Supplementary Materials

for

"A Targeted Quantitative Proteomics Strategy for Global Kinome Profiling of Cancer Cells and Tissues"

by

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Supplementary Materials and Methods

Preparation of the Desthiobiotinylated Nucleotide Affinity Probe

The desthiobiotinylated nucleotide affinity probes were prepared according to previously published procedures with minor modifications (1, 2). In this regard, we first prepared light or heavy form of desthiobiotinyl-aminobutyric acid (desthiobiotin-C4) following previously published procedures. Isobutyl chloroformate (0.19 mL) was added to a solution containing 150 mg desthiobiotin, 15 mL DMF, and 0.38 mL tri-*n*-butylamine. After being incubated at room temperature for 10 min, the mixture was slowly added to a suspension of 230 mg light or heavy γ-aminobutyric acid (Sigma) in DMF (7.5 mL) at 5 °C. After stirring at 5 °C for 2 h, the solvent was removed under reduced pressure and the crude product was dissolved in 5 mL water at 40 °C. The solution pH was adjusted to 2 with 2.0 N HCl, and the mixture was kept at 0 °C for 12 h. The desired desthiobiotin-C4 was precipitated out of solution. The precipitate was filtered, washed with water, and dried under vacuum.

To render nucleotide soluble in organic solvent, the commercially available sodium salt form of ATP was first converted to the tributylammonium form by passing the nucleotide through a cation-exchange column packed with Spectra/Gel IE 50×8 resin (40-75 μm) at 4 °C once. Fractions containing the tributylammonium form of ATP were collected and lyophilized. Desthiobiotin-C4 (8 mg), dissolved in a 1-mL solvent mixture of ice-cold dry CH_2Cl_2 and DMF (4:1, v/v), was mixed with tri-*n*-butylamine (11 μL) and ethyl chloroformate (5 μL). After stirring at 0 °C for 5 min, the mixture was stirred at room temperature under argon atmosphere for another 60 min. Tributylammonium form of ATP (50 mg), dissolved in a 1.25 mL solution of CH_2Cl_2 and DMF (4:1, v/v), was then added to the above reaction mixture. The reaction was continued at room temperature and under argon atmosphere for 18 h. The CH_2Cl_2 was then removed by argon purging for 10 min and the remaining 200 μL solution was directly subjected to HPLC purification using a YMC ODS-AQ column (4.8×250 mm, 120 Å in pore size, 5 μm in particle size, Waters). The flow rate was 0.8 mL/min, and a 45-min linear gradient of 0-30% acetonitrile in 50 mM triethylammonium acetate (pH 6.8) was used for the purification. A UV detector was set at 265 nm to monitor the effluents. Appropriate HPLC fractions were pooled, lyophilized, and stored at -80 °C. The structures of the products were confirmed by ESI-MS analysis (3).

Protein lysate preparation from cultured cells and human lung tissues

HeLa-S3 cells were purchased from the National Cell Culture Center (Minneapolis, MN). IMR-90, WM-115 and WM-266-4 cells (ATCC; Manassas, VA) were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and penicillin (100 IU/mL). K562 cells (ATCC) were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum and penicillin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Approximately 2×10^7 cells were harvested, washed with cold PBS for three times, and lysed in a 1-mL lysis buffer, which contained 0.7% CHAPS, 50 mM HEPES (pH 7.4), 0.5 mM EDTA, 100 mM NaCl, and 10 μL (1:100) protease inhibitor cocktail on ice for 30 min. The cell lysates were centrifuged at 16,000g at 4 °C for 30 min and the resulting supernatants were collected.

Human lung tumor tissues and paired adjacent normal tissues were purchased from National Disease Research Interchange (NDRI, Philadelphia, PA). Frozen tissue samples were thawed, minced with razor blades, and weighed to obtain about 0.2 g of tissue prior to protein extraction. In the presence of liquid nitrogen, tissue samples were grounded into fine powders and then homogenized on ice with the same cell lysis buffer described above in a Dounce homogenizer for 2-3 min. The same experimental procedures and conditions were applied for the protein extracts of all the examined tissue samples to achieve comparable extraction efficiency. Cell debris was removed by centrifugation at 16,000 g at 4 °C for 30 min.

Approximately 1 mL of the resulting protein extracts from either cultured cells or human tissues were subjected to gel filtration separation using NAP-25 columns (Amersham Biosciences) to remove free endogenous nucleotides. Cell lysates were eluted into a 2-mL buffer, containing 50 mM HEPES (pH 7.4), 75 mM NaCl, and 5% glycerol. The amounts of proteins in the lysates were quantified using Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA) and stored at –80 °C. Prior to the labeling reaction, $MgCl_2$, $MnCl_2$, and $CaCl_2$ were added to the concentrated cell lysate until their final concentrations reached 50, 5, and 5 mM, respectively.

On-line 2D-LC-MS/MS analysis on an LTQ-Orbitrap Velos for discovery mode analysis

On-line 2D-LC-MS/MS analysis was performed on an LTQ-Orbitrap Velos mass spectrometer equipped with a nanoelectrospray ionization source (Thermo Fisher Scientific, San Jose, CA). The fully automated 7-cycle on-line two-dimensional LC-MS/MS was set up as described (4). Briefly, samples were automatically loaded from a 48-well microplate autosampler using an EASY-nLC II system (Thermo) at 3 µL/min onto a biphasic precolumn (150 µm i.d.) comprised of a 3.5-cm column packed with 5 µm C18 120 Å reversed-phase material (ReproSil-Pur 120 C18-AQ, Dr. Maisch) and 3.5-cm column packed with Luna 5 µm SCX 100 Å strong cation exchange resin (Phenomenex, Torrance, CA). The biphasic trapping column was connected to a 20 cm fused silica analytical column (PicoTip Emitter, New Objective, 75 µm i.d.) with 3 µm C18 beads (ReproSil-Pur 120 C18-AQ, Dr. Maisch). Ammonium acetate at concentrations of 0, 25, 50, 75, 125, 200 and 500 mM were then sequentially injected using a 48-well autosampler from the sample vial to elute bound peptides from precolumn to the analytical column with reversed-phase separation. The peptides were then separated with a 180-min linear gradient of 2-35% acetonitrile in 0.1% formic acid and at a flow rate of 250 nL/min. The LTQ-Orbitrap Velos was operated in a data-dependent scan mode. Full-scan mass spectra were acquired using the Orbitrap analyzer with a resolution of 60,000 with lock mass option enabled for the ion of *m/z* 445.120025 (5). Up to 20 most abundant ions found in MS with charge state $\geq$2 were sequentially isolated and sequenced in the linear ion trap with a normalized collision energy of 35, an activation Q value of 0.25 and an activation time of 10 ms.

Data processing and analysis for discovery mode results.

The raw data were first converted to mzXML files and DTA files using ReAdW (http://sourceforge.net/projects/sashimi/files/) and MzXML2Search (http://tools.proteomecenter.org/wiki/index.php?title=Software:MzXML2Search) programs, respectively. Bioworks 3.2 was used for protein identification by searching the DTA files against the human IPI protein database version 3.68 (87,062 entries) and its reversed

complement. Initial precursor mass tolerance of 10 ppm and fragment mass deviation of 0.8 Th were set as the search criteria. The maximum number of miss-cleavages for trypsin was set at two per peptide. Cysteine carbamidomethylation was considered as a fixed modification, whereas methionine oxidation and light or heavy desthiobiotinylation of lysine (+281.17394 Da and +287.21160 Da, respectively) were included as variable modifications. The search results were then filtered with DTASelect (6) to achieve a peptide false discovery rate of 1%. Census was employed for peptide and protein quantification (7). Extracted-ion chromatograms were first generated for peptide ions based on their *m*/*z* values and peptide intensity ratios were subsequently calculated in Census from peak areas found in each pair of extracted-ion chromatograms.

MRM spectral library generation for the kinome peptides

Skyline (version 1.4.0.4421) (8) was used to generate the spectral libraries for kinome peptides. All raw files generated in discovery-mode proteomic analysis on an LTQ-Orbitrap Velos were searched by using Maxquant (Version 1.2.2.5) (9) against the human IPI protein database version 3.68 (87,062 entries) using the same search parameters as described above. The resulting MS/MS files were then imported into Skyline and filtered with a threshold score of 0.9. An interactive Skyline spectral library file containing tandem mass spectra of all desthiobiotin-labeled kinome peptides was then generated.

Retention time (RT) extraction and iRT calculation for targeted peptides

To calculate the iRT score (10) for each kinome peptide in the MRM kinome library, 10 peptides derived from the tryptic digestion mixture of bovine serum albumin (BSA) were selected to constitute the reference peptides for a new iRT scale. iRT of these 10 BSA standard peptides were calculated using the empirically measured RT from shotgun LC-MS/MS analysis by setting iRT scores of peptides AEFVEVTK and DAFLGSFLYEYSR as 0 and 100, respectively. The BSA peptide mixture was then added to desthiobiotin-labeled kinase peptide mixture from IMR-90 and K562 cell lysates and measured by LC-MS/MS on the Orbitrap system with a regular 130-min linear gradient. RTs were extracted for all BSA standard peptides as well as kinase peptides using the Skyline MS1 filtering workflow (11). The transformed iRT for all the newly identified kinase peptides were calculated based on linear regression of iRT and empirically measured RT of peptides with previously determined iRT score. With the accumulation of iRT scores for targeted kinase peptides, it is not necessary to include the BSA standard mixture for RT extraction and iRT calculation. Any common kinase peptides that yield a regression with correlation of 0.99 or higher were used as standard peptides to calculate the new iRT score for newly identified kinase peptides by Skyline.

Western blot

WM-115 and WM-266-4 cells were cultured in 75 cm^2 cell culture flasks until 70-80% confluency and lysed as described above. The concentrations of the resulting protein lysates were determined by Bradford Assay (Bio-Rad). The whole cell lysate for each sample (20 μg) was denatured by boiling in Laemmli loading buffer and then electrophoresed on a 12% SDS-PAGE gel with a 4% stacking gel. Subsequently, proteins were transferred onto the

nitrocellulose membrane at 4 °C overnight. The resulting membrane was blocked with PBS-T (PBS with 0.1% Tween 20) containing 5% milk (Bio-Rad) at 4 °C for 6 h. Next, the membrane was incubated with primary antibody at 4 °C overnight and then secondary antibody at room temperature for 1 h. After thorough washing with PBS-T buffer, the HRP signals were detected with Pierce ECL Western Blotting Substrate (Thermo).

EphA2 (D4A2) XP® Rabbit mAb (Cell Signaling # 6997, with dilution ratio 1:5000) and CDK2 (78B2) Rabbit mAb (Cell Signaling #2546, with dilution ratio 1:1000) were employed as primary antibodies, and horseradish peroxidase-conjugated anti-rabbit IgG were used as secondary antibodies. Membranes were also probed with anti-actin antibody (1:10,000) to confirm equal protein loading.

MTT cell proliferation assay

For MTT assay, WM-115 and WM-266-4 cells were placed in a 96 well plate at a density of 4×10^3 cells per well with 100 μL EMEM medium. After a 24-h pre-incubation, cells were treated with dasatinib or flavopiridol (Santa Cruz Biotechnology) at concentrations ranging from 32 nM to 1 μM in serum-free EMEM medium for 24 h. The medium was then supplemented with 10 μL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Roche, Basel, Switzerland] reagent (0.5 g/L) to each well and incubated at 37 °C for 4 h. After this incubation period, purple formazan salt crystals were formed. Thereafter 100 μL of solubilizing solution was added to each well. The plates were incubated at 37 °C overnight to dissolve the formazan crystals. The solubilized formazan product was spectrophotometrically quantified using the Victor 2 plate reader (Perkin Elmer, Waltham, MA). Absorbance readings were performed at 570 nm with reference of 630 nm.

Supplementary References:

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Supplementary Figures

Figure S1. A schematic diagram showing the reaction between isotope-coded ATP affinity probe with an ATP-binding protein.

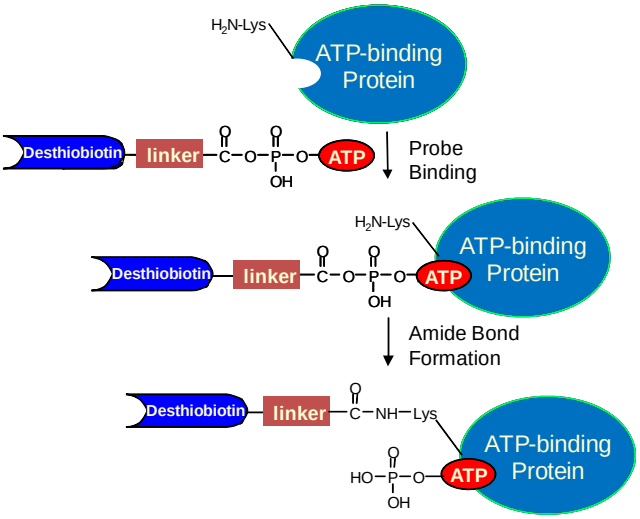
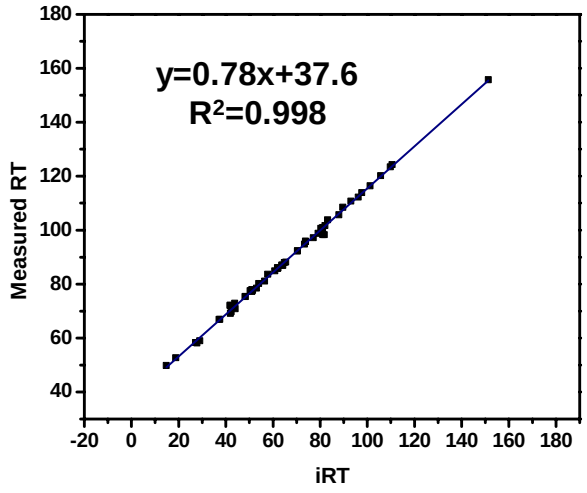
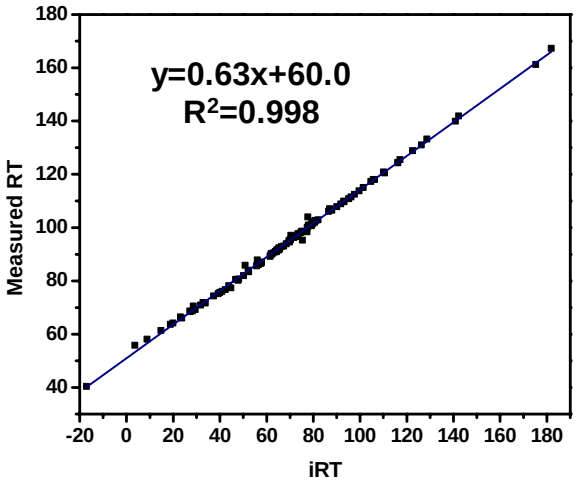


Figure S2. Linearity of iRT and measured RT on different instrument and experiment platforms. (A) iRT values predict measured RT in 1D LC-MS/MS experiment using Jurkat-T cell lysates on an Orbitrap Velos (180 min linear gradient) with a very high correlation coefficient (R^2=0.998); (B) iRT values predict measured RT in online 2D LC-MS/MS experiment using K562 cell lysates on an Orbitrap Velos (180 min linear gradient) with a very high correlation coefficient (R^2=0.998) (C) iRT values predict measured RT in 1D LC-MS/MS experiment using HeLa-S3 cell lysates after offline HPLC separation on Orbitrap Velos (130 min linear gradient) with a very high correlation coefficient (R^2=0.997).

(A)



(B)



(C)

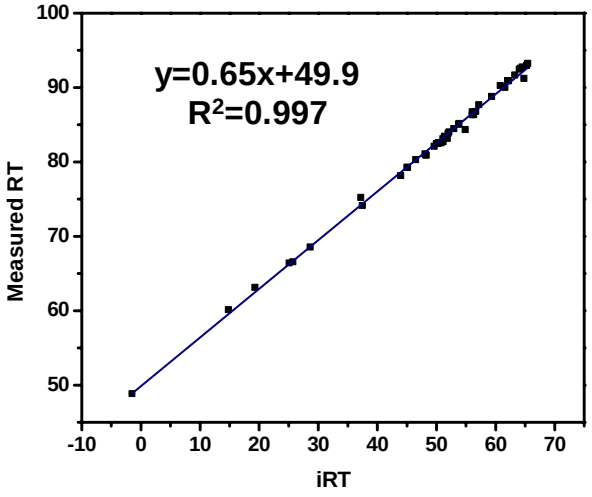

y=0.65x+49.9
R2=0.997
Measured RT
iRT
100
90
80
70
60
50
-10
0
10
20
30
40
50
60
70

Figure S3. Quantitative results by DDA shotgun proteomics analysis for peptide ETSVLAAAK#VIDTK from SLK kinase.

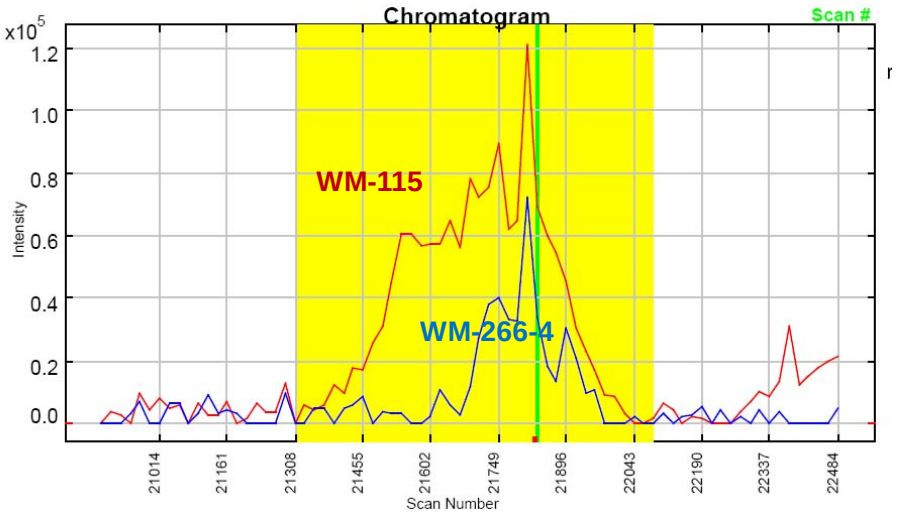


Figure S4. (A, B, C) Quantitative results obtained from MRM assay for peptide FLEDDTSDPTYTSALGGK#IPIR from Ephrin type-B receptor 2: Extracted ion chromatograms for three transitions monitored for light- (Red) and heavy-labeled (Blue) peptides in forward (A) and reverse (B) labeling reactions; (C) The distribution of the peak areas observed for each monitored transition from light- and heavy- labeled peptides in forward and reverse labeling reactions along with the theoretical distribution derived from MS/MS acquired from discovery mode analysis and stored in the kinome MRM library; (D) Quantitative results by DDA shotgun proteomics analysis for peptide FLEDDTSDPTYTSALGGK#IPIR.

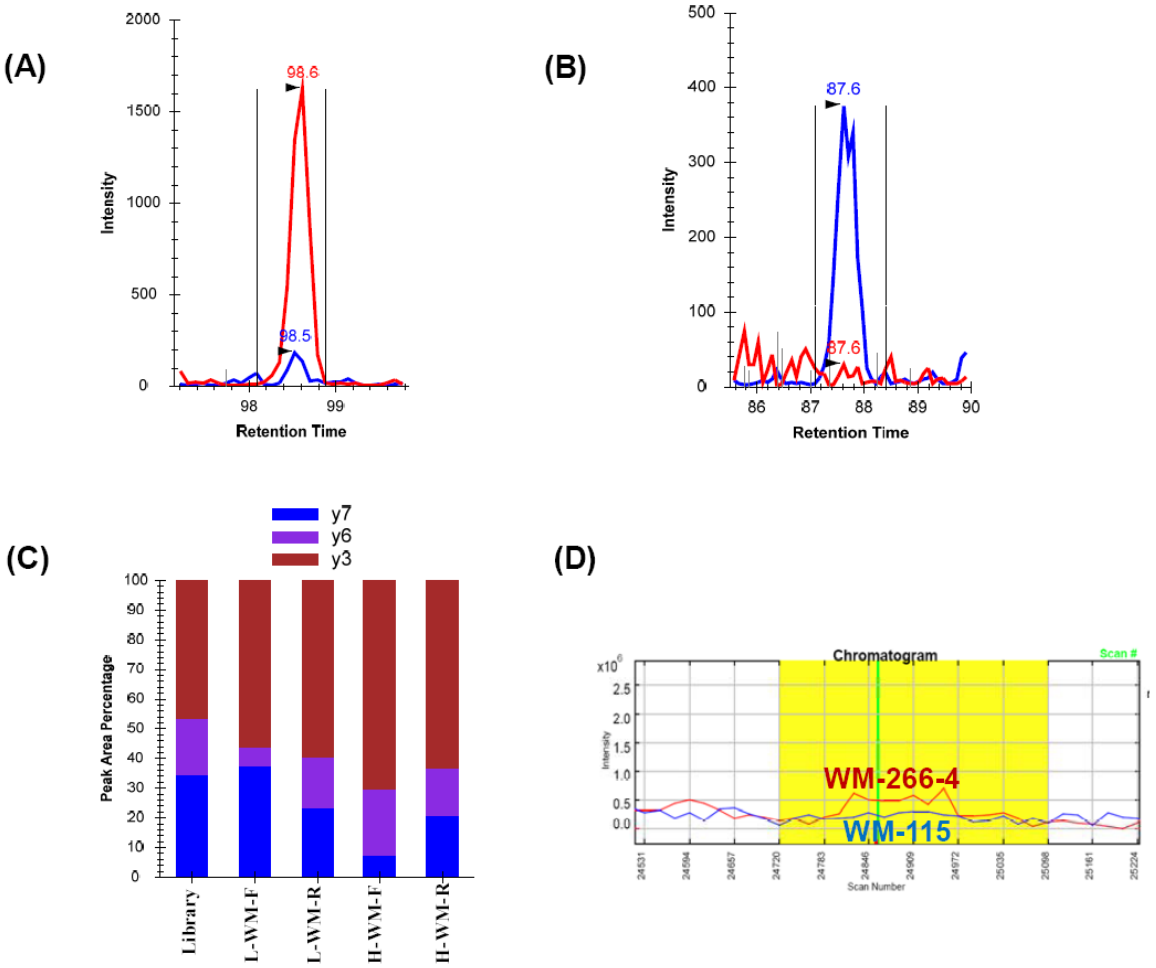


Figure S5 A heatmap showing the differential expression of kinases in WM-115 and WM-266-4 cells based on $R_{WM\text{-}115/WM\text{-}266\text{-}4}$ ratio. Dark red and white boxes represent kinases that are upregulated in WM-115 cells and WM-266-4 cells, respectively, as indicated by the scale bar above the heatmap.

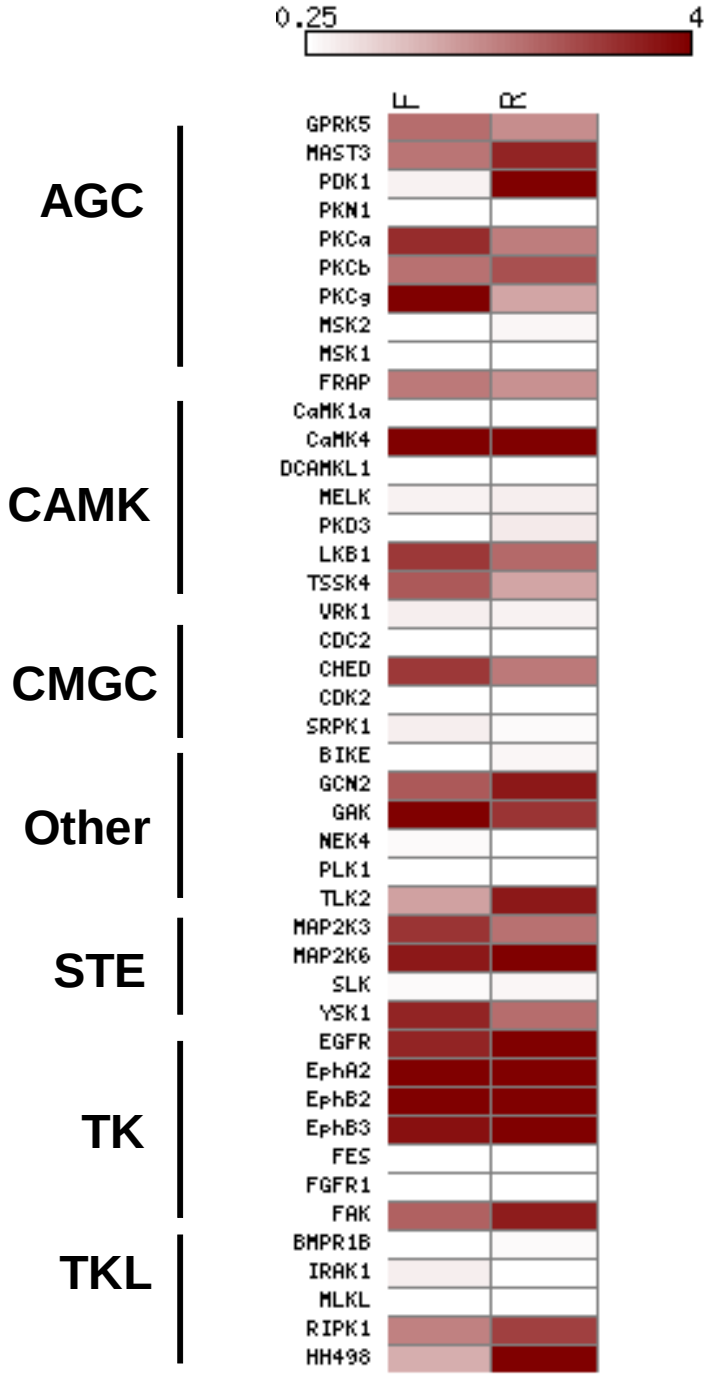


Figure S6 (A) Quantified protein kinases from WM-115 and WM-266-4 cells mapped in the dendrogram of the human kinome. Filled red circles indicate the kinases that are upregulated in WM-115 cells; filled blue circles indicate the kinase that are upregulated in WM-266-4 cells; Red shadow covers the targeted kinases for dasatinib; blue shadow covers the targeted kinases for flavopiridol. (B) Percentage of differentially expressed kinases from WM-115 and WM-266-4 cells for the seven major kinase groups as well as the atypical and other kinases. White bar represents the percentage of kinases upregulated in WM-115 cells; grey bar represents the percentage of kinases upregulated in WM-266-4 cells; black bar represents the percentage of kinases differentially expressed in either of these two cells.

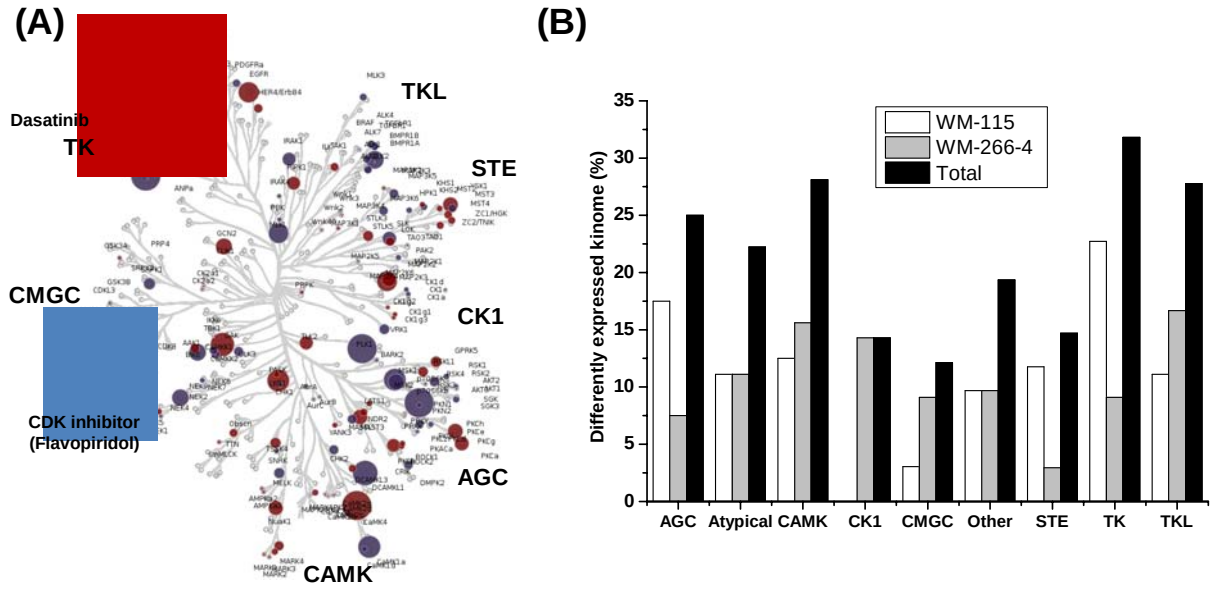
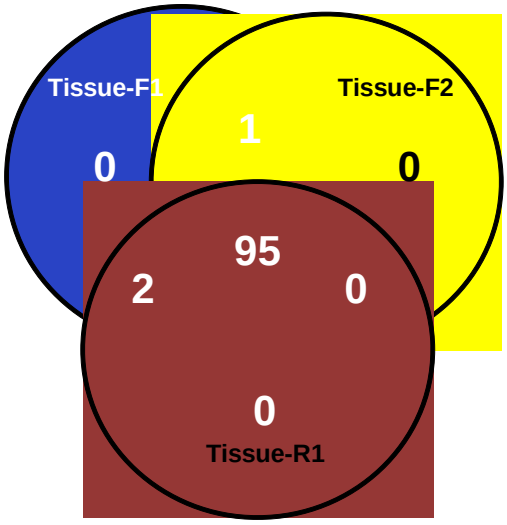


Figure S7 The Venn diagrams showing the overlap of quantified kinase peptides from human tumor and normal lung tissues by triplicates of MRM analysis.



A list of Supplementary Tables:

Table S1. Targeted kinases and kinase peptides in human MRM kinome library. The "refined kinase" table listed all the targeted kinases. The sequence and function annotations for protein kinases were adopted from reference (Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science. 2002;298:1912-34.). The "refined kinase peptides" Table contains sequence and modification information of all 383 kinase peptides. All peptide sequences are mapped to the human IPI and UniProt protein identifier. For kinase peptides, which can be mapped to multiple kinases, all the corresponding kinases are listed. The class of each targeted peptides is also assigned according to the manuscript.

Table S2. A list of identified peptides as well as kinases in human MRM kinome assay for IMR-90 cell lysates.

Table S3. A list of quantified peptides as well as kinases in human MRM kinome assay for protein lystates of WM-115 and WM-266-4 cells. The results were based on one set of forward and one set of reverse labeling experiments. Shown also are a list of quantified peptides as well as kinases in discovery-mode shotgun proteomics analysis for protein lystates of WM-115 and WM-266-4 cells, which is based on two sets of forward and two sets of reverse labeling experiments.

Table S4. A list of quantified peptides as well as kinases in human MRM kinome assay for protein extracts of human lung tissue. The results were based on two sets of forward and one set of reverse labeling experiments.

Supplementary Document (MCP-kinase library-skyline.zip): MCP-kinase library-skyline.zip file is the exported Skyline document file containing the MS/MS spectra and iRT information for 386 targeted kinase peptides and 10 standard BSA peptides. To open the skyline document file, extract MCP-kinase library-skyline.zip file into single folder and open kinase-1.sky file using Skyline software developed in MacCoss lab.