CHAPTER 3
METHODOLOGY

3.1 INTRODUCTION

Sample preparation is key to any mass spectrometric experiment and it is very vital especially in signal transduction experiments for investigating phosphorylation a transient reaction, in order to capture the spectrum of molecules that are differentially phosphorylated on treatment with the drug and in order to view changes in the phosphorylation status triggered by the drug protocol optimization is necessary. A rapid lysis protocol in a buffer that preserves phosphorylation on the tyrosine, serine and threonine residues is vital. In order to identify such post-translational modifications (PTMs) mass spectrometers with higher sensitivity is key to the whole experiment.

3.2 CELL CULTURE AND PREPARATION OF LYSATES

H3255 cell was obtained from ATCC and cultured in RPMI medium at 37°C, 5% CO2 and high humidity in accordance with the previously described protocol SILAC95,97,102. Briefly, SILAC media were prepared by supplementing Arginine/Lysine free RPMI medium with “light” [normal Arg (200mg/l)/Lys (60mg/l)], “medium” [13C6Arginine (254mg/l)/D4 Lysine (61mg/l)], or “heavy” [13C6,15N4Arginine (262mg/l)/13C6,15N2 Lysine (62mg/l)] labeled amino acids. All amino acids were purchased from either Cambridge Isotope laboratories Inc, Andover MA or from Sigma-Aldrich, Inc. St. Louis, MO. The cells were passaged five times to ensure complete labeling. Cell lysates were prepared to check for labeling efficiency and only when complete labeling was observed, between 5x10^7-10^8 cells were seeded onto 15 cm dishes. The cells were serum starved for 14 hours prior to experimentation. Light cells were left untreated as the control, the medium labeled cells were stimulated with 100 ng/ml EGF (Cat#01-107, Millipore) for 3 minutes and the heavy cells were treated with Erlotinib (Genentech) to a final concentration of 100nM for two hours before stimulating the cells with 100 ng/ml EGF for 3 minutes. The cells were lysed with Urea lysis buffer (20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium
orthovanadate 2.5 mM sodium pyrophosphate and 1 mM ß-glycerophosphate). Protein concentrations were determined by the Modified Lowry method (BioRad), and equal amounts of protein from lysates of each state were mixed together. For Western blots, unlabeled cells were lysed with modified RIPA buffer (50 mM Tris-HCl, pH 7.4 containing 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, protease inhibitor mixture tablets (Roche), 1 mM Na3VO4, NaF 1 mM). For the phospho kinase arrays lysates were prepared by rinsing nearly 1x10⁷ cells with PBS and were lysed in Lysis Buffer 6 (R&D Systems).

3.3 Tryptic Digestion of Peptides and Purification of Digested Peptides

Pooled lysates from all three SILAC states were combined to constitute either 10 or 20mg of the pooled lysate that were homogenized and centrifuged at 15,000g followed by reduction and alkylation with 45 mM dithiothreitol (Sigma Aldrich, MO) and 100mM iodoacetamide(Sigma Aldrich, MO) respectively. Modified sequencing grade Trypsin (Promega, Madison, WI, US) was used to carry out digestion of the lysate at 37°C for 16 hrs. The digest was then acidified using 0.1% TFA and lysates were loaded onto the column followed by a wash of 0.1% TFA. The bound peptides were eluted with 0.1% TFA and 40% acetonitrile (ACN). The lysates were dried in a lyophilizer to remove excess ACN and TFA. The lyophilized lysate was used either for SCX chromatography for enrichment of phosphorylated serine or threonine residues or for Phospho-Scan enrichment of phosphorylated tyrosine residues

3.4 Strong Cation Exchange (SCX) Chromatographic Fractionation

SCX was performed as described earlier using an Agilent 1100 HPLC system (Agilent Technologies) with C18 guard columns and a polysulfoethylA SCX column (PolyLC, Columbia, MD; 200 mm 2.1 mm inner diameter, 5 m, 200-Å). Dry peptides were resuspended in SCX Buffer A (5 mM KH₂PO₄, 30% ACN and 0.05% FA, pH 2.7), loaded onto the SCX column with 80% ACN and 0.05% formic acid and eluted over a gradient of SCX buffer B (350 mM KCl, 5 mM KH₂PO₄, 30% ACN and 0.05% FA, pH
2.7). The gradient was performed as follows: 0% B for 5 min, 0–50% B in 30 min, 50–100% B in 7 min and 100% B for 3 min. A gradient time of 50 min was followed. A total of 96 fractions were collected in a 96-well plate and were dried in a vacuum centrifuge.

3.5 TiO₂ Enrichment

5µm TiO₂ beads (Titansphere, GL Sciences) were incubated with DHB containing wash Solution A (3% 2,5,-Dihydroxybenzoic acid, 80% ACN and 1%TFA) for two hours. The SCX fractionated samples were pooled into 30 fractions in wash Solution B (80% ACN and 1% TFA). 50µl of beads were added to each of the fractions and they were incubated overnight at room temperature on rotation. The beads were then washed thrice with Solution A, twice with Solution B and once with 40% ACN. The bound peptides were eluted into tubes with NH₄OH in 40% ACN. The peptides were dried and resuspended in SCX Buffer B prior to LC-MS/MS analysis.

3.6 Tyrosine Phosphopeptide Enrichment

The urea lysates were homogenized and centrifuged at 15,000g and 40 mg of protein was used for immunoaffinity purification. The lysates were reduced and alkylated with 45 mM dithiothreitol and 100mM iodoacetamide respectively. Trypsin (modified sequencing grade; Promega, Madison, WI, US) digestion was carried out at 37°C for 16 hrs. The digest was then acidified using TFA and cleaned to remove salts. Briefly, a C18 column was activated with 100% CAN and equilibrated using 0.1% TFA and lysates were loaded onto the column followed by a wash with 0.1% TFA. The bound peptides were eluted with 0.1% TFA and 40% ACN. Phosphopeptide enrichment was carried out using the P-Tyr-100 antibody from Cell Signaling Technologies. Briefly, the lyophilized peptides were resuspended in Immunoaffinity purification buffer (IAP) containing 50 mM MOPS pH 7.2, 10 mM sodium phosphate and 50 mM NaCl and pH 7.2. The sample was centrifuged to remove precipitate. The peptide solution was then mixed with phosphotyrosine antibody beads and incubated for 30 minutes. The bound phosphopeptides were eluted in 0.1% TFA. The enriched phosphopeptides were
concentrated for LC-MS/MS using a ZipTip, dried and stored at -80°C until LC-MS/MS analysis.

3.7 LC-MS/MS ANALYSIS

The mixture of phosphopeptides enriched from SCX fractions or PhosphoScan protocol were analyzed on an LTQ Orbitrap XL ETD (Thermo Scientific), interfaced with a dual nano pump (Eksigent) and an Agilent 1100 microwell plate autosampler. Phosphopeptides were loaded onto a trap column (75µm x 2 cm, Magic C18AQ 5 µm, 100Å, Michrom Bioresources), separated on an analytical column (75µm x 15 cm, Magic C18AQ, 5 µm, 100Å, Michrom Bioresources,) at 300nL/min flow rate with a running time of 75 minutes. The MS data was acquired at a resolution of 60,000 at m/z 400 and MS/MS data was acquired on an ion trap. For each cycle of data dependent analysis the 8 most abundant precursors were selected for MS/MS analysis with normalized collision energy of 35%. Multistage activation mode was enabled with neutral loss masses of 32.66, 48.99 and 97.97. Selected ions for fragmentation were excluded dynamically for 90 seconds.

3.8 DATA ANALYSIS

Raw MS/MS spectra were processed by using the freely available MaxQuant software\textsuperscript{167-168}. The derived peak list was searched against the Refseq 40 human protein database V40 as deemed necessary to which a set of normally contaminants and all reverse sequences was added. The parameters used for data analysis include trypsin as a protease with allowed one missed cleavage. Carbamidomethyl cysteine was specified as a fixed modification. Phosphorylation at serine, threonine and tyrosine, deamidation of asparagine and glutamine, oxidation of methionine and protein N-terminal acetylation were specified as variable modifications. The precursor mass tolerance was set to 20 ppm and fragment mass tolerance to 0.5 Da. False discovery rate was calculated using a decoy database and a 1% cutoff was applied.
3.9 IMMUNODETECTION ASSAYS

For Western blot analysis 100 µg of lysate was separated by SDS-PAGE (Invitrogen) and transferred to nitrocellulose membrane. After blocking in 5% BSA in PBST for one hour membranes were incubated with the appropriate antibody [CBL (Y774) Cat#3555, SHP2 (Y580) #3703, SHP2 #3397, SHIP2 (Y986/987) #2008, SHIP2 #2839, mTOR Cat#2972, pmTOR (S2248) Cat#5536, RSK2 Cat#5528, pRSK2 (S380) Cat#9341, ERK1/2 Cat#4695, pERK1 (T202/Y204) Cat#4376, AKT1 Cat#4685 and pAKT1 (S473) Cat#4058 were purchased from Cell Signaling Technology, MA, Actin Cat#A2228 was purchased from Sigma Aldrich, MO and HRS Cat#sc-271925 was purchased from Santa Cruz, CA.] followed by secondary antibody coupled with horseradish peroxidase (Cell Signaling Technology/Sigma Aldrich). Membranes were incubated with ECL (Amersham) for 5 minutes prior to exposing to X-ray film.

For antibody arrays, cells were washed with PBS and lysed with Lysis Buffer 6 (R&D systems) and centrifuged to remove cell debris. The human Phospho-MAPK array (R&D systems) was used to according to the manufacturer’s instructions and the lysate was incubated with the membrane post blocking. The membrane was then exposed to X-ray film for 5 min. The following are the kinases that were investigated with these arrays. Cells were washed with PBS and lysed with Lysis Buffer 6 (R&D systems) and centrifuged to remove cell debris. The human Phospho-MAPK array (R&D systems) was used to according to the manufacturer’s instructions and the lysate was incubated with the membrane post blocking. The membrane was then exposed to X-ray film for 5 min.

3.10 IPA ANALYSIS TO IDENTIFY CANONICAL PATHWAYS

Data was analyzed using IPA (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com). First, molecules were divided into nine categories based on expression dynamics in response to EGF activation and Erlotinib treatment. A comparative analysis was done among the different groups of molecules to select specific signaling pathways that were enriched for each pathway. A threshold value
of 0.05 was selected and a B-H Correction was done to determine FDR. The ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway was determined and the B-H P-value was calculated using B-H test determining the probability that the association between the genes in the data set. The IPA knowledge base database was used with respect to pathways that were enriched to identify other interacting molecules that could be involved in signaling.