4. Materials and Methods

4.1 Materials

4.1.1. Cell lines:

HEK293, HeLa and MCF-7 cells were cultured in Dulbecco's modified Eagle's media (DMEM) (Life Technologies, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, USA), 1% penicillin, streptomycin and neomycin (PSN) antibiotic mixture (Life Technologies, USA). The cells were maintained in 5% CO_2 at 37°C. The mitochondrial DNA depleted cells in HEK293 was prepared and maintained as described previously (Galimov, et al., 2014). MDA-MB231 cells and their derivatives were grown on DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml streptomycin, 100 U/ml penicillin.

4.1.2. Generation of stable cell lines:

To generate HEK293-MTRFP stable cell line, 1.5×10^5 cells of HEK293 were plated in 24 well plate. After overnight incubation, mitochondrial targeted red fluorescent protein vector (pHcRed1-Mito) (Clonetech, USA) was transfected using calcium phosphate transfection method (Tomar et al., 2015). Media was changed with DMEM supplemented with G418 (500 μg/ml) after 24 hrs of transfection and every alternate day until stable clones were clearly visible. 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. Chemicals:

Tunicamycin, rotenone, H_2O_2, oligomycin, trypaflavin, tricine, tris, glycine, triton-X 100, Digitonin, poly-L-lysine and N-acety-L-cysteine were purchased from Sigma-Aldrich, USA.TNF-α (Tumor Necrosis Factor- alpha)) and Mito-TEMPO was procured from Enzo Life Sciences, Inc. USA. CM-H_2DCFDA 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 2000, tetramethyl rhodamine methyl ester (TMRM), Opti-MEM, 4485-mimic, inhibitor, control oligos, Yeast tRNA, Dynabeads, SUPERase•In, MyOne Streptavidin C1 were purchased from Life technologies, USA. The validated primers (KiCqStart™ Primers) for HK2, PKM2, PGAM1, GPD2, NUP50, MDM4, STAT5B, MEIS2 and WDFY3 were ordered from Sigma-Aldrich, USA. HiPerfect Transfection Reagent was purchased from (Qiagen, USA). Biotin tagged miR-4485 was custom designed from Exiqon, Denmark. The mimic and inhibitors of miR-320a, miR-4485 and control were purchased from Ambion, USA. miR-293m80059 mimic and Cy5-miR-4485 was synthesized at Eurofins Genomics, USA. Nitro blue trizolium was purchased from SRL. 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 and qPCR primers for hsa-let-7b, hsa-let-7g, hsa-miR-107, hsa-miR-181a, hsa-miR-221, hsa-miR-320a, hsa-miR-145, U6 snRNA and 5S rRNA were purchased from Exiqon.

Rabbit polyclonal NDUFS2, RPS9 and cytochrome c antibody respectively were gifted by Dr. Jassie Cameron (Dept. of Genetics and Genome Biology, Hospital for Sick Children, Toronto, Canada), Mikael S. Lindstro¨m (Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska University Hospital in Solna, Stockholm, Sweden). Mouse monoclonal Ago2 antibody was gifted by Dr. Nicolas Charlet Bergraund, (Institut de génétique et de biologie moléculaire et cellulaire, Strasbourg, France). MapMi alignments were assisted by Jose Afonso Guerra-Assunc¸a˜o (European Bioinformatics Institute, Welcome Trust Genome Campus, Hinxton, Cambridge, UK). pEGFPC1-Ago2/3 and HA-Flag-Ago2/3 was kind gift of Dr. Hank Qi (Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa). Flag-CLUH was gifted by Dr. Elena I Regulari, Institute for Genetics, CECAD Research Center, University of Cologne, Köln, Germany.

4.1.4. Patient tissue/ethics statement:

Human breast tumor and extra-tumoral tissue specimens were collected from patients undergoing surgery. Human studies were performed following the norms of 1964

Declaration of Helsinki. Ethical approval from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, UP, India ethical committee was taken prior to collection of tissue. The prior consent of each patient was taken. Tissues were collected from the tumor zone (tissue within the tumor boundary), and normal zone (distal normal tissue at least 10 mm from the outer tumor boundary). A fraction of all tissues was fixed in formalin and embedded in paraffin for routine histopathological analysis. The rest of the fractions were frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

4.2 Methodology

4.2.1. Mitochondrial preparation:

Mitochondria were isolated using Qproteome Mitochondria Isolation Kit (Qaigen, USA). Briefly, the cells (7 X 10^6) were resuspended in 700 µl 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 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. The purity of the mitochondrial fraction was assessed by western blotting as described earlier (Tomar et al., 2012) with minor modifications. Briefly, the mitochondria and total cells were solubilized in solubilizing buffer (50 mM Bis-Tris (pH 7.0), 750 mM e-aminocaproic acid, 0.1% TritonX-100 and 10 ug/ml protease inhibitor). The concentration of total cellular and mitochondrial lysate was determined by Bradford assay, normalized and resolved on 12.5% SDS-PAGE. The proteins were electroblotted on PVDF membrane at 100 V for one hour. The membrane was blocked with 5% non-fat dried milk and 0.1% Tween-20 in PBS for 1 hour at room temperature. The membrane was incubated with rabbit polyclonal antibody against NDUFS2 (mitochondrial complex I subunit). After incubation membrane was washed three times with PBS-T (PBS containing 0.1% Tween 20) for 10 minutes each and incubated with a secondary antibody at room temperature for 1 hr. The membrane was again washed three times with PBS-T and signal visualized by using EZ-ECL chemiluminescence detection kit (Biological Industries, Israel) by exposing to X-ray film. The membrane was restriped and further probed with polyclonal antibody against RPS9 (ribosomal protein S9). All primary antibodies were

used at 1:1000 dilutions. The nuclear contamination and mitochondrial enrichment were checked at RNA level by RT-PCR. RNA was isolated from mitochondria and first strand cDNA was synthesized. The β-actin was taken as nuclear control whereas mitochondrial DNA encoded mRNA as positive control.

4.2.2. Mitoplast preparation:

The mitoplast was prepared from purified mitochondria as described earlier (Atorino L *et. al.*, 2011) with minor modifications. The mitochondria (protein concentration of 1.0 mg/ml) were incubated in 10 mM KH2PO4 with 2.7 mg/ml digitonin for 20 min on ice. The solution was centrifuged at 10,000 g for 10 min at 4˚C. The pellet was washed twice with PBS. The quality of preparation was confirmed by analyzing the presence/absence of cytochrome c by western blotting as described earlier.

4.2.3. RNase A treatment:

The mitochondria were treated with RNase A to check the association of miRNAs and mRNA with outer surface of mitochondria as described previously (Barrey et al., 2011). The purified mitochondrial pellet was resuspended in 20 µl mitochondrial storage buffer and mixed with 300 ml buffer P1 (QIAprep Spin Miniprep Kit, Qiagen, USA) containing 40 mg/ml RNase A and incubated at 37˚C for 1 hr. The reaction was stopped by adding Proteinase K (100 mg/ml) resuspended in PBS. RNase A treated mitochondria were pelleted down at 8000 rpm for 10 min and washed twice in mitochondrial storage buffer prior to RNA isolation.

4.2.4. PROTEINASE K protection assay:

For the PK accessibility test, mitochondria from HEK293 and HeLa cells were resuspended at a final concentration of 0.5 mg/ml in mitochondria digest buffer (25mM Tris-HCl (pH 7.4), 125mM sucrose and 1mM $CaCl_2$) and PK was added to a final concentration of 50 mg/ml, and the mixture was incubated on ice for 20 min. PMSF (2 mM) was then added to inhibit the PK activity and incubated to 10 min on ice.

Subsequently, the mitochondrial suspension was centrifuged at 10000g for 10 min. The pellet was washed twice with mitochondria isolation buffer and RNA isolation was performed using trizol method.

4.2.5. Small RNA isolation:

RNA was extracted from purified mitochondria and total cell using miRCURY^{TM} RNA Isolation Kit according to manufacturer's instructions (Exiqon, Germany). Briefly, the purified mitochondria were resuspended in RNA lysis buffer and incubated at room temperature in column for 5 min to allow binding. The column was washed thrice with washing buffer at 14000 rpm for 1 min. The RNA was eluted in nuclease free water from the column. Purity and concentration of RNA was determined using NanoPhotometer (Implen, Germany). The integrity of mitochondrial and total cellular RNA was checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA).

4.2.6. RNA preparation from tumor tissue:

The breast tumor and non-tumorous adjacent tissue (NAT) were collected following the norms of institute's ethical committee for each sample and stored as described previously (Rubio et al., 2011). RNA was isolated from frozen tumors and NAT using miRCury RNA isolation Kit (Exiqon, Denmark) following manufacturer's instruction with minor modifications. Briefly, the tissues were disrupted in liquid nitrogen and collected in lysis buffer supplemented with β-mercaptoethanol. The lysate was homogenized by passing 15 times through 25G syringe. The lysate was incubated with Proteinase K at 56˚C for 15 minutes (min) and centrifuged at 1000 rpm for 10 min. The cells and subcellular fractions were lysed in 300 µl lysis buffer supplemented with β-mercaptoethanol. The supernatant was collected and mixed with 700 µl absolute alcohol passed through columns, the column were washed thrice with washing solution, dried at 10000 rpm for 1 min at room temperature. The RNA was eluted in 50-100 µl nuclease free water at 55˚C.

4.2.7. sRNA library preparation and sequencing:

RNA library preparation and sequencing was performed by (BGI) at ShenZhen according to the manufacturer's instructions. Briefly, sRNAs ranging from 18 to 30 nt were gel

purified, ligated to the 3' and 5' adaptor. The ligated products were used for cDNA synthesis, followed by acrylamide gel purification and PCR amplification to generate libraries (Rubio et al., 2011). One µl of each library was loaded on an Agilent Technologies 2100 Bioanalyzer to check size, purity, and concentration. Libraries were sequenced on an Illumina HiSeq 2000 (Illumina, USA). Sequencing data was processed with the Illumina pipeline v1.3.2.

4.2.8. Bioinformatics analysis of sRNA libraries from mitochondria:

All the 50 nt sequence tags generated from Illumina HiSeq 2000 went through data cleaning process which included getting rid of low quality tags and all contaminants. The clean tags lengths were then summarized. The clean tags were also mapped to the UCSC hg19 human assembly using SOAP (Short Oligonucleotide Alignment Program) (Li et al., 2008) with the following options: soap -v 0 -r 2 -s 7 -p 7 -a. Sequences with perfect match or one mismatch were retained for further analysis. The presence of known miRNA were analyzed by aligning clean tags to pre-miRNA (1424), mature miRNA (1539) and miRNA* (194) of miRBase17.0 (Puranam et al., 2011). The other populations of non coding sRNA (rRNA, scRNA, snoRNA, snRNA, tRNA and piRNA) were all identified by aligning the sequences to Genbank (Hernandez et al., 2011) and Rfam (www.sanger.ac.uk/resources/databases/rfam.html) using following program and parameters: blastall -p blastn -F F -e 0.01. The repeat associated RNA and piRNA were identified using tag2repeat and tag2piRNA respectively at BGI. sRNA tags were also aligned to exons and introns of mRNA to find the degraded fragments of mRNA in the sRNA tags. All annotations were summarized using tag2annotation software in the following order of preference: rRNA (Genbank > Rfam) > known miRNA > repeat > exon > intron.

4.2.9. Analysis of differential association of miRNA:

To compare the differential miRNA expression between two libraries (HEK293 and HeLa), first, the expression of miRNAs in two samples were normalized to obtain the expression of transcripts per million. If the normalized expression value of a given

miRNA is zero, its expression value was modified to 0.01. Then, the fold-change and P-value were calculated from the normalized expression using the following formulas.

Normalized expression = (Actual miRNA sequencing reads count / Total clean reads count) ×1,000,000.

Fold change = Log2 (HEK293-NE /HeLa-NE)

P-value:

$$p(x|y) = \left(\frac{N_2}{N_1}\right)\frac{(x+y)!}{x!\,y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y \le y_{min}|x) = \sum_{y=0}^{y \le y_{min}} p(y|x)$$

$$D(y \ge y_{max}|x) = \sum_{y \ge y_{max}}^{\infty} p(y|x)$$

The N1 and x represent total count of clean reads and normalized expression level of a given miRNA in HeLa sRNA library, respectively. The N2 and y represent total count of clean reads and normalized expression level of a given miRNA in HEK293 sRNA library, respectively. A cross-species comparative function was performed to analyze the distribution of identified miRNAs in different species (as deposited in the miRBase) using DSAP (Deep-sequencing smallRNA analysis pipeline) (http://dsap.cgu.edu.tw).

4.2.10. Prediction of putative novel miRNA:

All unannotated sRNA tags that aligned to UCSC hg19 were employed to screen novel miRNAs using Mireap [http://sourceforge.net/projects/mireap/] algorithm under these parameter settings : miRNA sequence length (18-26nt); miRNA reference sequence length (20-24nt); Minimal depth of Drosha/Dicer cutting site (3) ; Maximal copy number of miRNAs on reference (20); Maximal free energy allowed for a miRNA precursor (-18 kcal/mol) ; Maximal space between miRNA and miRNA* (35) ; Minimal base pairs of miRNA and miRNA* (14) ; Maximal bulge of miRNA and miRNA* (4) ; Maximal asymmetry of miRNA/miRNA* duplex (5); Flank sequence length of miRNA precursor (10). The base bias on the first position and different position was also determined.

4.2.11. Clustering of miRNA targets:

To characterize biological processes that may be regulated by identified miRNA in mitochondria; we planned to analyze the targets of the miRNA by different tools. The miRNA identified in mitochondria were divided in two classes based on the number of reads: Class-I (count>5000) and Class II (count = 500 - 5000). The targets were analyzed by using StarBase, a database that has been developed to avoid false positive outputs and has experimentally verified miRNA–target interactions by CLIP-Seq (HITS-CLIP, PAR-CLIP) and degradome sequencing (Degradome-Seq, PARE) data(Yang et al., 2011). Given that miRNA targets prediction tools give high number of false positive, only the target identified by three independent tools (PicTar, miRanda and TargetScan) and overlapped with CLIP-Seq data were taken into further consideration. The targets of novel miRNA were also identified using CLIP search tool based on CLIP -Seq database Starbase (Sano et al., 2008). CLIP search scans peak Clusters overlapping with 3UTR for potential miRNA targets (6mer-8mer). To characterize the biological processes regulated by targets of identified miRNA in mitochondria we used we used Gene Ontology (GO) and the functional annotation clustering feature of DAVID. This tool measure measures the similarities among related GO terms based on the extent of their associated genes and assembles the similar and redundant GO terms into annotation clusters. Fisher Exact p-value is assigned to each GO term that represents the degree of enrichment of the GO term in the input gene list. Finally each cluster is given enrichment score to measure the biological significance. The resulting clusters were further curated to keep only GO terms with p-values > 0.05. The genes with FDR≤0.05 were considered as significantly enriched in target gene candidates and were mapped in relevant signalling pathways in KEGG database.

4.2.12. Alignment of sRNAs to mitochondrial genome:

The clean reads from both the libraries were aligned to human mitochondrial genome NC_012920 (Schon et al., 2011) through MapMi (EMBL) (www.ebi.ac.uk/enright-srv/MapMi) and SOAP to check if these sRNA molecules were originated from mtDNA. Further the alignment of novel miRNA was also done by BLASTN 2.2.20 to investigate their matching sequence in mitochondrial genome.

4.2.13. Validation of miRNAs and their targets associated with mitochondria:

For small ncRNA expression analysis, poly-A tailing of small RNA was performed using E. coli Poly-A Polymerase (New England Biolabs, UK) at 42˚C for 20 min, enzyme inactivation at 65˚C for 5 min followed by cDNA synthesis using cDNA synthesis kit (Takara, Japan) and pre-designed universal degenerate primer (Balcells et al., 2011).

The cDNA for gene expression analysis (of mRNA) was performed using random primers from same kit in two steps. The initial primer binding was performed at 65˚C for 5 min followed by addition of reverse transcriptase and incubation at 37˚C for 60 min. qPCR was performed to validate the targets. The initial denaturing was done at 95˚C for 30 sec followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The specificity of each amplicon was checked by melt curve analysis. The reactions were performed using StepOnePlusTM (Applied Biosystem, USA). The reactions were performed in triplicates, ΔΔCT calculated and relative association with standard error was plotted using GraphPad Prism v.5.0.

4.2.14. Transfection of mimic:

The mimic, inhibitor of miR-320a and control were transfected using Lipofectamine 2000 following manufacturer's instructions. Briefly, the miRNA mimic (final concentration of 30 nM) and Lipofectamine 2000 reagent was diluted in OptiMEM (Life Technologies, USA). The miRNA mix was added to Lipofectamine mix and incubated for 5 min before adding to cells. The media was replaced after 10 hrs, further incubated/treated as per requirement in different assays.

4.2.15. ATP Assay:

The cellular ATP level was measured using ATP determination kit (Molecular Probes/Life Technologies, Canada) by adding 1 µl cell lysate in 100 µl ATP determination master mix containing 25 mM Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 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.

4.2.16. Mitochondrial Complex-1 assay:

The mitochondrial complex I activity was performed as described previously (Prajapati et al., 2014). Briefly, the cells were permeabilized by three freeze-thaw cycles in buffer containing 25 mM sucrose, 20 mM Tris, pH 7.2, 40 mM KCl, 2 mM EGTA, 1 mg/ml BSA, 0.01% digitonin and 10% percoll. The cells were washed thrice in buffer devoid of BSA, digitonin and percoll. The permeabilized cells were suspended in complex I assay buffer (35 mM Potassium Phosphate (pH 7.4), 1mM EDTA, 2.5 mM NaN_3,1 mg/ml BSA, 2 µg/ml Antimycin A, 5 mM NADH). Complex I activity was measured following the decrease in absorbance of NADH at 340 nm after the addition of 2.5 mM acceptor decylubiquinone for 3 min.

4.2.17. Mitochondrial Complex-IV assay:

For mitochondrial Complex-III/IV assay, the mitochondria pellets were resuspended in solubilisation buffer (50 mM NaCl, 50 mM Imidazole/HCl, 2 mM 6-Aminohexanoic acid, 1mM EDTA, (pH-7.0) 10% digitonin at detergent/protein ratio of 6.0 (g/g) was added to pellet. The suspension was incubated on ice for 45 min with occasional vortexing and was clarified by centrifugation at 12000 rpm for 1 hour. Following centrifugation, supernatant was mixed with loading buffer (50% w/v glycerol, 0.1% w/v Ponceau S solution). Non gradient clear native gel and in-gel activity staining were performed as described previously (Yan and Forster, 2009). All gel images were documented using an Epson Perfection 1670 scanner.

4.2.18. ROS estimation:

The mitochondrial membrane potential and ROS levels were determined by staining with CM-H_2DCFDA (10 µM) for 15 min followed by quantification at 490/510-550 by flourimeter (Hitachi High-Technologies Corp., Japan). The protein content was determined by Bradford assay in each assay for normalization.

4.2.19. Mitochondrial trans membrane potential determination:

The mitochondrial membrane potential was determined by staining cells with TMRM (2.5 nM) for 15 min followed by quantification at 510/570–600 respectively by flourimeter (Hitachi High-Technologies Corp., Japan). The protein content was determined by Bradford assay in each assay for normalization.

4.2.20. BN-PAGE and IGA assay:

The effect of miR-320a on mitochondrial supercomplex assembly and complex-I activity was determined by Blue native PAGE followed by In-gel activity and colloidal coomassie blue staining. Briefly, HEK293 cells were seeded in 10 cm^2 dishes, transfected with control and miR-320a mimic followed by TNF-α treatment. Cells were disrupted in diluted sucrose buffer (83 mM sucrose, 6.6 mM imidazole/HCl, pH 7.0) to isolate mitochondrial fraction. Mitochondrial protein concentration was determined using the Bradford method, using BSA as a standard. 50 ug mitochondrial protein were solubilized in 20 ul of 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 25 min. After incubation, solubilized mitochondria pellet were centrifuged for 20 min at 20,000g. 20 ul supernatant was mixed with 5 ul 50% glycerol and 5% Coomassie blue G-250 (8 g/g detergent to dye ratio). The sample was loaded to 12% acrylamide non-gradient gel for BN-PAGE, run at 4-7 ˚C and In-gel activity for mitochondrial complex-I was performed as described previously (Yan and Forster, 2009). For visualizing individual mitochondrial respiratory chain subunits, 100 ug mitochondrial protein was processed and run as described above and stained with colloidal coomassie blue G-250.

4.2.21. Mitochondrial DNA copy number:

The mitochondrial copy number was determined by analysing the relative quantitation of mitochondrial genome. The cells were collected post transfection and treatment. The genomic DNA was isolated by phenol choloroform purification. The relative quantity of mitochondrial genome w.r.t nuclear genome was analysed using RNaseP as marker for

nuclear genome and region from HVR of mitochondrial genome (sequences are provided in Table 4.1).

4.2.22. Analysis of mitochondrial RNA processing:

The mitochondrial transcriptional processing and maturation defects were analyzed by PCR. RNA was isolated from control and cells expressing miR-4485 mimic. The regions flanking adjacent mitochondrial genes were amplified to determine the accumulation of unprocessed transcripts and intermediates. The reaction was performed in 15 µl system comprising of primer combinations as described in Table S2 and 2X EmeraldAmp GT PCR Master mix (Takara, Japan) at 95˚C, 5 min; 35 cycles of 95˚C for 20s, 56˚C for 20s and 72˚C for 40s; 72˚C for 5 min. (sequences for processing intermediates are provided in Table 4.2)

4.2.23. Mitochondrial protein synthesis analysis:

The cells were seeded and transfected as described above. The effect on mitochondrial translation was determined using Click iT Protein Synthesis Assay Kit (Life technology, USA) following manufacturer's instructions. The cells were conditioned in methionine free RPMI for 15 min and treated with 100 µg/ml cyclohexamide for 30 min. The cells were then pulse labeled with 40 µM of methionine analogue, L-Azidohomoalanine (AHA) for 2 hrs. The Click-IT chemistry was performed to conjugate biotin with AHA. The cell lysate was resolved in 12% polyacrylamide SDS gel and blotted on PVDF. The signal of nascent protein synthesis was obtained using anti biotin antibody.

4.2.24. RNA Immunoprecipitation:

HEK293 cells transfected with pCMV-tag-4a, Flag-CLUH and HA-Flag Ago2. After 48 hours of transfection, the cells were suspended in mitochondria isolation buffer (MIB) (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM $MgCl_2$, 100 U/ml RNase inhibitor, 1% NP-40, and complete protease inhibitors) and incubated on ice for 40 min, with occasional vortexing. The lysate was centrifuged at 20,000 g at 4˚C for 45 min and supernatant was collected. Further, anti-FLAG M2 affinity beads (Sigma) was washed thrice with in (MIB)

and blocked with 1% BSA and 100 mg/ml yeast tRNA. The supernatant was incubated with anti-FLAG M2 affinity beads with rotation at 4°C for 2-4 h. The bead complex was washed three times with MIB and then supplemented with 5 mM EDTA and 1ug yeast tRNA. RNA isolation was performed from bead complex using trizol method.

4.2.25. Biotin-tagged mirNA pull-downs and target validation:

The targets of miR-4485 were determined by immunoprecipitation of biotin tagged miR-4485 (Orom et al., 2007). Briefly, HEK293 were transfected with 200 pmoles of synthetic biotin-labeled miR-4485 duplexes (custom designed from Exiqon, Denmark) using HiPerFect Transfection Reagent (QIAGEN, Melbourne, VIC, Australia). After 24 hours, cells were harvested and lysed in hypotonic lysis buffer (10 mM KCl, 1.5 mM MgCl2,10 mM Tris-Cl pH 7.5, 5 mM DTT, 0.5% NP-40, 60U/ML SUPERase•In (Ambion, Austin, TX, USA) and 1X protease inhibitor (Roche, Brisbane, QLD, Australia)). Cell lysate was obtained at 10,000 g at 4°C for 2 minutes and calibrated to 1M NaCl in fresh vial and incubated with 25 µl of Dynabeads™ MyOne™ Streptavidin C1 for 30 min at room temperature. The beads were washed and pre-blocked with 1 µg/µl bovine serum albumin and 1 µg/µl yeast tRNA (Invitrogen) prior to incubation with cell lysate. The beads were washed thrice after incubation with hypotonic lysis buffer, 1 M NaCl and RNA extracted for target identification.

4.2.26. Confocal microscopy:

The co-localization of Ago2 and Ago3 with mitochondria was analyzed by confocal microscopy. HEK293-MTRFP cells were plated in 24 well optical bottom plate (Greiner Bio, USA) with cell density of 1.5×10^5 per well. After 12 hrs of plating, the cells were transfected with pEGFPC1-Ago2, pEGFPC1-Ago3 and pAcGFP-N1 as described earlier. After 24 hrs of transfection, nucleus was stained with Hoechst. The cells were analyzed by Leica TCS SP5-II confocal microscope (Leica Microsystems, Germany) by sequential imaging of Hoechst, GFP and RFP fusion proteins using 63X objective with 3.0 X zoom.

4.2.27. Cell viability and death assays:

The cellular viability was determined by MTT assay as described previously (Prajapati et al., 2014). Briefly, 5 X 10^3 cells were plated in 96 well plate and transfected with mimic/inhibitor as described above. MTT was performed by incubating cells with 0.1 mg/ml MTT for 2 h at 37 ˚C. The formazan crystals were then dissolved in DMSO and absorbance was measured at 510 nm using microplate reader μQuant (Biotech Instruments, USA).

The caspase 3/7 activity was determined using Caspase-GloR 3/7 Assay kit as described previously (Bhatelia et al., 2014). Briefly, 4000 cells were plated in 96 well optical bottom plates and transfected with mimic/inhibitor after 16 h. After 24 h of transfection, Caspase-GloR 3/7 (10 μl) reagent was added to each well and luminescence was measured using TriStar² LB 942 Multimode Microplate Reader, Berthold Technologies, Germany.

The PARP cleavage was determined by western blotting as described previously. To study the cell death, HEK293 cells were seeded at a density of 4.5×10^5 per well in six well plate and miR-320a was transfected. After 48 h of transfection, cells were harvested, washed with ice cold PBS and lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 1% NP40, 1 mM PMSF). Protein concentration was determined by Bradford assay and equal protein resolved on 10% SDS-PAGE. Protein was electroblotted on PVDF membrane at 100 V for 1 h at 4 °C. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in PBS) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation membrane was washed three times with PBS-T (PBS containing 0.1% Tween 20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with PBS-T and signal visualized by using EZ-ECL chemiluminescence detection kit for HRP (Takara, Japan) by exposing to X-ray film.

4.2.28. Colony formation assay:

The clonogenic activity was performed as described previously (Tomar et al., 2013). Briefly, the cells were plated in 48 well plate and transfected with mimic/inhibitor after 16 h. After 24 h, the cells were trypsinised counted and 3000 cells were plated in 30 mm dishes and incubated for one week. The colonies were fixed in chilled methanol and

stained with 0.2% crystal violet dissolved in DPBS. The stained colonies were washed in DPBS and counted. Similarly, the CFU assays were performed in the presence of NAC. Plating efficiency was calculated as percent of colonies formed to the number of cells formed.

4.2.29. Migration assay:

The role of miR-320a in migration was determined by scratch assay as described previously (Tomar et al., 2012). Briefly, 2.5 X 10^5 cells were plated in 12 well plate, transfected with miR-320a mimic and after 24 hours, a vertical wound was created using a sterile P200 micropipette tip (Axygen Inc., USA). The wells were washed and replaced with fresh medium, first image was acquired at zero time point using inverted phase contrast microscope (1X81 Olympus, Japan) at 10X magnification and the same scratch area was examined, photographed and analyzed using T-Scratch program (www.cse-lab.ethz.ch).

4.2.30. LDH assay:

To determine the role of miR-320a on glycolytic shift, the cells were transfected with mimic. After 24h, the cells were lysed and incubated with buffer containing NAD^+-catalyst, Iodo-tetrazolium chloride -dye and sodium lactate for 30 min and the absorbance was recorded at 490 nm as per manufactures instructions provided in cytotoxicity detection kit (LDH), Roche, Germany.

4.2.31. Proliferation assay:

The cells were plated in 24 well plate and transfected with mimic. After 24 hrs, log phase cells were counted at seeded in 24-well dishes (Corning), with initial seeding density of 5,000 cells per well. After overnight incubation, replica of adhered cells was counted to determine initial count at time of treatment. Cells were washed twice in phosphate buffered saline (PBS) and treated with Glycolysis Media (DMEM supplemented with 4mM glutamine and 10mM glucose, 10% FBS) and OXPHOS medium (DMEM

supplemented with 4mM glutamine and 10mM galactose, 10% FBS) (Aguer et al., 2011). Final cell counts were measured 4 days after treatment, and proliferation rate was calculated.

Proliferation Rate = {log2 (Final cell count (day 5)/Initial cell count (day 1))}/4 (days) (Doublings per day)

4.2.32. Analysis of tumorigenic potential in nude mice:

miR-4485 was cloned in pEP-miR-puro (Cell Biolabs Inc., San Diego, CA). Briefly, a fragment encoding miR4485 precursor was PCR amplified from human DNA with the following primers containing BamHI (5'-primer) and Nhe1 (3'-primer) restriction nuclease sites: 5'- GAGAGGATCCGCATGCCCACACGGCCCGGT

and 5'- GAGAGCTAGCGGGCAGGTCAATTTCACTGG.

The 254 bp fragment contained 57 bp stem-loop miRNA precursor, plus 97 bp upstream and 100bp downstream regions. The fragment was digested with BamHI and NheI restriction nucleases and cloned into micro RNA expression plasmid pEP-miR-puro (Cell Biolabs Inc., San Diego, CA). An empty vector pEP-miR-Null) was used as a control. The plasmids pEP-miR-4485 and pEP-miR-Null were transfected into MDA-MB231 (triple-negative breast carcinoma) using BioTool transfection reagent. Four days after the transfection the cells were serially diluted and seeded onto 96-well plates in the medium containing 2 µg/ml puromycin. Individual puromycin-resistant clones were harvested in 2 weeks. To avoid clonogenic variations six clones from each transfection series were pooled together and the combined cultures were used for xenografts experiments. Subconfluent cell cultures (two days after seeding by splitting confluent cultures at 1:6) were trypsinized, pipetted in complete growth medium, washed in PBS, counted and used immediately for inoculations into nude mice.

Animal studies were performed according to the rules and protocols approved by the Bioethical committee of the Engelhardt Institute of Molecular Biology, Moscow, Russia.MDA-MB-231 sublines containing either pEP-miR-4485-puro or control pEP-Null-puri plasmids were inoculated subcutaneously into right and left hind limbs of 5 weeks old male athymic Balb/c nu/nu mice, 5 x 10^6 cells in 0.1 ml. Twelve mice (24 tumor sites) were injected with either MDA-MB-231 subline. As soon as tumors become

visible (by day 18-21) the mice were inspected weekly and tumor size was measured using electronic calipers. Tumor volume was calculated according to the formula: ellipsoid volume: 1/6 ∗ pi ∗ a ∗ b ∗ c, where a, b, and c are linear sizes of the tumor in three dimensions.

4.2.33. Statistical analysis:

The data is represented as SEM of 2-4 independent experiments. The data sets were normalized considering the values of controls as 100%. The comparisons between data sets were performed by unpaired two-tailed Student's t test using GraphPad Prism 5. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to be statistically significant.

4.2.34. List of primers:

Table 4.1 Details of primers used in qPCR.

S. No.	Gene	Forward primer	Reverse Primer
1	12SrRNA	GGTACACACCGCCCGTC	AGTTTTTTTTTTTTTTGTTCGTCCAAGT
2	5SrRNA	GGTCTACGGCCATACCACC	CAGTTTTTTTTTTTTTTAAAGCCTACAG
3	U6snRNA	GGTGCTCGCTTCGGCA	TCCAGTTTTTTTTTTTTTTTAAAAATATGGAAC
4	miR-4485	AGUAACGGCCGCGGUAC	TCCAGTTTTTTTTTTTTTTTTAGG
5	pre-miR-4485	GCAGAGAGGCACCGCCUGCCCA	GTCCAGTTTTTTTTTTTTTTTTTTTGCACAGTTAGGGTA
6	miR-320a	CGCAGAAAAGCTGGGTTGA	GTCCAGTTTTTTTTTTTTTTTTCGCCCT
7	miR-23a	CATCACATTGCCAGGGAT	CGTCCAGTTTTTTTTTTTTTTTGGAA
8	miR-103	AGAGCAGCATTGTACAGGG	GTCCAGTTTTTTTTTTTTTTTTCATAG
9	let-7g	GGCAGTGAGGTAGTAGTTTGT	GGTCCAGTTTTTTTTTTTTTTTAACTGTAC
10	293m8m0165	GCTTTAACCCTTCCCCAGGTTCCCATT	GTGGGCAGGGGCTTATTG
11	293m8m0134	GTCGGGCGGGAGTGGT	TCCAGTTTTTTTTTTTTTTTAAAAGCCA
12	293m8m0056	CAGCGACTGGACTGGAGCGG	GGTCCAGTTTTTTTTTTTTTTCACCCGGC
13	293m8m099	GCAGCAAAATGATGAGGTACC	GGTCCAGTTTTTTTTTTTTTTTTAGCAGG
14	293m8m0022	GCAGATCAGGCTGTGTAGTAT	CAGTTTTTTTTTTTTTTTAACCCC
15	293m8m0195	AGAAGAGAAAGGCTGAAGG	AGTTTTTTTTTTTTTTTCATCCC
16	293m8-m0007	GCAGACAAGGAAGGACAAGA	GTCCAGTTTTTTTTTTTTTTTTTTCACAC
17	293m8-m0018	GCAGCCAGGCTGGCT	TCCAGTTTTTTTTTTTTTTTTTCTCCTC
18	293m8-m0025	CAGAGGGAGGAGGCCT	GTTTTTTTTTTTTTTTTCGGCG
19	293m8-m0046	GCAGAGGGTAGGAGTCAGT	AGTTTTTTTTTTTTTTTTTCCCAA
20	293m8-m0059	AGCAGGGGAAGGGAAG	AGTTTTTTTTTTTTTTTTTGCTTCA
21	293m8-m0071	CAGAAGGAAGATGGCGG	CCAGTTTTTTTTTTTTTTTTTTCTACTG
22	293m8-m0072	GCAGAGTAGGATGATGGGGT	AGTTTTTTTTTTTTTTTTTTCCGTGA
23	293m8-m0089	AGCTAGAGGGGAAGGGAA	CAGTTTTTTTTTTTTTTTTTTTCCTAT

24	**293m8-m0095**	GCGGGAGTGGGTGG	AGTTTTTTTTTTTTTTTTTTTCTGACT
25	**293m8-m0098**	CAGAAATGGGAGGGGAA	CCAGTTTTTTTTTTTTTTTTCATCA
26	**293m8-m0169**	GCAGCTAGGGAATGGGA	CCAGTTTTTTTTTTTTTTTTTGCTAC
27	**293m8-m0182**	AGAGGGCCGAAGGGT	AGTTTTTTTTTTTTTTTTTCAGCTT
28	**ND4**	ACAAGCTCCATCTGCCTACGA	GGCTGATTGAAGAGTATGCAATGA
29	**CYB**	AACCGCCTTTTCATCAATCG	AGCGGATGATTCAGCCATAATT
30	**16SrRNA**	GAAACCAGACGAGCTACCTAAG	CGCCTCTACCTATAAATCTTCCC
31	**ND1**	ATACCCCCGATTCCGCTACGAC	GTTTGAGGGGGAATGCTGGAGA
32	**ND2**	ATTCCATCCACCCTCCTCTC	TGGGGTGGGTTTTGTATGTT
33	**ND3**	CCCTCCTTTTACCCCTACCA	GGCCAGACTTAGGGCTAGGA
34	**ND4L**	TAACCCTCAACACCCACTCC	GGCCATATGTGTTGGAGATTG
35	**ND5**	CAAAACCTGCCCCTACTCCT	GGGTTGAGGTGATGATGGAG
36	**ND6**	GGGTGGTGGTTGTGGTAAAC	CCCCGAGCAATCTCAATTAC
37	**ATP6**	CGCCACCCTAGCAATATCAA	TTAAGGCGACAGCGATTTCT
38	**ATP8**	ATGGCCCACCATAATTACCC	GCAATGAATGAAGCGAACAG
39	**COX1**	CGATGCATACACCACATGAA	AGCGAAGGCTTCTCAAATCA
40	**COX2**	TGAAGCCCCCATTCGTATAA	ACGGGCCCTATTTCAAAGAT
41	**COX3**	GGCATCTACGGCTCAACATT	CGAAGCCAAAGTGATGTTTG
42	**GAPDH**	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
43	**Actin**	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
44	**NDUFS1**	TACTCGCTGCATCAGGTTTG	CATGCATACGTGGCAAAATC
45	**NDUFA10**	CACCTGCGATTACTGGTTCAG	GCAGCTCTCTGAACTGATGTA
46	**MITA**	CGCCTCATTGCCTACCAG	ACATCGTGGAGGTACTGGG
47	**MFN2**	ATGTGGCCCAACTCTAAGTG	CACAAACACATCAGCATCCAG
48	**STAT3**	TTCTGGGCACAAACACAAAAG	TCAGTCACAATCAGGGAAGC
49	**XIAP**	GCACGGATCTTTACTTTTGGG	GGGTCTTCACTGGGCTTC
50	**TRIM4**	CACCAGACTCACGCCATG	ACGAGATTACGCTGAGACTTAAG
51	**RNP**	CCCCGTTCTCTGGGAACTC	TGTATGAGACCACTCTTTCCCATA
52	**MITO**	CACTTTCCACACAGACATCA	TGGTTAGGCTGGTGTTAGGG

Table 4.1. Details of primers used in qPCR. *The list of primers along with their sequence details used in the study are tabulated.[1.]Name of gene; [2.]Sequence of forward primer; [3.]Sequence of reverse primer.*

Table 4.2 Details of primers used for mitochondrial RNA processing

S. No	Flanking Region[1]	Forward Primer [2]	Reverse Primer [3]	amplicon (in bp) [4]
1	16SrRNA-ND1	GAAACCAGACGAGCTACCTAAG	GTTTGAGGGGGAATGCTGGAGA	**2353**
2	ND1-ND2	ATACCCCCGATTCCGCTACGAC	TGGGGTGGGTTTTGTATGTT	**1307**
3	ND2-COI	ATTCCATCCACCCTCCTCTC	AGCGAAGGCTTCTCAAATCA	**2135**
4	COI-COII	CGATGCATACACCACATGAA	ACGGGCCCTATTTCAAAGAT	**1021**
5	COXII-ATP8	TGAAGCCCCCATTCGTATAA	GCAATGAATGAAGCGAACAG	**535**
6	ATP8-ATP6	ATGGCCCACCATAATTACCC	CGCCACCCTAGCAATATCAA	**762**
7	ATP6-COIII	CGCCACCCTAGCAATATCAA	CGAAGCCAAAGTGATGTTTG	**858**
8	COIII-ND3	GGCATCTACGGCTCAACATT	GGCCAGACTTAGGGCTAGGA	**590**
9	**ND3-ND4L**	**CCCTCCTTTTACCCCTACCA**	**GGCCATATGTGTTGGAGATTG**	**442**

Table 4.2. Details of primers used for mitochondrial RNA processing. *The list of primers used to amplify the flanking regions of adjacent mitochondrial genes is tabulated.1.Name of flanking region; 2.Sequence of forward primer; 3. Sequence of reverse primer;4. Size in bp*