclear inclusion

The intracellular expression profile of FMRpolyG is dependent on size of repeat region. FMRpolyG inclusions formed from long >90 CGG repeats mostly localized to cytoplasm and within nucleus, whereas FMRpolyG of short repeats of premutation range were diffuse throughout the cytoplasm (This means repeats of lower premutation range are known to form cytosolic inclusions). To confirm the sub cellular localization of FMRpolyG, we transfected U87MG cells with both premutation constructs (ATG FMRpolyG-GFP and 5UTR *FMR1* CGG99X) along with pEGFP-C1(control). After 24 hours of transfection, cells were fixed and observed under confocal microscope. As anticipated, our both the plasmids were expressing FMRpolyG and forming nuclear inclusion (Fig.6.1A).Consistent with a decrease viability upon FMRpolyG expression, cells with nuclear inclusions showed altered morphology as they are rounded, vacuolated and have less projections compared to control transfected cells (Fig. 6.1B).

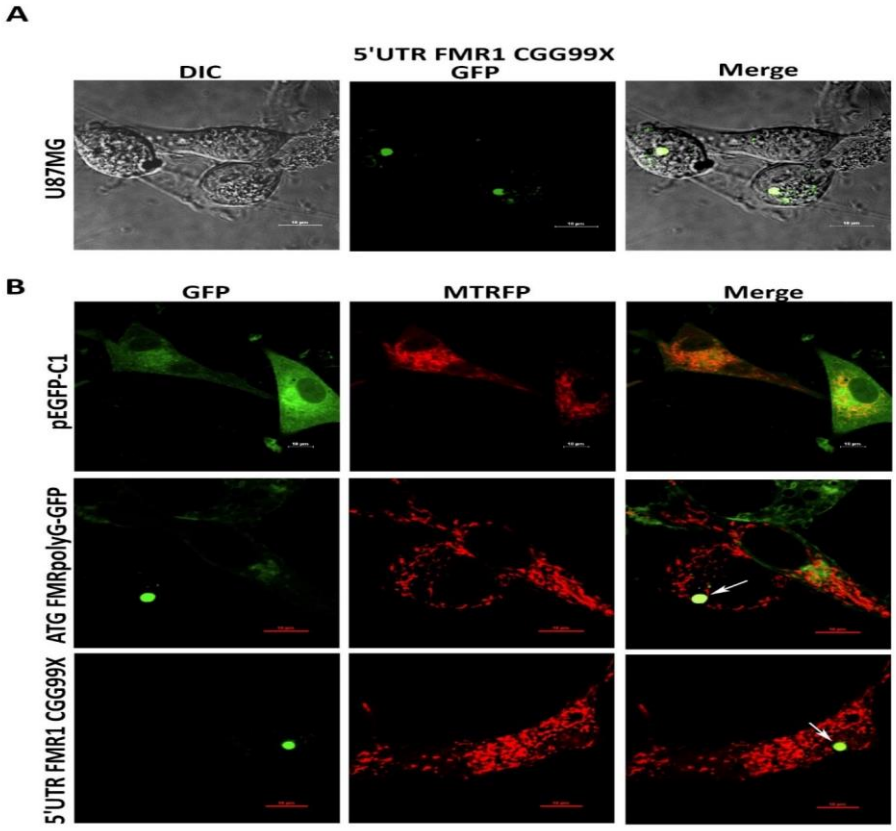

Figure6.1.Translation of CGG repeats forms FMRpolyG nuclear inclusions. U87MG cells were transfected with both CGG repeats constructs (ATG FMRpolyG-GFP, 5'UTR *FMR1* CGG99X) along with pEGFP-C1 (control) with the co-transfection of MTRFP for all conditions. After 24 hours of transfection cells were analysed under confocal microscope for sub cellular localization of FMRpolyG (A). Translation of CGG repeats lead to formation of nuclear aggregates (FMRpolyG), visible as intra nuclear green puncta in most of the cells (B).

6.2 FMRpolyG forms cytosolic inclusions that interacts with mitochondria

Our previous results have showed that the expanded CGG repeats embedded in their natural *FMR1* sequence are RAN translated into the FMRpolyG protein, which in turn forms nuclear inclusion. However, their subcellular dynamics of aggregate formation and its interaction with another subcellular organelle before translocation to nucleus is not understood. Hence, we analysed sub cellular localization of FMRpolyG aggregates. Importantly, we observed that

beside formation of large nuclear inclusions, most of CGG repeats or FMRpolyG-GFP transfected cells also show presence of smaller cytosolic aggregates of FMRpolyG in U87MG (Fig. 6.2A) and HEK293 (Fig. 6.3A) cells. We further investigated the localization of these small cytosolic FMRpolyG inclusions. Co-transfection of expanded CGG repeats with a mtRFP plasmid that labels mitochondria followed by confocal microscopy indicated that the cytosolic FMRpolyG puncta are in contactor on the surface of mitochondria in U87MG cells (Fig. 6.2B). Identical results were observed with HEK293 cells co transfected with mCherry-Mito-7 along with CGG repeats constructs(Fig. 6.3B).

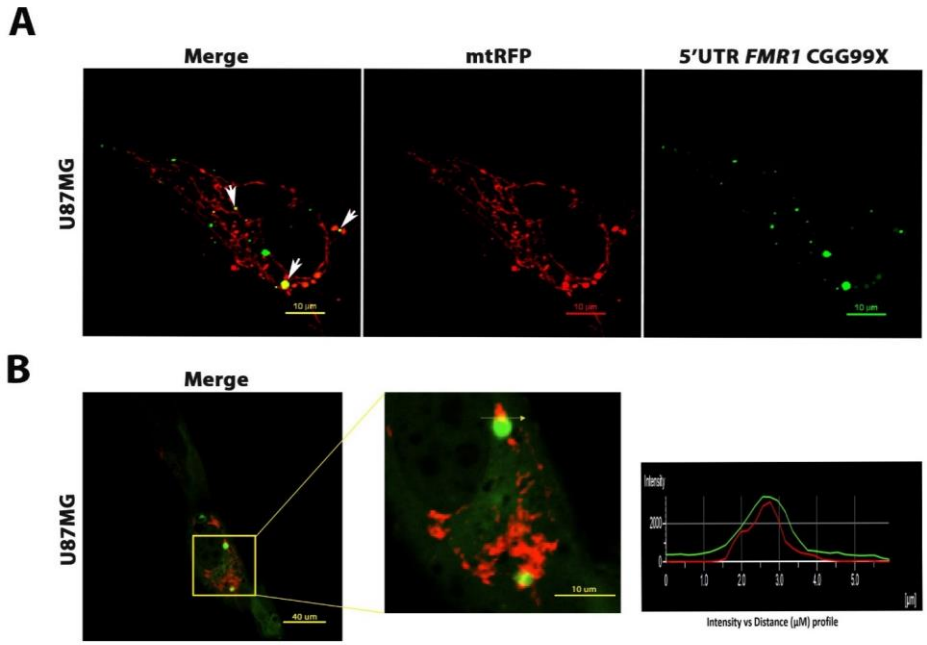

Figure6.2: FMRpolyG forms small cytosolic inclusions. U87MG cells co-transfected with CGG repeats constructs along with MTRFP showed formation of cytosolic aggregates of FMRpolyG. White arrows (Merge) inthe image shows extra nuclear inclusions (A). These extra nuclear aggregates (GFP) ~~specifically~~ colocalize~~d~~ to mitochondria (Red) (Magnified Image) (B). Graph of intensity v/s distance indicates overlapping peaks for green and red channels suggests FMRpolyG and mitochondrial interaction.

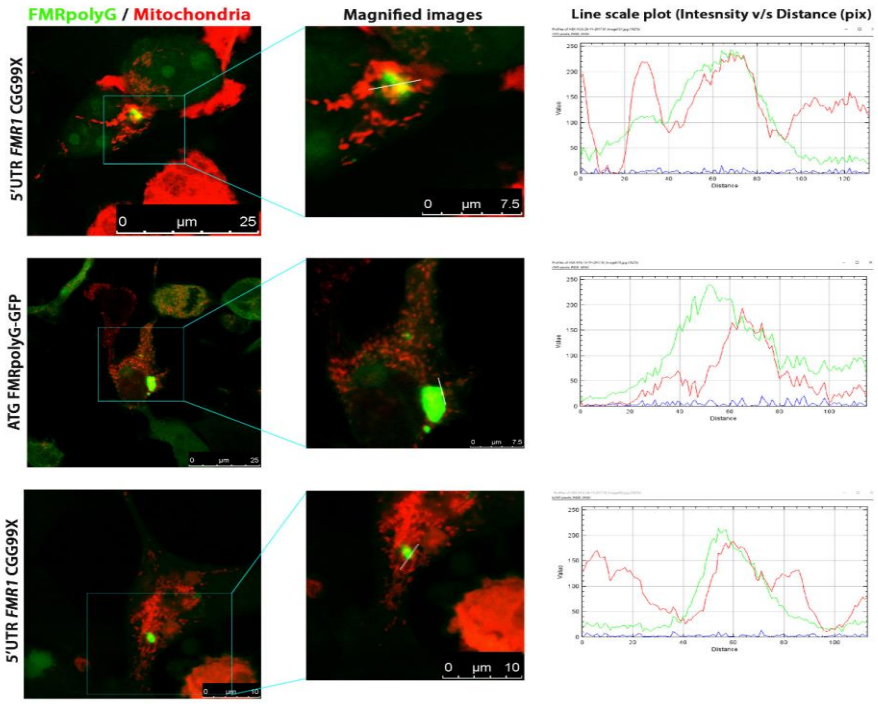

Figure6.3.Cytosolic inclusions of FMRpolyG are in the vicinity of the mitochondria in HEK293 cells. HEK293 cells co-transfected with CGG repeats constructs along with mCherry-Mito-7 show formation of cytosolic aggregates of FMRpolyG. These extra nuclear aggregates (GFP) specifically localized to mitochondria (Red) (Magnified Image). Line plot of intensity v/s distance indicates overlapping peaks for green and red channels further confirms FMRpolyG and mitochondrial interaction.

To further validate the interaction of FMRpolyG with mitochondria, mitochondrial fractions were prepared and analyzed by western blotting. Interestingly, FMRpolyG was observed in the mitochondrial fraction, whereas it was not detected in control GFP transfected cells (Fig. 6.4A). Finally, presence of small FMRpolyG cytosolic aggregates along with large nuclear inclusions was also observed in FXTAS mouse model. Confocal imaging of FMRpolyG in cortical sections of 2 months old transgenic mice expressing the CGG permutation indicated that the small cytosolic aggregates of FMRpolyG (red) co-localize with AIF (green), a protein marker of

mitochondria (Fig. 6.4B). These observations suggest that small aggregates of FMRpolyG interacts withmitochondria in cell and animal model of FXTAS.

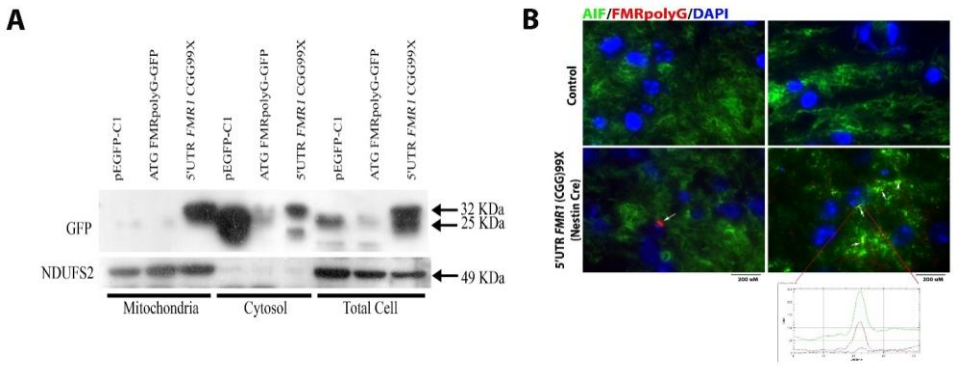

Figure6.4. FMRPolyG forms cytosolic small aggregates and show association with mitochondria. Immunoblotting against GFP antibody in the mitochondria, cytosolic and whole cell fraction of HEK293 cells transfected with ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X. NDUFS2 has been used as mitochondrial marker (A). FMRpolyG inclusion in FXTAS transgenic mice brain tissue section visible as red puncta (8FM) in the nucleus and smaller aggregates showed by white arrows show association with mitochondria (green, AIF) under different resolution. Line intensity profile with both (red/green) channels indicates association of FMRpolyG with mitochondria (B).

6.3 Expression of FMRpolyG affects mitochondrial functions

The expression of the CGG premutation allele known to alter mitochondrial functions. Hence, several mitochondrial functional parameters were analyzed in in HEK293 and SH-SY5Ycells expressing CGG repeats. We observed decreased cellular ATP levels in both cells and animal (CGG transgenic mouse) models of FXTAS compared to controls (Fig. 6.5A). Interestingly, a similar cellular ATP decreased was observed in HEK293 cells upon expression of FMRpolyG-GFP cloned under an artificial ATG (Fig. 6.5A). As, mitochondrial membrane potential is direct indicator of ATP production, we further monitored mitochondrial membrane potential using the fluorescent dye TMRM [4]. The expression of either the CGG premutation or expression of ATG FMRpolyG-GFP in both non-neuronal HEK293 and neuronal SH-SY5Y cells (Fig. 6.5B) decreased mitochondrial membrane potential. The mitochondrial content was also analysed in the cells expressing FMRpolyG by quantifying mitochondrial/ nuclear DNA ratio by quantitative PCR. The qPCR results indicated that there is no alteration in mitochondrial mass upon expression of FMRpolyG or CGG premutation both in HEK293 and SH-SY5Y cells (Fig. 6.5C).

These results confirm that the CGG premutation alters mitochondrial activities which could be mediated by FMRpolyG direct/indirect interaction with mitochondria.

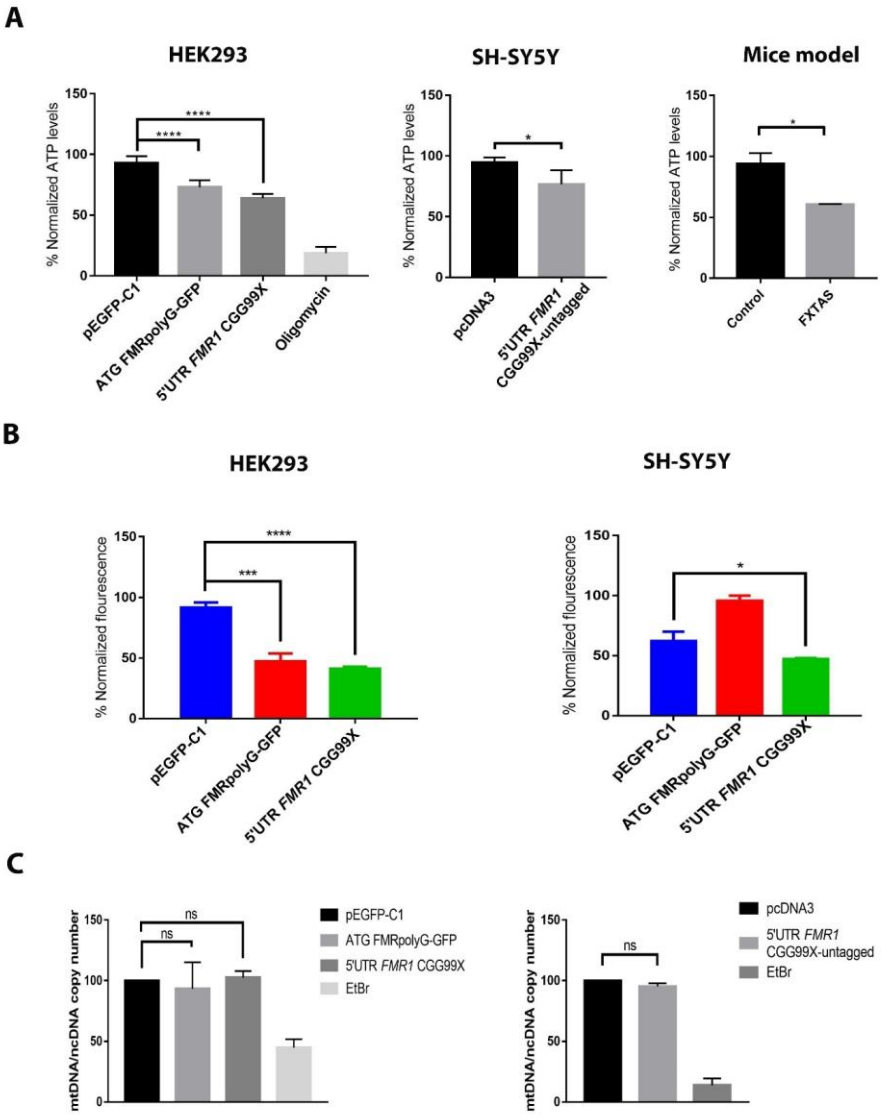

Figure 6.5. Mitochondrial functions altered by expression of premutation allele.ATP levels were monitored in ATGFMRpolyG-GFP and 5'UTR *FMR1* CGG99X transfected condition in HEK293, SH-SY5Y cells and in cortical neurons derived from FXTAS transgenic mice (n=3) (A). TMRM staining showing decreased mitochondrial membrane potential in presence of expanded CGG repeats in HEK293 and SH-SY5Y cells (n=3) (B). mtDNA/ncDNA ratio quantified by qRT-PCR to monitor mitochondrial mass in both the cell lines HEK293 and SH-SY5Y expressing FMRpolyG (C). (n=3) $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

6.4 CGG permutation alters mitochondrial supercomplexes assembly and affects respiratory chain complex activity

The individual respiratory chain complexes CI, CIII, and CIV associate to form intermediate supramolecular assemblies known as mitochondrial supercomplexes (mSCs), which are required for efficient electron transport and coupling (ETC) [5], [6]. The impairment of assembly of supercomplexes may show decline in ETC capacity, cellular respiration and ATP production[7]. BN PAGE followed by colloidal blue staining indicated that expression of the CGG premutation disrupts assemblies of the mitochondrial supercomplexes in both SH-SY5Y (Fig. 6.6A) and HEK-293 (Fig. 6.6B) cells. Interestingly, expression of FMRpolyG cloned under an artificial ATG start codon also disrupted respiratory chain super complexes assembly (Fig. 6.6A,B). Densitometry analysis was done for intensity quantification of each band in the respective lane (Fig.6.7A). The detailed densitometry analysis in SH-SY5Y and HEK293 suggest decreased levels of SCs labelled as n1-n5 and individual complex II, III, IV in 5'UTR *FMR1* CGG99X compared to control (pEGFP-C1) transfected cells (Fig. 6.7B). Mitochondria isolated from cortical region of FXTAS mice model also showed alteration in the levels of supercomplexes intermediate (n1-n5) (Fig. 6.6C). To further determine the specific effect of CGG repeats and FMRpolyG on individual complexes of mitochondrial respiratory chain, we examined the in-gel activity of individual respiratory chain complexes in HEK-293 and in SH-SY5Y cells. Expression of the CGG premutation significantly decreased complex I (NADH dehydrogenase) activity compared to control conditions (Fig. 6.6D,E). As compared to control, complex I activity is also decreased in cortex regions of mice expressing the CGG premutation (Fig. 6.6F). Densitometry analysis further confirms the decreased complex I activity as shown by decreased area of peak intensities in the graph (Fig. 6.7C). Similarly, complex IV (Cytochrome Oxidase) activity is also decreased in cells (Fig. 6.6G,H) and mice (Fig. 6.6I) expressing the CGG

premutation. These results further confirmed by decreased area of peak intensities under CGG premutation condition in HEK293, SH-SY5Y cells and mice expressing CGG premutation (Fig. 6.7D). These observations suggest that the expression of the CGG premutation in cell and animal models induces defects in respiratory chain assembly and individual complex activity.

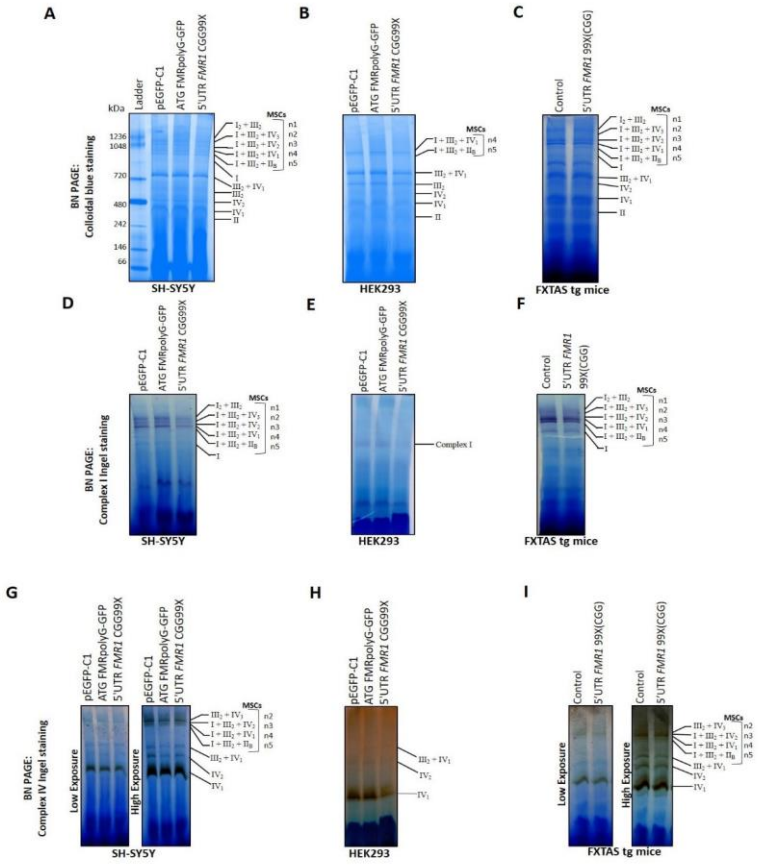

Figure 6.6.FMRpolyG formed due to expanded CGG repeats disrupts mitochondrial respiratory chain supercomplexes (SCs) assembly and decreases activity of individual complexes. SH-SY5Yand HEK293 cells were transfected with ATG FMRpolyG-GFP and 5'UTR*FMR1* CGG99Xand mitochondrial supercomplexes (n1-n5) and individual complex levels were detected by gradient BN-PAGE followed by colloidal Coomassie blue staining in SH-SY5Y (A) (n=3),HEK293 (B) (n=3) and cortical tissue from FXTAS transgenic mice (n=2) (C).

Activity of CI containing supercomplexes and individual CI were determined by BN-PAGE followed by in-gel activity staining specific for CI in SH-SY5Y (n=3) (D), HEK293 cells (E) (n=4) and in transgenic mice (n=3) (F). Assembly and activity of CIV containing supercomplexes and individual CIV were determined by gradient BN-PAGE followed by in-gel activity staining specific for CIV in FMRpolyG expressing SH-SY5Y (n=3) (G), HEK293 (n=3) (H) and in transgenic mice model (n=2) with low as well as high time exposure (I).

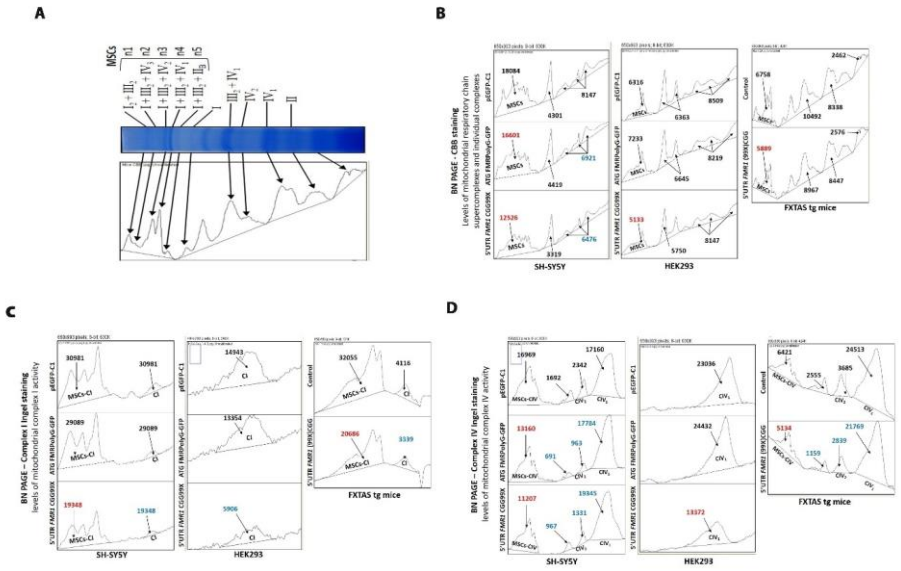

Figure 6.7. Quantification of the area for band intensities of mitochondrial supercomplexes and individual complexes. Schematic representation of densitometry analysis (A). Densitometry analysis for the levels of mitochondrial supercomplexes (B), complex I activity (C) and complex IV activity (D) in CGG repeats transfected SH-SY5Y, HEK293 cells and neuronal cells derived from cortical region FXTAS transgenic mice.

6.5 FMRpolyG expression alters the level of mitochondrial DNA encoded transcripts

Critical components of the OXPHOS are encoded by the mitochondrial DNA (mtDNA). Thirteen transcripts are encoded by mtDNA: seven transcripts (ND1–ND6 and ND4L) of complex I (NADH: ubiquinone oxidoreductase); one (apocytochrome B) of the complex III (ubiquinol: cytochrome C oxidoreductase) subunits; three (COX1–COX3) of the complex IV (cytochrome c oxidase) and two (ATP6 and ATP8) of the complex V (ATP synthase)subunits[8]. Hence,

critical levels of mitochondrial transcripts should be maintained for optimal mitochondrial functions. To understand how the CGG premutation affects mitochondrial complexes assembly, we analyzed the levels of mitochondrial encoded transcripts. As shown in Figure 3C, there is no significant change in mitochondrial DNA content in cells expressing the CGG premutation. However, levels of mitochondrial DNA encoded transcripts such as 16S rRNA, ND1, ND3, ND4L, ND4, COX2, COX3 and ATP6 are all reduced in HEK-293 cells expressing both CGG permutation compared to control (Fig 5A). Similar results were observed in neuronal SH-SY5Y cells (Fig B). Interestingly, we confirmed these results in brain tissue of FXTAS patients. Quantitative PCR revealed decreased expression of the 16S rRNA, ND1, ND2, ND3, ND5, COX1, COX2, COX3 and ATP6 transcripts in cerebellum samples of individuals with FXTAS compared to control individuals (Fig. 5C). These results suggest that mitochondrial respiratory chain dysfunctions in FXTAS is paralleled by decreased mitochondrial transcripts levels.

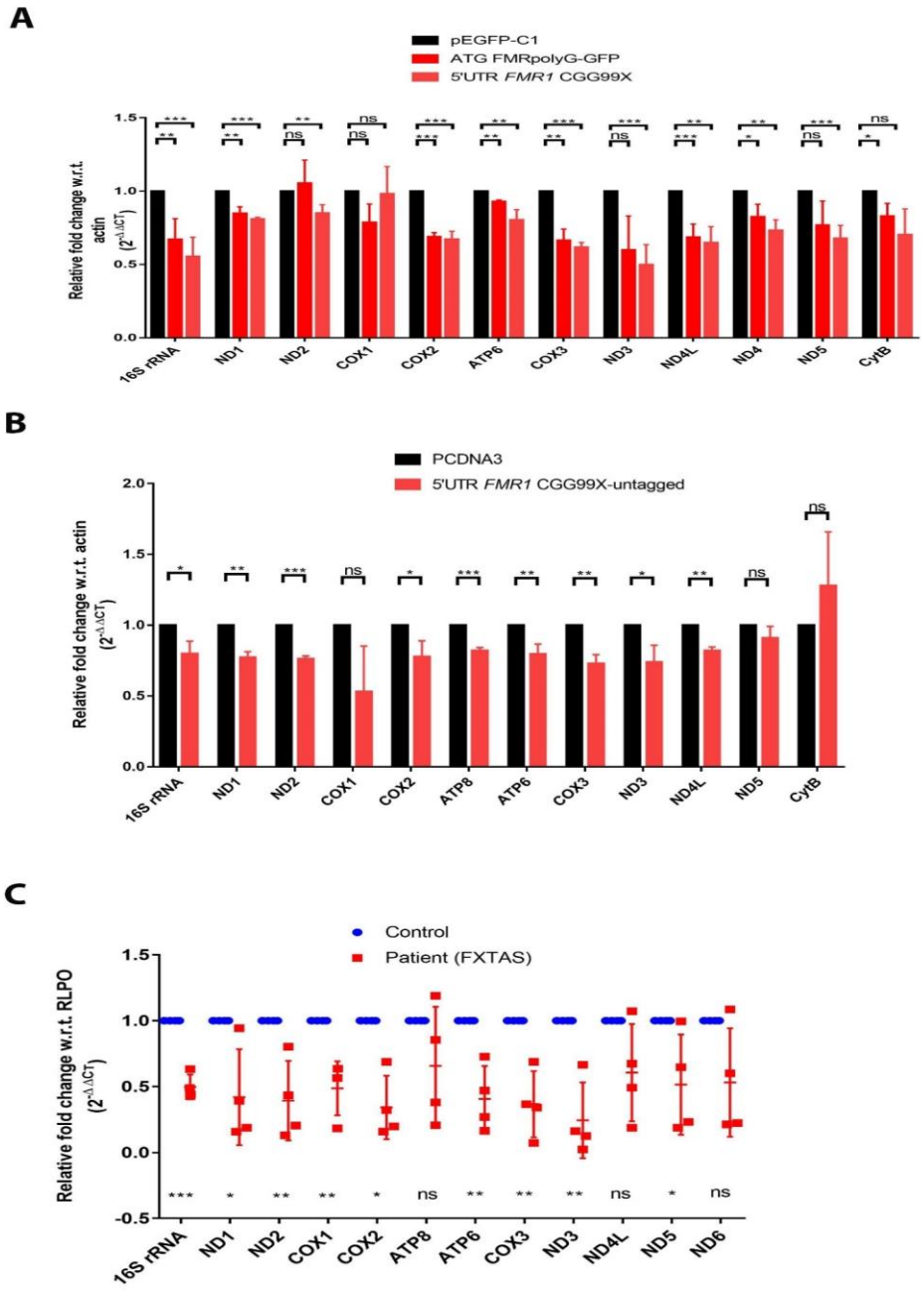
A
pEGFP-C1
ATG FMRpolyG-GFP
5'UTR FMR1 CGG99X
Relative fold change w.r.t. actin
16S rRNA
ND1
ND2
COX1
COX2
ATP6
COX3
ND3
ND4L
ND4
ND5
CytB
B
PCDNA3
5'UTR FMR1 CGG99X-untagged
Relative fold change w.r.t. actin
16S rRNA
ND1
ND2
COX1
COX2
ATP8
ATP6
COX3
ND3
ND4L
ND5
CytB
C
Control
Patient (FXTAS)
Relative fold change w.r.t. RLPO
16S rRNA
ND1
ND2
COX1
COX2
ATP8
ATP6
COX3
ND3
ND4L
ND5
ND6

Figure 6.8. FMRpolyG alters transcripts encoded by mitochondrial genome. HEK293 andSH-SY5Y cells were transfected with ATG FMRpolyG-GFP and 5'UTR*FMR1* CGG99X. Mitochondrial transcript levels were analysed by qPCR in HEK293 (A) and SH-SY5Y (B) cells (n=3). Actin was used as endogenous control. Similarly, mitotranscripts level were checked in FXTAS patients (C), RLPO (large ribosomal protein) was used for endogenous control. (n=4), $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

6.6 Discussion

FMRpolyG is a toxic protein, and it sequesters several RNA binding proteins (RBPs) involved in neuronal development and maturation. However, the molecular basis of pathogenicity of FMRpolyG in neurons is unclear. Apparently, there are many reports which show mitochondrial alteration in neurodegenerative diseases. In FXTAS, mitochondrial dysfunction was associated with a Warburg-like shift[9], resulting into lower OXPHOS capacity as described in cultured skin fibroblasts from FXTAS patients [10]. Deficit of OXPHOS capacity enhances ROS generation, which are a collection of highly unstable molecules that attack cellular structures and cause oxidative stress damage, triggering apoptotic mechanisms in FXTAS[11]. Regarding FXTAS, mitochondrial dysfunction has also been described in cultured fibroblasts and in postmortem brain tissue from FXTAS patients [12]–[15]. Although several studies support a role for mitochondrial dysfunction in FXTAS pathogenesis, the implication of several mitochondrial processes like mitochondrial dynamics, nuclear encoded mitochondrial mRNA transport, mitochondrial super complex assemblies and individual complex activity in FXTAS pathogenesis have not been studied. In the current study, we confirmed that expression of the CGG premutation alters various mitochondrial functions in cell and animal models which may have implication in FXTAS pathogenesis.

Apart from nuclear inclusions, FMRpolyG also forms smaller cytoplasmic aggregates which interact with mitochondria. Importantly, expression of FMRpolyG under an artificial ATG start codon reproduces the deleterious effect of the CGG premutation and leads to multiple alterations of mitochondrial activities. Of technical interest, we used a CGG construct that form little CGG RNA foci but encodes FMRpolyG expressed under an artificial ATG codon. This constructs have also shown similar results suggesting FMRpolyG is responsible for causing altered mitochondrial functions. The decrease in mitochondrial transmembrane potential and

bioenergetic capacity was also observed in cells expressing FMRpolyG. This is in consonance with previous observation where mitochondrial functions were altered in fibroblast derived from the FXTAS patients [12], [15], [16]. Strikingly, results here also suggest the global decrease in mitochondrial DNA encoded transcripts in FMRpolyG expressing cells and in brain samples of FXTAS patients. This could be explained by FMRpolyG proteomics analysis performed in an earlier study[3], where FMRpolyG was known to sequester several proteins important for mitochondrial RNA processing (FASTKD5, DHX30) and ribosomal biogenesis [17], [18]. Hence, this raises the possibility that FMRpolyG may interact and sequester the proteins which translocate to mitochondria and regulate RNA processing and translation of mitochondrial DNA encoded transcripts. This hypothesis supports our observation which showed decreased level of mitochondrial DNA encoded transcripts in FMRpolyG expression in cells, transgenic mice and brain of FXTAS patients. Moreover, FMRpolyG also altered mitochondrial architecture, which further confirms mitochondrial deformity as previously reported from FXTAS patients [19].

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In conclusion, this study provides several evidences suggesting that CGG repeats RAN translated into the FMRpolyG protein forms cytosolic small aggregates that interacts with mitochondria and potentially alters mitochondrial activities. Given the importance of mitochondrial dysfunctions in neurodegenerative diseases, including Parkinson's disease, and some overlap of symptoms between FXTAS and parkinsonism, it will be important to further study the cause and evolution of mitochondrial dysfunctions in FXTAS. The modulation in assembly of supercomplexes by FMRpolyG as observed here, should be further explored in large cohort of FXTAS patients so that therapeutics targeting mitochondria can be evaluated.