4. Materials and Methods

4.1. Cells and cell culture

MCF7, T47D and HBL100 breast cancer cell lines were obtained from National Center for Cell Sciences, Pune, India. MDA-MB-231 was a gift of Prof. R. P. Singh. (Central University of Gujarat, India). MCF-7, ZR-75-1 and T47D cells were cultured in RPMI 1640 (Life Technologies, USA), HBL100 in Dulbecco's modified Eagle's medium (Life Technologies, USA) and MDA-MB-231 in Leibovitz's L-15 media (HI-MEDIA, India). The media used were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). Cells were incubated at 37°C, 5% CO2 in specified media. MCF 10A cells were cultured in DMEM F12 (INVTROGEN) base media supplemented with (10%horse serum) along with the following supplements: 1) cholrea toxin (100ng/ml) 2) EGF (20ng/ml) 3) Hydrocortisone (500ng/ml) 4) insulin (cell culture tested) (10ug/ml).

4.2. Plasmids and reagents

MITA cloned in pCMV6-ENTRY plasmid was a gift from Dr. Hong-Bing Su (Wuhan University, China). p65shRNA (RelA1 shRNA and RelA2 shRNA) and control shRNA were provided by Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). MITA shRNA was a generous gift by Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). FDEVDG$_2$ construct was gifted by Dr. Brian Seed (Department of Genetics, Harvard Medical School, Cambridge Street, Boston). TFEB-GFP was provided by Prof. Andrea Ballabio (Scientific Director, TIGEM. LC3-GFP was provided by Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan), LC3-mCherry-GFP, p62-mCherry- GFP and p62-GFP by Dr. Terje Johansen (Dept. of Biochemistry, Institute of Medical Biology, University of Tromsø). MITA-mcherry (mouse) and MITA-mcitrine (human) constructs were gifted by Dr. Veit Hornung (University Hospital of Bonn, Sigmund-Freud-Strasse 2553127 Bonn, Germany)[115].

Primary antibodies used were MITA (Proteintech, USA), Caspase-8, PARP, p65 (Cell Signaling Technology, Inc, USA), β-Actin and GAPDH (Abcam, USA), IκBα (Cell Signaling Technology, Inc, USA), NDP52 (Cell Signaling Technology, Inc, USA), TFEB

and GFP(Abclonal Technology, USA), LC-3 (Sigma-Aldrich, USA) and p62(GeneTax, USA). HRP-conjugated anti-rabbit and anti-mouse antibodies (Thermo Scientific, USA) were used. The reagents used were TNF-α (Tumor necrosis factor) (Biovision, USA), PDTC (Pyrrolidine dithiocarbamate) (Sigma Aldrich, USA) zVAD-fmk (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone) (Biovision, USA), IETD-fmk (Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone) (Clontech, USA), rapamycin, NH_4Cl and bafillomycin (Sigma-Aldrich, USA).

4.3. Transfection

MCF-7 cells were transfected using standard calcium phosphate method [118] or with Biotool DNA transfection reagent (Biomake, USA) as per manufacturer's protocol. MCF-7 cells and T47D were transfected using X-treme gene transfect reagent (Roche, Germany). HBL100 cells were transfected with X-tremeGENE 9 DNA transfection reagent (Roche, Germany) as per the manufacturer's protocol.

4.4. Collection of tissues

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 and Protein extraction. Ethical approval from institute's ethical committee was taken prior to collection of sample for each of the patients. Details of the tissue specimen used are given in following Table-1.

4.5. Immunohistochemistry

After de-paraffinization in xylene and hydration by gradient alcohol series, antigen retrieval was done by heat treatment in citrate buffer (10 mM, pH 6.0). The sections were incubated in 10 % NSS (Normal sheep serum) for 20 min to block non-specific binding

and further incubated with antibodies against MITA (1:1000) in 0.1 % BSA overnight at 4°C. Sections were stained using Quick Universal ABC KIT (Vector) followed by peroxidase staining reaction with DAB/H_2O_2 as chromogen. The stained sections were observed under bright field light microscope (Nikon Eclipse 80i; Nikon Instech Co. Ltd., Kawasaki, Kanagawa).

Tissue Specimen	Details
1 : Normal(A)	Infiltating ductal carcinoma (nottingham grade II),no evidence of malignancy
2: Tumor	MRM specimen ,infiltrating ductal carcinoma (nottingham grade II) with axillary node metastasis (2/18),ER,PR-positive , HER 2 -negative
3: Tumor	Incisional biopsy specimen ,atypical medullary carcinoma, ER,PR,HER- negative
4: Tumor(A)	Infiltating ductal carcinoma (nottingham grade II),no evidence of malignancy, MRM spcimen
5:Tumor	Infiltating ductal carcinoma (nottingham grade II),positive for malignant cells,MRM specimen,ER,PR-positive , HER 2 -negative
6:Tumor	MRM specimen ,infiltrating ductal carcinoma (nottingham grade II) with axillary node metastasis (3/18),ER,PR-40% positivity , HER 2 -negative
7:Normal(B)	infiltrating ductal carcinoma (nottingham grade II)) with axillary node metastasis (2/18),ER,PR,HER 2 -negative
8:Tumor(B)	infiltrating ductal carcinoma (nottingham grade II)) with axillary node metastasis (2/18),ER,PR , HER 2 -negative
9:Tumor	infiltrating ductal carcinoma positive for malignant cells (2/18),ER,PR-positive , HER 2 -negative

Table-1 Details of the tissue specimen used for the study

4.6. Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life technologies, USA) and was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) or SuperScript VILO cDNA Synthesis Kit (Life technologies, USA) according to the manufacturer's instructions. Real time PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) or SYBR mix (life technologies, USA) or Applied Biosysyems as per the manufacturer's instructions.

Specific primers of the genes are listed below.

1. MITA: Fwd 5′- CGCCTCATTGCCTACCAG -3′;

   Rev, 5′-ACATCGTGGAGGTACTGGG-3′;

2. TNF-α: Fwd 5′-CCCAGGGACCTCTCTCTAATCA-3′;

   Rev 5′-GCTACAGGCTTGTCACTCGG-3′;

3. β-Actin: Fwd 5′-TCGTGCGTGACATTAAGGGG-3′

   Rev 5′-GTACTTGCGCTCAGGAGGAG-3′;

4. 16s rRNA: Fwd 5′-GAAACCAGACGAGCTACCTAAG-3′;

   Rev 5′-GCCTCTACCTATAAATCTTCCC-3′;

5. GAPDH: Fwd 5'-AGAAGGCTGGGGCTCATTTG-3′;

   Rev 5′AGGGGCCATCCACAGTCTTC 3′

6. PGC1: Fwd 5'- GAGCCGAGCTGAACAAGCAC-3′;

   Rev 5′ -AGACACATTGAACAATGAATAGGATG - 3′

7. NRF1: Fwd 5'- GGCACTGTCTCACTTATCCAGGTT-3′;

   Rev 5′ -CAGCCACGGCAGAATAATTCA- 3′

8. NRF-2: Fwd 5'- AAACCAGTGGATCTGCCAAC-3′;

Rev 5′ - GACCGGGAATATCAGGAACA -3′

9. Lysosomal protein transmembrane 4α: Fwd 5'- GCCTGTGTTCTTTTTGCCGT-3′;

Rev 5′- GGCAACTGAGGACGAAGTCA- 3′

10. ATPase: Fwd 5'- TCTGGTGATGACTTGAAACTGC-3′;

Rev 5′ - GTCTAGGAAGCTGGCGAGTG -3′

11. mtDNA: Fwd 5'- CACTTTCCACACAGACATCA -3′;

Rev 5′ - TGGTTAGGCTGGTGTTAGGG -3′

12. RNAse P: Fwd 5'- CCCCGTTCTCTGGGAACTC -3′;

Rev 5′ - TGTATGAGACCACTCTTTCCCATA -3′

4.7. Western blot

Cells were plated at a density of 4.5×10^5 cells/well in the six well plate and transfected with indicated expression plasmid or shRNAs using calcium phosphate method. After 48 hr of transfection, the cells were harvested, washed with ice cold PBS and lysed in buffer A (150 mM NaCl, 30 mM Tris–Cl, 10% Triton X-100, 10% Glycerol, 1X Protease Inhibitor (Roche, Germany). The equal protein was loaded and resolved on 11% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1 % Tween-20 in TBS-0.02M Tris-Cl, 0.15M NaCl) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation, the membrane was washed three times with TBS-T (TBS 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 TBS-T and signal was visualized by using EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing it to X-ray film.

For the western blotting from tissue samples, the tissue samples obtained from breast cancer patient was snap frozen in liquid nitrogen. The tissue was homogenized to fine powder in the presence of liquid nitrogen and lysed in RIPA lysis buffer(50 mM Tris [pH 7.4], 50 mM NaCl, 5mM EDTA, 1mM EGTA, 0.1%SDS, and 1% Triton-x100, 0.2% protease inhibitor cocktail, 1mM PMSF, 2mM NaF and 2.5 mM Sodium pyrophosphate). The lysates were freeze thawed three times in liquid nitrogen. After 15 min of centrifugation (4,000 rpm at 4°C), the supernatant was saved to use as a whole-cell lysate. The protein was analyzed by western blotting as described above.

4.8. NF-κB luciferase assay

To assess NF-κB activity, MCF-7 cells were plated at density of $1X10^5$ cells/well in 24 well plate and luciferase assay was performed using Dual-Glo luciferase assay system (Promega, USA). MCF-7 cells were seeded at density of $1.5X10^5$ cells per well in 24 well plate. The cells were transfected with NF-κB luciferase reporter plasmid together with indicated expression vector. Renilla luciferase expressing plasmid was used for normalizing transfection efficiencies. The total amount of DNA (500 ng) was kept constant by inclusion of vector. The luciferase activity was determined using Dual-GloH luciferase assay system according to the manufacturer's instructions (Promega) and measured with a Centro LB 960 Luminometer (Berthold Technologies).

4.9. Caspase 3/7 and caspase-8 activity assay

The activity was performed using Caspase-Glo^R 3/7 Assay kit (Promega, USA) or Caspase- Glo^R 8 Assay kit (Promega, USA). Cells were plated at the density of $4X10^4$ cells per well in 96 well white clear bottom plate and transfected with indicated expression plasmids or shRNAs and respective controls. Caspase-GloR 3/7 (10 µl) reagent or caspase-8 Glo reagent was added to each well and luminescence was measured with a Centro LB 960 Luminometer (Berthold Technologies, Germany).

4.10. Secreted GLUC activity assay for caspase activation in culture supernatant

Cells were plated in 24 well plate and co-transfected with MITA and a reporter construct FDEVDG2 [119]. The construct has a DEVD site placed in between GLUC reporter (*Gaussia* luciferase) and β-actin, so once the substrate site is cleaved by the caspases,

luciferase will be secreted in the supernatant. After 24 hours of transfection, the cells were treated with specific inducer of cell death. The supernatant (SN) was collected and centrifuged at 14000 rpm for 5min. Supernatant was diluted in 1:10 in 100 µl 1X lysis buffer. The substrate was added. (50 µl) of substrate was added to 10 µl of this mixture and was analyzed with Centro LB 960 Luminometer (Berthold Technologies, Germany). Attached cells were lysed in 1X lysis buffer 100 µl per well for 15 minutes. 10 µl of lysate was added to 50 µl of 1X substrate to detect that cellular GLUC activity luminescence was measured with a Luminometer. Total caspase activity was calculated in the SN as well as in cell lysate, and total caspase activity was calculated as the ratio of the caspase activity in SN verses (vs) cellular caspase activity.

4.11. Trypan blue exclusion assay

Cells were plated at the density of 1 $x10^5$ cells/well in 24 well plate and transfected with the specific constructs. After 24 hours of transfection, the cells were treated with TNF-α (10 ng/ml) for 24 hours and stained with trypan blue. Minimum 100 cells per view were counted and percentage of cell survival was plotted.

4.12. MTT assay

The cellular proliferation was analyzed by MTT assay. MCF7 cells were plated in 24-well plate at a density of $1X10^5$ cells/well. The cells were transfected with MITA as well as vector. After 24 hours of transfection, 20 µl of MTT solution (5 mg/ml) (Serva, Germany) was added to each well and incubated for 2 hours. After incubation, 500 µl of solubilization buffer (2% w/v SDS, 18.5% w/v formaldehyde) was added to dissolve the precipitate of purple colored formazan and color intensity was monitored using colorimetric microplate reader (BioTek Instruments, Inc. USA) at 595 nm wavelength.

4.13. Colony formation assay and scratch assay

Clonogenic activity of cells and migration ability of cells were determined as described previously [120, 121].

4.14. Analysis of reactive oxygen species (ROS) and mitochondrial potential

Cells were plated in 12-well plate at the density of $2X10^5$cells/per well. Cells were transfected with MITA as well as vector control. Intracellular ROS production was measured by CM-H_2DCFDA (10 μM) and mitochondrial potential was measured using tetramethylrhodamine, methyl ester (TMRM). The cells were stained with CM-H_2DCFDA (10 μM)/TMRM (100 μM) in DMEM for intracellular ROS quantification. The cells were washed thrice using DPBS. ROS levels were quantified by spectrofluorometric analysis using F-7000 Fluorescence Spectrophotometer (Hitachi, Japan).

4.15. Sub cellular fractionation

a. Isolation of mitochondria

MCF-7 cells were plated at the density of 2×10^6 cells/90 mm^2 dish and transfected with indicated constructs using Biotool transfection reagent. Cells were treated with NH_4Cl for 4 h and washed with cold PBS, harvested by centrifugation at 600 ×g for 10 min. Cell pellets were resuspended in mitochondria isolation buffer (200 mM mannitol, 70mMsucrose, 1 mMEGTA, 10mMHEPES; pH 7.4, 1× protease inhibitor cocktail (Sigma-Aldrich, USA) and incubated on ice for 5 min. The cells were disrupted by passing through a 24G sterile syringe needle and centrifuged at 600 ×g for 10 min to separate nuclei and cell debris. The supernatant (cytosolic fraction) was collected and centrifuged again at 10,000 ×g for 10 min. The pellet (mitochondrial fraction) were washed twice with isolation buffer and lysed with mitochondria lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP40 and 1× Protease Inhibitor Cocktail). The mitochondrial and cytosolic protein concentrations were assessed using Bradford protein estimation assay. Equal proteins were loaded and resolved on 12% SDS-PAGE and analyzed by Western blotting using indicated antibodies.

b. Preparation of Nuclear fraction

MCF-7 cells were plated at density of $1X10^6$ in 60-mm^2 dish and transfected with MITA. After 24 hours of transfection, the cells were treated with indicated chemical. After incubation, the cells were washed with chilled DPBS (GIBCO, Invitrogen, USA), resuspended in three-fold volume of buffer-A (10 mM HEPES buffer, pH 7.9, 0.1 mM

EDTA, 10 mM KCl, 0.4% (v/v) NP40, 0.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 30 min. Cell lysates were centrifuged at 15,000 g for 15 min and the resulting supernatant was collected as cytosolic fraction, re-centrifuged as above to remove nuclear remnants. Pellets were washed three times with buffer-A and resuspended in 2 fold volumes of ice cold buffer-B (20 mM HEPES buffer, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and incubated in ice for 20 min. The lysate were centrifuged at 15000 g for 15 min to obtain nuclear fraction in the supernatant. The fractions were analyzed for the presence of indicated proteins using western blotting as described above.

4.16. Complex-I activity assay

MCF-7 cells were plated at the density of 5×10^5 cells/well in the 6-well plate. After overnight incubation, the cells were transfected and treated as indicated. The cells were harvested and washed with cold DPBS. The cells were subjected to 2–3 freeze–thawcycles in a freeze–thaw complete solution (0.25Msucrose, 20 mM Tris–HCl (pH 7.4), 40 mM KCl, 2 mM EDTA supplemented with 1 mg/ml fatty acid-free BSA, 0.01% Digitonin and 10% Percoll). The cells were washed again with the freeze–thaw solution devoid of digitonin and resuspended in Complex I assay buffer (35 mM potassium phosphate (pH 7.4), 1 mM EDTA. 2.5 mM NaN3,1 mg/ml BSA, 2 μg/ml antimycin A, 5 mM NADH). The reaction was started by adding 80 μg of cell lysate to 500 μl of assay buffer in 1 ml quartz cuvette. Complex I activity was measured for 3 min by monitoring the decrease in absorbance at 340 nm after the addition of 2.5mM acceptor decylubiquinone indicating the oxidation of NADH.

Complex-I activity was also performed using In-gel activity assay. The cells were plated in 60 mm dish at density of $5 \text{ X } 10^6$ and cultured for 24 hours. The cells were transfected with specific constructs and complex-I activity was determined by BN-PAGE (Blue-Native Page). Acrylamide (50% w/v)/ Bisacrylamide (0.5% w/v) solution was prepared such that it yielded highly porous gels on polymerization, for the separation of higher order protein complexes. The final concentration of the stacking gel was 4% and that of the resolving gel was 10%. Purified mitochondrial fraction was mixed with sample buffer and non-gradient BN-PAGE was performed at room temperature as described previously

[122]. The gel was stained with complex-I staining solution (50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH).

4.17. ATP assay

MCF-7 cells were plated and transfected with indicated constructs and treated with indicated chemicals. The cells were lysed in 0.1 % TritonX-100 and ATP levels were analyzed using ATP Bioluminescence kit CLS II (ThermoFisher Scientific, USA) as per the manufacturer's protocol.

4.18. Microscopic analysis for autophagy assays

Cells were plated on coverslips and transfected with specified constructs. After 36 h of transfection, cells were fixed with 4% para-formaldehyde and stained with DAPI. The cells were analyzed by Leica DMI 8000 fluroscent microscope for LC3-GFP puncta analysis at 40X. All the other experiments were analyzed using Leica SP8 confocal microscope (Leica Microsystems, Germany) by sequential imaging using 63X objective with 3X zoom (Confocal microscopy).

4.19. Statistical analysis

Data are shown as mean ± SEM for no. of times experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. The experiments were performed minimum two times independently and $p<0.05$ was considered as statistically significant. GraphPad Prism was used to perform all the statistical analysis.