7. MITA regulates mitophagy in breast cancer

7.1. MITA initiates autophagosome formation in breast cancer cells

MITA acts as a tumor suppressor in breast cancer and it also induces cell death but what contributes to MITA induced cell death is not well understood. The increased level of autophagy in cancerous cells facillitate the increasing nutrient demand[80, 138-140]. Though autophagy increases survival of cancerous cells; it is also known to induce cell death. Moreover, as discussed in section 6.3, MITA activates NF-κB which is reported to regulates essential autophagy proteins as discussed in 2.3.2. We hence investigated role of MITA in the regulation of autophagy process. MITA was expressed in MCF-7 cells and autophagy was monitored using LC3 western blotting. LC3 is a marker of autophagy which specifically marks autophagosome[141]. MITA transfected cells were collected and lysed after 24hrs of transfection. The lysate was than subjected to western blot analysis. There was a significant increase in 16 KDa band corresponding to LC3-II protein in MITA transfected cells as compared to control was observed (Fig 7.1A). This suggest that autophagosome formation is induced in the presence of MITA. The result was further confirmed by microscopy analysis of LC3 protein. MCF-7 cells were cotransfected with mMITA-mcherry and LC3-GFP and autophagosome formation was analyzed using fluroscent microscopy analysis. There was a significant increase in green coloured puncta was observed in the presence of MITA as compared to control (Fig 7.1B and C). p62 is an adaptor protein of autophagy and marker of autophagosome[142]. p62 puncta formation was hence analyzed using confocal microscope. MCF-7 cells were cotransfected with p62-mcherry and hMITA-mcitrine and autophagosome formation was analyzed under microscope. There was a significant increase in red coloured p62 puncta observed in the presence of MITA as compared to control (Fig 7.1 D and E). The results clearly indicated that MITA initiates the autophagy process and increases the autophagosome synthesis.

7.2. MITA inhibits autophagy flux

In several conditions such as Parkinson's disease and other lysosomal storage disease, autophagosomes are synthesized but they are not degraded by lysosome. It is hencefotrh essential to understand the autophagy flux to interprete autophagy regulation. To understand the autophagy flux, autophagy inhibitors were used such as Bafilomycin and NH_4Cl. Bafilomycin A1 is a specific inhibitor of V-ATPase; hence it inhibits

autophagosome and lysosome fusion and induces lysosomal dysfunction by increasing the pH of lysosome[143]. MCF-7 cells were

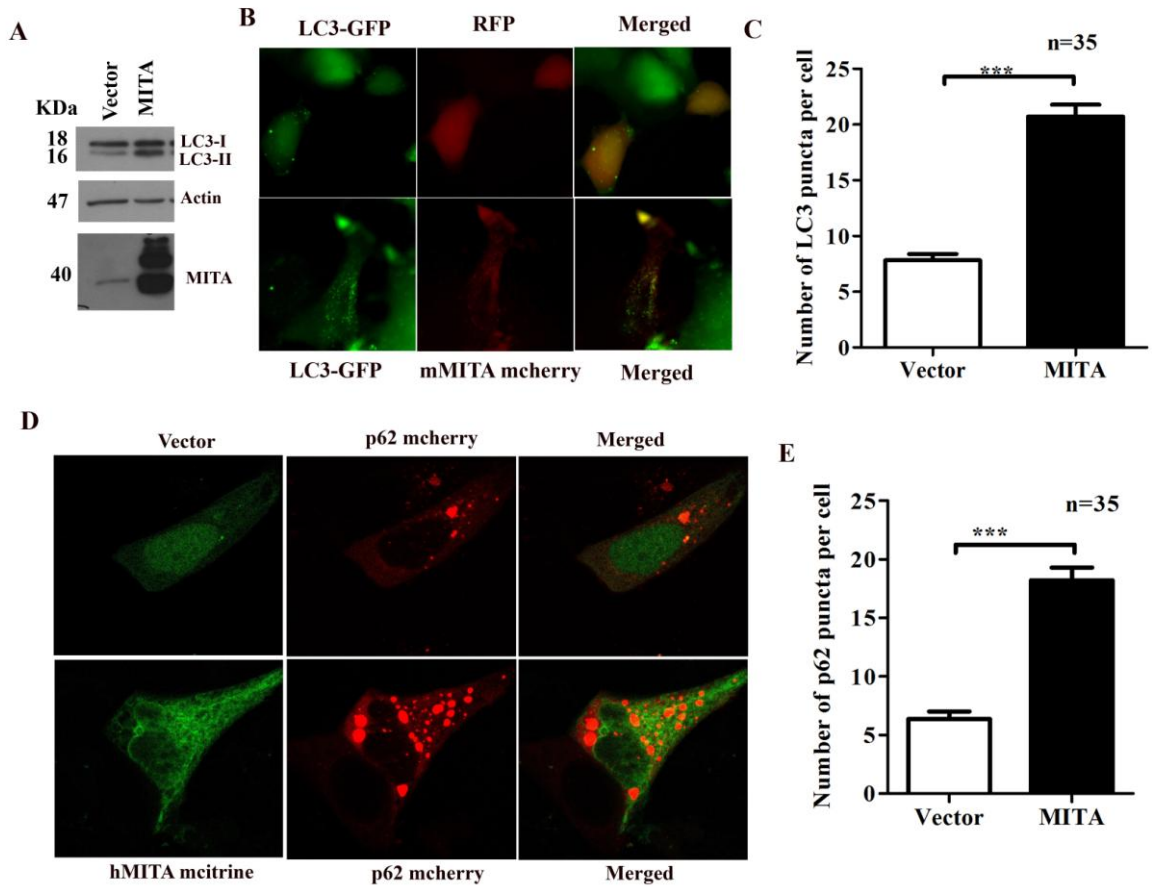


Figure-7.1. MITA initiates autophagosome formation in MCF-7:

(A) MCF-7 cells were transfected with MITA. After 24 hr of transfection cells were collected and lysed. The lysate was subjected to LC3 western blot analysis. The LC3-II protein was analyzed using LC3 specific antibody. (B) MCF-7 cells were cotransfected with LC3-GFP and mMITA-mcherry and green colored LC3 puncta (autophagosomes) formation was analyzed under fluorescent microscope. (C) Number of green puncta per cell was counted and graph was plotted for number of LC3 puncta formed inside the cell representing Fig 7.1B. (D) MCF-7 cells were cotransfected with p62-mcherry and mMITA-mcitrine and red colored p62 puncta (autophagosomes) formation was analyzed under fluorescent microscope. (E) Number of red puncta per cell was counted and graph was plotted for number of p62 puncta formed inside the cell representing Fig 7.1D.

transfected with MITA and treated with Bafilomycin. The cells were collected and lysed. The lysate was subjected to LC3 western blot analysis. Bafilomycin treatment did not increase the 16 KDa LC3-II band further in the presence of MITA than untreated MITA expressing MCF-7 cells (Fig 7.2A). The result suggests that though MITA initiates the synthesis of autophagy but the autophagosomes are either not degraded by lysosomes or the autophagosome lysosomes fusion is inhibited. The result was further confirmed by LC3-GFP cleavage assay. LC3-GFP is a fusion protein. During the process of autophagy, LC3-GFP is incorporated on the menbrane of autophagosome and will further fuse with lysosomes in normal autophagy flux condition. The fusion protein will be degraded and free GFP will be released. GFP being stable at lysosomal ph will not be degraded and hence will be detected by western blot analysis. MITA was expressed in MCF-7 cells and treated with rapamycin and/or NH_4Cl and analyzed using western blot analysis. Rapamycin is a positive regulator of autophagy[144, 145] whereas NH_4Cl alters the lysosomal pH thus inhibiting lysosomal fuction[146]. GFP cleavage assay clearly showed significant decrease in the 25 KDa cleaved GFP band in the presence of MITA than vector control (Fig 7.2 B). Rapamycin was taken as a positive control. Rapamycin treatment did not increase 25 KDa cleaved GFP band further in the presence of MITA; whereas clear increase in 25 KDa cleaved GFP band in the vector control was observed (Fig 7.2 B). NH_4Cl treatment inhibited the GFP cleavage in the absence or presence of MITA (Fig 7.2 B). LC3-GFP cleavage assay confirmed the defect in the autophagy flux. For further validation, tandem constructs (LC3-GFP-mcherry and p62-GFP-mcherry) were used. The tandem constructs flurosce yellow or orange in autophagosomes. The autophagosomes once fuses with lysosomes the GFP fluroscence is quenched in acidic pH conditions of lysosomes and mature autolysosome are detected as red puncta[146]. MCF-7 cells were cotransfected with LC3-GFP-mcherry and MITA and puncta formation was analyzed using confocal microscope. Number of orange as well as red colored puncta per cell were counted. Increased number of LC3 orange coloured puncta were observed as compared to red colored punta in MITA transfected cells as compared to vector control (Fig 7.2C and D). Similarly, MCF-7 cells were cotransfected with p62-GFP-mcherry and MITA and puncta formation was analyzed using confocal microscope. Similar to LC3 tandem construct, orange coloured p62 puncta increased significantly as compared to redcoloured puncta in thepresence of MITA (Fig 7.2E and F). These results further confirms that the

process of autophagy is defective. Autophagosomes formed are either not able to fuse with lysosomes or lysosomal functions are compromised. Rapamycin treatment increased the number of red coloured LC3 as well as p62 puncta as compared to orange one (Fig 7.2C-F).

7.3. MITA regulates mitophagy in breast cancer cells

MITA is an essential regulator of IFN signaling. Mitochondria provides a platform to recruit regualtors of inflammatory pathways. Mitophagy henceforth is an essential process to analyze in MITA overexpressing cells. Mitophagy is essential to maintain the mitochondrial homeostasis[147-149]. Mitophagy removes the damaged mitochondria and the cell then tires to replenish itself with healthy mitochondria[150, 151]. Therefore it is necessary to study mitochondrial turnover. The inhibtion of autophagy flux by MITA may lead to accumulation of defective organelle or reduced nutritent tunrnover which may negatively effect the tumor growth; hence we further analyzed mitophagy in presence of MITA. MCF-7 cells were transfected with MITA and treated with NH_4Cl. Mitochondrial fraction was isolated and analyzed for LC3-II western blotting. 16 KDa band corresponding to LC3-II increased in the presence of MITA in mitochondrial fraction as compared to vector control (Fig 7.3A). No significant difference was observed in LC3-II band in the presence or absence of MITA in NH_4Cl treated cells. NDP52 and p62 are mitochondrial marker of mitophagy and gets degraded as the autophagy progresses via lysosomal degradation[152, 153]. Intrestingly, the band corresponding to NDP52 *i.e* 52 KDa and p62 *i.e.* 62 KDa increased instead of decreasing in MITA expressing cells than in vector control cells in mitochondrial fraction. Increased level of LC3-II in mitochondrial fraction but no difference was observed in the presence or absence of MITA in NH_4Cl treated cells. This suggests the formation of mitophagosome but the mitophagy flux is inhibited. At the same time increased level of p62 and NDP52 confirms the defect in mitophagosome to lysosome fusion or lysosomal functions are compromised. Recruitment of LC3 and p62 on mitochondria was further confirmed by microscopic analysis. MCF-7 cells were cotransfected with p62-GFP or LC3-GFP and hMITA-mcitrine and stained with TMRM. Green colored p62 or LC3 GFP pucta colocalized on red coloured mitochondria as observed under confocal micropscope (Fig 7.3B-E). Confocal

microscopic analysis confirmed the recruitment of LC3 and p62 on mitochondria suggesting role of MITA in the regulation of mitophagy.

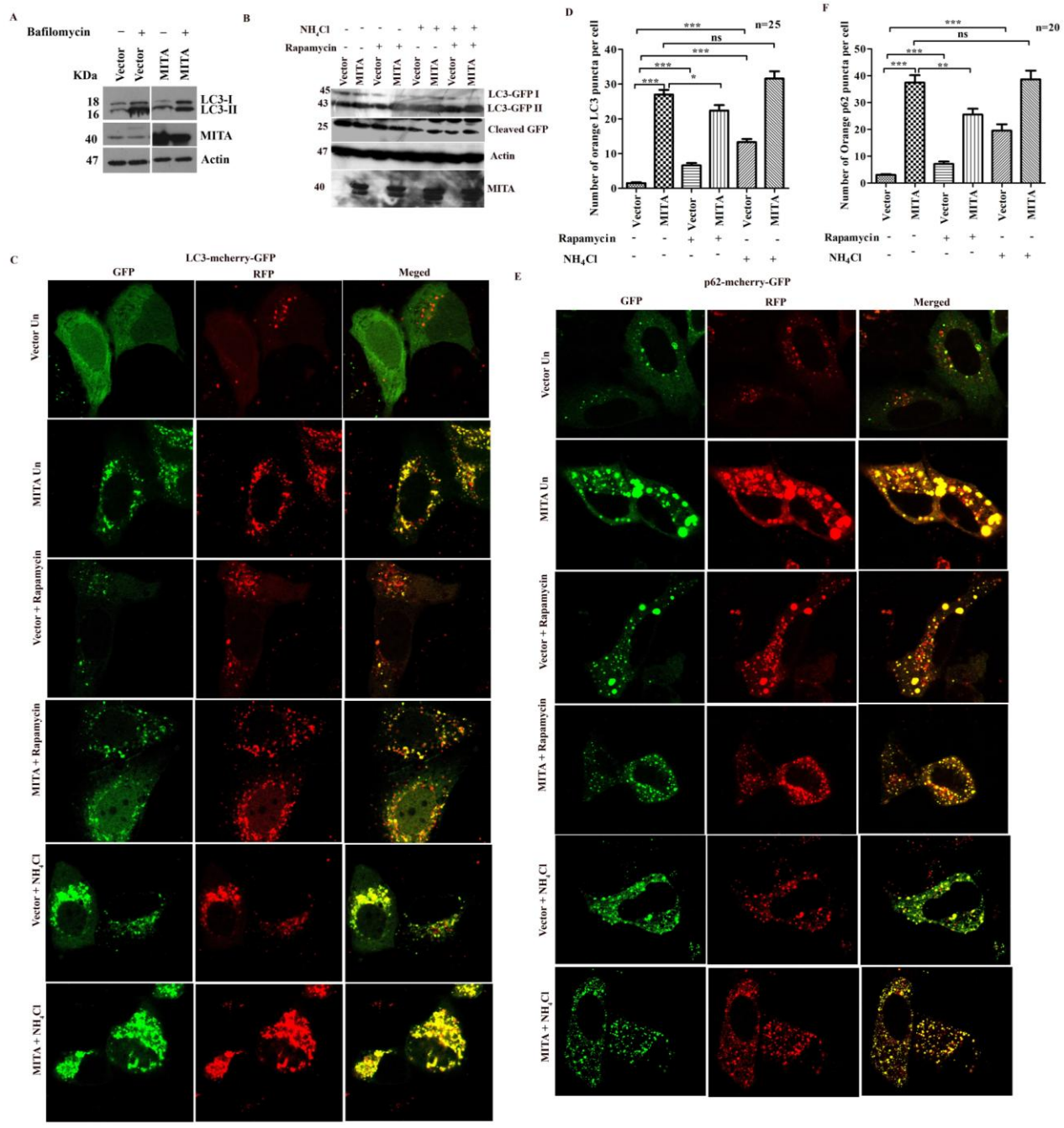
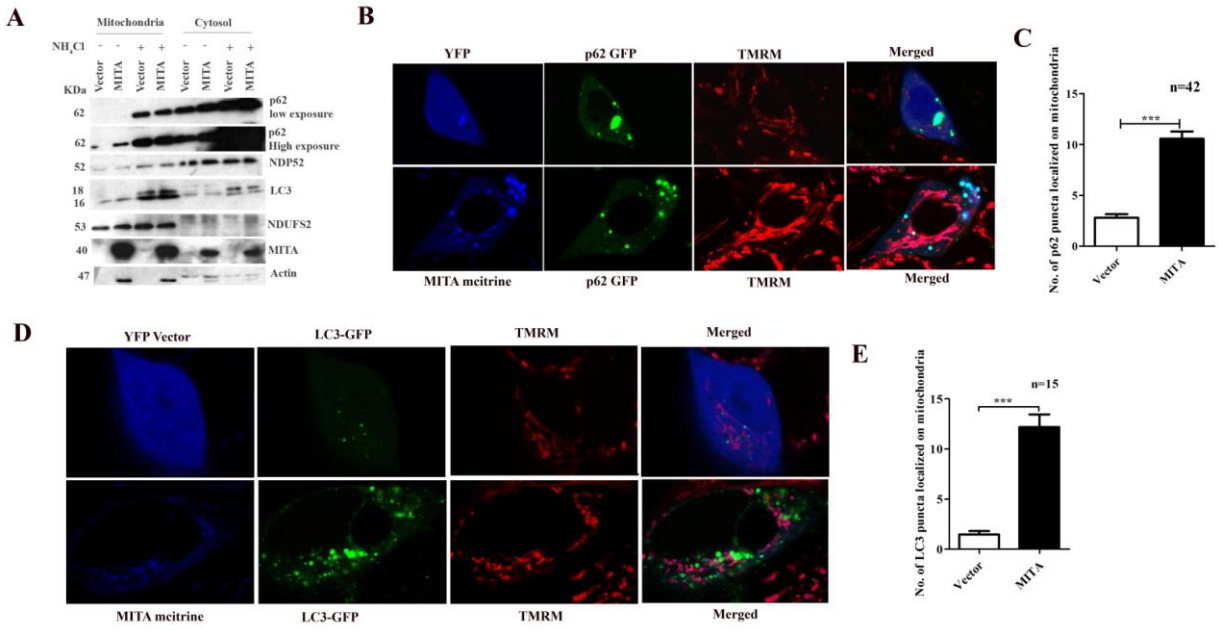


Figure-7.2. MITA inhibits the autophagy flux in breast cancer cells:

(A) MCF-7 cells were transfected with MITA and treated with Bafilomycin. Cells were collected and lysed. The lysate were subjected to western blot analysis and LC3-II band

was detected using LC-3 specific antibody. (B) MCF-7 cells were cotransfected with LC3-GFP and MITA and treated with Rapamycin and NH_4Cl for GFP cleavage assay. The cells were collected and lysed. The lysate were subjected to western blot analysis and GFP band was detedted using GFP specific antibody. (C) MCF-7 cells were cotransfected with LC3-GFP-mcherry and MITA and orange as well as red colored LC3 puncta formation was analyzed under fluorescent microscope. (D) Number of orange colored puncta per cell was counted and graph was plotted for number of orange colored LC3 puncta formed inside the cell representing Fig 7.2C. (E) MCF-7 cells were cotransfected with p62-GFP-mcherry and MITA and orange as well as red colored p62 puncta formation was analyzed under fluorescent microscope. (F) Number of orange clored p62 puncta per cell was counted and graph was plotted for number of orange colored p62 puncta formed inside the cell representing Fig 7.2E.



Figur-7.3: MITA regulates mitophagy:

(A) MCF-7 cells were transfected with MITA and treated with NH_4Cl. Mitochondrial fraction was isolated and subjected to western blot analysis. Mitophagy markers were analyzed using antibodies speciofic to LC3-II, p62 and NDP52. MCF-7 cells were cotransfected with p62-GFP (B) or (D) LC3-GFP and hMITA-mcitrine. Mitochondria were stained with TMRM and cells were observed under confocal microscope. (C and E)

The number of green puncta recruited on mitochondria were counted in cotransfected cells and graph was plotted representing Fig 7.3 B and D respectively.

7.4. MITA inihibts the turnover of mitochondria

Defective mitophagy might lead to the accumulation of defective mitochondria that might further affect mitochondrial biogenesis or turnover. To understand the role of MITA in mitochondrial biogenesis, expression of mitochondrial transcriptional regulators (PGC1α, NRF-1 andNRF-2) was monitored. PGC1α is a transciptional activator and stimulator of mitochondrial biogenesis [154]. The transcripts levels of PGC1α were hence analyzed in MITA trasfected cells using quantitative real time PCR. The transcript levels of PGC1α significantly decreased in MITA transfected cells as compared to control (Fig 7.4A). NRF1 and NRF2 are also transcriptional regulator of mitochondrial biogenesis[154, 155] and act downstream of PGC1α; hence their levels were also analyzed. The increased levels of NRF-1 expression was observed in MITA transfected MCF-7 cells as compared to control (Fig 7.4B). There was no significant difference observed in case of NRF-2 expression (Fig 7.4C). These evidences suggest that the mitochondrial biogenesis is altered. PGC-1 α, an transcriptional activator, acts upstream to NRF-1 and NRF-2. Thus decrease in PGC-1 alpha suggest that possibly it is further downregulating the mitochondrial biogenesis. The NRF-1 and NRF-2 will not show its effect as they act downstream to PGC-1 α. The decrease in mitochondrial biogenesis was further confirmed by analysing mitochondrial DNA content. Significant decrease in mitochondrial DNA content was observed in the presence of MITA as compared to vector control (Fig 7.4D). This suggests that MITA induces the defective mitophagy and at the same time inhibits further mitochondrial biogenesis. This might lead to accumulation of damaged mitochondria generating chaos inside the cells ultimately leading to cell death.

7.5. MITA regulates mitochondrial functions in breast cancer cells

Accumulation of damged mitochondria are known to generate ROS[107]; hence ROS genration was monitored using CM-H_2DCFDA stain. MITA was expressed in MCF-7 cells and ROS generation was monitored. MITA expression led to increased ROS generation as compared to vector control (Fig 7.5A). For further validation, MITA was knocked down inHBL100 cells and ROS genration was monitored using CM-H_2DCFDA. MITA knockdown significantly decreased ROS generation in HBL100 cells (Fig 7.5B).

Complex-I is a major source of ROS generation[156]. Mitochondrial complex-I activity was hence measured using oxidation of NADH by spectrophotometric analysis and BN-PAGE in the presence of MITA. The complex-I activity increased significantly in the presence of MITA as compared to control both spectrophotometrically and in gel activity assay (Fig 7.5C and D). Complex-I activity was also monitored in the MITA knockdown condition in HBL100 cells. MITA knockdown led significant decrease in complex-I activity of HBL100 cells (Fig 7.5 E). Mitochondrial membrane potential is also altered when there is impaired complexactivity or ROS generation. Mitochondrial membrane potential was measured hence monitored using TMRM stain in the presence of MITA. MCF-7 cells were transfected with MITA and stained with TMRM. Mitochondrial potential was then analyzed using fluorimeter. Significant decreased in the TMRM fluroscence level was observed in MITA transfected cells as compared to vector (Fig 7.5 F). MITA knockdown in HBL100 significantly increase mitochondrial potential as compared tocontrol cells further validating the result (Fig 7.5G). Mitochondria is a power house of cell and ATP generation is the central role of mitochondria. The altered mitochondrial biogenesis might also reflect to ATP production. ATP levels were hence measured in MITA transfected MCF-7 cells. The ATP levels significantly decreased in the presence of MITA as compared to control (Fig 7.5H). These evidences suggest that the defect in mitophagy flux is leading to accumulation of damaged mitochondria and thus leads to altered mitochondrial functions. We therefore wanted to investigate the reason of defective autophagy/mitophagy in MITA expressing cells. The autophagy might be defective at the autophagosome to lysosome fusion or the lysosomal function or lysosomal biogenesis themselves might be defective.

7.6. MITA regulates nuclear translocation of TFEB

To understand the defect in autophagy flux, nuclear translocation of TFEB was monitored. TFEB is a master regulator of CLEAR network of lysosomal biogenesis[82]. The nuclear fractionation analysis of MITA transfected MCF-7 cells was performed. The levels of 53 KDa band corresponding to TFEB was less intense in the nucleus of MITA transfected cell as compared to vector control (Fig 7.6A). Rapamycin is chemical inducer of nuclear translocation of TFEB[82]; hence it was used as a positive control. Rapamycin treatment leads to accumulation of TFEB inside the nucleus in the presence or absence of MITA as

expected. ZKSCAN3 is an inhibitor of lysosomal biogenesis as well as autophagy. The expression of ZKSCAN3 was monitored in the MITA transfected cells using quantitative PCR. The ZKSCAN3 expression increased significantly in the presence of MITA (Fig 7.6B). Surprisingly, the expression of lysosomal proteins LAMP1, lysosomal protein transmembrane 4 alpha and ATPase levels were observed to be increased in the presence of MITA expressing cells as compared to vector control (Fig 7.6C, D and E). The results suggest that possibly further lysosomal synthesis is inhibited but at the same time the degradation of old lysosomes are also inhibited. The increased lysosomal proteins suggest that there is no defect in lysosomal activity but possibly the fusion of autophagosome to lysosome is inhibited. We confirmed the same using lysosensor green and lysotracker blue (Data not shown). We did not observe any difference in lysosomal number or functions in the presence of MITA. TFEB is master regulator of lysosomal biogenesis but at the same time it regulates autophagy. No difference in lysosomal number as observed in FACS analysis indicates that TFEB nuclear inhibition might be contributing to defective autophagy regulation by some unknown mechanism.

7.7. MITA induced cell death is rescued by normalizing the autophagic flux

It is hence possible that MITA induces autophagy and/or mitophagy, but they are unable to get degraded. This leads to accumulation of defective mitochondria and hence increases in ROS levels; this might lead to cell death in MCF-7 cells. This might be because of the inhibition of the autophagy flux. We henceforth hypothesize that if we rescue the autophagic flux using rapamycin, MITA induced cell death might be rescued. It was observed that rapamycin treatment rescues the autophagy flux (Fig 7.2C-E). We hence treated MITA transfected MCF-7 cells with rapamycin and measured caspase activity in MCF-7 cells. There was a significant increase in the caspase activity of MITA transfected cells; but the caspase activity decreased significantly in the presence of rapamycin. This suggests that MITA induced increase in the caspase activity was rescued upon rapamycin treatment or enhanced autophagic flux (Fig 7.7A). Rapamycin is chemical inducer of nuclear translocation of TFEB[82]; hence TFEB overexpression might rescue defective autophagy flux similar to rapamycin. We co-transfected hMITA-mcitrin and TFEB-GFP in MCF-7 cells and looked for the nuclear translocation of TFEB using confocal microscopy. We observed increased nuclear translocation of TFEB inside the nucleus in

co-transfected cells confirming TFEB overexpression increases its own nuclear translocation (Fig 7.7B and C). We further assessed caspase activity in MITA and TFEB co-transfected cells MCF-7 cells. Similar to rapamycin treatment, MITA induced increase in the caspase activity was rescued upon TFEB overexpression (Fig 7.7D). The result further confirms that enhancement of autophagy flux rescues from MITA induced cell death.

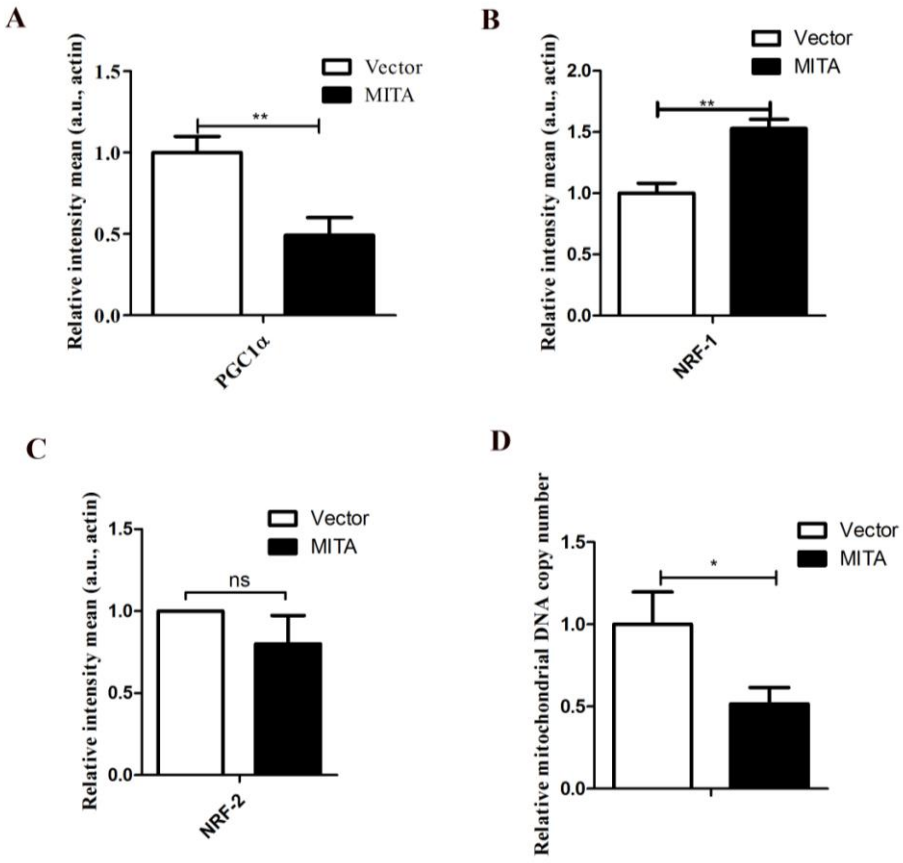


Figure-7.4: MITA inhibits mitochondrial turnover:

MCF-7 cells were transfected with MITA. After 24 hrs of transfection cells were collected, RNA was isolated and reverse transcribed to cDNA. The levels of PGC1α (A) NRF1 (B) and NRF2 (C) were analyzed using quantitative real time PCR using specific primers. (D) MCF-7 cells were transfected with MITA. After 24 hrs of transfection cells were collected, total cellular DNA was isolated and mtDNA content was analyzed using quantitative real time PCR using specific primers to unique mitochondrial DNA fragment relative to a single copy region of the nuclear gene RNase P.

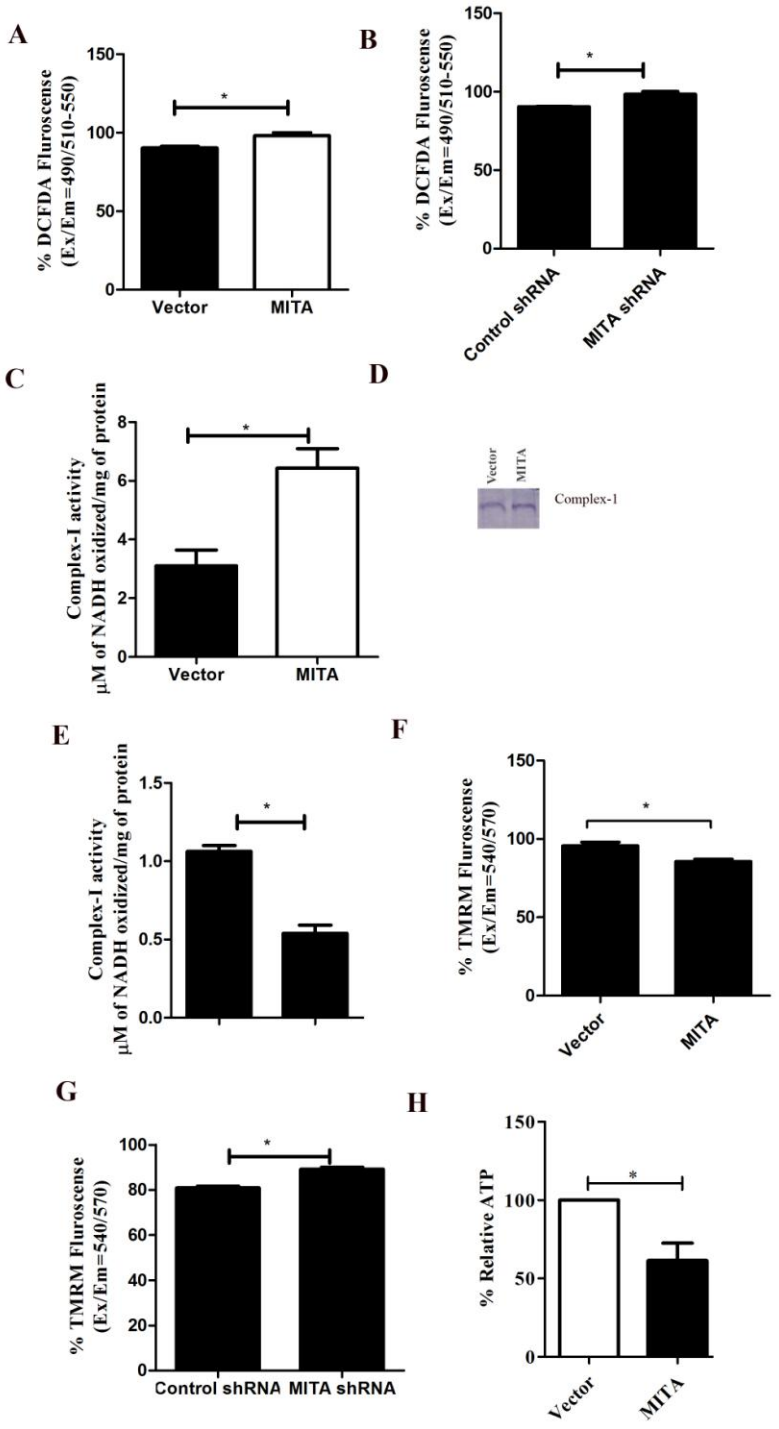


Figure-7.5: MITA regulates mitochondrial function.

(A) MCF-7 cells were transfected with MITA and stained with CMH_2DCFDA, an indicator of ROS. ROS levels were measured using flurimeter. (B) HBL-100 cells were transfected with MITA shRNA and stained with CMH_2DCFDA, an indicator of ROS. ROS levels were

measured using fluorimeter. (C) MCF-7 cells were tran sfected with MITA and complex-I activity was measured (C) spectrophotometricaly as well as using (D) BN-PAGE. (E) HBL-100 cells were transfected with MITA shRNA and complex-I activity was measured spectrophotometricaly. (F) MCF-7 cells were transfected with MITA and stained with TMRM. Mitochondrial potential was analyzed fluorometricaly. (G) HBL-100 cells were transfected with MITA shRNA and stained with TMRM. Mitochondrial potential was analyzed flurometricaly. (H) MITA was transfected in MCF-7 cells and ATP levels were measured using luminescence based ATP assay.

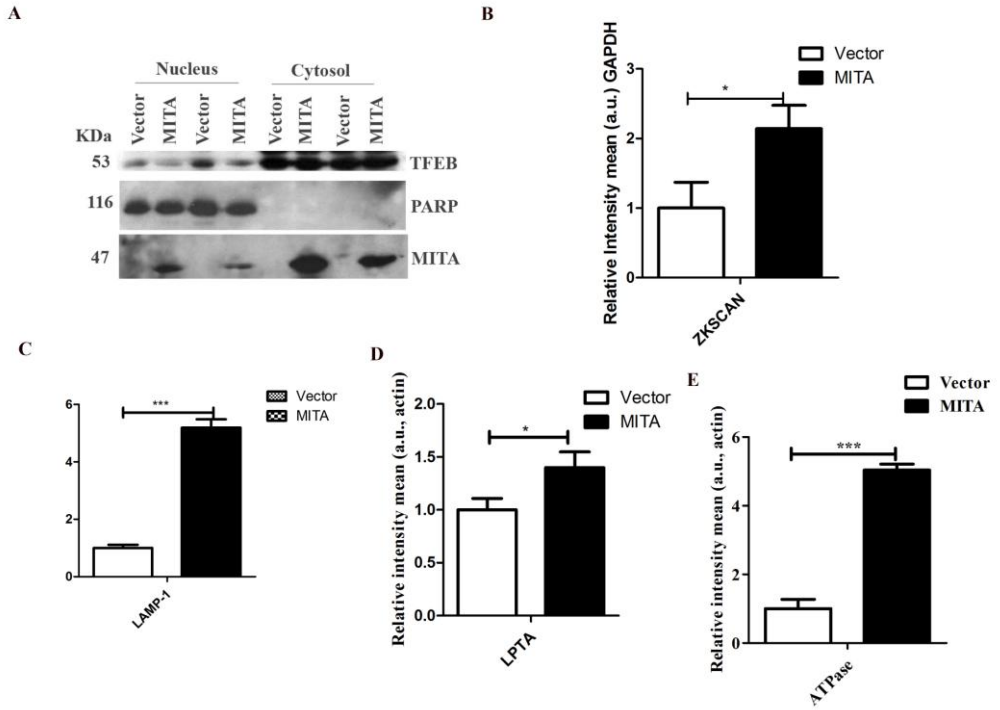


Figure-7.6: MITA regulates lysosomal biogenesis:

(A) MCF-7 cells were transfected with MITA and treated with rapamycin. Cells were collected and nuclear fraction was isolated. Nuclear fraction was subjected to western blot analysis and TFEB levels were analyzed using specific antibodies against TFEB. (B) MCF-7 cells were transfected with MITA. After 24 hrs of transfection, cells were collected, RNA was isolated and reverse transcribed to cDNA. Expression level of (B) ZKSCAN3 (C) LAMP-1 (D) lysosomal protein transmembrane 4 alpha and (E) ATPase were monitored using quantitative real time PCR.

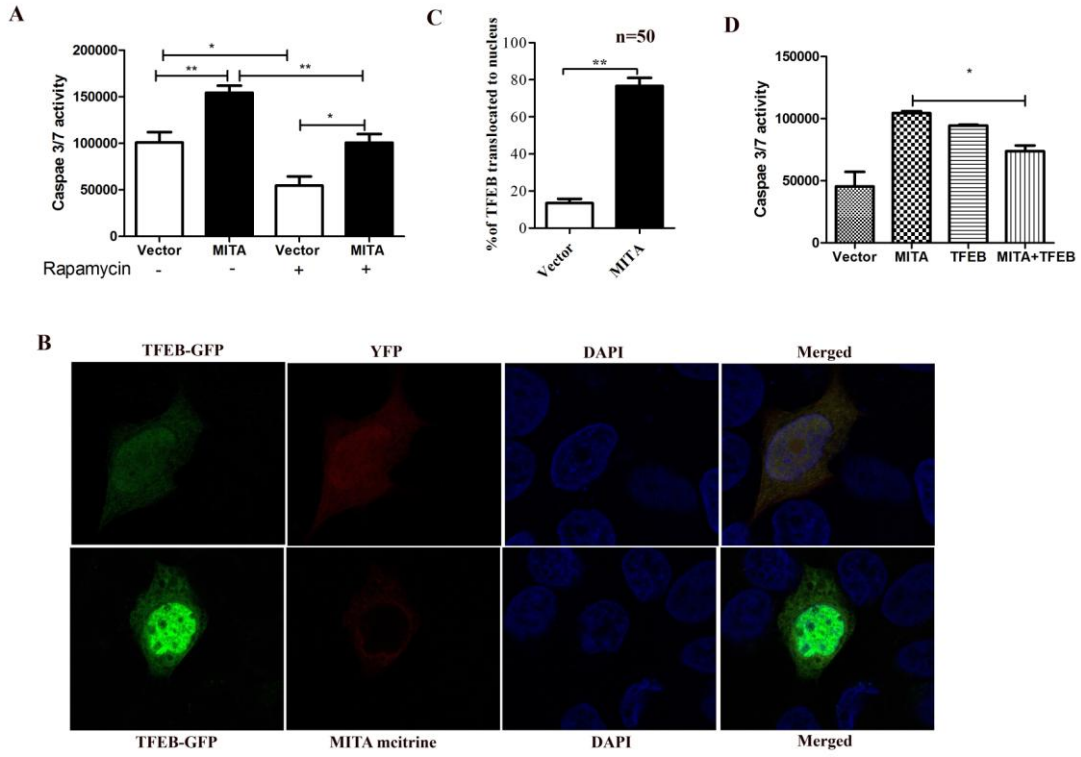


Figure-7.7: Normalizing the autophagy flux rescues from MITA induced cell death

(A) MCF-7 cells were transfected with MITA and treated with rapamycin. The caspase activity was measured using luminometer. (B) MCF-7 cells were cotransfected with hMITA-mcitrine and TFEB-GFP and nuclear translocation of TFEB was analyzed using confocal microscopy. (C) The number of cells showing nuclear translocation of TFEB was counted and the graph was plotted. (D) The cells were cotransfected with MITA and TFEB-GFP. After 48 hours of transfection caspase activity was measured using luminometer.

The evidences in the current study suggest that MITA leads to induction of defective mitophagy and inhibition of further healthy mitochondrial biogenesis. This leads to accumulation of damaged mitochondria and subsequent ROS generation and defective mitochondrial function. Rescue of the autophagy flux using rapamycin rescues from

MITA induced cell death suggesting the defective flux contributes to MITA induced cell death and its tumorigenic potential.

7.8. Discussion

Abundant evidences suggest that inflammation and breast cancer are linked to each other as discussed in section 2.2.1. The detailed mechanism of how they are linked to each other is under investigation. In our previous report, we have shown that inflammatory mediator NF-κB regulates tumorigenic potential of MITA in breast cancer cells[61]. Other reports also suggest that it acts as tumor suppressor in other cancerous conditions[62-64]. Direct activation of MITA in tumor microenvironment systematically regress the tumor[65]. Autophagy is another physiological process that plays an important role in tumorigenesis[84, 86]. Evidences also suggest that autophagy links inflammation to cancer[13]. NF-κB is recently shown to regulate p62 adaptor protein essential for mitophagy to remove the damaged mitochondria[22]. We hence started looking at the role of MITA in regulation of autophagy. In the current report we suggest that MITA leads to the accumulation of damaged mitochondria and defective mitophagy process. This leads to the cell death in breast cancer.

Current evidences suggest that MITA leads to the increased autophagosome synthesis as evident from the increase in LC3-II band and LC3/p62 puncta. Further experiments suggested that even though autophagosome synthesis is enhanced, they are not going to lysosomal degradation. MITA also affects mitophagy process and leads to accumulation of damaged mitochondria. The condition like this occurs in many neurodegenerative diseases such as lysosomal storage disease or Parkinson disease where lysosomal dysfunction further leads to defective autophagosome to lysosome fusion[9, 81]. The other example is Niemann-Pick Type C1 Disease, where amphisome formation is blocked[157]. In neurodegenerative disorder defective mitophagy leads to neurodegeneration. We suggest that MITA leads to similar condition in breast cancer and thus leads to cell death. The complete autophagy process may help tumor cells replenish with nutrients and helps survival; but defective autophagy lead to nutrient deprived condition inside the cell and subsequently cell death.

The defect in autophagy process leads to accumulation of damaged cell organelle. Defective autophagy may also lead to the accumulation of damaged mitochondria that may severely affect the tumor cell metabolism [23, 140, 158]. LC3 and the adaptor proteins having LC3 interaction region like p62/NDP52 are recruited on mitochondria in MITA transfected cells suggesting induction of mitophagy. The further experiments suggest that though the mitophagosomes are synthesized; they are unable to fuse to lysosome. The expression of MITA showed increased ROS levels as well as complex-I activity. ROS may be generated due to the reverse electron transfer in complex-I which may have role in amplifying NF-κB activity due to MITA. It had been observed earlier that mitochondrial complex-I generated ROS activates NF-κB and helps in differentiation of muscle cells[159]. ROS are also an integral part of TNF mediated cell death. MITA is also known to sensitize TNF mediated cell death as discussed in previous chapter. The generation of ROS might further initiate mitochondrial mediated cell death pathway and further establishes its role as tumor suppressor.

MITA also seems to be downregulating further mitochondrial biogenesis by downregulating PGC-1α expression. PGC1-α is known to enhance mitochondrial biogenesis and providing necessary energy to the cell thus contributes to metastasis of cancer cells[160]. There is strong correlation between high levels of PGC-1α and increased metastasis in breast cancer cells[160]. Moreover, silencing of PGC1α diminishes the invasive and metastatic potential of breast cancer cells[160]. This is also observed in other tumors like melanoma and prostate cancer[161-163]. Our observation also suggests decrease in PGC1α levels in the presence of MITA. This might contribute to the decreased tumorigenic potential of MITA. PGC1α is also known to regulate oxidative phosphorylation and ATP synthesis. The results confirm that MITA expression leads to decreased ATP production. Cancerous cell with high energy demand faces problem with decrease in ATP levels. This can be an additional reason contributing to cell death. Our results also suggest increased ROS production, indicator of presence of damaged mitochondria inside the cell. Activity of complex-1, major source of ROS, is also increased in the presence of MITA contributing the ROS production. Thus MITA seems to be regulating mitochondrial functions by regulating the levels of PGC1α. PGC1α though does not directly bind to DNA, it binds to transcription factors such as NRF-1 and NRF-2

and leads to increased mitochondrial biogenesis[154]. Though NRF1 levels are increased and NRF2 levels are not affected, PGC1α downregulation affects mitochondrial biogenesis and decreased mitochondrial DNA content as it acts upstream to NRF-1 and NRF-2[154]. The notion is further supported by recent report suggesting reduced expression of PGC1α suppresses melanoma metastasis, acting through a pathway distinct from that of its bioenergetic functions[161, 164]. MITA thus on one hand accumulates damaged mitochondria and on the other hand inhibits replenishment of healthy mitochondria to cell. This leads to bioenergetic crisis and cell death.

Further investigation of defective autophagy flux suggested inhibition of nuclear translocation of TFEB inside the nucleus. TFEB is an important regulator of autophagy flux as well as lysosomal biogenesis[83]. Normalization of autophagy flux by rapamycin or overexpression of TFEB rescues the MITA induced cell death. TFEB as discussed is a nuclear transcription factor, which translocates to nucleus in the presence of rapamycin. The treatment of MITA transfected cells with rapamycin decreased caspase activity in the presence of MITA by normalizing the autophagy flux. During normal conditions, TFEB is retained in the cytosol by phosphorylation and subsequent binding of 14-3-3 proteins to TFEB[82]. If TFEB is overexpressed, the 14-3-3 might not be sufficient inside the cell to sequester complete amount of TFEB and hence free TFEB would migrate inside the nucleus and subsequently rescued from MITA induced cell death.

Our results suggested that even though TFEB nuclear translocation is inhibited it does not affect the lysosomal content of the cell. Instead, MITA transfected cells show increased expression of lysosomal proteins. Possibly, there are adequate numbers of lysosomes present already in the cell as MITA inhibits the lysosomal degradation process of autophagy. The cell hence comes in a balance state where cell is neither degrading the lysosomes nor further synthesiszing; whereas in normal condition the lysosomes synthesized are utilized in the flux and are degraded by autophagy process. Increase in the lysosomal content also suggests indirectly that the lysosomal number and activity are optimal but somehow the autophagosome to lysosome fusion might be affected; though this needs to be further investigated. The similar type of condition is observed in case of Niemann-Pick Type C1 Disease, a neurodegenerative disease. Defective autophagy in Niemann-Pick type C1 (NPC1) disease leads cholesterol accumulation because of the

defective fusion of autophagosomes to lysosomes; whereas the lysosomal proteolytic function remains unaffected[157]. Further evidences are needed for the validation of the hypothesis.

MITA is an important link which can connect inflammation, autophagy and cancer. MITA upregulated NF-κB might lead to accumulation of p62 protein and thus increase of mitophagosome formation. MITA at the same time inhibits nuclear translocation of TFEB and thus defective flux and accumulation of defective mitochondria. This leads to energy crisis as well as ROS production in cell, leading to cell death in breast cancer cells. Damaged mitochondria might release damaged pieces of mitochondrial DNA inside the cytoplasm, which may act as DAMPs. DAMPs might induce cGAS/MITA mediated IFN signaling. This is also one of the possible mechanisms of MITA induced cell death. This may be the reason why cGAS/MITA mediated IFN signaling is kept under check by caspase 3/7 under physiological condition. Tumor formation is an evolutionary process, where tumor evolves itself by downregulating unfavorable genes (known as tumor suppressors) and upregulating tumor promoting genes. MITA is hence downregulated in many of the tumors including breast cancer.