## 4.1 Materials

### 4.1.1 Cell culture and reagents

HEK293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, USA), SH-SY5Y cells were grown in DMEM-F12 (Gibco, Invitrogen, USA), and U87MG cells were grown in MEM (Minimal Essential Medium, Gibco, Invitrogen, USA) at 37 °C, 5% CO2 and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen, USA), 1% penicillin, streptomycin and neomycin (PSN) antibiotic mixture (Gibco, Invitrogen, USA)

### 4.1.2 Generation of stable cell lines

HEK293-MTRFP stable cell line was prepared by seeding $1.5\times10^5$ cells of HEK293 in 24 well plate. After 24 hours, cells were transfected mitochondrial targeted red fluorescent protein vector (pHcRed1-Mito) (Clonetech, USA) using calcium phosphate transfection method [1]. G418 was used as screening marker to selectively grow the cells expressing pHcRed1-Mito. After 24 hours of transfection media was changed by adding DMEM containing G418 (500 μg/ml) and every alternate day until stable clones achieved. The stable cells were harvested from 24 well plate and transferred to 96 well plate to obtain single clone using serial dilution method. The single clones were transferred and maintained in 12 well plates. After incubation for 7 days, the cells were transferred to 25 $cm^2$ culture flask and maintained in DMEM supplemented with 200 μg/ml of G418.

### 4.1.3 Kits and reagent

Primers for all miRNAs, actin, 5S RNA, and mitotranscripts were purchased from IDT, USA. XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and CM-H2DCFDA was purchased from Thermofisher, USA. Antibodies used for all immunoblotting were purchased from Cell Signaling Technology (CST), USA. RNase A and buffer P1 were used from QIAprep Spin Miniprep Kit (Qiagen, Netherlands). SYBR green and cDNA isolation kits were purchased from Takara, Japan. MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Film, Lipofectamine 2000, Opti-MEM was procured from Thermofisher, USA.

$H_2O_2$, rotenone, tricine, tris, glycine, triton-X 100, and digitonin were purchased from Sigma-Aldrich, USA. CM-$H_2$DCFDA and MitoSOX™ Red were purchased from Molecular Probes Inc., USA. Qproteome Mitochondrial Isolation Kit, RNase and buffer P1 were used from QIAprep Spin Miniprep Kit (Qiagen, Netherlands). SYBR green and complementary DNA (cDNA) isolation kits were purchased from Takara, Japan. MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Film, Lipofectamine 3000, RNAiMAX, tetramethyl rhodamine methyl ester (TMRM), Opti-MEM, Yeast tRNA, Dynabeads, SUPERase•In, MyOne Streptavidin C1 were purchased from Life technologies, USA. The mimic and inhibitor of miR-320a and control were purchased from Ambion, USA. Coomassie brilliant blue was purchased from Merck, USA. NADH, Protease inhibitor and Proteinase K were purchased from Roche. Glo Caspase 3/7 assay kit was procured from promega. miRCURY$^{TM}$ RNA Isolation Kit

### 4.1.4 Construct details

Mainly three CGG repeats constructs ATG FMRpolyG-GFP, 5'UTR *FMR1* CGG99X (fused with GFP) and 5'UTR *FMR1* CGG99X untagged were used for transfection for mimicking FXTAS condition in vitro (Fig S1 A). mCherry-Mito-7 was a gift from Michael Davidson (Addgene plasmid # 55102). ATG FMRpolyG-GFP, which has canonical start codon (ATG) before the expanded CGG repeats constitutively expresses FMRpolyG while 5'UTR *FMR1* CGG99X contain non canonical codon (ACG) before the CGG repeats leads to formation of FMRpolyG mainly due to RAN translation, more resembles premutation condition. pEGFP-C1 was used as control for all the experiments.

### 4.1.5 FXTAS transgenic mouse model

Double transgenic Nestin-cre + Full 5'UTR *FMR1* mice were described previously [2]. Briefly, these mice overexpress 99 CGG repeats embedded in the 5'UTR natural sequence of the human *FMR1*gene. Expression of the premutation is restricted to the nervous system due to the Nestin-cre that removes a LoxP stop polyadenylation cassette located between the CAG promoter and the 5'UTR *FMR1* construct. All mouse procedures were done according to protocols approved by the Committee on Animal Resources of the ICS and IGBMC animal facilities and under the French and European authority guidelines.

### 4.1.6 Patient brain tissue samples

Frozen postmortem human cerebellum tissue from four premutation cases, diagnosed with FXTAS, (mean age at death 77.2 yrs, range 67-85 yrs. Mean CGG repeat number = 92.7; range 86-100 CGG repeats), were obtained from the Medical Investigation of Neurodevelopmental Disorders Institute Brain Repository at the University of California at Davis in Sacramento, CA, under approved IRB protocols (University of California, Davis). Frozen postmortem human cerebellum tissue from four age matched controls were obtained frozen from the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development at the University of Maryland in Baltimore, MD. CGG repeat sizing was determined on genomic DNA isolated by kit-based method (Puregene Kit; Gentra Inc., Minneapolis, MN). A combination of polymerase chain reaction (PCR) and southern blot (SB) analysis was performed as described previously [3], [4]. Total RNA was isolated using TRIzol reagent, quantified and cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara). Realtime PCR was performed to assay the levels of all mitochondrial DNA encoded transcripts.

## 4.2 Methods

### 4.2.1 Cell death assay and Caspase 3/7 activity

HEK293 and SH-SY5Y cells were plated at density of $5 \times 10^4$ cells/well in 96 well plate and transfected with indicated constructs. After 24 h of transfection, MTT assay was performed to check cellular viability [5] and LDH release assay was performed to check cytotoxicity as described previously [6]. Caspase 3/7 activity was measured by using Caspase-Glo® 3/7 Assay Systems (Promega) as per the described protocol.

### 4.2.2 Immunohistochemistry and Confocal Microscopy

The subcellular localization of FMRPolyG, and interaction of cytosolic FMRPolyG with mitochondria was analyzed by confocal microscopy. Briefly, HEK293 cells were plated at density of $1.5 \times 10^5$ cells per well in glass bottom 24 well plate (Greiner Bio-One, USA). Cells were co-transfected with mCherry-Mito-7 with ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X fused with GFP using Lipofectamine® 2000 (Thermofisher, USA). After 24

h of transfection, cells were fixed and intracellular localization and colocalization of FMRpolyG with mitochondria was analyzed using Leica TCS-SP5II confocal microscope (Leica Microsystems, Germany).

Mouse brain sections were deparaffinized for 20 min in Histosol Plus (Shandon) and dehydrated as follows: twice in ethanol 100% (5 min), twice in ethanol 95% (5min), once in ethanol 80% (5 min), once in ethanol 70% (5 min) and rinsed in DPBS. Glass coverslips containing brain sections treated as described above, fixed in PFA for 10 min and washed three times with PBS. The coverslips or slides were incubated for 10min in PBS plus 0.5% Triton X-100 and washed three times with PBS and incubated with primary antibody against FMRpolyG (8FM, 1/100), AIF (1/100, Cell Signaling (USA)). Slides or coverslips were washed twice with PBS and incubated with goat anti-rabbit or goat anti-mouse conjugated with Cyanine 3 (1/500 dilution; Fisher) for 60 min; incubated for 2 min in PBS 1X-DAPI (1/10 000 dilution) and rinsed twice with PBS 1X before mounting in Pro-Long media (Molecular Probes). Slides were examined using a fluorescence microscope (Leica). Minimum 30 cells were examined in different conditions for cellular morphology, FMRPolyG localization and interaction with mitochondria. Representative image panel was prepared by Adobe Photoshop CS.

**4.2.3 Western blotting and antibodies**

To analyze the presence of FMRPolyG in mitochondria, subcellular fractionation was performed followed by western blotting. Briefly, HEK293 cells were plated at density of $2 \times 10^6$ in 90 mm dishes and transfected with indicated constructs using Lipofectamine® 2000 (Thermofisher, USA). After 24 hours of transfection, cells were washed with cold PBS and harvested. Mitochondrial fraction and cytosolic fraction were prepared using Qproteome Mitochondria Isolation Kit (37612, QIAGEN) as per the protocol with minor modifications. Mitochondrial fraction and total cell were resuspended in Triton X-100 IP buffer (150 mM NaCl, 50 mM Tris-HCl, 10% Glycerol, 1% Triton X-100, containing complete protease inhibitor cocktail (Roche, Germany)), incubated on ice for 1 hours and centrifuged at 13,000 rpm for 10 min at 4 °C. Protein concentration was quantified by Bradford reagent (BioRad). Protein lysates were separated on 12% SDS-PAGE and analyzed by western blotting using

specific antibodies. The primary antibodies used were: Rabbit polyclonal against GFP (AE0011, ABclonal), NDUFS2 (Cell Signaling, USA). Secondary antibodies: HRP-conjugated anti-rabbit and anti-mouse antibodies (Open Biosystems, USA) were used in the study.

### 4.2.4 ATP assay

The cellular ATP level was measured using ATP determination kit (Molecular Probes/Life Technologies, Canada) by using 1:10 diluted cell lysate in ATP determination master mix (25 mM Tricine buffer, pH 7.8, 5 mM MgSO4, 0.5 mM D-luciferin, 1.25 µg/mL firefly luciferase, 100 µM EDTA and 1 mM DTT). The luminescence intensity was measured using TriStar² LB 942 Multimode Microplate Reader, Berthold Technologies, Germany. The protein content was determined by Bradford assay and equal protein was used for different assays.

### 4.2.5 Membrane Potential

The mitochondrial membrane potential was determined by staining cells with TMRM (5µM) (Tetramethylrhodamine, Methyl Ester, Perchlorate, Molecular Probes) for 15 min followed by quantification of fluorescence at 510/570–600 by fluorimeter (Hitachi High-Technologies Corp., Japan). The Protein content was determined by Bradford assay and normalized for assay.

### 4.2.6 BN-PAGE, Colloidal Coomassie Blue Staining and Ingel activity of mitochondrial respiratory chain complexes

Mitochondrial supercomplexes (SCs) assembly and complex-I and complex-IV activity was determined by Blue Native PAGE followed by colloidal coomassie blue staining and in-gel activity [7]. Briefly, HEK293 and SH-SY5Y cells were seeded at the density of 3 X $10^6$ in 10cm dishes, transfected with pEGFP-C1 (control), ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X. After 24 hours of transfection, cells were collected. Similarly, from the brain, cortical region of control as well as FXTAS transgenic mice was collected and homogenized. Isolation of mitochondrial fraction was performed using Qproteome Mitochondria Isolation Kit (37612, QIAGEN). Briefly, the cells (7 X $10^6$) were resuspended in 700 µl lysis buffer.

The cells were lysed in disruption buffer using 24G needle and centrifuged at 1000×g for 10 min at 4 ◦C and supernatant collected. The mitochondrial fraction was collected by centrifugation at 6000×g for 10 min at 4°C and purified from the interface of disruption buffer and purification buffer. Mitochondrial protein concentration was determined using the Bradford method and 50 µg mitochondrial protein were solubilized in solubilization buffer (50 mM NaCl, 50mM Imidazole/HCl pH 7.0, 2 mM 6-aminohexanoic acid, 1mM EDTA, 6.0 g/g digitonin (10%)) and kept on ice for 30 min. After incubation, solubilized mitochondria were centrifuged for 20 min at 20,000g. 20 µl supernatant was mixed with 3µl 50% glycerol and 5% Coomassie blue G-250 (8 g/g detergent to dye ratio). The sample was loaded to 3-12% acrylamide gradient gel for BN-PAGE, run at room temperature and in-gel activity for mitochondrial complex I and complex IV was performed. For visualizing SCs and individual mitochondrial respiratory chain subunits, 100 µg mitochondrial protein was processed and separated by BN-PAGE followed by colloidal coomassie blue (G-250) staining.

### 4.2.7 Analysis of relative mtDNA content and mitochondrial transcripts by qPCR

HEK293 and SH-SY5Y cells were plated at the density of $5 \times 10^5$ cells/well in 6 well plate and transfected with pEGFP-C1 (control), ATG FMRpolyG-GFP and 5'UTR *FMR1* CGG99X plasmids using by Lipofectamine® 2000. After 24 hrs of transfection, cells were collected, and genomic DNA was isolated using phenol: chloroform method [8]. Mitochondrial DNA quantification was done by qPCR using RNaseP as endogenous control [9]. RNA was isolated using TRIzol reagent, quantified and cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara). Realtime PCR was performed to assay the levels of all

mitochondrial DNA encoded transcripts. Primer sequences used for mouse mitochondrial transcripts are given in Table S1 and for human mito transcripts are given in Table S2.

### 4.2.8 Transfection of mimic

The control RNA (scrambled sequence) and mimic of miR-320a (mirVana, Thermofisher, USA) were transfected using Lipofectamine RNAiMAX(Thermofisher, USA)following manufacturer's instructions. Briefly, the miRNA mimic and control (final concentration of 30 nM) and Lipofectamine RNAiMAX reagent was diluted in OptiMEM(Life Technologies, USA). The miRNA mix was added to Lipofectamine mix and incubated for 15 min and added to the cells. The media was replaced after 10 hrs and further incubated/treated as per requirement in different assays.

### 4.2.9 Isolation of mitochondria(MT), mitoplast(MP) and inter membrane space (IMS)

Mitochondria were isolated using Qproteome Mitochondria Isolation Kit (Qiagen, USA). Briefly, the cells (7 X $10^6$) were resuspended in 700µl lysis buffer. The cells were disrupted in disruption buffer using 24G needle and centrifuged at 1000×g for 10 min at 4 ◦C and supernatant was collected. The mitochondrial fraction was collected by centrifugation at 6000×g for 10 min at 4◦C and purified from the interface of disruption buffer and purification buffer. To prepare mitoplasts, the mitochondria were incubated in 10mM potassium phosphate buffer supplemented with 2.7mg/ml digitonin for 20 min in ice followed by centrifugation 10000×g for 10 min at 4ºC. Supernatant containing IMS (intermembrane space) fraction was collected in other tube and the obtained mitoplast pellet was then washed thrice with PBS [10].

### 4.2.10 RNase Treatment

The purified mitochondria were treated with RNase A (40µg/ml) to remove bound non-specific RNA at 37 ◦C for 1 hr in 300µl buffer P1 (QIAprep Spin Miniprep Kit, Qiagen, USA) followed by treatment with 100 mg/ml proteinase K to stop the reaction.

### 4.2.11 miRNAs expression analysis by next generation sequencing and qPCR

Next generation sequencing (NGS) was performed to investigate mitochondrial and cellular miRNAs expression in HEK293 cells transfected with expanded CGG repeats. Small RNAs were isolated using miRCURY™ RNA Isolation Kit (Exiqon). Total RNAs were normalized using DESeq2 method followed by cDNA library preparation. QC analysis of library further confirms purity and integrity of RNAs. RNA samples were analysed on Illumina HiSeq platform. For qPCR expression analysis, total RNAs were isolated using TRIzol reagent (Takara, Japan).Poly-A tailing of small RNA was done using E. coli Poly-A Polymerase (New England Biolabs, UK) at 37◦C for 20min, enzyme inactivation at 65 ◦C for 5 min followed by cDNA synthesis using cDNA synthesis kit (Takara, Japan).The cDNAs were synthesized using oligo dTs from same kit in two steps. For, miRNA expression analysis in FXTAS patients, the same RNAs were used which was mentioned in the previous study [11]. Primers for miRNAs were designed as previously [12].The levels of target transcripts were determined by $2^{-\Delta\Delta Ct}$ method using suitable endogenous control whereas 5S rRNA/U6 snRNA were endogenous controls for miRNA analysis. The reaction conditions were 95 ◦C for 2 min followed by 35 cycles of 95 ◦C for 5sec and 60◦C for 34 sec (for mRNA) / 1 min (for miRNA) (the data was acquired at this step).The melt curves were also acquired.

### 4.2.12 Droplet Digital PCR workflow

EvaGreen chemistry was used for all the ddPCR performed in the study. Primer optimization and annealing temperature was standardized for all the targets. cDNA concentration was also optimized for each experiment to maintain ratio of positive and negative population to follow Poisson's distribution at 95% confidence of upper limit. A no template control (NTC) was included in every assay. Briefly, each ddPCR system of 20µl was loaded into a disposable droplet generator cartridge (BioRad) followed by addition of 70µl of droplet generation oil for EvaGreen (Bio-Rad)into each of the eight oilwells. The droplets were generated by QX200 droplet generator (Bio-Rad) and transferred toa 96-well PCR plate (Bio-Rad) and the plate was heat-sealed with foil and placed in a conventional thermal cycler (Bio-Rad). The PCR condition for EvaGreen assays were as follows: 95ºC for 5 minutes, then 40 cycles of 95ºC for 30 seconds and 58ºC (depending on annealing temperature) for 1 minute, and three final steps at 4ºCfor5 minutes, 90ºC for 5 minutes, and a 4ºC for 30 minutes to enhance dye stabilization [13]. After completion of PCR cycle plate was kept in QX200™ Droplet Reader and analysed further using

QuantaSoft™ software. The results were plotted in two ways by showing Ch1Amplitute and copies/µl, where yellow lines suggest partitions of droplets for each group which is also denoted by curly braces at the top. There are two population of droplets, (1) blue droplets, which are positive droplets for their respective targets and (2) grey droplets, which are negative droplets showing absence of their respective target (droplets which appear black are just bunch of grey droplets). Pink line in the graph indicates threshold intensity (Ch1: green) set to discriminate positive and negative droplets.

### 4.2.13 ROS Estimation

To determine ROS levels, cells were stained with CM-H2CFDA (10µM)for 15 min followed by quantification at 510/570–600nm(ex/em) by fluorimeter (Hitachi High-Technologies Corp., Japan). The protein content was determined by Bradford assay in each assay for normalization.

### 4.2.14 Bioinformatics analysis and validations

The putative targets of altered miRNAs including miR-320a from Next Generation Sequencing (NGS) data were analyzed by miRDB and StarBase [14],[15]. The targets with present in more than two target prediction software were shortlisted and submitted to DAVID annotation platform for clustering into meaningful groups [16]. The miRNAs which were showing high relevant targets to mitochondria, cell death and neuronal functions were selected for qPCR validation.

### 4.2.15 Details for primer sequences

| Sr No | Gene name | | Primer sequence (5'-3') |
|---|---|---|---|
| **1** | hsa-miR-320a-3p | miR Seq | AAAAGCUGGGUUGAGAGGGCGA |
| | | F | CAGAAAAGCTGGGTTGAGAG |
| | | R | GTTTTTTTTTTTTTTTCGCCC |
| **2** | hsa-miR-181a-5P | miR Seq | AACATTCAACGCTGTCGGTGAGT |
| | | F | GCATTCAACGCTGTCGGT |
| | | R | CAGGTCCAGTTTTTTTTTTTTTTTACTC |
| **3** | hsa-mir-221-3p | miR Seq | AGCTACATTGTCTGCTGGGTTTC |
| | | F | AGCTACATTGTCTGCTG |
| | | R | CAGGTCCAGTTTTTTTTTTTTTTTGAAA |
| **4** | hsa-miR-4485-3P | miR Seq | AACGGCCGCGGTACCCTAA |
| | | F | AGTAACGGCCGCGGTAC |
| | | R | TCCAGTTTTTTTTTTTTTTTTAGG |
| **5** | hsa-mir-148a-3p | miR Seq | UCAGUGCACUACAGAACUUUGU |

| | | | |
|---|---|---|---|
| | | F | CGTCAGTGCACTACAGAACT |
| | | R | CAGGTCCAGTTTTTTTTTTTTTTTACAA |
| **Mitotranscripts (Human)** | | | |
| **1** | 16S rRNA | F | GAAACCAGACGAGCTACCTAAG |
| | | R | CGCCTCTACCTATAAATCTTCCC |
| **2** | ND1 | F | ATACCCCCGATTCCGCTACGAC |
| | | R | GTTTGAGGGGGAATGCTGGAGA |
| **3** | ND2 | F | ATTCCATCCACCCTCCTCTC |
| | | R | TGGGGTGGGTTTTGTATGTT |
| **4** | COX1 | F | CGATGCATACACCACATGAA |
| | | R | AGCGAAGGCTTCTCAAATCA |
| **5** | COX2 | F | TGAAGCCCCCATTCGTATAA |
| | | R | ACGGGCCCTATTTCAAAGAT |
| **6** | ATP8 | F | ATGGCCCACCATAATTACCC |
| | | R | GCAATGAATGAAGCGAACAG |
| **7** | ATP6 | F | CGCCACCCTAGCAATATCAA |
| | | R | TTAAGGCGACAGCGATTTCT |
| **8** | COX3 | F | GGCATCTACGGCTCAACATT |
| | | R | CGAAGCCAAAGTGATGTTTG |
| **9** | ND3 | F | CCCTCCTTTTACCCCTACCA |
| | | R | GGCCAGACTTAGGGCTAGGA |
| **10** | ND4L | F | TAACCCTCAACACCCACTCC |
| | | R | GGCCATATGTGTTGGAGATTG |
| **11** | ND4 | F | ACAAGCTCCATCTGCCTACGA |
| | | R | GGCTGATTGAAGAGTATGCAATGA |
| **12** | ND5 | F | CAAAACCTGCCCCTACTCCT |
| | | R | GGGTTGAGGTGATGATGGAG |
| **13** | CytB | F | AACCGCCTTTTCATCAATCG |
| | | R | AGCGGATGATTCAGCCATAATT |
| **Endogenous controls** | | | |
| **1** | 5S rRNA | F | GGTCTACGGCCATACCACC |
| | | R | CAGTTTTTTTTTTTTTTAAAGCCTACAG |
| **2** | Actin | F | CCTCGCCTTTGCCGATCC |
| | | R | CGCGGCGATATCATCATCCAT |
| **3** | GAPDH | F | TGCACCACCAACTGCTTAGC |
| | | R | GGCATGGACTGTGGTCATGAG |
| **4** | U6 snRNA | F | GGTGCTCGCTTCGGCA |
| | | R | TCCAGTTTTTTTTTTTTTTAAAAATATGGAAC |

### 4.2.16 Statistical Analysis

Most of the statistical tests and graphs of the results are generated by GraphPad Prism: version 7.Experimental data are shown as mean ± SEM for the number of observations. Student's unpaired t test was performed wherever comparisons of two groups for repeated measurements to determine the levels of significance for each group. One-paired ANOVA test is used to calculate degree of significance wherever there are more than two groups (Dunnett's multiple comparison test).Each experiment has been repeated minimum two times independently and probability values of $p < 0.05$ were considered as statistically significant.