# Chapter Eight:
# Summary and Conclusion

## 8.1 Summary:

Chronic inflammation, a hallmark of solid tumor, is intricately associated with initiation and progression of many cancer types, including breast cancer. The specific role of inflammation in providing a survival advantage to tumor cells is not well understood. An increased level of proinflammatory cytokines, including TNF-α in TME regulates the bioenergetic capacity, immune response and survival of cancer cells. Emerging evidences suggest the role of mitochondria-localized immune adaptor proteins including MAVS and STING in sensing  stress has been co-opted as an indirect mechanism to regulate the inflammatory response and metabolic adaption resulting from intrinsic cellular damage.NLRX1, a mitochondria-localized NOD-like receptor (NLR) family protein, regulates NF-κB/type-I IFN signaling and autophagy to limit acute inflammatory response during infection. However, its role in modulating metabolic functions to regulate cell survival or death is not well understood. In the present study, we systematically investigated the role of NLRX1 in modulating the TNF-α-regulated mitochondrial function and autophagy flux to regulate cell death and tumorigenic potential of breast cancer cells. We further characterized the mechanism of NLRX1-mediated regulation of OxPhos capacity and bioenergetic adaptation of breast cancer cells.

### 8.1.1 NLRX1 acts as a tumor suppressor by modulating TNF-α-regulated mitochondrial function and apoptosis in cancer cells

Here, we identified NLRX1 as a crucial regulator of TNF-α induced cell death and reveal its potential role as a tumor suppressor. The major findings of the study are summarized as below:

*8.1.1.1 NLRX1 sensitizes TNF-α-induced cell death by promoting the activation of caspase-8*

- Ectopic expression of NLRX1 specifically sensitizes TNF-α induced death.
- Conversely, knockdown of NLRX1 increases cell survival in the presence of TNF-α/CHX.

- NLRX1 increases TNF-α-induced cell death by promoting the early activation of caspase-8.
- Inhibition of caspase-8 activation by z-IETD-fmk rescues TNF-α induced apoptosis in NLRX1 over-expressing cells.

*8.1.1.2 NLRX1 promotes caspase-8 activation by associating with TNF-α-induced complex-II*

- NLRX1 regulates caspase-8 activation through its interaction with TRAF2, a key component of TNF-α-induced complex-II.
- The association of NLRX1 with TRAF2 stabilizes the formation of pro-death complex-II and increases caspase-8 activation in the presence of TNF-α/CHX.

*8.1.1.3 NLRX1 localizes to mitochondria and regulates TNF-α-induced ROS generation*

- Both NLRX1 and activated caspase-8 are significantly enriched in mitochondrial fraction in the presence of TNF-α/CHX.
- Ectopic expression of NLRX1 augments mitochondrial ROS generation which depends on the activation of caspase-8 in the presence of TNF-α/CHX.
- Squelching mitochondrial ROS levels rescues TNF-α induced apoptosis in NLRX1 over-expressing cells.

*8.1.1.4 NLRX1 alters cellular ATP levels by modulating TNF-α-regulated mitochondrial function*

- Ectopic expression of NLRX1 decreases mitochondrial CI enzyme activity and ATP levels which depends on the activation of caspase-8 in the presence of TNF-α.
- Conversely, knockdown of NLRX1 increases mitochondrial CI and CIII enzyme activity and ATP levels in the presence of TNF-α.

*8.1.1.5 NLRX1 suppresses tumorigenic potential of cancer cells both in vitro and in vivo.*

- NLRX1 expression is downregulated in MCF-7 and T47D (ER and PgR positive) cells and upregulated in MDA-MB-231 and HBL-100 (ER and PgR negative) cells.

- Ectopic expression of NLRX1 in MCF-7 cells decreases clonogenic ability, anchorage-independent growth and migration property in the presence of TNF-α.
- Expression of NLRX1 in cancer cells of different origin suppresses tumorigenesis in nude mice.

These results strongly suggest that NLRX1 acts as a potential tumor suppressor by modulating TNF-α-regulated mitochondrial metabolism and apoptotic cell death in cancer cells (Figure 8.1). The evidences presented here suggest that loss of NLRX1 may confer dual advantage to ER and PgR positive breast cancer cells in TME by acquiring resistance to TNF-α induced cell death and reprograming mitochondrial metabolism to meet increased bioenergetic and anaplerotic demands.

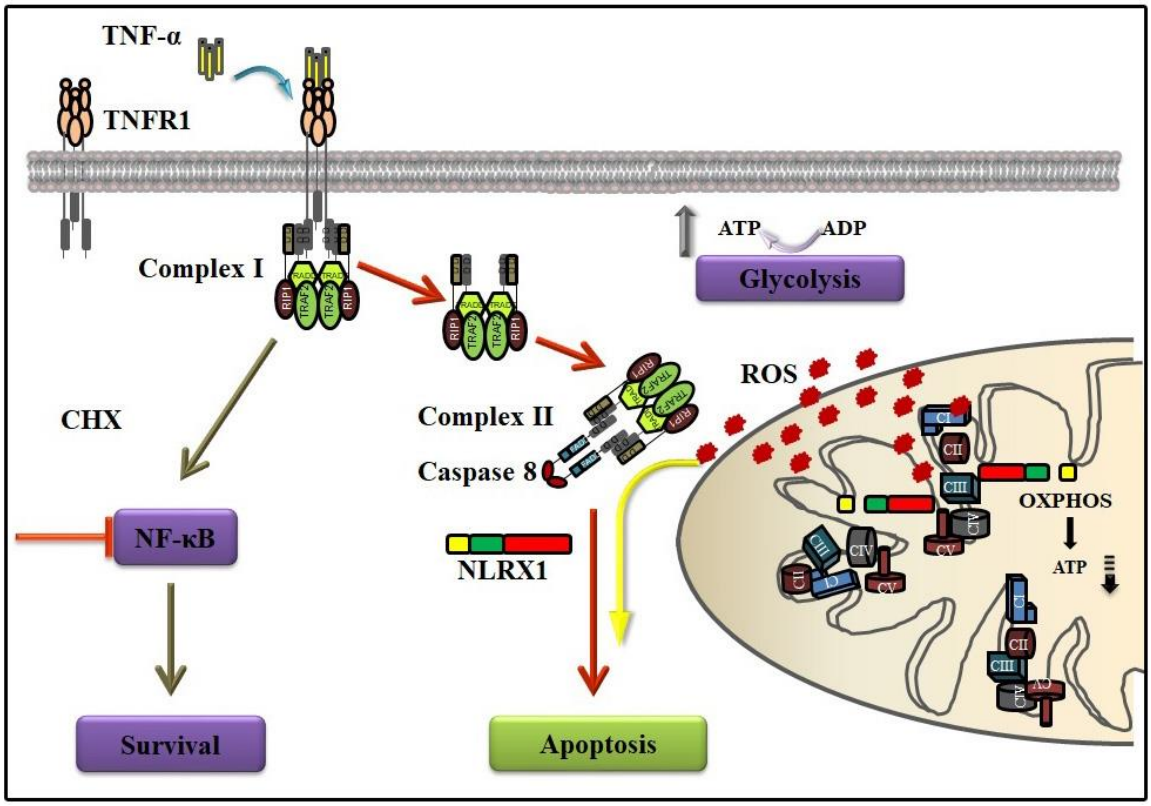


***Figure 8.1: NLRX1 acts as a tumor suppressor in cancer cells of different origin.*** *NLRX1 is downregulated in ER/PgR positive breast cancer cells. Ectopic expression of NLRX1 in these cells sensitizes TNF-α-induced apoptosis by promoting caspase-8 activation. NLRX1 localizes to mitochondria and increases ROS generation by regulating mito-*

*chondrial CI and CIII activity. This in turn amplifies apoptotic signals via formation of TNF-α-induced complex-II and caspase-8 activation.*

### 8.1.2 NLRX1 localizes to mitochondrial RNA granules and regulates mitochondrial RNA processing and metabolic adaptation

Here, we systematically characterized the sub-mitochondrial localization of NLRX1 and identified the mechanism through which NLRX1 may regulate mitochondrial function and metabolic adaptation of cancer cells. The major findings of the study are summarized as below:

*8.1.2.1 NLRX1 localizes to the mitochondrial matrix*

- N-terminal addressing sequence of NLRX1 is essential mitochondrial localization.
- NLRX1 displays a punctate distribution and colocalizes with matrix-targeted RFP in mitochondria.

*8.1.2.2 NLRX1 interacts with FASTKD5 and colocalizes with mitochondrial RNA granules*

- The pull down of endogenous FASTKD5 specifically precipitates NLRX1 as FASTKD5-associating protein.
- Both NLRX1 and FASTKD5 colocalizes as distinct puncta across the tubular distribution of matrix-targeted CFP.
- NLRX1 and FASTKD5 are concentrated in discrete focal structures ranging from 30-100 nm in size.
- NLRX1 colocalizes with BrU-labelled nascent mitochondrial RNAs identified as mitochondrial RNA granules.

*8.1.2.3 NLRX1 regulates the processing and maturation of non-canonical precursor transcripts in mitochondria*

- Ectopic expression of NLRX1 selectively decreases the levels of mature mitochondrial RNA including 16S rRNA and ATP8, ATP6, COX III, ND5 and cyt b mRNAs.
- Knockdown of NLRX1 upregulates the levels of non-canonical mitochondrial transcripts.
- NLRX1 expression does not alter the relative copy number of mtDNA.
- NLRX1 expression causes the accumulation of ATP8+COX III and ND5+cytbnon-canonicalprecursor transcripts.

*8.1.2.4 NLRX1 bind to non-canonical precursor transcripts and negatively regulates FASTKD5-mediated processing*

- RNA immunoprecipitation of ectopically expressed NLRX1selectively enriches ND5 and cyt b mRNA and inhibits the binding of mitochondrial transcripts to FASTKD5.
- Knockdown of FASTKD5 results in loss of enrichment of non-canonical mRNAs.
- RNA immunoprecipitation of endogenous NLRX1 enrichesATP8, ATP6, COX III, ND5 and cyt b mRNAs with high affinity in the absence of FASTKD5.

*8.1.2.5 NLRX1 interacts with FASTKD5 and binds to mitochondrial RNA through its LRR domain*

- NLRX1 lacking LRR domain could not precipitate FASTKD5 as NLRX1-binding protein.
- RNA immunoprecipitation of NLRX1 lacking LRR domain did not showed the binding of non-canonical mitochondrial mRNAs.

*8.1.2.5 NLRX1 regulates the translation of mtDNA-encoded proteins and assembly of OxPhos supercomplexes*

- Ectopic expression of NLRX1 specifically decreases the levels of nascent mitochondrial protein subunits encoding for ND5,cyt b and COXIII.
- Conversely, knockdown of NRX1 upregulates the levels of these mtDNA-encoded proteins subunits

- NLRX1 expression decreases the supramolecular organization and activity of CI, CIII, and CIV.
- NLRX1 expression decreases the enzyme activity of individual CI and CIV but does not affect CII activity.
- Knockdown of NLRX1 increases enzyme activity of CI and CIV.

*8.1.2.7 NLRX1 limits the proliferation of respiration-competent cells*

- Ectopic expression of NLRX1 decreases the proliferation of breast cancer cells in galactose-containing medium as primary carbon substrate.
- Knockout of NLRX1 rescues the growth of breast cancer cells in galactose-containing medium.

These findings establish a critical role of NLRX1in regulating the post-transcriptional processing of mitochondrial precursor mRNAs to modulate the steady state levels of mature mitochondrial RNAs, thus, controlling the activity and organization of OxPhos complexes (Figure 8.2). This study provides the new paradigm by which NLRX1 participates in metabolic reprogramming during innate immune response, and oncogenic transformation.

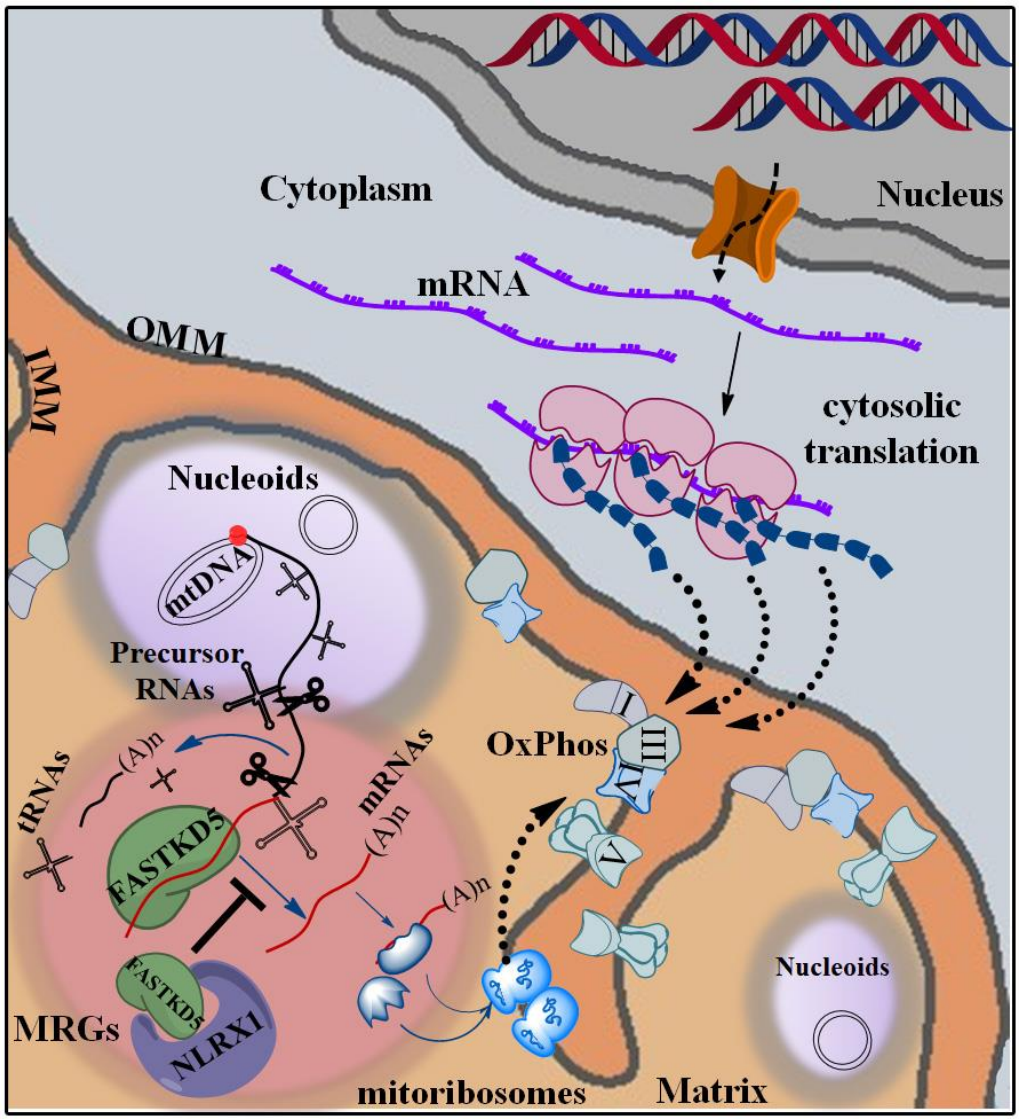


***Figure 8.2: NLRX1 localizes to mitochondrial RNA granules and regulates RNA processing and OxPhos assembly.*** *NLRX1 localizes to MRGs and interacts with FASTKD5, a bonafide MRG protein. Ectopic expression of NLRX1 regulates the post-transcriptional processing, maturation and translation of non-canonical heavy strand transcripts (shown in red) by either directly binding to mitochondrial RNA or sequestering FASTKD5. This in turn controls the activity and organization of OxPhos complexes formed by both mitochondrial and nuclear-encoded genes.*

### 8.1.3 NLRX1 regulates TNF-α-induced mitochondria-lysosomal crosstalk to maintain the tumorigenic potential of breast cancer cells

NLRX1 expression is differentially regulated inhuman breast cancer cells of different origin suggesting a complex role of NLRX1-regulated mitochondrial function in controlling metabolic adaptation and tumorigenicity of cancer cells. Here, we investigated the role of NLRX1-regulated mitochondrial function in modulation of TNF-α-induced autophagy flux and hence the tumorigenic potential of breast cancer cells. The major findings of the study are summarized as below:

*8.1.3.1 NLRX1 expression is upregulated in invasive breast cancer cell lines and metastatic tumors*

- The mRNA expression and protein levels of NLRX1 is strongly upregulated in ER and PgR negative cells (MDA-MB-231 and HBL100) as compared to ER and PgR positive cells (MCF-7, ZR-75-1, BT-474 and T47D).
- The upregulated NLRX1 expression directly corelates with metastatic and ER and PgR negative breast tumors.
- Meta-analysis of NLRX1 expression profiles of breast tumors from TCGA database display a strong association of upregulatedNLRX1 expression levels with ER/PR negativity and advanced stage tumors.

*8.1.3.2 Knockdown of NLRX1 expression represses TNF-α-induced autophagy in ER and PgR negative breast cancer cells*

- Depletion of NLRX1 increases accumulation of GFP-LC3 containing autophagosomes and LC3 levels in the presence of TNF-α in MDA-MB-231 and HBL100 breast cancer cells.
- The knockdown of NLRX1 reduces p62 turnover and autophagosome maturation and inhibits TNF-α-induced autophagy flux.
- NLRX1 depletion increased the accumulation of p62 and LC3I/II levels in the presence of TNF-α

*8.1.3.3 NLRX1 depletion impairs mitochondrial function in the presence of TNF-α in ER and PgR negative breast cancer cells*

- Knockdown of NLRX1 in breast cancer cells increases TNF-α-regulated mitochondrial ROS levels.
- Knockdown of NLRX1 decreases the level and activity of mitochondrial SC-CI+CIII+CIV and CIII+CIV as well as individual CI and CIV in the presence of TNF-α.
- NLRX1 silencing result in a decreased activity of NADH-dependent electron transfer from CI to CIII in the presence of TNF-α while FADH2-dependent electron transfer from CII to CIII remains unchanged.
- NLRX1-KD cells shows reduced mitochondrial ATP synthesis and NADH levels in the presence of TNF-α.

*8.1.3.4 NLRX1 silencing alters mitochondrial dynamics and inhibits TNF-α-induced mitophagy in breast cancer cells*

- Depletion of NLRX1 induces fragmentation of mitochondrial network in the presence of TNF-α.
- NLRX1-KD cells exhibits an increased colocalization of mCherry-p62 and mCherry-LC3 with mitochondria in the presence of TNF-α.
- Depletion of NLRX1 leads to increased translocation of p62/NDP52-autophagy receptors and conjugated LC3II to mitochondria in the presence of TNF-α.

*8.1.3.5 Depletion of NLRX1 causes abnormal accumulation of lysosomal vacuoles and increases lysoso-mal biogenesis in the presence of TNF-α*

- Knockdown of NLRX1 results in accumulation of large LAMP1-positive lysosomal vesicles containing damaged mitochondria in the presence of TNF-α.
- Knockdown of NLRX1 increases transcriptional activation of TFEB-responsive genes of lysosome biogenesis in breast cancer cells in the presence of TNF-α.

*8.1.3.6 NLRX1-regulated mitochondrial function modulates lysosomal activity in the presence of TNF-α in breast cancer cells*

- Knockdown of NLRX1 decreases lysosomal acidification as well as cathepsin B, lysosomal lipase and acid phosphatase activity in the presence of TNF-α.

- Ectopic expression of full length NLRX1 completely restores defective lysosomal function in NLRX1-KD breast cancer cells in the presence of TNF-α.
- Exogenous supply of NAM restores mitochondrial respiratory chain deficiency and hence the lysosomal functions in NLRX1-KD cells in the presence of TNF-α.

*8.1.3.7 Loss of NLRX1 inhibits OxPhos-dependent cell proliferation, clonogenic ability and migration of breast cancer cells*

- Loss of NLRX1 expression limits the proliferation of MDA-MB-231 cells in galactose-containing medium as primary carbon source in the presence of TNF-α.
- Depletion of NLRX1 decreases the clonogenic ability of MDA-MB-231 and HBL100 cells in the presence of TNF-α.
- Loss of NLRX1 decreases the migration ability of MDA-MB-231 cells in the presence of TNF-α.

These results strongly suggest that NLRX1 is an essential mitochondrial protein of metastatic breast cancer cells, which preserves the mitochondrial homeostasis by regulating lysosomal function in the presence of TNF-α (Figure 8.3). This further maintains bioenergetic capacity and organelle function regulating tumorigenic activity of aggressive breast cancer cells.

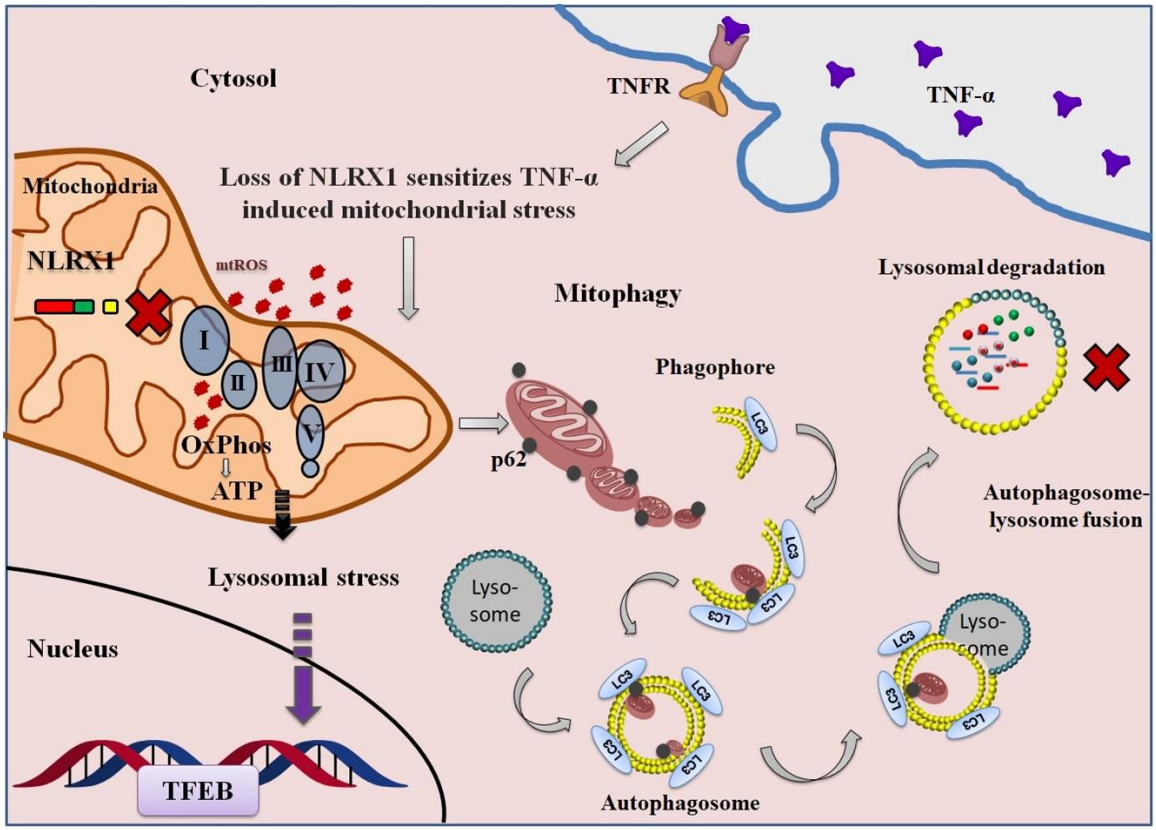


***Figure 8.3: NLRX1 regulates TNF-α-induced mitochondria-lysosomal crosstalk to maintain the tumorigenic potential of breast cancer cells.*** *NLRX1 is an essential mitochondrial protein which is upregulated in ER/PgR negative, metastatic breast cancer cells. Depletion of NLRX1 expression in these cells decreases mitochondrial function and its turnover during TNF-α-induced autophagy. Dysregulation of mitochondrial function impairs lysosomal activity during TNF-α induced autophagic flux to negatively regulate tumorigenicity of breast cancer cells.*

## 8.2 Conclusion:

Immuno-metabolic crosstalk regulated by the cell-intrinsic inflammatory pathways or its immune microenvironment, is a strong driver of tumor heterogeneity observed within various solid tumors including breast cancer. The selective amplification or loss of genes involved in regulation of inflammatory pathways represents an adaptive evolutionary mechanism to evade immune recognition and promote survival of the malignant cells in the heterogenous TME. This also constitute an important mechanisms in tumor progression from primary neoplasms to metastatic tumors. The first line of immune defense also plays important role in immune recognition of cancer cells. The differential expression of innate immune regulators in these two mutually exclusive states of breast cancer cells controls the outcome of innate immune signaling from lethal to a survival mechanism in inflammatory microenvironment. An emerging concept is that mitochondria are the central participants in regulating the metabolic adaptation and chronic inflammatory response during tumor growth and development. In the present study, we demonstrated that NLRX1 is a potential regulator of immunometabolic functions which may regulate TNF-α-induced cell death and mitochondrial functions to control the tumorigenic potential of breast cancer cells of different subtypes (Figure 8.4).

NLRX1 is differentially expressed in ER, PgR-positive, luminal subtype (primary breast neoplasms) and ER, PgR-negative, basal subtype (invasive breast tumors) suggesting a complex role of NLRX1-regulated immunometabolic pathways associated with breast tumor development. NLRX1-mediated regulation of mitochondrial gene expression and OxPhos capacity may play differential role in metabolic reprogramming and bioenergetic adaptation of glycolytic primary breast tumors and oxidative metastatic tumors. Increased levels of TNF-α observed in TME and loss of NLRX1 expression may confer dual advantage for the primary breast cancer cells by acquiring resistance to TNF-α-induced programmed cell death and reprogramming mitochondrial metabolism to meet high energy and anaplerotic demands. On the other hand, the upregulated expression of NLRX1 may support the tumorigenic potential of aggressive breast cancer cells by maintaining mitochondrial homeostasis and function during TNF-α-induced autophagy (Figure 8.4). Thus, NLRX1-regulated immunometabolic function may have different outcome in the

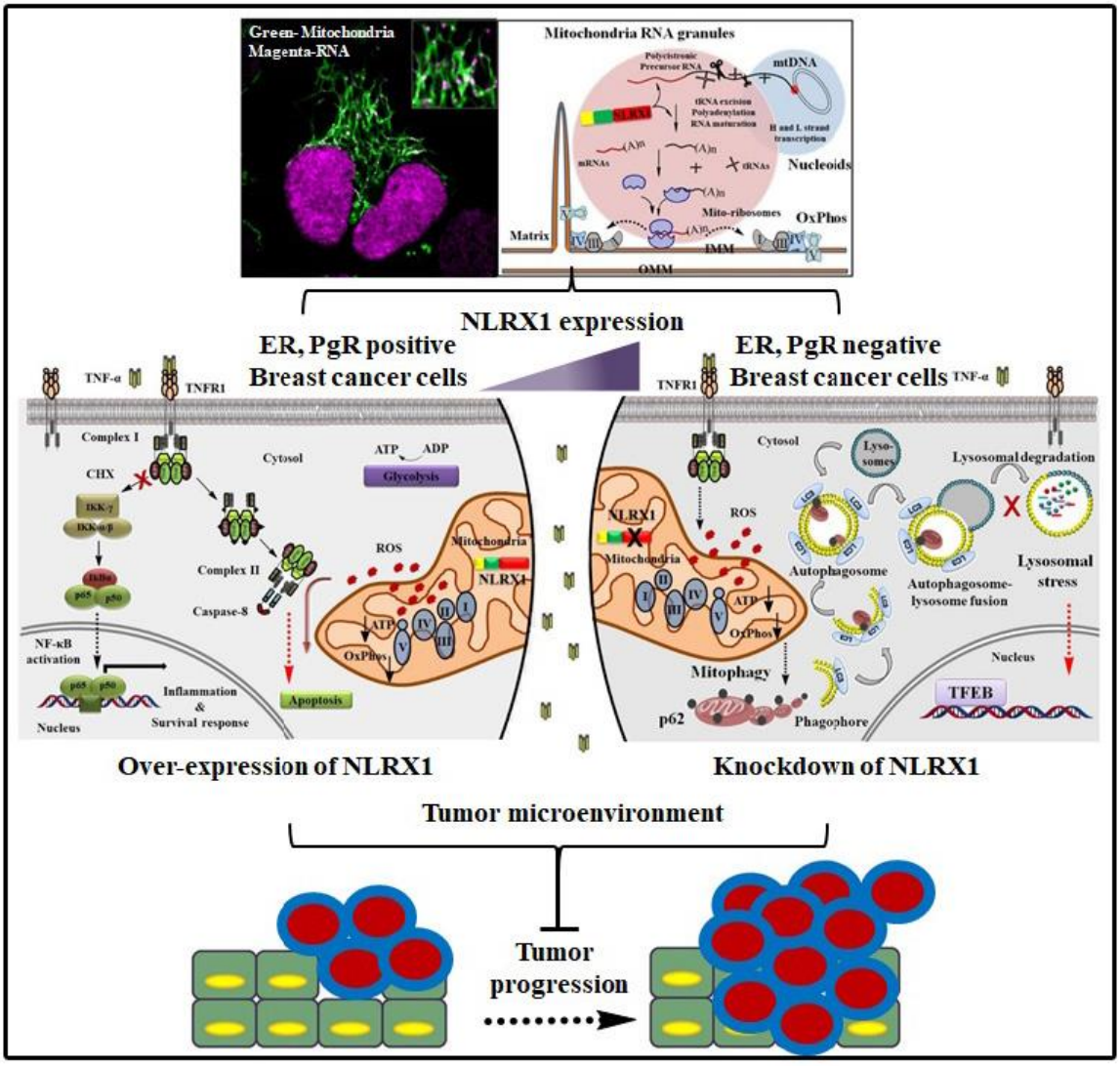


***Figure 8.4: NLRX1-mediated control of mitochondrial gene expression and OxPhos capacity regulates the tumorigenicity of breast cancer cells.*** *NLRX1 expression is differentially regulated in heterogenous breast tumors. NLRX1-mediated control of mitochondrial gene expression and hence the OxPhos capacity may differentially regulate TNF-α-induced innate immune responses including apoptosis and autophagy in different subclonal population of breast tumors. Thus, modulating NLRX1 expression and its function could be therapeutically targeted for controlling breast cancer.*

altered TME. The tumor suppressor role of NLRX1 may be breast cancer cell subtype-dependent, highly specific to the TME. The subclonal heterogeneity observed in TME of

different breast tumors supports this conclusion. The study further suggests that the cross-talk between NLRX1-regulated mitochondrial metabolism and TNF-α-induced innate immune responses may engage in a form of immune mimicry to maintain the chronic activation of inflammatory pathways associated with tumor growth and metastasis in a feed-forward fashion. Additional *in vivo* studies are required to further confirm this observation. Moreover, large cohorts of breast cancer patients are also required to confirm our preliminary finding. The patient showing the differential NLRX1 phenotype, as observed here, can be targeted for OxPhos capacity in primary and metastatic conditions.

### 8.3 Limitations of the study:

In the present study we convincingly demonstrated the role of NLRX1-regulated mitochondrial metabolism in modulation of TNF-α-induced autophagy flux and programmed cell death to control the tumorigenic potential of breast cancer cells. However, following are the limitations of the study:

- *Validation of TME-specific role of NLRX1*: The study suggests that NLRX1 may have a different role in the altered tumor microenvironment. Therefore, tumor-promoting function of NLRX1 could be further studied using mouse models of metastatic breast cancer.
- *Validations using NLRX1-KO mice*: The role of NLRX1 in regulating the post-transcriptional processing of mtDNA-encoded genes and hence the OxPhos assembly and activity could be confirmed in cells isolated from complete or tissue-specific NLRX1$^{-/-}$ mice.
- The post-transcriptional processing of mtDNA-encoded transcripts and its maturation in NLRX1-KD MDA-MB-231 cells in the presence of TNF-α should have been addressed.

- The mechanism of NLRX1-regulated mitochondrial function in modulating lysosomal activity during TNF-α-induced autophagy flux should be further investigated in detail.

- *Limited sample size*: The expression analysis of NLRX1 was performed on small group of patient samples due to limited availability of breast tumor tissues. Therefore, the study could be further extended by including a large group of patient samples and a more diverse panel of ER, PgR positive/negative breast cancer cell lines.

### 8.4 Future perspective:

The future studies in this direction would help in deciphering the role of NLRX1 in regulating the crosstalk of inflammation and metabolism during different patho-physiological conditions.

- NLRX1 may support the tumorigenic potential of aggressive breast cancer cells by immunometabolic functions to provide survival advantage and immune evasion within tumor microenvironment. This hypothesis needs to be tested in mouse models of metastatic breast cancer as well as NLRX1-KO mice models.
- Given, the role of NLRX1 in innate immune signaling, it may also regulate mitochondrial metabolism during infection by controlling OxPhos organization and assembly via alteration of mt-mRNA processing within MRGs. Thus, NLRX1 may act as a critical link which couples host mitochondrial bioenergetic capacity to innate immune signaling during infection. This hypothesis needs to be further tested in different models of viral infections.
- Similar to NLRX1, STING , an upstream innate immune regulator which acts as tumor suppressor, is upregulated in metastatic breast cancer cells. A previous study demonstrated the crosstalk between STING and NLRX1 innate immune signaling through direct interaction during viral infection. Hence, the regulation of STING/NLRX1 pathway may provide survival advantage by limiting inflammatory response and cell death and enhancing autophagy flux in metastatic cancer cells. This hypothesis needs to be further investigated in different models of breastcancer.