# 3. Materials and Methods

# Chapter 3

### 3.1 Cells and cell culture:

MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, USA), and mouse 4T1 breast cancer cells and E0771 cells were cultured in DMEM high glucose (Sigma Life Science, USA). Media were supplemented with 10% v/v heat-inactivated fetal bovine serum (Life Technologies), 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies). Cells were incubated at 37 °C, 5% CO2 in specified media.

### 3.2 Mouse breast cancer model:

Five-week-old female BALB/c mice were from Charles River (Kingston, ON, Canada). Mice were kept at 21°C, 55% humidity and a 12h light/dark cycle. The mice were given free access to 4% fat laboratory diet and water. All procedures were performed in accordance with the Canadian Council of Animal Care approved by University of Alberta Animal Welfare Committee. Mouse 4T1 breast cancer cells were cultured in high glucose DMEM at 37ºC, 5% $CO_2$ and 95% humidity. Cells were suspended at 400,000 cells/ml and mixed with Matrigel (BD Biosciences, Mississauga, ON, Canada) and 100 ul (20,000 cells) were injected using 30-gauge needle in $4^{th}$ inguinal mammary fat pad. After 4-5 days, tumor volumes were measured for the next 21 days using calipers. Mice were euthanized at Day 24.

### 3.3 Animals:

Female BALB/c mice, 5 weeks old, were from Charles River (Kingston, ON, Canada). Mice were kept at 21°C, 55% humidity and a 12h light dark cycle. The mice were fed with 4% fat laboratory diet and water ad libitum. Procedures were performed in agreement with the Canadian Council of Animal Care and approved by University of Alberta Animal Welfare Committee.

### 3.4 Breast cancer patient samples:

Tumor samples were taken from breast cancer patients at the University of Alberta Hospital. The procedure was approved by the Health Research Ethics Board of Alberta (HREBA 26195).

### 3.5 Tumor cells injection in mice:

Mouse 4T1 breast cancer cells cultured in DMEM high glucose (Sigma Life Science, USA) and kept at 37$^{o}$C, 5% $CO_2$ and 95% humidity. 2,00,000 cells/ml were suspended and mixed with Matrigel (BD Biosciences Mississauga) and 20,000 cells were injected using 30-gauge needle in 4$^{th}$ inguinal mammary fat pad of the mice. After 4-5 days, tumor volumes were measured with callipers for the next 21 days. Mice were euthanized at Day 24.

### 3.6 Infliximab treatment in mice model:

Infliximab was administered intraperitoneally at a dose of 10mg/kg on day 7 after tumor cell inoculation and then on days 13 and 21. Untreated mice received saline by intraperitoneal injection. Each group included 9 mice.

### 3.7 Kits and Reagents:

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), TMRM, MitoSOX$^{TM}$ and CM-H2DCFDA was purchased from Thermofisher, USA. Recombinant Human TNF-α was purchased from Milteny Biotech, Germany and PeproTech, USA. SYBR green and cDNA isolation kits were purchased from Takara, Japan. MicroAmp Fast Optical 96-Well Reaction Plate, MicroAmp Optical Adhesive Film, Lipofectamine 2000, Opti-MEM was procured from Thermofisher, USA. $H_2O_2$, tricine, glycine, triton-X 100, tris, and digitonin were purchased from Sigma-Aldrich, USA. NADH, Protease inhibitor and Proteinase K were purchased from Roche. Immun-Blot® PVDF Membrane, Protein Assay Dye Reagent Concentrate and Clarity Western ECL Substrate were purchased from Bio-Rad Laboratories, USA.

**3.8 Antibodies:** Antibodies: Anti-p65, Anti-p-IκBα and Anti-TOM20 (Cell Signaling, ON, Canada), Anti-LYRM7, Anti-UQCRC2, Anti-SDHA (Thermofisher Scientific, Ottawa, ON, Canada), Anti-Actin (GenScript, Piscataway, NJ, USA), Secondary antibodies HRP-conjugated anti-rabbit and anti-mouse antibodies (Jackson Immuno Research, Baltimore, PA, USA).

### 3.9 CRISPR Cas9 knockout constructs:

sgRNA clones were generated by protocol described in Ran et al. (ref). The guide RNA targeting the exon of LYRM7 and p65 were designed using GPP sgRNA Designer tool (Broad Institute). Top and bottom sgRNA strand were synthesized as per the above-mentioned protocol. Oligos were annealed and cloned into BbsI-linearized pSpCa9(BB)-2A-Puro (PX459) V2.0 vector. LYRM7-sgRNA and p65-sgRNA clones were transformed into competent cells and transformed colonies were screened by colony PCR using U6 sequencing primer and sg-RNA bottom. Sanger sequencing was used to confirm the clones.

**LYRM7:** 5'AGCTCTTTAAAACACTGCAC3'

3'TCGAGAAATTTTGTGACGTG5'

**p65:** 5'TCAATGGCTACACAGGACCA3'

3'ACCAGGACACATCGGTAACT5'

### 3.10 Mitochondria isolation:

Cells were seeded at $3X10^6$ density and after overnight incubation cells were treated as indicated. The cells were passed through 24GX1" syringe 50-60 times using Sucrose-Tris mitochondria isolation buffer (0.25M Sucrose, 10mM Tris HCl, and 1X protease inhibitor). After centrifugation at 600g for 10 min, the supernatant was collected and centrifuged at 8000g. The obtained pellet (mitochondrial fraction) was washed thrice with the isolation buffer and lysed in RIPA lysis buffer.

### 3.11 Sample preparation and digestion

Isolated mitochondrial fractions were lysed in RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA) with protease inhibitor (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail (Roche Diagnostics), followed by a brief sonication on ice. The cells were sonicated and centrifuged for 15 min at 24,000g at 4 °C and the supernatant was transferred to a new tube. Protein concentration was determined using BCA assay kit (Thermo Scientific). Protein samples were fractionated on 4–12% Bis-Tris Gels (Invitrogen, Carlsbad, CA, USA) and stained with Coomassie Brilliant Blue (SigmaAldrich, St. Louis, MO, USA). Each gel lane

was cut into ten pieces and subjected to in-gel tryptic digestion following the general protocol[13]. Briefly, protein bands were excised, destained, washed, and further reduced with 20 mM DTT and alkylated with 55 mM iodoacetamide. After dehydration, the proteins were digested with 13 ng/ml sequencing-grade modified porcine trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted from the gel slices in 50% (v/v) ACN and 5% (v/v) formic acid and dried under vacuum.

### 3.12 Mass spectrometry analysis

Peptides were resuspended in 25 µL Solvent A (0.1% formic acid in water, pH 2.0) and 5 µL sample was loaded onto an analytic column (PepMap, 75µm ID*50 cm 3µm, ES803, Thermo Fisher Scientific, San Jose, CA, USA) interfaced with a nano-ultra-HPLC system (EasynLC, Thermo Fisher Scientific)and separated with a linear gradient of 5–32% Solvent B (0.1% formic acid in ACN), time (B%) 0~12 min (5% solvent B), 97 (40%), 105 (70%), 117 (70%), 120 (2%), for 120 min at a flow rate 300 nL/min. MS spectra were recorded on a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The standard mass spectrometric condition of the spray voltage was set to 2.2 kV and the temperature of the heated capillary was set to 250 °C. The full MS scans were acquired in the mass analyzer at 400–1400m/z with a resolution of 70,000 and the MS/MS scans were obtained with a resolution of 17,500 by normalized collision energy of 27 eV for high-energy collisional dissociation fragmentation. The automatic gain control target was $1\times10^5$, the maximum injection time was 120ms, and the isolation window was set to 2.0*m/z*. The Q-Exactive was operated in a data-dependent mode with one survey MS scan followed by ten MS/MS scans, and the duration time of dynamic exclusion was 20s.

### 3.13 Database search for proteomics data

Collected MS/MS data were searched against the decoy UniProt human database (version 3.83, 186 578 entries) by Proteome Discoverer 2.2 (PD 2.2, Thermo Scientific) software. Precursor and fragment ion tolerance were set to 10 ppm and 0.5 Da, respectively. Trypsin was chosen as the enzyme with a maximum allowance of up to two missed cleavages. Carbamidomethyl (+57.02) of cysteine was considered as the fixed modification, while the variable modification was set for methionine oxidation (+15.99). The result filtration parameters of PD 2.2 were set

as follows: peptide and protein identifications were accepted if they could be established at greater than 95% and 99% probability, respectively, as specified by the Peptide and Protein Prophet algorithm and if the protein identification contained at least two identified peptides with a false discovery rate ≤0.1%.

### 3.14 Relative protein quantification and bioinformatics analysis

Relative protein quantitation was accomplished using spectral counting. The MS/MS data were normalized to compare the abundances of proteins between samples using PD 2.2 software. The normalized spectral counts from triplicate analyses of the MCF-7 and MDA-MB-231 cells treated or untreated with TNF-α were compared using the R program with power-law global error model (PLGEM; version 1.50.0) software used to determine signal-to-noise ratio and P-value. We filtered statistically significant differentially expressed proteins (DEPs) using 0.01 as a p-value threshold. Then we refined spectral count readouts for the proteins within the range of 0.01≤ p-value ≤0.05 using the Moment Adjusted Imputation (MAI) equation to identify DEPs with statistical significance with more sensitivity. After the MAI refinements, we have determined the p-value with PLGEM and filtered statistically significant DEPs using 0.01 as a p-value threshold. The subcellular localization and functional annotation of the identified proteins were classified using Ingenuity Pathway Analysis (IPA, QIAGEN Inc., Valencia, CA, US) and Protein Analysis through Evolutionary Relationships Classification System ((PANTHER, version 7.2,). Kaplan–Meier survival analysis was used to estimate the association of the gene's expression with survival of patients.

### 3.15 BN-PAGE

MCF-7 and MDA-MB-231 cells were seeded at density $3X10^6$/100 mm dish. After overnight incubation cells were treated as required. Mitochondria from MCF-7 and MDA-MB-231 cells were isolated in Tris-Sucrose buffer as described above and 50 µg pellets were solubilized as per manufacturer's protocol (Thermo Fisher Scientific) and BN-PAGE was performed on Native PAGE Novex 3%–12% Bis-Tris Protein Gels (ThermoFisher Scientific). In-gel enzyme activity of different OXPHOS complexes was analysed on gradient Bis-Tris gel. For complex, I- 1mg NADH and 25mg NTB was used in 2mM Tris-HCl (pH-7.4), and for complex IV- 5mg DAB and 10mg cytochrome C in 50mM potassium phosphate buffer (pH-7.4) was used for in-

gel activity. For complex III and complex IV combined- 10mg 3,3' diaminobenzidine tetrachloride (DAB) and 25mg Cytochrome C in 25ml of 50mM sodium phosphate buffer (pH 7.2) was used.

### 3.16 Spectrophotometric analysis of mitochondrial Complex I and Complex II assays

MCF-7 and MDA-MB-231 cells were seeded at the density of $5\times10^5$ cells/well in the 6-well plate. The cells were treated as indicated, harvested, and washed with cold DPBS. The cells were subjected to 2–3 freeze-thaw cycles in a freeze-thaw complete solution (0.25 M sucrose, 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 $NaN_3$,1 mg/ml BSA, 2 µg/ml antimycin A, 5 mM NADH). Complex I activity was measured by monitoring the decrease in absorbance at 340 nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH. Similarly, for Complex II activity, cells were seeded at a density of $1.5x10^6$/60mm dish. The cells were harvested and washed with cold DPBS. The cells were suspended in 0.5 ml of 20 mM hypotonic potassium phosphate buffer (pH 7.5) and lysed using a 24G sterile syringe and subjected to freeze–thaw cycle. The cell lysate (80µg) was added to the 1ml of Complex II assay buffer (0.1M potassium phosphate (pH 7.5), 50mg/ml BSA, 100mM $NaN_3$, 200mM succinate) and incubated at 37ºC. Complex II activity was measured for 6min by monitoring the decrease in absorbance at 600nm after the addition of 2.5mM acceptor decylubiquinone and DCPIP.

### 3.17 ATP Assay

MCF-7 and MDA-MB-231 cells were seeded in a density of $5x10^4$ in 24 well plates. ATP levels were measured in control and treatment conditions by an ATP dependent luciferase assay using an ATP determination kit (Molecular Probes/Life Technologies, ON, Canada)

### 3.18 Assay of Intracellular and mitochondrial ROS:

Microscopy: ROS levels and mitochondrial ROS were measured by CM-$H_2$DCFDA (10 µM) and MitoSOX$^{TM}$ Red (5 µM) staining, respectively. Briefly, MCF-7 and MDA-MB-231 cells were plated at the density of $1.5 \times 10^5$ cells/well in 24-well plates. The cells were treated and stained with indicated reagent and monitored under a fluorescence microscope (Ti2-E inverted fluorescence microscope, Nikon, Japan). For the mitochondrial membrane potential, cells were treated and stained with 5 µM tetramethyl rhodamine methyl ester (TMRM) and monitored by fluorescence microscope. A minimum of 10 images and 80–100 cells were used for these analyses. Fluorometry: ROS levels were also quantified by fluorometry. Briefly, MCF-7 and MDA-MB-231 cells were treated and stained with CM-$H_2$DCFDA (12.5 µM) in DPBS for intracellular ROS quantification and MitoSOX Red (2.5 µM) in DMEM for mitochondrial ROS quantification. The cells were washed with DPBS and normalized to $1\times10^6$ cells/ml. Fluorescence intensity was quantified by a fluorometer (Hitachi High-Technologies Corp., Japan) with excitation/emission at 495/520–540 nm and 510/570–600 nm, respectively.

### 3.19 MTT assay

MDA-MB-231 were seeded (5000 cells/well) in 96-well plate. The cells were treated as indicated and cell viability was determined using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromideassay. The purple formazan crystals were dissolved in DMSO, transferred in a 96 well plate (100 µL/well) and the absorbance was recorded on a microplate reader at a wavelength of 570nm.

### 3.20 Scratch assay

Scratch assay was performed in MCF-7 and MDA-MB-231 cells. Cells were seeded at a density of 2.5X105 cells per well in 12 well plates. After overnight incubation cells were treated and a vertical wound was created using a sterile P200 micropipette tip. At zero time point, images of each scratch were taken using Nikon Ti-2 eclipse inverted fluorescent microscope at 10X and were analysed after 24 hours of treatment. Migration rate was measured using ImageJ software which measures open area at a different time interval. The percentage of open area in each condition was plotted.

**3.21 Cancer cell invasion assay:**

MDA-MB-231 cells were grown on an 8 µM pore size 24 well system (Costar, Corning, ME, USA) in DMEM serum-free medium. The lower chamber was filled with DMEM with serum. Cells were incubated for the required time and the cells on the inner chamber were removed using cotton swab. Invading cells were stained with crystal violet and quantified by microscopy.

**3.22 Colony forming assay:**

Cells were transfected with required vectors and kept for 24 h post media changes. Later, transfected cells were seeded at 2000 cells/well in 6 well. Cells were subjected to required treatments at 12 h post seeding. The formation of colonies was monitored for 12-14 days, and media were changed every 3 days until termination. Later, the cells were stained using crystal violet and colonies were counted.

**3.23 Migration assay:**

Cell migration was analysed by wound healing assay. Briefly, MDA-MB-231 and MCF-7 cells were seeded in 12 well plate. After 90% confluency, a scratch was created vertically in the middle of the wells using 200 µl tip to form a wound zone. Detached cells were removed by changing the media. The scratch was photographed for specific times using an inverted light microscope. The distance between the two layers was measured and migration was analysed.

**3.24 3D culture:**

Cells were seeded in 3D culture plates using 500 cells/well. After incubating for 24 h, cells were treated with required reagents and images of the spheroids formed were taken every day for 14-15 days by an inverted light microscope at 10X magnification.

**3.25 Boyden chamber assay**:

MDA-MB-231 cells transfected with control vector and sgRNA resuspended in serum-free DMEM medium were counted and adjusted to a concentration of 5x105 cells/ml. A 96 well

ChemoTx microplate with a 8µM filter (Neuro Probe, Inc., Gaithersburg, MD (Boyden chamber) was assembled and chemoattractant was added to the lower chamber. Cells(5X104) were added to the upper chamber (100µl). The assembly was incubated at 37℃ tissue a culture incubator for 4h. The filter is than fixed with methanol and stained with crystal violet. After wiping away the cells on the top of the filter, the membrane was scanned for image of migrated cells.

### 3.26 Immunohistochemistry:

IHC was performed on 5 µm paraffin-embedded tumor sections using Dako LSAB+ Universal Kit. Antigen retrieval was performed by microwaving hydrated slides in 10 mM citric acid for 20 min. Primary antibodies against LYRM7 was used for IHC staining. Ten images per section were taken by Zeiss Axioskop 2 imaging system. Mean intensity was measured using imageJ software.

### 3.27 Lung nodules measurement:

Formalin injected lungs were stained with India ink. Visible metastatic nodules were counted on each lobe.

### 3.28 Realtime PCR:

Total RNA was isolated using Biobasic Molecular Biology kit followed by cDNA synthesis by BlasTaq 2X qPCR MasterMix. Primers for gene of interest were designed by IDT primer designing tool for PCR. The levels of mRNAs were determined by $2^{-\Delta\Delta CT}$method using Cyclin A as endogenous control. The melt curves were also acquired.

**Table No. 2 Primer sequence**

| | |
|---|---|
| Mouse RELA Forward | ACCCGAAACTCAACTTCTGTC |
| Mouse RELA Reverse | TTGATGGTGCTGAGGGATG |
| | |
| Mouse TNF-α Forward | CTTCTGTCTACTGAACTTCGGG |
| Mouse TNF-α Reverse | CAGGCTTGTCACTCGAATTTTG |
| | |
| Mouse IL-6 Forward | CAAAGCCAGAGTCCTTCAGAG |
| Mouse IL-6 Reverse | GTCCTTAGCCACTCCTTCTG |
| | |
| Mouse IL-18 Forward | GCCTCAAACCTTCCAAATCAC |
| Mouse IL-18 Reverse | GTTGTCTGATTCCAGGTCTCC |
| | |
| Mouse ENPP2 (ATX) Forward | GAAAGCAGAGCATTCAGGGC |
| Mouse ENPP2 (ATX) Reverse | GGATATTACCTGGTATGACCCGAAA |
| | |
| Mouse LYRM7 Forward | AGCTGTTATTCAAGGAATTCACAC |
| Mouse LYRM7 Reverse | AAGGAGCAGAGATGGGTTTATC |
| | |
| Mouse cyclophilin A Forward | CACCGTGTTCTTCGACATCAC |
| Mouse cyclophilin A Reverse | CCAGTGCTCAGAGCTCGAAAG |
| | |
| Mouse GAPDH Forward | ACTTTGTCAAGCTCATTTCC |
| Mouse GAPDH Reverse | TCTTACTCCTTGGAGGCCAT |
| | |
| Mouse β-actin Forward | ACCTTCTACAATGAGCTGCG |
| Mouse β-actin Reverse | CTGGATGGCTACGTACATGG |

### 3.29 ATX assay:

ATX activity was measured in plasma, fat pads or in the primary tumor by the choline released from Lysophosphatidylcholine (LPC) (ref). Primary tumor and fat pads were homogenized in 500 µl of buffer A using the Qiagen Tissue Lyser II system (Qiagen, Toronto, ON, Canada). Activity measurements were normalized to protein content by the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA). Five µl of plasma or normalized tissue lysate was mixed with 19 µl of buffer A (100 mM Tris-HCl, pH 9.0; 500 mM NaCl; 5 mM $MgCl_2$; and 0.05% v/v Triton X-100). The blank value was established by adding 1 µl of 10 mM ONO-8430506 (ONO pharmaceuticals, Japan) to inhibit ATX activity and samples were incubated at 37°C for 30 min. Samples were then mixed with 25 µl of 6 mM LPC in buffer A and incubated for 2 h for plasma and 48 h for the primary tumor lysate at 37°C. Then 20 µl samples were added into a 96-well plate and incubated at 37°C for 2 h with 90 µl of buffer C [For 24 samples: 2.2 ml of buffer B (100 mM Tris-HCl, pH 8.5, and 5 mM $CaCl_2$) mixed with 1µl 1000U/ml Horseradish

peroxidase 7 µl 10 mM Amplex Red and 7 µl 100U/ml choline oxidase. Choline formation was measured at fluorescence Ex544/Em590 nm.

### 3.30 Cytokine/chemokine measurements

Cytokines and chemokines were measured in plasma and primary tumor tissue by Eve Technologies Corp. (Calgary, AB, Canada), with a Milliplex Mouse Cytokine/Chemokine 32-plex kit (Millipore, MO), according to the manufacturer's protocol, on a Luminex 100 system (Luminex, Austin, TX). Tissue specimens (25-30 mg) were homogenized in 200 µl of 20 mM Tris-HCl (pH 7.5) buffer with 0.5% Tween 20, 150 mM NaCl, and protease inhibitor cocktail (HB9081, Hello Bio, USA) and centrifuged for 10 min at 4 °C, and the supernatant was transferred to a fresh tube. Protein content was measured using the Pierce BCA protein assay Kit (Thermo Fisher Scientific, USA). Samples were normalized before analyses. Plasma samples were diluted with 25 µl of PBS before measurements.

### 3.31 Databases:

The TIMER database is a web resource used for systemic analysis and evaluation of clinical impacts of different immune factors in diverse cancer types. Hence, we analysed transcript levels of genes of interest using the correlation tool in breast cancer patients. Differential expression of genes in tumor versus adjacent normal tissue was analysed using Gene_DE module from TIMER database. The Human Protein Atlas was explored to understand the gene expression in breast cancer tissue of patients. The BCGENEX Miner database was used to investigate the expression of genes in different subtypes of breast cancer patients. Kaplan Meier plotter was used to estimate the effects of genes on survival of breast cancer patients. The survival analysis gives overall survival rates in high and low expression of genes. The Hazard ratio (HR) and 95% confidence were calculated automatically by website tool. The Eukaryotic promoter database was used to explore the binding sites of transcription factors on promoter region of gene of interest. The TIMER database (http://timer.cistrome.org/) is a web resource used for systemic analysis and evaluation of clinical impacts of different immune factors in diverse cancer types. Hence, we analysed transcript levels of genes of interest using the correlation tool in breast cancer patients (TCGA-BRCA data through GDC data portal). The bc-GenExMiner database (http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php) was

used to investigate the expression of genes in different subtypes of breast cancer patients. The GEPIA2 data base (http://gepia2.cancer-pku.cn/) was used for correlation of genes of interest in breast tumors (TCGA and GTEX datasets). Spearman's rho value was used as the degree of their correlation with values lying between -1 to +1.

### 3.32 Statistics

Data are shown as mean ± SEM for number of times the experiment was repeated. Comparisons between two groups were performed using student t-test or ANOVA for repeated measurements to determine the levels of significance for each group. For correlation studies regression analysis was used as a statistical method to examine the relation between genes of interest as described above. The experiments were performed minimum three times independently and $p<0.05$ was considered as statistically significant. GraphPad Prism (version 9) software was used to perform all the statistical analysis.