

A QUANTITATIVE PHOSPHOPROTEOMIC ANALYSIS OF THE T-CELL
RECEPTOR SIGNALING PATHWAY USING NOVEL HIGH-THROUGHPUT
APPROACHES

By

Vinh Hai Nguyen

B.S., University of California, San Diego, La Jolla, CA, 2004

M.S., University of California, San Diego, La Jolla, CA, 2005

A Dissertation Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in the Department of
Molecular Biology, Cell Biology, and Biochemistry at Brown University

Providence, Rhode Island

May 2011

© Copyright 2010 by Vinh Hai Nguyen

All Rights Reserved

This dissertation by Vinh Hai Nguyen is accepted in its present form by the Department of Molecular Biology, Cell Biology, and Biochemistry as satisfying the dissertation requirements for the Degree of Doctor of Philosophy.

Date _____

Dr. Arthur R. Salomon, Director

Recommend to the Graduate Council

Date _____

Dr. Laurent Brossay, Reader

Date _____

Dr. Philip A. Gruppuso, Reader

Date _____

Dr. Gary M. Wessel, Reader

Date _____

Dr. Karsten Sauer, Outside Reader

Approved by the Graduate Council

Date _____

Dean of the Graduate School

Curriculum Vitae

Vinh Hai Nguyen was born in San Diego, California in 1981. After completing his high school degree at James Madison Senior High School in 2000, Vinh went on to attend the University of California, San Diego (UCSD) as an undergraduate student. In 2004, he received his Bachelor of Science degree in Biochemistry and Cell Biology with a minor in Music. During his senior year, he was accepted into the five year B.S./M.S. program at UCSD, where he completed a Master's thesis in the laboratory of Dr. Julian I. Schroeder. There he studied the role of Reactive Oxygen Species (ROS) as a second messenger in abscisic acid stomatal guard cell signaling in *Arabidopsis thaliana*.

In 2005, Vinh moved to Providence, Rhode Island to pursue his Ph.D. in Molecular Biology, Cell Biology, and Biochemistry at Brown University. His interest in signal transduction led him to join the laboratory of Dr. Arthur R. Salomon. His thesis project focused on developing a quantitative phosphoproteomic approach to measure the changes in phosphorylation in response to the removal of essential proteins in receptor stimulated T-cells.

Publications:

1. Kwak JM, **Nguyen V**, and Schroeder JI. The Role of Reactive Oxygen Species in Hormonal Responses. Plant Physiol. 2006 141: 323-329.
2. Robert N, Merlot S, **N'guyen V**, Boisson-Dernier A and Schroeder JI. A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA

- signaling in *Arabidopsis*. FEBS letters 2006 580:1919, 4691-4696.
3. Cao L, Yu K, Banh C, **Nguyen V**, Ritz A, Raphael BJ, Kawakami Y, Kawakami T, and Salomon AR. Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. J Immunol. 2007 179(9):5864-76.
 4. **Nguyen V***, Cao L*, Lin JT*, Hung N, Ritz A, Jianu R, Ulin, SP, Raphael BJ, Laidlaw DH, Brossay L and Salomon AR. A New Approach for Quantitative Phosphoproteomic Dissection of Signaling Pathways Applied to T-cell Receptor Activation. Molecular Cellular Proteomics 2009 8:2418-2431.
 5. Jianu R, Yu K, Cao L, **Nguyen V**, Salomon AR and Laidlaw DH. Effective visual integration of quantitative proteomic data, pathways and protein interactions. IEEE Transactions on Visualization and Computer Graphics 2010 16(4):609-620.
 6. Wang YF, Bloom RE, **Nguyen V**, and Schroeder JI. A role for Reactive Oxygen Species in Activation of Store-Operated Ca^{2+} Channels in RBL-1 Cells. The Journal of Biological Chemistry 2010. *In Revision*

Abstract

Reversible protein phosphorylation plays a pivotal role in the regulation of cellular signaling pathways. Current approaches in phosphoproteomics focus on analysis of the global phosphoproteome in a single cellular state or of receptor stimulation time course experiments, often with a restricted number of time points. Although these studies have provided some insights into newly discovered phosphorylation sites that may be involved in pathways, they alone do not provide enough information to make precise predictions of the placement of individual phosphorylation events within a signaling pathway. Protein disruption and site-directed mutagenesis are essential to clearly define the precise biological roles of the hundreds of newly discovered phosphorylation sites uncovered in modern proteomics experiments.

We have combined genetic analysis with quantitative proteomic methods and recently developed visual analysis tools to dissect the tyrosine phosphoproteome of isogenic Zap-70 tyrosine kinase null and reconstituted Jurkat T-cells. In our approach, label-free quantitation using normalization to co-purified phosphopeptide standards is applied to assemble high density temporal data within a single cell type, either Zap-70 null or reconstituted cells, providing a list of candidate phosphorylation sites that change in abundance after T-cell stimulation. SILAC ratios are then used to compare Zap-70 null and reconstituted cells across a time course of receptor stimulation, providing direct information about the placement of newly observed phosphorylation sites relative to Zap-70. These methods are adaptable to any cell culture signaling system in which isogenic wild type and mutant cells have been or can be derived using any available

phosphopeptide enrichment strategy. These studies here have revealed quantitative phosphoproteomic signatures, which may be indicative of the ERK positive feedback pathway and Zap-70-dependent FYN regulation. Follow-up studies were performed and discussed in order to further examine these results. Finally, our group has engaged in our own method development, with the aim of improving our sample preparation procedure for LC/MS/MS analysis. A battery of experiments was performed on pervanadate-stimulated Jurkat T-cells and modifications to our sample preparation procedure were discussed.

Acknowledgements

I would first like to thank my graduate advisor, Dr. Arthur R. Salomon, for his supervision, support, and guidance. He has been a kind mentor that has not only helped me to become a better scientist, but also a better person. Thank you for being supportive of my decisions and working with me to achieve my goals.

Next, I would like to thank my thesis committee members, Dr. Laurent Brossay, Dr. Philip A. Gruppuso, and Dr. Gary M. Wessel for their valuable advice and support. I cannot thank them enough for always taking time out of their busy schedules to meet with me whenever I needed their guidance. I am lucky to have such incredible people looking out for me.

I would also like to thank Dr. Karsten Sauer for traveling all the way from San Diego to be my outside reader. This means a lot to me.

To the Salomon lab graduate students and former lab members: Lulu Cao, Yiyuan Ding, Qinqin Ji, Ynes Helou, Jayameenakshi Manivannan, Sam Ulin, Richard Park, John Lung, and Anthony Sabelli- thank you for making this lab a special place to be these last five years. I really enjoyed my time with you guys.

Specifically, I want to thank former graduate student Kebing Yu for his mentorship in the lab and always assisting me when I needed help. I could not have done this without you. You have been a good friend.

Acknowledgements

I would to like to give a special thank you to my students, Jonathan Lin and Norris Hung. When I look back at my time as a graduate student, my best memories come from my experiences mentoring and working with you guys. Thank you for making me realize that educating and working directly with people on a daily basis would have to be an integral part of the rest of my life. You guys were not just great students, but also great friends.

To all the friends that I have made at Brown: Thank you for making this an unforgettable place to be for the past 5 years.

Finally, I would like to thank my family and Cindy Banh for their unconditional love and support. Without them, this could never be possible.

Table of Contents

INTRODUCTION	1
1.1 Mass Spectrometry-Based Proteomics.....	2
1.2 Phosphoproteomics.....	7
1.3 Quantitative Approaches To Unravel Phosphorylation Networks	10
1.4 T-cell Receptor Signaling and Activation	13
1.5 Zap-70: Activation, Development and Defects	19
1.6 Reference	22
 A NEW APPROACH FOR QUANTITATIVE PHOSPHOPROTEOMIC DISSECTION OF SIGNALING PATHWAYS.....	 31
2.1 Introduction.....	32
2.2 Materials And Methods	37
2.2.1 Cell culture, SILAC labeling and T-cell OKT3/OKT4 stimulation.....	37
2.2.2 Cell lysis, protein reduction, alkylation, digestion, and peptide immunoprecipitation.....	38
2.2.3 Western blotting for Zap-70 and phospho-ERK 1/2	39
2.2.4 Automated desalt-IMAC/nano-LC/ESI-MS.....	40
2.2.5 Database analysis	41
2.2.6 Quantitation of relative phosphopeptide abundance.....	42
2.2.7 Flow cytometric analysis	44
2.3 Results.....	44
2.3.1 Overall scheme.....	44
2.3.2 Representation of label-free and SILAC quantitation	49
2.3.3 Phosphoproteomic profiling of phosphorylation sites identified in receptor stimulated Zap-70 reconstituted Jurket cells	52
2.3.4 Clustering of phosphorylation sites by Zap-70 null/reconstituted SILAC ratios	55
2.3.5 Novel phosphorylation sites identified.....	58
2.4 Discussion.....	60
2.5 Reference	69

PHOSPHOPROTEOMIC AND TRADITIONAL ANALYSIS OF ERK POSITIVE FEEDBACK AND ZAP-70-DEPENDENT REGULATION OF FYN BY PTP ALPHA75

3.1 Disruptions in Erk Positive Feedback.....	76
3.1.1 Introduction.....	76
3.1.2 Materials and Methods	78
3.1.2.1 U0126 MEK 1/2 inhibition	78
3.1.2.2 Cell culture, SILAC labeling, T-cell OKT3/OKT4 stimulation, and cell lysis	79
3.1.2.3 Protein reduction, alkylation, digestion, and peptide immunoprecipitation	79
3.1.2.4 Automated nano-LC/ESI-MS, database analysis, and quantitation of relative phosphopeptide abundance	81
3.1.2.5 Western blotting for phospho-ERK 1/2 and GAPDH	81
3.1.3 Results and Discussion	82
3.1.4 Reference.....	88
3.2 Enhanced phosphorylation of FYN	91
3.2.1 Introduction.....	91
3.2.2 Materials and Methods	93
3.2.2.1 Protein immunoprecipitation	94
3.2.2.2 Colloidal gold membrane protein stain	94
3.2.2.3 Western blotting for FYN.....	95
3.2.3 Results and Discussion	95
3.2.4 Reference.....	98

METHOD DEVELOPMENT WITH THE AIM OF IMPROVING THE SAMPLE PREPARATION PROCEDURE..... 101

4.1 Introduction.....	102
4.2 Materials And Methods	103
4.2.1 Cell culture, T-cell pervanadate stimulation, and cell lysis	103
4.2.2 Protein reduction, alkylation, digestion, and peptide immunoprecipitation .	104
4.2.3 Automated nano-LC/ESI-MS, database analysis, and quantitation of relative phosphopeptide abundance.....	105
4.3 Results and Discussion	105
4.4 Reference	117

CONCLUSIONS	118
5.1 Significance and Implications.....	119
5.2 Summary of Results.....	121
5.3 Future Directions	126
5.3.1 <i>Applying the hybrid quantitative approach towards further studies of TCR signaling</i>	126
5.3.2 <i>Erk positive feedback in TCR signaling</i>	128
5.3.3 <i>Zap-70-dependent FYN regulation through PTPα</i>	129
5.4 Reference	130
APPENDIX.....	133
A) Comparison of the phosphorylation sites enriched by the various anti-phosphotyrosine antibodies.....	134
B) SILAC ratio heatmap comparing the differences in phosphorylation profiling of Zap-70 reconstituted to wild type Jurkat cells in response to TCR stimulation	185
C) Updated protocol for SILAC labeling, Jurkat T-cell OKT3/OKT4 co-stimulation, and sample preparation for LC/MS/MS analysis.....	192
D) Protocol for 1G2 antibody immunoprecipitation with 1.0 mM phenyl phosphate elution and antibody regeneration.....	196

Table of Figures

Figure 1.1: Nomenclature of peptide fragment ions	6
Figure 1.2: An annotated MS/MS spectrum acquired using CID fragmentation	7
Figure 1.3: Schematic representation of reversible protein phosphorylation	8
Figure 1.4: General schematic of the SILAC procedure.....	12
Figure 1.5: Canonical T-cell signaling pathway.....	18
Figure 1.6: Generalized procedure for selection and analysis of Jurkat mutants	19
Figure 1.7: Schematic of Zap-70 domains and phosphorylated tyrosine residues	20
Figure 1.8: Schematic of the different stages in T-cell development	21
Figure 2.1: Canonical TCR signaling pathway with SILAC heatmaps.....	36
Figure 2.2: Growth rate comparison of wild type Jurkat JE6-1 cells labeled with heavy or light isotopic amino acids	45
Figure 2.3: TCR-CD3 expression of Zap-70 null and reconstituted Jurkat clones.....	46
Figure 2.4: Disruption of Zap-70 from Jurkat-cells and phosphorylation of ERK1/2.....	47
Figure 2.5: Experimental procedure.	49
Figure 2.6: Quantitative phosphoproteomic analysis of known TCR signaling proteins in wild type cells.	50
Figure 2.7: Zap-70 null to reconstituted SILAC ratio heatmap of proteins not known to function in TCR signaling in wild type cells	52
Figure 2.8: Quantitative phosphoproteomic analysis of proteins not known to function in TCR signaling in wild type cells.....	54
Figure 2.9: Classification of ZAP-70 null/reconstituted SILAC ratios.	55
Figure 2.10: LCK phosphorylation of the CD3 ITAM motif requires Zap-70.....	57
Figure 2.11: Dynamic effects of Zap-70 removal on novel phosphorylation sites discovered	59

Figure 2.12: Theoretical model distinguishing direct inhibition from feedback regulation	63
Figure 2.13: Custom-made exploration software for navigation through protein-protein interactions in the context of the TCR signaling pathway.....	66
Chapter 2 Supplemental Material 5: Table of SILAC $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg and $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys incorporation into peptides to verify complete SILAC labeling in Jurkat T-cells	http://tcellpathway.com
Chapter 2 Supplemental Material 6: Table of peptide sequence and phosphorylation site assignment statistics (standard deviation and p-values) and quantitative analysis for all Zap-70 data described in this thesis	http://tcellpathway.com
Chapter 2 Supplemental Material 7: Equations for color representations of relative peptide abundance for label-free and SILAC heatmaps	http://tcellpathway.com
Figure 3.1: ERK positive feedback.....	77
Figure 3.2: Schematic representation of MEK 1/2 inhibition by U0126.....	78
Figure 3.3: SLP-76 null to reconstituted SILAC ratio heatmap of known upstream T-cell signaling proteins.....	83
Figure 3.4: Titration to determine the optimal conditions for U0126 MEK 1/2 inhibition	84
Figure 3.5: Experimental procedure	85
Figure 3.6: MEK1/2 inhibition by U0126 treatment	88
Figure 3.7: Regulation of FYN signaling	93
Figure 3.8: Determining FYN antibody specificity for use in protein immunoprecipitation and immunoblotting.....	98
Figure 4.1: Titration of pTyr100 antibody to determine optimal antibody concentration for peptide immunoprecipitation.....	107
Figure 4.2: Selectivity of various amino acid-independent phosphotyrosine specific antibodies	109
Figure 4.3: Phosphotyrosine peptide yields of various amino acid-independent phosphotyrosine specific antibodies	110
Figure 4.4: Phosphotyrosine peptide yields of 0.15 % TFA elution vs. 1 mM phenyl phosphate elution during peptide immunoprecipitation	111
Figure 4.5: Phosphotyrosine peptide yields of Promega vs. Worthington trypsin	112

Figure 4.6: Phosphotyrosine peptide yields of Top 5 vs. Top 9 ion detection methods.	115
Figure 4.7: Phosphotyrosine peptide yields of LTQ-FT vs. LTQ-Obitrap for peptide sequence analysis	116
Figure 5.1: SLP-76 expression constructs	127

Abbreviations Page

2D-gel: two-dimensional gel electrophoresis
ACBD6: Acyl-CoA binding domain 6
ADAM9: A disintegrin and metalloproteinase domain 9
ADAP: adhesion and degranulation adaptor protein
AKT: acutely transforming retrovirus AKT8 in rodent T-cell lymphoma
Arg: arginine
ATP1 A1/A3: sodium/potassium-exchanging ATPase alpha chain 1/3
BDH: D-beta-hydroxybutyrate dehydrogenase
C10orf28: chromosome 10 open reading frame 28
C9orf78: hepatocellular carcinoma associated antigen 59
c-cbl: casitas B-lineage lymphoma
CCT8: cytosolic chaperonin theta chain subunit 8
CD: cluster of differentiation
CDC2: cell division cycle 2 protein
CID: collision-induced dissociation
CLH-17: Clathrin heavy polypeptide
Crk: CDC2 related kinase
CRKL: Crk like protein
CRMP2: collapsin response mediator protein 2
Csk: C-terminal Src kinase
CV: Coefficient of Variation
CYFIP1: cytoplasmic FMR1-interacting protein 1
DAG: diacylglycerol
DDX: dead box X isoform
DHB: dihydroxybenzoic acid
DN: double negative
DOK: downstream of tyrosine kinase
DP: double positive
Drf1: diaphanous related forming
Drf1: diaphanous 1
DTT: dithiothreitol
DYRK 1A/1B: dual-specificity tyrosine-regulated kinase 1A/1B
ECD: electron capture dissociation
EF-1- α -1: elongation factor alpha 1
ELMO1: engulfment and cell motility 1
ELP3: elongation protein 3
EMS: ethylmethanesulphonate
EphA3: Ephrin type-A receptor 3
EPRS: glutaminyl-tRNA synthetase
Erk1/2: extracellular signal-regulated kinase-1/2

ETD: electron transfer dissociation
FACS: fluorescence-activated cell sorting
FDR: false discovery rate
Fer: Fes related
FTMS: Fourier transform mass spectrometry
G6PD: glucose-6-phosphate dehydrogenase
GA17: B5 receptor
GAB2: Grb2-associated binding protein 2
GDP: Guanidine Diphosphate
Grlf1: glucocorticoid receptor DNA-binding factor 1
GSK3 β : glycogen synthase kinase 3 beta
GSPG3059: Transmembrane and immunoglobulin domain-containing protein 2 precursor
hARP11: actin related protein 10
HILIC: hydrophilic interaction chromatography
HIP14: huntington-interacting protein 1
hnRNP U like 1/H3: heterogeneous nuclear ribonucleoprotein U like protein 1/H3
HPLC: High performance liquid chromatography
HPRD: Human Protein Reference Database
Hrs: hepatocyte growth factor 1
HSP: heat shock protein
ICAT: Isotope-Coded Affinity Tags
IFN- γ : interferon gamma
IL-2: interleukin 2
IMAC: immobilized metal affinity chromatography
IP3: inositol trisphosphate
ITAM: immunoreceptor tyrosine-based activation motif
ITK: IL2-inducible T-cell kinase
ITMS: ion trap mass spectrometry
iTRAQ: isobaric tag for relative and absolute quantitation
KIR: killer immunoglobulin-like receptor
KIR2DL2: killer immunoglobulin-like receptor 2 domain, long cytoplasmic tail, 2
KIAA0711: kelch repeat and BTB domain containing protein 11
KLRG1: killer cell lectin-like receptor G1
LAT: linker for activation of T-cells
LAX: LAT like membrane associated protein
LC: liquid chromatography
Lck: lymphocyte-specific protein tyrosine kinase
LDH: lactate dehydrogenase
LTQ: linear trap quadrupole
Lys: lysine
MudPIT: Multidimensional Protein Identification Technology
MPZL1: myelin protein zero-like 1
MS: mass spectrometry
MS/MS tandem mass spectrometry
MSL2-like 1: male specific lethal 2 like 1
m/z: mass over charge

NFAT: nuclear factor of activated T-cells
NF- κ B: nuclear factor kappa B
NK: natural killer
NTBA: natural killer-, T- and B-cell antigen
p38 MAPK: mitogen activated protein kinase 14
PAG: phosphoprotein associated with glycolipid-enriched membrane protein
PAR3: partitioning-defective 3 homolog
PBS: phosphate buffer saline
PGAM-B: phosphoglycerate mutase 1
PGM 1: phosphoglucomutase 1
PH domain: pleckstrin homology domain
PHLDB3: pleckstrin homology like domain, family B
PI3K: phosphoinositide 3-kinase
PI4K: phosphoinositide 4-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
PIP3: phosphatidylinositol (3,4,5)-trisphosphate
PKC: protein kinase C
PLC- γ 1/2: phospholipase C gamma 1/2
PRP4: pre-mRNA processing factor 4
PTEN: phosphatase and tensin homologue deleted on chromosome 10
PTM: post-translational modification
PTP α : protein tyrosine kinase alpha
pTyr: phosphotyrosine
RA-GEF-2: Rap guanine nucleotide exchange factor 6
RAN: ras-related nuclear protein
Rap-1 GTPase: rho GTPase-1
RCC1 like: regulator of chromosome condensation 2
RF: radio frequency
RhoGEF2: Rho guanine exchange factor 2
SAE2: SUMO1 activating enzyme subunit 2
SAH Hydrolase: S Adenosylhomocysteine hydrolase
SCID: severe combined immunodeficiency
SCX: strong cation exchange
SHC: Src homologous and collagen-like protein
SHIP: SH2-domain-containing inositol polyphosphate 5' phosphatase
SHP-1/2: SH2 domain-containing protein tyrosine phosphatase-1/2
SIC: selected ion chromatogram
SILAC: stable isotopic labeling of amino acids in cell culture
SIT: SHP2-interacting transmembrane adaptor protein
SKAP55: src kinase-associated phosphoprotein of 55kDa
SLP-76: SH2 domain containing leukocyte protein of 76kDa
snRNP70: small nuclear ribonucleoprotein 70kDa
SP: single positive
STAM2: signal transducing adaptor molecule 2
STS1: suppressor of T-cell signaling 1
TCR: T-cell receptor

TFA: trifluoroacetic acid
TfR: transferrin receptor
TiO₂: titanium dioxide
TSG101: Tumor susceptibility gene 101
WASP: Wiskott-Aldrich syndrome protein
Zap-70: zeta-chain-associated protein kinase 70
ZNF: Zinc finger protein

Chapter 1

INTRODUCTION

1.1 MASS SPECTROMETRY-BASED PROTEOMICS

Proteomics was first introduced in the late 1990s as an approach to investigate large-scale protein identifications, structures and functionalities (39-42). Although systems biology at the time had made great strides in genomics-based research, scientists still faced many obstacles in effectively deciphering and understanding various signaling pathways based on genomic data alone (43). Some information had to be gathered at the protein level, such as post-translational modifications (PTMs) and protein-protein interactions, which is not encoded in genes. Recognizing this limitation, proteomics gained popularity as an alternative approach to provide scientists with a wealth of information to aid in their studies of physiological functions.

Early stages in proteomics utilized two-dimensional gel (2D-gel) electrophoresis as its main method to separate and analyze complex protein mixtures (44, 45). Protein mixtures were separated on SDS gels, first by molecular weight in one dimension, and then by isoelectric point in a second dimension. This method relied primarily on silver staining to visualize the proteins, and Edman degradation for sequencing (46). Soon after, mass spectrometry-based proteomics quickly became the favored choice among scientists, as it was found to be more efficient, sensitive, and precise when compared to traditional approaches (47-49). Proteins or peptides are first ionized via electrospray (50) or MALDI (51, 52), and mass analyzers are used to determine mass-to-charge ratios (m/z) (66-69). Tandem mass spectrometry (MS/MS) methodology further fragments isolated peptide ions to provide sequence information (49).

Many mass spectrometers combine two or more mass analyzers for MS/MS spectrometry, thereby providing a greater dynamic range of mass accuracy and sensitivity (49, 65). In particular, combining linear trap quadrupole (LTQ) (66) with Fourier transform mass spectrometry (FTMS) (67, 68) or Orbitrap (69) has been shown to be a powerful tool to study PTMs in various signaling pathways (13, 16, 17, 20). The LTQ utilizes static DC and oscillating AC radio frequency (RF) electric fields to trap ions in a two-dimensional quadrupole field. Ions are then expelled from orbit sequentially by ramping the RF potential, so that the ion with the lowest mass becomes unstable and ejects onto the detector (66, 70). In contrast, FTMS measures the m/z of ions based on the cyclotron frequency of the ions in a fixed magnetic field (67, 68). Instead of measuring the deflection of ions with a detector, the ions are injected into a Penning Trap (an electromagnetic ion trap), and detectors at fixed positions in space measure the electrical signal of orbiting ions as they pass near them over time, thus producing a periodic signal. The m/z can be determined for each ion by performing a Fourier transform (71) on this signal. Because ions are counted more than once, FTMS is highly sensitive and very accurate in its measurement of masses (67). Unlike the LTQ or FTMS, the Orbitrap does not require RF potential or magnetic fields to trap ions (69, 72). Instead, ions are injected tangentially into an electric field and electrostatically trapped in an orbit around a central, spindle-shaped electrode. Orbitraps not only cycle ions around the central electrode, but also oscillate them back and forth along the axis of the central electrode. The frequency of oscillations for each ion is measured by detector plates (similar to FTMS), and this information is used to determine m/z . The main advantage of using an Orbitrap, as opposed to the FTMS, is the rapid MS/MS scan speed, allowing for

more acquisitions of MS/MS spectra in the same given time. In addition, combining the Orbitrap with the LTQ Velos can deliver greater sensitivity, due to the LTQ Velos' unique dual-pressure ion trap technology (www.thermofisher.com). These two discrete pressure regions in the LTQ Velos allow for the decoupling of ion manipulation and detection, enabling the use of optimal pressures during analysis. This results in increased scan speed and resolution. Moreover, from a two hour gradient analysis of SILAC-labeled Hela cytoplasmic extract, it was reported that the LTQ-Orbitrap Velos acquired 2-fold more MS/MS scans than the LTQ-FT ultra (LTQ-Orbitrap Velos: 33,138 MS/MS scans; LTQ-FT ultra: 17091 MS/MS scans), leading to one third more peptide and protein identifications (LTQ-Orbitrap Velos: 11306 Peptide IDs, 1581 Protein IDs; LTQ-FT ultra: 8036 Peptide IDs, 1177 Protein IDs) (65).

In general, mass spectrometry-based proteomics utilizes two complementary approaches to analyze complex protein mixtures: “top-down” and “bottom-up” proteomics. “Top-down” proteomics analyzes samples at the protein level to provide direct information about each protein’s molecular weight. Protein ions are fragmented by various approaches to yield protein fragments that contain protein sequence information. Electron capture dissociation (ECD) (53) and electron transfer dissociation (ETD) (54) are two common methods of fragmentation used in this approach, which involves the direct introduction of low energy electrons to trapped gas phase ions. A drawback to using “top-down” proteomics is that the MS/MS spectra acquired from these protein fragments are typically very difficult to interpret with respect to protein sequence, due to the large number of possible ways to break-up protein backbones, thus giving rise to complexities in data analysis. “Bottom-up” proteomics is another approach to analyze

protein mixtures, which focuses on the use of small pre-cleaved peptide fragments to identify proteins and their PTMs. Protein mixtures are digested at specific amino acid residues with use of proteases, such as trypsin (55), to form short peptide fragments. Although pre-cleavage of proteins increases the number of molecules that must be analyzed, for certain types of analyses, “bottom-up” proteomics has distinct advantages over “top-down” proteomics. In particular, MS/MS spectra acquired from short pre-cleaved peptides are much easier to interpret than those generated directly from proteins.

The MS/MS spectra acquired from “bottom-up” proteomics experiments are very accurate in the determination of peptide sequences, as well as the identification of PTM locations. In theory, substituting one amino acid in the peptide fragment would result in corresponding ion mass shifts, which would lead to the generation of a completely different spectrum. Additionally, different fragmentation methods used to break different chemical bonds could also lead to the generation of different spectra from the same precursor peptide ion (Figure 1.1) (58). Collision-induced dissociation (CID) (56) mainly produces b and y ions (57), while ETD produces c and z ions (54). CID is the most popular fragmentation method utilized in standard MS/MS spectrometry, and consists of the acceleration of molecular ions by some electrical potential in the vacuum of a mass spectrometer and the collision of these ions with neutral gas molecules, often helium (56). An example of an annotated MS/MS spectrum generated from CID fragmentation is shown in Figure 1.2. Once the MS/MS spectra have been acquired, peptide sequence information is assembled using database-matching programs, such as Sequest (59) and Mascot (60). Theoretical MS/MS spectra of predetermined cleavages are generated for all possible peptides contained in a genomic protein database and matched to the

empirical MS/MS spectra collected. Quality scores are computed to indicate the matching confidence between the theoretical and empirical MS/MS spectra. To locate PTMs within the peptide sequence, the exact molecular weight of the modification can be entered as a searching criterion.

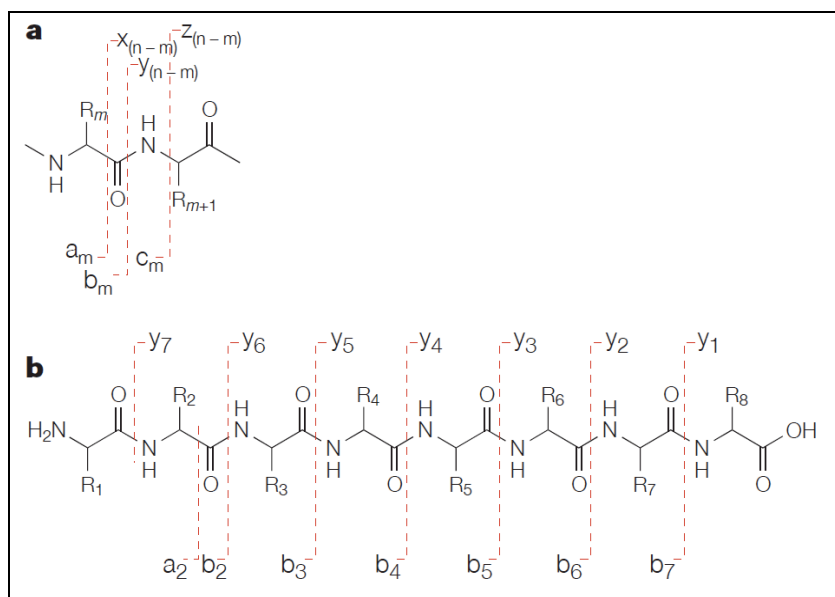


FIGURE 1.1: Nomenclature of peptide fragment ions. Peptides are ionized, either through electrospray for LC/MS or triggered by laser beam for MALDI, and forced into chemical bond breakage using various fragmentation methods. a) a, b, c and x, y, z ions are defined according to the chemical bond breakage and the charge location. N-terminal charged fragments are labeled as a, b, c ions, while C-terminal charged fragments are labeled as x, y, z ions. b) Ions are labeled consecutively along the amide bond. b ions are read from left to right, while y ions are read from right to left. (Figure adopted from Steen and Mann, 2004) (58)

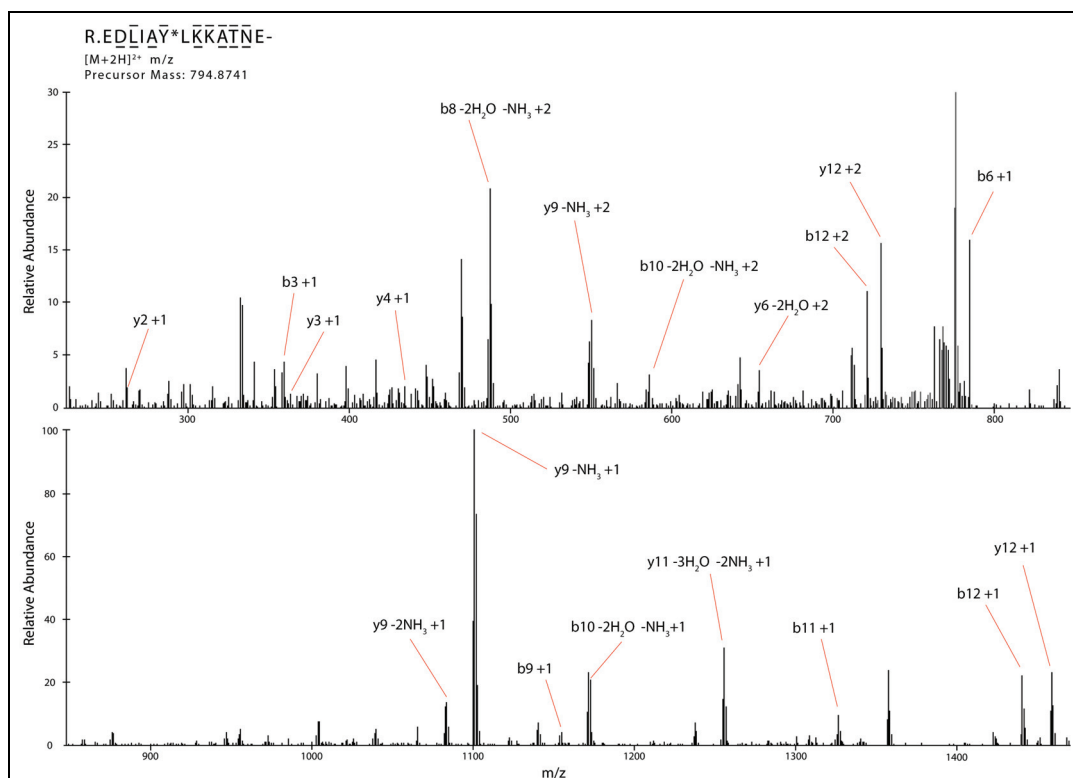


FIGURE 1.2: An annotated MS/MS spectrum acquired using CID fragmentation. Peptide fragment ions are assigned to singly- or doubly-charged b and y ions.

1.2 PHOSPHOPROTEOMICS

Reversible phosphorylation is an essential PTM that functions in nearly all cellular processes, which include cell growth, apoptosis, proliferation, differentiation, and intercellular/intracellular communication (1) (Figure 1.3). It is estimated that at least 30% of proteins are phosphorylated in mammalian cells (2). Reversible phosphorylation is highly regulated and defects can lead to the development of many physiological diseases, such as cancer. As a result, there have been many therapeutic discovery efforts focused on targeting kinases and phosphatases, the enzymes responsible for adding and

removing these protein modifications to/from substrate proteins. A better fundamental understanding of this process will allow for the development of improved treatments.

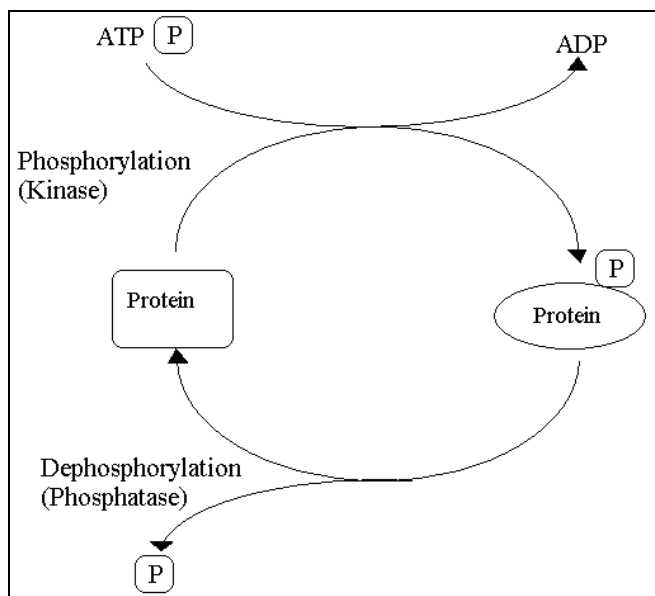


FIGURE 1.3: Schematic representation of reversible protein phosphorylation. (Figure from <http://herkules.oulu.fi>)

Proteomics was initially used in biological research to identify proteins and investigate protein-protein interactions. These studies were mainly accessed by 2D-gel electrophoresis analysis due to its ability to perform high-resolution separation of proteins from large mixtures (3). However, this technique is relatively inefficient and results in a low yield of protein extraction from gels, thus making it unsuitable for PTM studies (3, 4).

Investigators faced many challenges in applying mass spectrometry-based proteomics to studies of protein phosphorylation because of the lack of instrument

sensitivity and method specificity in detecting phosphopeptides. Furthermore, phosphopeptides are present at extremely low levels and are barely detectable without enrichment prior to MS analysis. This was especially the case for phosphotyrosine (pTyr)-containing peptides. In normal growing cells, the majority of phosphorylation occurs on serine (90%) and threonine (10%) residues, while only a small fraction occurs on tyrosine (0.05%) residues (7). Recent developments in enrichment techniques have further advanced LC/MS technology, allowing for high-throughput phosphorylation studies (5, 6).

Several enrichment strategies have been developed in the past few years to overcome these challenges. An approach based on the high affinity and specificity of chelation of phosphate groups to certain metal ions, represented by Fe^{3+} -immobilized metal affinity chromatography (IMAC) (8), Ga^{3+} -IMAC (9), TiO_2 (10), and Nb_2O_5 (11), has been very successful in the enrichment of phosphoproteins and phosphopeptides. One complication of using this approach is the competitive binding of non-phosphopeptides containing multiple acidic amino acids, due to their chemically similar carboxyl functional group found on Asp and Glu residues. Peptide conversion to methyl esters for IMAC (5) or the addition of an organic competitor, such as dihydroxybenzoic acid (DHB) (10), for TiO_2 has been used to minimize the binding of non-phosphorylated, acidic peptides. Another enrichment strategy specific for phosphotyrosine is based on immunoaffinity binding between tyrosine-phosphorylated proteins or peptides and anti-pTyr antibodies (11-13). Antibodies are conjugated to agarose beads, and through immunoprecipitation techniques, selectively capture phosphotyrosine-containing peptides or proteins from complex mixtures. Strong cation exchange (SCX) (14) and hydrophilic

interaction chromatography (HILIC) (15) have also shown promise as phosphopeptide enrichment strategies. These methods enrich for phosphopeptides based on their charge state and hydrophilicity, respectively. Combining these enrichment strategies has also been found to provide enhanced selectivity in enrichment for phosphopeptides (11, 16, 17).

1.3 QUANTITATIVE APPROACHES TO UNRAVEL PHOSPHORYLATION NETWORKS

Identification and determination of phosphorylation sites is not sufficient to investigate phosphorylation networks; rather, it is critical to resolve the temporal change of phosphorylation in response to various stimulations. Reversible phosphorylation events are transient and dynamic, thus requiring a quantitative approach to capture the intermediate states.

Quantitative proteomics has been used to measure the relative abundance of proteins in cell or tissue samples under various conditions. This approach has been used to compare cancerous and normal tissue (18), drug-treated vs. untreated cells (19), and to analyze the changes in cellular components in response to receptor activation (20). Several approaches have recently gained popularity. Label-free LC/MS proteomics is a cost-effective, but possibly less precise, quantitation method (13). One approach to label-free quantitation involves the use of phosphopeptide standards that are co-purified in parallel with the peptide mixtures and used to normalize peptide chromatographic peak

areas. This approach provides the relative abundance of a peptide at different cellular conditions, such as through a time course of receptor stimulation (11, 16).

Stable heavy isotopic labeling is an alternative quantitative method shown to minimize experimental error through its ability to simultaneously compare various cell states in a single MS spectrum. Heavy stable-isotope containing amino acids or covalent modifications to peptides are incorporated either through chemical or metabolic labeling. Chemical labeling relies on the covalent attachment of heavy isotope-containing chemical functionalities to peptide functional groups, such as amines, hydro-sulfuric or carboxylic acid groups. Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) labeling, for instance, has the capability to quantitatively compare up to 8 conditions in a single MS/MS spectra (24). Different isobaric mass tags are first attached to amine groups of peptide mixtures deriving from various conditions. Peptides mixtures are then combined, purified, and processed through LC/MS/MS analysis. Peptides from different conditions are indistinguishable in the MS scan, but are distinguished and quantified in the MS/MS scan via dissociation of a reporter group from the isobaric mass tag. In contrast, quantitation of peptides using other chemical labels are performed at the MS level (ICAT (21), GIST (22), ICPL (23)).

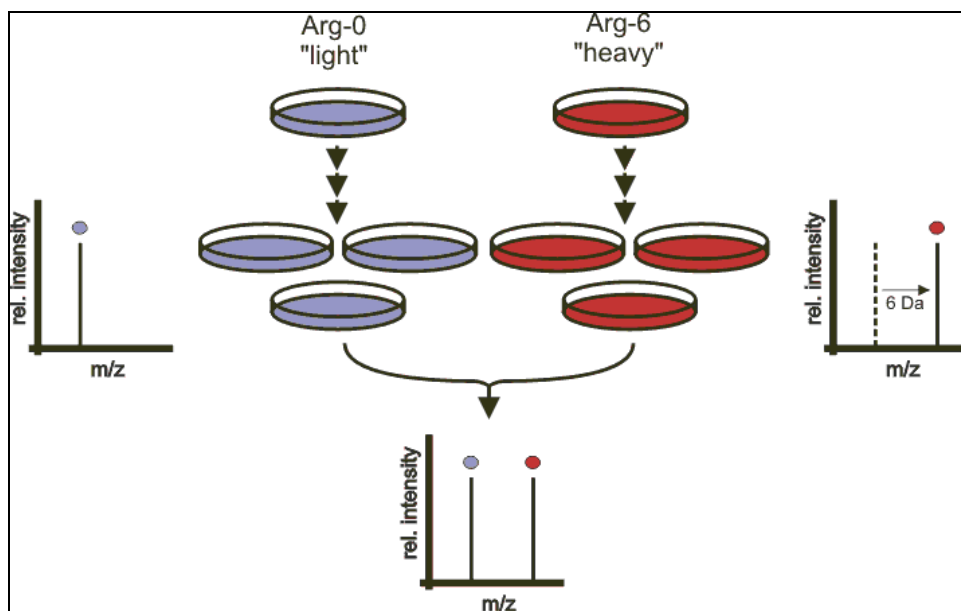


FIGURE 1.4: General schematic of the SILAC procedure. (Figure adopted from Ong et al., 2002) (25)

Metabolic labeling using Stable Isotope Labeling with Amino acids in Cell culture (SILAC) can provide a more accurate form of quantitation than chemical labeling due to the normalization of errors resulting from the entire process, including culturing of cells, protein purification, and MS data acquisition (Figure 1.4). Prior to cellular stimulation, one population of cells is incubated in media containing normal (light) amino acids, while a second population is incubated in media containing stable isotope incorporating (heavy) amino acids, resulting in the incorporation of labeled amino acids into cellular proteins. Proteins from both cell populations can then be combined, digested, purified, and sequenced through LC/MS/MS analysis. Pairs of chemically identical peptides of different stable-isotope composition can be differentiated in a mass spectrometer owing to their differences in mass (26, 27). The ratio between heavy and light peptide chromatographic peak areas provides the relative peptide abundance. Lys and Arg are the most common substituted amino acids. This is because trypsin, which

cleaves after Lys and Arg residues, is used to digest proteins in most “bottom-up” style proteomics experiments. In a general sense, the positioning of a basic amino acid at the peptide C-terminus leads to more complete fragmentation of the peptide, and better MS/MS sequence coverage. It is worth noting that when choosing isotopic reagents, ^{15}N , ^{14}C and ^{18}O incorporated into heavy Arg or Lys are more favorable than ^2H . This is due to the deuterium effect that leads to a chromatography shift in retention times (28).

1.4 T-CELL RECEPTOR SIGNALING AND ACTIVATION

T-cells are lymphocytes which play a critical role in adaptive immune response. In general, there are two types of T-cells: CD4^+ (eg. Helper T-cells) and CD8^+ (eg. Cytotoxic T-cells). The main difference between these cells and other types of lymphocytes is that they express the T-cell receptor (TCR) on their cell surface. This receptor is composed of an $\alpha\beta$ heterodimer along with $\text{CD3}\delta/\epsilon/\zeta/\gamma$ subunit chains. It has been well-established that the TCR associates with antigen-derived peptides presented on major histocompatibility complexes (MHCs) found on Antigen Presenting Cells (B-cells, dendritic cells, and macrophages), leading to the secretion of cytokines that regulate the immune response. CD4^+ cells associate with antigen-derived peptides presented on MHC class II, while CD8^+ cells associate with antigen-derived peptides presented on MHC class I (29).

T-cells are generally activated via the two-signal model, which requires the interaction between the antigen-MHC molecule and the TCR- $\text{CD3}\delta/\epsilon/\zeta/\gamma$ subunit chains,

along with an additional co-stimulatory receptor, such as CD4, for full activation (29, 30) (Figure 1.5). Once the MHC molecule presents the processed antigen to the TCR, CD4 then binds to the MHC molecule, resulting in the juxtaposition of a lymphocyte-specific protein tyrosine kinase, LCK, which is tethered with CD4 (31). LCK is now in close proximity to CD3 $\delta/\epsilon/\zeta$ chains and thereby phosphorylates specific tyrosine residues found in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 chains, initiating the recruitment and phosphorylation of a SYK-family protein tyrosine kinase, Zap-70. Phosphorylated Zap-70 is then activated, resulting in further phosphorylation and activation of known adaptor proteins (LAT, SLP-76), allowing for the docking and activation of signaling proteins VAV1, ITK, PLC γ -1, and PI3K (73). Both PLC γ -1 and PI3K act on membrane-bound lipid PIP₂ to initiate distinct mechanisms (74, 75). PLC γ -1 hydrolyzes PIP₂ to yield the soluble intracellular messenger IP₃, and DAG, an endogenous lipid stimulator of PKC. IP₃ then binds to receptors on the endoplasmic reticulum, which induces the release of intracellular calcium, which then activates phosphatase calcineurin to activate transcription factor NFAT (74). DAG activates PKC, leading to the transactivation of transcription factors, NF- κ B and AP-1 (74). PI3K phosphorylates PIP₂ to form PIP₃. AKT, a serine/threonine kinase, and ITK, a Tec family kinase, then bind with their PH domain to PIP₃ to become active, which eventually leads to transactivation of NF- κ B (75, 76). Other downstream signaling events, such as the activation of MAPK and Ras-Raf pathways, result in an increased production of IL-2, a cytokine that promotes cell growth and proliferation (73, 29).

Research efforts to study the T-cell signaling pathway were initially carried out on primary cells of human or mouse origin. *In vitro* model systems, such as transformed cell

lines, have been used in addition to *in vivo* studies and have led to major advances in the field (32-34). Of the transformed cell lines that are currently being used by T-cell immunologists, it can be argued that the most popular and historically significant of the group is the human Jurkat leukemic T-cell line. The Jurkat cell line gained its popularity as a model system for studies in TCR signaling due to its low maintenance and ability to secrete large amounts of IL-2 (35). In culture, these cells proliferate rapidly (doubling in cell population each day), and do not require the addition of external cytokines to initiate growth, thereby making these cells an ideal model for large-scale studies of TCR signaling.

The use of Jurkat cells has led to the identification of many TCR signaling proteins (32-34, 37-38), and numerous protein-deficient Jurkat clones have been isolated through genetic screens (Figure 1.6). Jurkat wild type cells were first mutagenized by ionizing radiation, frameshift mutagen ICR-191, or point mutagen ethylmethanesulphonate (EMS). The cells were then subjected to a high-throughput selection procedure involving resistance to a death-inducing stimulus (for example, excessive TCR ligation) or collection of unresponsive TCR-stimulated cells by fluorescence-activated cell sorting (FACS). Clonal cell populations were then isolated and re-screened to verify that they still retained their defective phenotype. To determine the molecular basis of the TCR-signaling defect, the selected clones were profiled for TCR-dependent Ca^{2+} response, tyrosine phosphorylation patterns, and expression of candidate signaling proteins by immunoblotting or RNA analysis (36). Upon determination of the deficient protein in these clones, restoration of normal T-cell signaling through reconstitution was performed to confirm the identity of the missing

protein. This procedure has led to the discovery and/or characterization of many essential TCR signaling proteins, which include LCK (32), LAT (34), Zap-70 (33), PLC- γ -1 (37), and SLP-76 (38). These isolated Jurkat clones are well-characterized models for elucidating TCR signaling events.

The use of the Jurkat T-cell line as a model system for studying TCR signaling also has its disadvantages. The question of physiological relevance behind performing *in vitro* experiments must first be asked when using this cell line, and other transformed T-cell lines, as a model system. In particular, it has been shown that the Jurkat T-cell line has defects in expression of two lipid phosphatases, PTEN and SHIP (61-63). Although the biological implications of SHIP's loss in T-cells are still poorly understood, PTEN is a known regulator of TCR signaling. Upon TCR engagement, PI3K phosphorylates PIP₂ to form PIP₃, which serves as a docking site for AKT and ITK for activation (64, 75). PTEN (as well as SHIP) inhibits PI3K signaling by mediating the conversion of PIP₃ back to PIP₂. Therefore, the loss of PTEN leads to constitutive activation of the PI3K signaling pathway, which includes the misregulation of AKT and ITK (64). Another disadvantage to using Jurkat cells is that they can change or adapt over time. Overgrowing or excess passaging of these cells is known to lead to the down-regulation of TCR-CD3 expression. In addition, it is possible that, over time, alternative pathways may be up-regulated in the protein-deficient Jurkat clones to compensate for the loss of the signaling protein. These factors must be strongly considered when choosing this cell line as a model system, as well as when interpreting data acquired from these cells.

An issue that arises when utilizing protein-deficient Jurkat clones is distinguishing whether the phenotypes observed are due to the loss of the signaling protein, or due to changes that occur in response to constitutive activation of the PI3K pathway and/or compensatory pathways that can become active over time. To address this, experiments performed in primary T-cells are required to verify phenotypes displayed in the Jurkat cells. Protein-deficient primary T-cells can be generated through extraction from transgenic KO mice, the use of RNAi, or drug inhibitors specifically targeting the protein of interest. If the phenotypes observed in these protein-deficient primary T-cells are similar to that observed in the protein-deficient Jurkat clones, this would suggest the loss of the protein is the cause, and not the physiological defects inherent in the Jurkat cells. The use of RNAi or drug inhibitors would also shorten the time of protein removal, thus decreasing the possibility of compensatory pathways becoming active. In addition, immunoblots and FACS analysis should be performed to confirm whether losses in protein expression are occurring in the Jurkat cells being utilized. It is critical to verify that the protein expression of essential signaling components is present at similar levels when comparing various Jurkat clones. Performing these follow-up studies are necessary before interpreting any data collected from the Jurkat T-cell line.

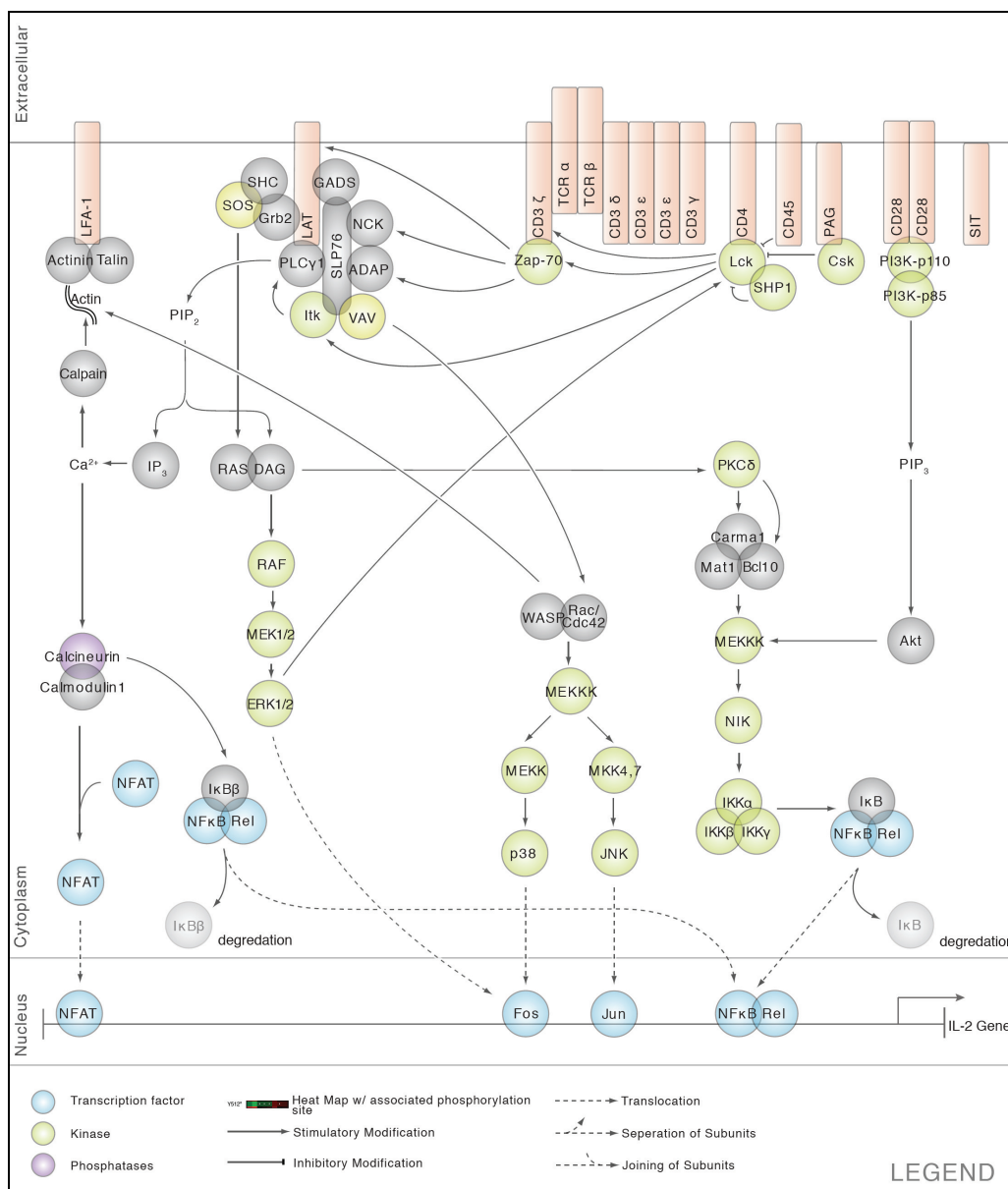


FIGURE 1.5: Canonical T-cell signaling pathway.

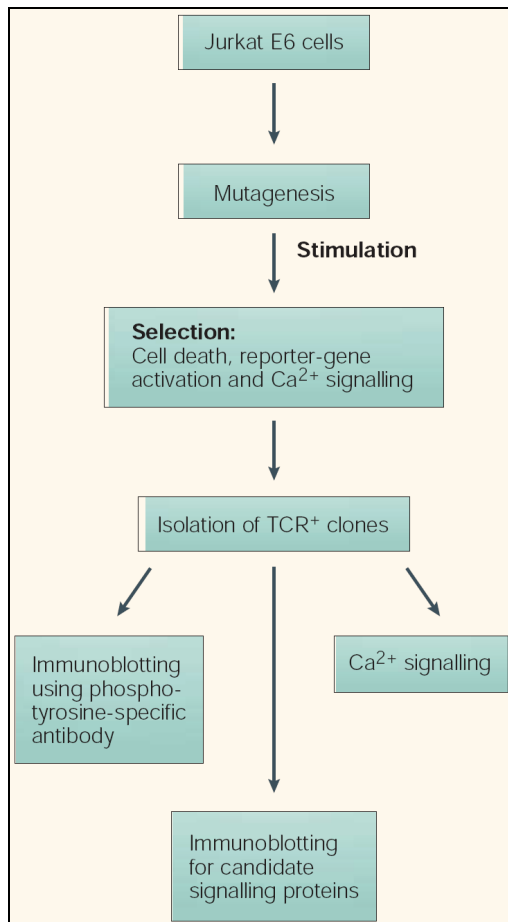


FIGURE 1.6: Generalized procedure for selection and analysis of Jurkat mutants. (Abraham and Weiss, 2004) (36)

1.5 ZAP-70: ACTIVATION, DEVELOPMENT AND DEFECTS

Zap-70 TCR- ζ chain-associated 70 kDa tyrosine phosphoprotein was initially identified in TCR-stimulated Jurkat cells (78). It is a member of the SYK tyrosine kinase family, and structurally, it consists of two tandem N-terminal SH2 domains and a C-terminal kinase domain (Figure 1.7). Although primarily expressed in T-cells (79) and natural killer (NK) cells (82), Zap-70 is also expressed developing and mature B-cells, as well as in tumoral cells from B-cell acute lymphoblastic leukemias (80). In T-cells, Zap-

70 is known to function in TCR-mediated signaling and development. Following TCR engagement and LCK-mediated phosphorylation of tyrosine residues in the ITAM, Zap-70 is recruited to bind to the doubly-phosphorylated TCR- ζ chain ITAM via its tandem SH2 domains, and is subsequently phosphorylated (by LCK and Zap-70 itself) at multiple tyrosine residues for activation of catalytic activity (79). A proposed model suggests that the binding of Zap-70 to the ITAM leads to the release of ZAP-70 from its auto-inhibited conformation, thus increasing the accessibility for LCK-mediated phosphorylation of regulatory sites Y³¹⁵ and Y³¹⁹ (81). For full activation, Zap-70 is further phosphorylated at sites Y⁴⁹² and Y⁴⁹³ in its activation loop, either by LCK or by Zap-70 itself (79). Active Zap-70 then phosphorylates LAT and SLP-76, which function as scaffolding proteins to recruit many downstream signaling proteins, eventually leading to T-cell activation, proliferation, and differentiation (73).

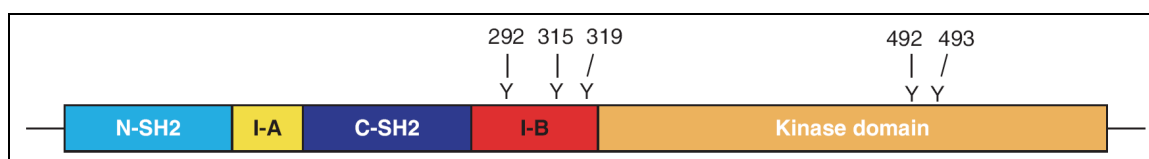


FIGURE 1.7: Schematic of Zap-70 domains and phosphorylated tyrosine residues. (Wang et al., 2010) (81)

Zap-70 is a major regulator of T-cell development and maturation. In order to become mature T-cells, thymic progenitors progress through four stages of CD4/CD8 double negative (DN1-4) before becoming CD4/CD8 double positive (DP), and finally CD4 or CD8 single positive (SP) mature thymocytes (Figure 1.8). During this process, thymic progenitors are required to pass through two critical checkpoints: pre-TCR mediated β -selection at the D3 stage, and TCR-mediated positive/negative selection at the

DP stage (83). Zap-70 is known to regulate later stages in T-cell development (84). It is highly expressed after the DN4 stage. In Zap-70 knock-out mice, it has been reported that positive/negative selection is completely arrested (84). Moreover, Zap-70 is responsible for sustained pre-TCR/TCR signaling and cellular proliferation from DN4 through DP stages (85).

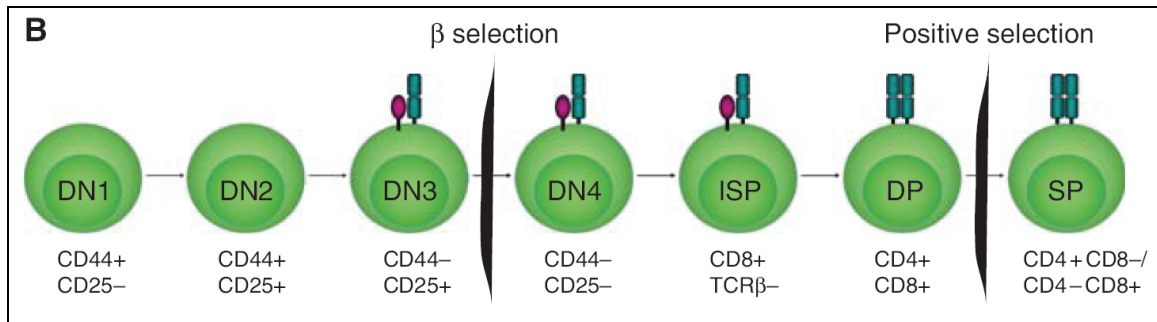


FIGURE 1.8: Schematic of the different stages in T-cell development. (Wang et al., 2010) (81)

It has been shown that Zap-70 loss leads to severe phenotypes in Jurkat cells, mice, and humans. As mentioned above, Zap-70 was first identified in Jurkat cells (78) and a Zap-deficient Jurkat clone (P116) was isolated through a genetic screen (33). Wild type Jurkat JE6-1 cells were mutagenized with ICR-191 and clones were isolated that failed to increase calcium in response to pervanadate stimulation. Although the exact location of the mutation is still unknown, P116 cells lacking Zap-70 expression (as well as SYK expression) displayed defects in TCR-induced signaling functions, which included protein tyrosine phosphorylation (on PLC-γ1, ITK, LAT, Erk1/2, and SLP76), stimulus-induced calcium mobilization, IL-2 production, and NFAT transcription activation (33, 86-88). In mice, complete knock-out of Zap-70 expression leads to a block in T-cell development at the DP stage, resulting in no mature CD4⁺ or CD8⁺ T-cells

in the periphery blood (84, 89). In addition, other Zap-70 mutant mice have been isolated that only display partial TCR signaling defects. For instance, SKG mice have a missense mutation (W163C) in the C-terminal SH2 domain of Zap-70, resulting in impairments in Zap-70 binding to the TCR- ζ chain, thus altering TCR-mediated signaling and T-cell development (90). These mice also develop autoimmune arthritis in response to innate immune activation. Humans that lose Zap-70 function or expression experience severe combined immunodeficiency (SCID), which is characterized by the presence of normal numbers of nonfunctional CD4⁺ T-cells (TCR signaling defects) and a selective absence of CD8⁺ T-cells in the periphery blood as well as in the thymus (91-93). The mechanism for preferential selection of CD4⁺ T-cells, however, remains unclear. It has been identified in SCID patients that the mutations that abolish Zap-70 expression mainly occur in the kinase domain (91-94). Phenotypically, SCID patients are more susceptible to opportunistic infections and have a failure to thrive. Many of these patients die in infancy unless treated by allogeneic hematopoietic stem cell transplantation (95).

1.6 REFERENCE

1. Cozzone, A. J. (1988) Protein phosphorylation in prokaryotes. *Annu Rev Microbiol* 42, 97-125.
2. Johnson, L. N., and Barford, D. (1993) The effects of phosphorylation on the structure and function of proteins. *Annu Rev Biophys Biomol Struct* 22, 199-232.
3. Lopez, J. L. (2007) Two-dimensional electrophoresis in proteome expression analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* 849, 190-202.

4. Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U S A* 97, 9390-9395.
5. Ficarro, S. B., McClelland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20, 301-305.
6. Ficarro, S. B., Salomon, A. R., Brill, L. M., Mason, D. E., Stettler-Gill, M., Brock, A., and Peters, E. C. (2005) Automated immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry platform for profiling protein phosphorylation sites. *Rapid Commun Mass Spectrom* 19, 57-71.
7. Hunter, T., and Sefton, B. M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A* 77, 1311-1315.
8. Andersson, L., and Porath, J. (1986) Isolation of phosphoproteins by immobilized metal (Fe³⁺) affinity chromatography. *Anal Biochem* 154, 250-254.
9. Posewitz, M. C., and Tempst, P. (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal Chem* 71, 2883-2892.
10. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jorgensen, T. J. (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* 4, 873-886.
11. Cao, L., Yu, K., Banh, C., Nguyen, V., Ritz, A., Raphael, B. J., Kawakami, Y., Kawakami, T., and Salomon, A. R. (2007) Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. *J Immunol* 179, 5864-5876.
12. Zhang, Y., Wolf-Yadlin, A., Ross, P. L., Pappin, D. J., Rush, J., Lauffenburger, D. A., and White, F. M. (2005) Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. *Mol Cell Proteomics* 4, 1240-1250.
13. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A* 100, 443-448.
14. Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A* 101, 12130-12135.

15. McNulty, D. E., and Annan, R. S. (2008) Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. *Mol Cell Proteomics* 7, 971-980.
16. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T-cell receptor activation. *Mol Cell Proteomics* 8, 2418-2431.
17. Schmelzle, K., Kane, S., Gridley, S., Lienhard, G. E., and White, F. M. (2006) Temporal dynamics of tyrosine phosphorylation in insulin signaling. *Diabetes* 55, 2171-2179.
18. Muller, U., Ernst, G., Melle, C., Guthke, R., and von Eggeling, F. (2006) Convergence of the proteomic pattern in cancer. *Bioinformatics* 22, 1293-1296.
19. Yan, F., Subramanian, B., Nakeff, A., Barder, T. J., Parus, S. J., and Lubman, D. M. (2003) A comparison of drug-treated and untreated HCT-116 human colon adenocarcinoma cells using a 2-D liquid separation mapping method based upon chromatofocusing PI fractionation. *Anal Chem* 75, 2299-2308.
20. Kim, J. E., and White, F. M. (2006) Quantitative analysis of phosphotyrosine signaling networks triggered by CD3 and CD28 costimulation in Jurkat cells. *J Immunol* 176, 2833-2843.
21. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17, 994-999.
22. Chakraborty, A., and Regnier, F. E. (2002) Global internal standard technology for comparative proteomics. *J Chromatogr A* 949, 173-184.
23. Kellermann, J. (2008) ICPL--isotope-coded protein label. *Methods Mol Biol* 424, 113-123.
24. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3, 1154-1169.
25. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1, 376-386.

26. Mann, M. (2006) Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol* 7, 952-958.
27. Olsen, J. V., Blagoev, B., Gnäd, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635-648.
28. Zhang, R., Sioma, C. S., Thompson, R. A., Xiong, L., and Regnier, F. E. (2002) Controlling deuterium isotope effects in comparative proteomics. *Anal Chem* 74, 3662-3669.
29. Robey, E., and Allison, J. P. (1995) T-cell activation: integration of signals from the antigen receptor and costimulatory molecules. *Immunol Today* 16, 306-310.
30. Szamel, M., and Resch, K. (1995) T-cell antigen receptor-induced signal-transduction pathways--activation and function of protein kinases C in T lymphocytes. *Eur J Biochem* 228, 1-15.
31. Kim, P. W., Sun, Z. Y., Blacklow, S. C., Wagner, G., and Eck, M. J. (2003) A zinc clasp structure tethers Lck to T-cell coreceptors CD4 and CD8. *Science* 301, 1725-1728.
32. Straus, D. B., and Weiss, A. (1992) Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T-cell antigen receptor. *Cell* 70, 585-593.
33. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol Cell Biol* 18, 1388-1399.
34. Finco, T. S., Kadlecsek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity* 9, 617-626.
35. Gillis, S., and Watson, J. (1980) Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T-cell line. *J Exp Med* 152, 1709-1719.
36. Abraham, R. T., and Weiss, A. (2004) Jurkat T-cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* 4, 301-308.
37. Irvin, B. J., Williams, B. L., Nilson, A. E., Maynor, H. O., and Abraham, R. T. (2000) Pleiotropic contributions of phospholipase C-gamma1 (PLC-gamma1) to

- T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC-gamma1-deficient Jurkat T-cell line. *Mol Cell Biol* 20, 9149-9161.
38. Yablonski, D., Kuhne, M. R., Kadlecsek, T., and Weiss, A. (1998) Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T-cell. *Science* 281, 413-416.
 39. James, P. (1997) Protein identification in the post-genome era: the rapid rise of proteomics. *Q Rev Biophys* 30: 279-331.
 40. Anderson, N. L., and Anderson, N. G. (1998) Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis* 19: 1853-1861.
 41. Liotta, L. A., and Petricoin, E. F. (2000) Beyond the genome to tissue proteomics. *Breast Cancer Res* 2: 13-14.
 42. Wang, C. C., and Tsou, C. L. (1998) Post-genome Study---Proteomics. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 30: 533-539.
 43. Dove, A. (1999) Proteomics: translating genomics into products? *Nat Biotechnol* 17: 233-236.
 44. Knopf, U. C., Sommer, A., Kenny, J., and Traut, R. R. (1975) A new two-dimensional gel electrophoresis system for the analysis of complex protein mixtures: application to the ribosome of E. coli. *Mol Biol Rep* 2: 35-40.
 45. Ames, G. F., and Nikaido, K. (1976) Two-dimensional gel electrophoresis of membrane proteins. *Biochemistry* 15: 616-623.
 46. Edman, P. (1949) A method for the determination of amino acid sequence in peptides. *Arch Biochem* 22: p. 475.
 47. Kuster, B., and Mann, M. (1998) Identifying proteins and post-translational modifications by mass spectrometry. *Curr Opin Struct Biol* 8: 393-400.
 48. McLafferty, F. W., Fridriksson, E. K., Horn, D. M., Lewis, M. A., and Zubarev, R. A. (1999) Techview: biochemistry. Biomolecule mass spectrometry. *Science* 284: 1289-1290.
 49. Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* 422:198-207.
 50. Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246: 4-71.
 51. Karas, M., and Hillenkamp, F. (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* 60: 2299-2301.

52. Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., and Yoshida, T. (1988) Protein and Polymer Analyses up to m/z 100 000 by Laser Ionization Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2: 151-153.
53. Sze, S. K., Ge, Y., Oh, H., and McLafferty, F. W. (2002) Top-down mass spectrometry of a 29-kDa protein for characterization of any posttranslational modification to within one residue. *Proc Natl Acad Sci U S A* 99: 1774-1779.
54. Syka, J. E. P., Coon, J. J., Schroeder, M. J., Shabanowitz, J., and Hunt, D. F. (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc Natl Acad Sci U S A*. 101: 9528-9533.
55. Keil-Dlouha, V. V., Zylber, N., Imhoff, J., Tong, N., and Keil, B. (1971) Proteolytic activity of pseudotrypsin. *FEBS Lett* 16: 291-295.
56. Wells, J. M., and McLuckey, S. A. (2005) Collision-induced dissociation (CID) of peptides and proteins. *Methods Enzymol* 402: 148-185.
57. Antoine, R., Broyer, M., Chamot-Rooke, J., Dedonder, C., Defrancois, C., Dugourd, P., Gregoire, G., Jouvet, C., Onidas, D., Poulain, P., Tabarin, T., and van der Rest, G. (2006) Comparison of the fragmentation pattern induced by collisions, laser excitation and electron capture. Influence of the initial excitation. *Rapid Commun Mass Spectrom* 20: 1648-1652.
58. Steen, H., and Mann, M. (2004) The ABC's (and XYZ's) of peptide sequencing. *Nat Rev Mol Cell Biol* 5: 699-711.
59. Eng, J. K., McCormack, A. L., and Yates, J. R. (1994) An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. *J Am Soc Mass Spectrom* 5: 976-989.
60. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551-3567.
61. Astoul, E., Edmunds, C., Cantrell, D. A., and Ward, S. G. (2001) PI3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol* 22, 490-496.
62. Shan, X., Czar, M. J., Bunnell, S. C., Liu, P., Liu, Y., Schwartzberg, P. L., and Wange, R. L. (2000) Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol. Cell. Biol.* 20, 6945-6957.
63. Wang, X., Gyorloff-Wingren, A., Saxena, M., Pathan, N., Reed, J. C., and Mustelin, T. (2000) The tumor suppressor PTEN regulates T cell survival and antigen receptor signaling by acting as a Phosphatidylinositol 3-Phosphatase. *J. Immunol.* 164, 1934-1939.

64. Seminario, M. C. and Wange, R. L. (2002) Signaling pathways of D3-phosphoinositide-binding kinases in T cells and their regulation by PTEN. *Semin. Immunol.* 14, 27-36.
65. Olsen, J. V., Schwartz, J. C., Griep-Raming, J., Nielsen, M. L., Damoc, E., Denisov, E., Lange, O., Remes, P., Taylor, D., Splendore, M., Wouters, E. R., Senko, M., Makarov, A., Mann, M., and Horning, S. (2009) A dual pressure linear ion trap Orbitrap instrument with very high sequencing speed. *Mol Cell Proteomics* 8, 2759-2769.
66. Schwartz, J. C., Senko, M. W., and Syka, J. E. P. (2002) A two-dimensional quadrupole ion trap mass spectrometer. *J Am Soc Mass Spectrom* 13, 659-669.
67. Comisarow, M. B., and Marshall, A. G. (1974) Fourier transform ion cyclotron resonance spectroscopy. *Chemical Physics Letters* 25, 282-283.
68. Marshall, A. G.; Hendrickson, C. L.; Jackson, G. S. (1998). Fourier transform ion cyclotron resonance mass spectrometry: a primer". *Mass Spectrometry Reviews* 17, 1-34.
69. Hu, Q., Noll, R. J., Li, H., Makarov, A., Hardman, M., and Cooks, R. G. (2005) The Orbitrap: a new mass spectrometer. *Journal of Mass Spectrometry* 40, 430-443.
70. Stafford, G. C., Kelley, P. E., Syka, J. E. P., Reynolds, W. E., and Todd J. F. J. (1984) Recent improvements in and analytical applications of advanced ion trap technology. *International Journal of Mass Spectrometry and Ion Processes* 60, 85-98.
71. Bracewell, R. N. (2000), *The Fourier Transform and Its Applications* (3rd ed.), Boston: McGraw-Hill.
72. Makarov A. (2000) Electrostatic axially harmonic orbital trapping: A high-performance technique of mass analysis. *Analytical Chemistry* 72, 1156-1162.
73. Wange, R. L., and Samelson, L. E. (1996) Complex complexes: signaling at the TCR. *Immunity* 5, 197-205.
74. Premack, B. A., and Gardner, P. (1992) Signal transduction by T-cell receptors: mobilization of Ca^{2+} and regulation of Ca^{2+} -dependent effector molecules. *Am. J. Physiol.* 263, 1119-1140.
75. Leervers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) Signaling through phosphoinositide 3-kinases: the lipids take centre stage. *Current Opinion in Cell Biology* 11, 219-225.

76. August, A., Sadra, A., Dupont, B., and Hanafusa, H. (1997) Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *PNAS* 94, 11227-11232.
77. Schneider, U., Schwenk, H., and Bornkamm, G. (1977) Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma". *Int J Cancer* 19, 621-626.
78. Chan, A. C., Irving, B. A., Fraser, J. D., and Weiss, A. (1991) The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. *Proc Natl Acad Sci* 88, 9166-9170.
79. Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992) ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell* 71: 649-662.
80. Crespo, M., Villamor, N., Gine, E., Muntanola, A., Colomer, D., Marafioti, T., Jones, M., Camos, M., Campo, E., Montserrat, E., and Bosch, F. (2006) ZAP-70 expression in normail Pro/Pre Cells, Mature B cells, and in B-cell acute lymphoblastic leukemia. *Clinical Cancer Research* 12, 726-734.
81. Brdicka, T., Kadlecsek, T. A., Roose, J. P., Pastuszak, A. W., and Weiss, A. (2005) Intramolecular regulatory switch in ZAP-70: Analogy with receptor tyrosine kinases. *Mol Cell Biol* 25, 4924-4933.
82. Rabinowich, H., Manciuola, M., Herberman, R. B., and Whiteside, T. L. (1996) β_1 Integrin-mediated activation of focal adhesion kinase and its association with Fyn and Zap-70 in human NK cells. *The Journal of Immunology* 157, 3860-3868.
83. Takahama, Y. (2006) Journey through the thymus: stromal guides for T-cell development and selection. *Nature Reviews Immunology* 6, 127-135.
84. Negishi, I., Motoyama, N., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995) Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376, 435-438.
85. Palacios, E. H. and Weiss, A. (2007) Distinct roles for Syk and ZAP-70 during early thymocyte development. *J Exp Med* 204, 1703-1715.
86. Shan, X., and Wange, R. L. (1999) Itk/Emt/Tsk activation in response to CD3 cross-linking in Jurkat T-cells requires ZAP-70 and Lat and is independent of membrane recruitment. *J Biol Chem* 274, 29323-29330.
87. Martelli, M. P., Lin, H., Zhang, W., Samelson, L. E., and Bierer, B. E. (2000) Signaling via LAT (linker for T-cell activation) and Syk/ZAP70 is required for

ERK activation and NFAT transcriptional activation following CD2 stimulation. *Blood* 96, 2181-2190.

88. Griffith, C. E., Zhang, W., and Wange, R. L. (1998) ZAP-70-dependent and -independent activation of Erk in Jurkat T-cells. Differences in signaling induced by H₂O₂ and Cd3 cross-linking. *J Biol Chem* 273, 10771-10776.
89. Kadlecsek, T. A., van Oers, N. S., Lefrancois, L., Olson, S., Finlay, D., Chu, D. H., Connolly, K., Killeen, N., and Weiss, A. (1998) Differential requirements for ZAP-70 in TCR signaling and T cell development. *J Immunol* 161, 4688-4694.
90. Sakaguchi, N., Takahashi, T., Hata, H., Nomura, T., Tagami, T., Yamazaki, S., Sakihama, T., Matsutani, T., Negishi, I., and Nakatsuru, S. (2003) Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* 426, 454-460.
91. Arpaia, E., Shahrar, M., Dadi, H., Cohen, A., and Roifman, C. M (1994) Defective T cell receptor signaling and CD8⁺ thymic selection in humans lacking zap-70 kinase. *Cell* 76, 947-958.
92. Chan, A. C., Kadlecsek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T., and Weiss, A. (1994) ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science* 264, 1599-1601.
93. Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., and Parslow, T. G. (1994) Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science* 264, 1596-1599.
94. Gelfand, E. W., Weinberg, K., Mazer, B. D, Kadlecsek, T. A., and Weiss, A. (1995) Absence of ZAP-70 prevents signaling through the antigen receptor on peripheral blood T cells but not on thymocytes. *J Exp Med* 182, 1057-1065.
95. Otsu, M., Steinberg, M., Ferrand, C., Merida, P., Rebouissou, C., Toberghien, P., Taylor, N., Candotti, F., and Noraz, N. (2002) Reconstitution of lymphoid development and function in ZAP-70-deficient mice following gene transfer into bone marrow cells. *Blood* 100, 1248-1256.

Chapter 2

A NEW APPROACH FOR QUANTITATIVE PHOSPHOPROTEOMIC DISSECTION OF SIGNALING PATHWAYS APPLIED TO T-CELL RECEPTOR ACTIVATION

2.1 INTRODUCTION

The reversible phosphorylation of serine, threonine, and tyrosine residues directly controls many cellular processes, leading to the activation of a coordinated network of additional phosphorylation events across multiple proteins over time. Clearly, there are benefits to individually identifying and characterizing specific components of a particular pathway, such as a phosphorylation site on a given protein, the kinase responsible for the modification, or the proteins interacting subsequently. However, a thorough understanding of these signaling pathways at the molecular level ultimately requires a global, simultaneous evaluation of these phosphorylation events as they occur over time.

Currently, the most common method for assessing wide-scale changes in the proteome is two-dimensional gel electrophoresis (1), but this methodology is relatively low-throughput and not optimal for the analysis of low-abundance and hydrophobic signaling proteins (2). Recent publications describe alternate approaches for assessing changes in phosphorylation patterns based primarily on LC/MS methodologies (3-8). A variety of promising purification approaches have been developed to discover hundreds to thousands of phosphorylation sites from complex cell lysates including strong cation exchange/titanium dioxide (SCX/TiO₂), IMAC, and IMAC in tandem with phosphotyrosine peptide immunoprecipitation (3, 4, 6, 9-12). These phosphoproteomic methods have been used to survey a large number of phosphorylation sites in a time course after receptor stimulation, where the magnitude of fold change in phosphorylation and the timing of phosphorylation suggest protein participation and placement within a pathway (6, 10, 12-14). For example, proteins phosphorylated late after receptor

stimulation are expected to represent downstream elements of a pathway while rapid phosphorylation is expected in the earlier stages of a pathway, especially at the receptor. Constitutive phosphorylation throughout a receptor stimulation time course is expected for proteins not involved in the pathway (10).

Although receptor stimulation time course experiments provide clues about placement of phosphorylation sites within a pathway relative to a stimulated receptor, a phosphoproteomic analysis comparing signaling protein null mutants and their reconstituted counterparts would allow for precise placement of novel phosphorylation sites within a signaling pathway relative to signaling landmarks within the canonical pathway. An ideal quantitative method to perform this analysis would provide both a dense temporal array of information about protein phosphorylation after receptor stimulation, as well as precise comparisons between isogenic matched normal cells and cells with altered signaling proteins.

Quantitation in proteomics experiments facilitates the comparison of proteins between various cellular states, such as a receptor stimulation time course experiment. Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) is an effective method for measuring the relative abundance of proteins in cell or tissue samples (15). In the SILAC technique, heavy or light essential amino acids are incorporated into cellular proteins through metabolic labeling in cell culture before cellular stimulation. This method allows for normalization of errors through the entire process of stimulation of cells, purification of proteins, and acquisition of LC/MS data, providing precise measurements of small differences between samples (15). However, the number of

comparisons possible in a single experiment is often limited in practice by the number of labeled amino acids available (16). The number of cellular state comparisons may be extended beyond the limits of available labeled amino acids by label-free quantitation using repetition of biological stimulations in separate SILAC experiments (6) or normalization to spiked phosphopeptide standards. Label-free quantitation between separate LC/MS experiments is facilitated by automation of peptide chromatography and data acquisition, resulting in enhanced reproducibility of chromatographic retention times and peak areas (17). The automated IMAC/nano-LC/ESI-MS system used here provides the necessary reproducible peak areas (8% relative standard deviation) and retention times (0.2% relative standard deviation) (17). The combination of SILAC and label-free quantitation allows for a greatly increased number of receptor stimulation time points while providing highly accurate comparisons between signaling protein null and reconstituted cells at each time point using SILAC.

Because of its high degree of prior characterization, the T-cell receptor (TCR) signaling pathway is an ideal model system for validating our approach for quantitative phosphoproteomic analysis of isogenic signaling pathway mutants. TCR signaling plays an essential role in regulating the adaptive immune response, and many proteins involved in the pathway have been identified (Figure 2.1) (18-20). The availability of the highly characterized Jurkat leukemic T-cell line has greatly facilitated investigations of TCR signaling by traditional and phosphoproteomic methods (13, 21). Furthermore, many isogenic disruption mutants of essential TCR signaling proteins have been isolated through genetic screens of mutagenized Jurkat clones, revealing severe phenotypic defects in TCR signaling and function (22-26). In particular, P116, a Zap-70 null clone

displays defects in stimulus-induced calcium mobilization, IL-2 production, NFAT transcription activation, and protein tyrosine phosphorylation on PLC- γ 1, ITK, LAT, Erk1/2, and SLP76 (25, 27-29). In the present study, a hybrid SILAC/label-free approach was applied to the P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted to wild type levels) Jurkat clones, and the placement of newly-discovered tyrosine phosphorylation sites relative to Zap-70 was determined.

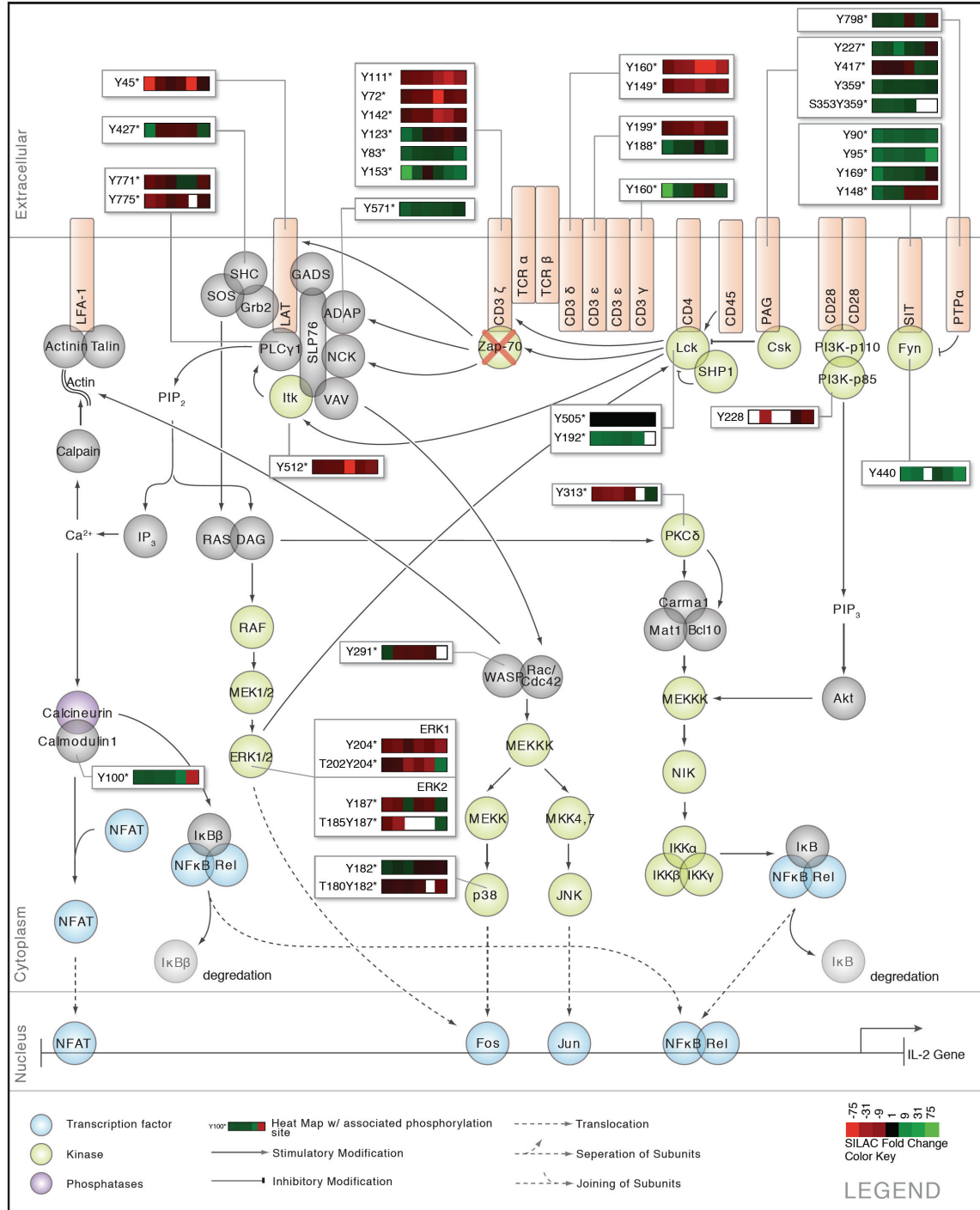


FIGURE 2.1: Canonical TCR signaling pathway with SILAC heatmaps. Established signaling cascades in activated T-cells with quantitative Zap-70 null/Zap-70 reconstituted SILAC ratio data represented as heatmaps beside individual proteins. Heatmaps represent averages of 5 replicate experiments. In the heatmap representation, green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Black represents no change. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. The utility of this visual representation is validated by the large

number of red heatmap bars downstream of Zap-70 in the canonical pathway. Note that the * next to the phosphorylation site signifies that this site previously has been described in the literature.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture, SILAC labeling and T-cell OKT3/OKT4 stimulation

Jurkat clones P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) were provided by L. Samelson at the National Institutes of Health. All cells were initially maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated undialyzed fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO₂ at 37 °C. After 5 days, all cell lines were washed twice with RPMI 1640 medium without Arg and Lys (Invitrogen) and reconstituted in RPMI 1640 medium containing either ¹²C₆, ¹⁴N₄ Arg and ¹²C₆, ¹⁴N₂ Lys (Sigma) or ¹³C₆, ¹⁵N₄ Arg and ¹³C₆, ¹⁵N₂ Lys (Cambridge Isotope Laboratories, Andover, MA) supplemented with 10% heat-inactivated dialyzed fetal bovine serum (Sigma), 2mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin in a humidified incubator with 5% CO₂ at 37 °C for 7 cell doublings. The concentration of Lys and Arg used in SILAC labeling of Jurkat cells in experiments described here was 0.22 mM and 0.38 mM, respectively.

Anti-CD3 and anti-CD4 (clones OKT3 and OKT4; eBioscience, San Diego, CA) stimulation was performed as described (13). Briefly, cells were washed once with 4 °C phosphate buffer saline (PBS), and reconstituted at a concentration of 1x10⁸ cells/ml in

PBS. For each time point, 1×10^8 cells were treated with OKT3 and OKT4 primary antibodies at a concentration of 2.5 $\mu\text{g/ml}$ of each antibody for 10 minutes at 4 °C. Cells were then crosslinked with 22 $\mu\text{g/ml}$ of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and incubated at 37 °C for 0, 2, 3, 5, 7, or 10 minutes.

2.2.2 Cell lysis, protein reduction, alkylation, digestion, and peptide

immunoprecipitation

To halt the stimulation, cells were placed in lysis buffer (8 M urea, 1 mM sodium orthovanadate, and 100 mM ammonium bicarbonate, pH 8.0) and incubated for 20 minutes at 4 °C. Lysates were then cleared at 12,000 xg for 15 minutes at 4 °C, and protein concentrations were measured by the DC Protein Assay (Bio-Rad, Hercules, CA). Once protein concentrations were determined, cell lysates from P116 and P116.c139 were combined in a 1:1 protein concentration ratio and reduced with 10 mM DTT for 1 hour at 56 °C, followed by alkylation with 55 mM iodoacetamide for 1 hour at room temperature. Cell lysates were then diluted 5-fold with 100 mM ammonium bicarbonate (pH 8.9) and digested with sequencing grade modified trypsin (Promega, Madison, WI) in a 1:100 (w/w) trypsin:protein ratio overnight at room temperature. Tryptic peptides were acidified to pH 2 with concentrated HCl, cleared at 2000 xg for 10 min at 22 °C, desalted using C18 Sep-Pak plus cartridges (Waters, Milford, MA) as described (14), and lyophilized in a Speed Vac plus (Thermo Fisher Scientific, Waltham, MA). A 100 pmol fraction of synthetic phosphopeptide LIEDAEpYTAK was added to each time point sample prior to peptide immunoprecipitation. Dry peptides from each time point were reconstituted and immunoprecipitated as previously described (10), except 20 μl of anti-phosphotyrosine

resin was used per 1×10^8 cells and eluted peptides were filtered through a 0.22 μ M filter (Millipore, Billerica, MA).

Results presented throughout this chapter are collected from the average of five total replicate analyses for each time point. Two biological replicates from separate frozen stocks of P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) cells were cultured in RPMI-containing isotopic heavy or light amino acids. These biological replicates were performed ten months apart. The first biological replicate contained two technical replicates while the second biological replicate contained three technical replicates. Cells were SILAC-labeled heavy (Zap-70 null) and light (Zap-70 reconstituted), stimulated, and lysed. Lysates were combined and digested with trypsin. For technical replicates, digested peptides from each time point were then divided into two or three equal fractions before desalting by C18 Sep-Pak plus cartridges and were kept separated throughout the rest of the experiment.

2.2.3 Western blotting for Zap-70 and phospho-ERK 1/2

Total cellular protein from 8M urea cell lysates was diluted 1:1 with gel loading buffer containing 4% SDS, 125 mM Tris-HCl (pH 6.8), 20% v/v glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8 from each proteomic sample. Equal amounts of protein (as measured by Lowry DC assay, Bio-Rad) were separated by 4-20% gradient SDS-polyacrylamide gel electrophoresis (Item 25204, Thermo Fisher Scientific), and electroblotted to an Immobilon membrane (Millipore). The membrane was blocked for 45 minutes in blocking buffer at 22 °C (PBS/Tween-20/5% milk) and then incubated for 12 hours at 4 °C with 1:1000 of either rabbit anti-human phospho-p44/p42 MAPK

(Thr202/Tyr204) or mouse anti-human Zap-70 antibody (Cell Signaling Technology, Danvers, MA). The membrane was washed 4x10 minutes at 22 °C in PBS/Tween-20. The membrane was then stained with 1:3000 of anti-rabbit IgG or anti-mouse IgG directly conjugated to horseradish peroxidase (Cell Signaling Technology) for 1 hour in blocking buffer at 22 °C and washed 5x15 minutes with PBS/Tween-20. Bands were visualized using chemiluminescence with the ECL+ kit (Amersham, Piscataway, NJ).

2.2.4 Automated desalt-IMAC/nano-LC/ESI-MS

Tryptic peptides were analyzed by a fully-automated phosphoproteomic technology platform integrating peptide desalting via reversed-phase chromatography, and Fe³⁺ IMAC enrichment of phosphopeptides as previously described (10). IMAC enriched phosphopeptides were eluted into the mass spectrometer (LTQ-FT; Thermo Fisher Scientific) through an analytical column (360 µm OD X 75 µm ID fused silica with 12 cm of 5 µm Monitor C18 particles with an integrated ~4 µm ESI emitter tip fritted with 3 µm silica; Bangs Laboratories) with a reversed-phase gradient (0-70% solvent B in 30 minutes). Static peak parking was performed via flow rate reduction from 200 nl/min to ~20 nl/min when peptides began to elute as judged from a BSA peptide scouting run, as described previously (17). Using a split flow configuration, an electrospray voltage of 2.0 kV was applied as described (30). Spectra were collected in positive ion mode and in cycles of one full MS scan in the FT (m/z: 400-1800), followed by data-dependent MS/MS scans in the LTQ (~0.3 s each) sequentially of the five most abundant ions in each MS scan with charge state screening for +1, +2, +3 ions and dynamic exclusion time of 30 s. The automatic gain control was 1,000,000 for the FTMS scan and 10,000 for the ion trap MS (ITMS) scans. The maximum ion time was 100 ms

for the ITMS scan and 500 ms for the FTMS full scan. FTMS resolution was set at 100,000.

2.2.5 Database analysis

MS/MS spectra were searched against the human National Center for Biotechnology Information non-redundant protein database using the SEQUEST algorithm provided with Bioworks 3.2 (SEQUEST v.27 rev12) (31). Peak lists were generated using Bioworks 3.2 (extract_msn.exe 11/27/06) using a mass range of 600-4500, precursor ion tolerance (for grouping) of 0.005 AMU, minimum ion count of 5, group scan of 0, minimum group count of 1. The NCBI human database contained 489,388 protein entries (50% forward, 50% reversed). SEQUEST was performed with the following parameters: trypsin enzyme specificity, 2 possible missed cleavages, 0.2 Da mass tolerance for precursor ions, 0.5 Da mass tolerance for fragment ions. Search parameters specified a differential modification of phosphorylation (+79.9663 Da) on serine, threonine, and tyrosine residues and a static modification of carbamidomethylation (+57.0215 Da) on cysteine. Search parameters also included a differential modification for arginine (+10.00827 Da) and lysine (+8.01420 Da) amino acids. To provide high-confidence phosphopeptide sequence assignments, SEQUEST results were filtered by Xcorr (+1 > 1.5; +2 > 2.0; +3 > 2.5), precursor mass error (<20 ppm), and a logistic spectral score (32) that assessed MS/MS spectral quality (> 0.7981), minimum peak area threshold of 500 SILAC and label-free quantitation were required, non-redundant phosphopeptides, and proteins with descriptors of "unnamed" or "unknown" were removed. Additionally, repeat observations of MS/MS spectra of each tyrosine-phosphorylated peptide in a minimum of three of six total time points were

required. False discovery rate was estimated with the decoy database approach after final assembly of nonredundant data into heatmaps (33). To validate the position of the phosphorylation site, the Ascore algorithm (34) was applied to all data, and the reported phosphorylation site position reflects the top Ascore prediction. Ascore probabilities are reported in the full data table in Supplemental Material 6 (Table can be found at <http://tcellpathway.com>).

2.2.6 Quantitation of relative phosphopeptide abundance

Relative quantitation of peptide abundance was performed via calculation of selected ion chromatogram (SIC) peak areas of heavy and light SILAC-labeled peptides. For label-free comparison of phosphopeptide abundance in the Zap-70 reconstituted Jurkat cells, individual time point SICs were normalized to the LIEDAEpYTAK peak area in the same time point. The standard exogenous LIEDAEpYTAK peptide accompanied cellular phosphopeptides through the peptide immunoprecipitation, desalt, IMAC, and reversed-phase elution into the mass spectrometer. Peak areas were calculated by inspection of SICs using recently developed software programmed in Microsoft Visual Basic 6.0 based on Xcalibur Development Kit 2.0 SR2 (Thermo Fisher Scientific). Quantitative data was calculated automatically for every assigned peptide using the ICIS algorithm available in the Xcalibur XDK with the following parameters: multiplet resolution of 8, noise tolerance of 0.1, noise window of 40, scans in baseline of 5, inclusion of RefExc peaks False. A minimum SIC peak area equivalent to the typical spectral noise level of 500 was required of all data reported for label-free and SILAC quantitation.

A label-free data heatmap was generated for comparison of phosphopeptides in Zap-70 reconstituted Jurkat cells through a time course of receptor stimulation. The magnitude of change of the heatmap color was calculated from the log of the ratio of the fold change of each individual peptide peak area compared with the geometric mean for that peptide across all time points (as described in detail in Supplemental Material 7; Figure can be found at <http://tcellpathway.com>). Any changes (either an increase or decrease of peptide abundance above the average) with greater than 75 maximal-fold change were displayed as the same color as the 75 maximal-fold change. In the heatmap representation, the geometric mean of a given phosphopeptide across all time points was set to the color black. A blue color represented below-average abundance, while yellow represented above-average abundance, for each unique phosphopeptide. Blanks in the heatmap indicated that a clearly-defined SIC peak was not observed for that phosphopeptide in any of the replicate analyses for that time point. The heatmap colors were generated from the average of the LIEDAEpYTAK standard peptide normalized SICs in the 5 replicate experiments. The coefficient of variation was calculated for each heatmap square. P-values were also calculated between the replicate distributions of each heatmap square compared to the time point with the minimum average value for that phosphopeptide (Supplemental Material 6; Table can be found at <http://tcellpathway.com>).

In the second type of heatmap, SILAC ratios corresponding to peptide abundance differences between Zap-70 null and reconstituted cell lines across the time course of receptor stimulation were represented. For the SILAC heatmaps, a black color represented a KO/WT ratio of 1 between the two cell lines for a given peptide at that time

point. A red color represented less abundance, and green represented higher abundance, of the given peptide in the Zap-70 null cells compared to the Zap-70 reconstituted cells. The magnitude of change of the heatmap color was calculated as described in detail in Supplemental Material 7 (Figure can be found at <http://tcellpathway.com>). The heatmap color was thresholded at a maximal fold-change of 75-fold increased or decreased in the Zap-70 null cells compared to reconstituted cells. The coefficient of variation percent was calculated for each SILAC ratio heatmap square amongst the 5 replicate analyses. P-values were also calculated between the Zap-70 null and Zap-70 reconstituted replicate measurements for each phosphopeptide and time point (Supplemental Material 6; Table can be found at <http://tcellpathway.com>).

2.2.7 Flow cytometric analysis

P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) Jurkat clones (1×10^6 cells from each population) were resuspended in staining medium (PBS supplemented with 3 % FBS and 0.02 % Azide), and preincubated with 100 μ l (1 test) of PE mouse anti-human CD3 antibody (BD Bioscience) for 15 minutes on ice. Cells were then washed 3 times with staining medium and resuspended in fixing solution (PBS supplemented with 0.5 % paraformaldehyde). TCR-CD3 expression of cells was evaluated on a FACScaliber (BD Biosciences) and analyzed with FlowJo software (TreeStar).

2.3 RESULTS

2.3.1 Overall scheme

Prior to analysis of Zap-70 null Jurkat clones, wild type Jurkat JE6-1 cells were incubated with either 0.38 mM $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg or 0.22 mM $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys in dialyzed serum for 7 doublings to test the SILAC labeling conditions. Tyrosine-phosphorylated peptides were enriched according to the standard peptide immunoprecipitation procedure described in the Materials and Methods. After desalt, IMAC, and LC/MS, the phosphopeptides were identified with SEQUEST and filtered as described in the Materials and Methods to compile a nonredundant list of tyrosine phosphopeptides. In the $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg and $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys-labeled samples, a total of 130 and 111 nonredundant phosphopeptides were observed, respectively. There were no unlabeled peptides observed. In the $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg-labeled sample, a single $^{13}\text{C}_5$, $^{15}\text{N}_1$ Pro was observed out of a total of 75 peptides that contained unlabeled proline (Supplemental Material 5; Table can be found at <http://tcellpathway.com>). In addition, growth rates were measured in wild type Jurkat JE6-1 cells to determine whether isotopic labeling would have an effect. Cells labeled with either heavy (0.38 mM $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg and 0.22 mM $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys) or light (0.38 mM $^{12}\text{C}_6$, $^{14}\text{N}_4$ Arg and 0.22 mM $^{12}\text{C}_6$, $^{14}\text{N}_2$ Lys) amino acids displayed no significant differences in growth over 7 days (Figure 2.2).

Day	Heavy-labeled (X 10^5 cells/ml)	Light-labeled (X 10^5 cells/ml)	Control (X 10^5 cells/ml)
1	1	1	1
2	3	2.7	2.5
3	6.2	5	7.3
4	10	11	13.2
5	2.3	3	2.2
6	5.2	7.3	5.5
7	10	11.5	10

FIGURE 2.2: Growth rate comparison of wild type Jurkat JE6-1 cells labeled with heavy or light isotopic amino acids. Cells were cultured for 7 days in RPMI 1640 medium containing either $^{12}\text{C}_6$, $^{14}\text{N}_4$ Arg and $^{12}\text{C}_6$, $^{14}\text{N}_2$ Lys (Light labeled) or $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg and $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys (Heavy labeled) amino acids. As a control, cells were also cultured in parallel with RPMI 1640 medium containing previously

incorporated endogenous Arg and Lys (Control) amino acids. All medium was supplemented with 10% heat-inactivated dialyzed fetal bovine serum (Sigma), 2mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin. At day 4, cells were passaged, and reseeded at a concentration of 1×10^5 cells/ml. Cell concentration was measured using a hemacytometer.

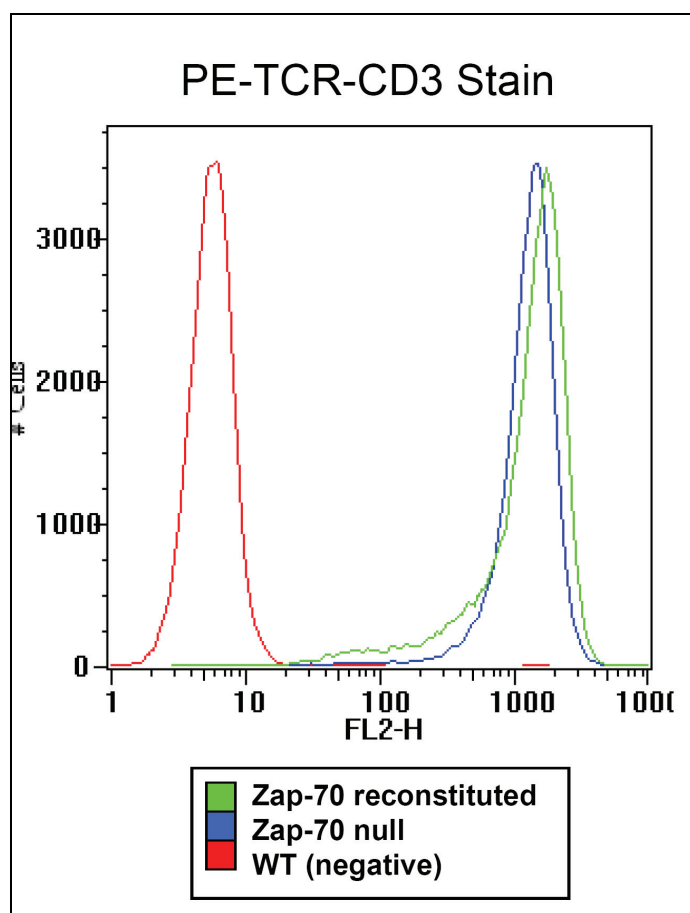


FIGURE 2.3: TCR-CD3 expression on Zap-70 null and reconstituted Jurkat clones. P116 (Zap-70 null) (Blue) and P116.c139 (Zap-70 reconstituted) (Green) Jurkat clones were resuspended in stained medium with PE mouse anti-human CD3 antibody and TCR-CD3 expression was evaluated by flow cytometry. Wild type JE6-1 Jurkat cells were unstained and used as a negative control. The histogram represents only live cells.

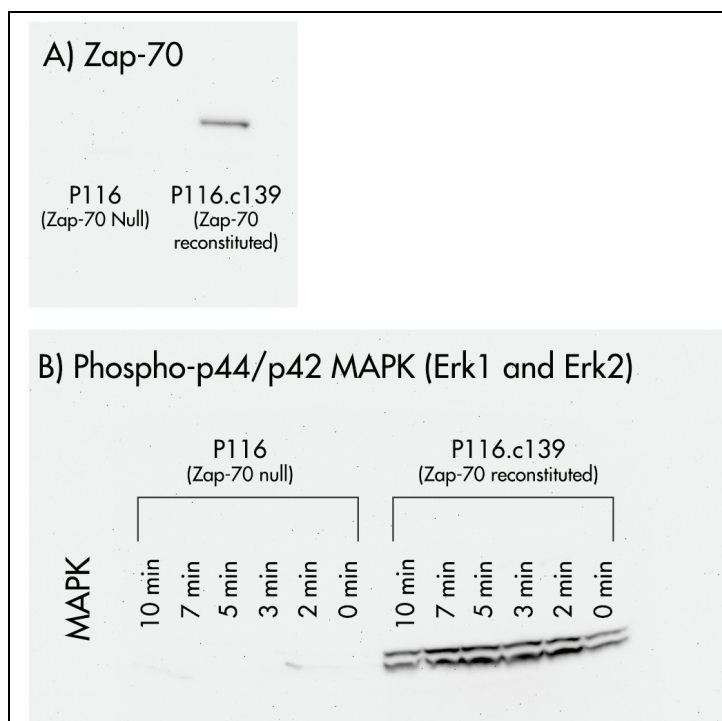


FIGURE 2.4: Disruption of Zap-70 from Jurkat-cells and phosphorylation of ERK1/2. A) Zap-70 expression in Jurkat T-cells. Protein lysates from P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) were separated by SDS-Page and immunoblotted with Zap-70-specific antibodies. B) ERK1 and ERK2 phosphorylation in OKT3/OKT4 stimulated Jurkat T-cells used in phosphoproteomic analysis. P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) cells were stimulated with anti-CD3 (OKT3) and anti-CD4 (OKT4) antibodies. Protein lysates were then separated by SDS-Page and immunoblotted with phospho-p44/p42 MAPK- (ERK1 and ERK2) specific antibodies.

To evaluate its utility, our quantitative phosphoproteomic approach was applied to the human Jurkat T-cell clones, P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted to wild type levels). The removal of Zap-70 protein and decrease in phosphorylation in ERK1/2 in Zap-70 null and reconstituted cells measured by Western blot was consistent with previous reports on these Jurkat clones (Figure 2.4) (25, 27-29). TCR-CD3 expression of Zap-70 null and Zap-70 reconstituted Jurkat cells was measured using FACS analysis to ensure that both cell populations had equivalent expression (Figure 2.3). A distribution of six time points was used to detect subtle fluctuations in the timing of phosphorylation. Zap-70 null to Zap-70 reconstituted SILAC phosphopeptide ratios

were calculated at each time point in a time course of TCR stimulation (Figure 2.5). Before peak area quantitation and SILAC ratio calculation, high-quality sequence assignments were first determined using stringent criteria ($X_{\text{corr}} +1 > 1.5$; $+2 > 2.0$; $+3 > 2.5$; precursor mass error < 20 ppm, logistic spectral score > 0.7981 (32). Additional requirements included: minimum SIC peak area threshold of 500 for SILAC and label-free quantitation was required, tyrosine-phosphorylated, and that observations of each peptide MS/MS spectra were present in at least three of the six time points). The false discovery rate (FDR) estimated from decoy database search was 2.22% after all filtering and assembly of non-redundant data into heatmaps. A total of 5 replicate experiments were performed (as described in Figure 2.5). Quantitative comparisons were generated from SIC peak areas, and heatmaps were generated from the average values from all replicates. The complete list of quantitative replicate data, calculated CV, and p-values are available (Supplemental Material 6; Table can be found at <http://tcellpathway.com>).

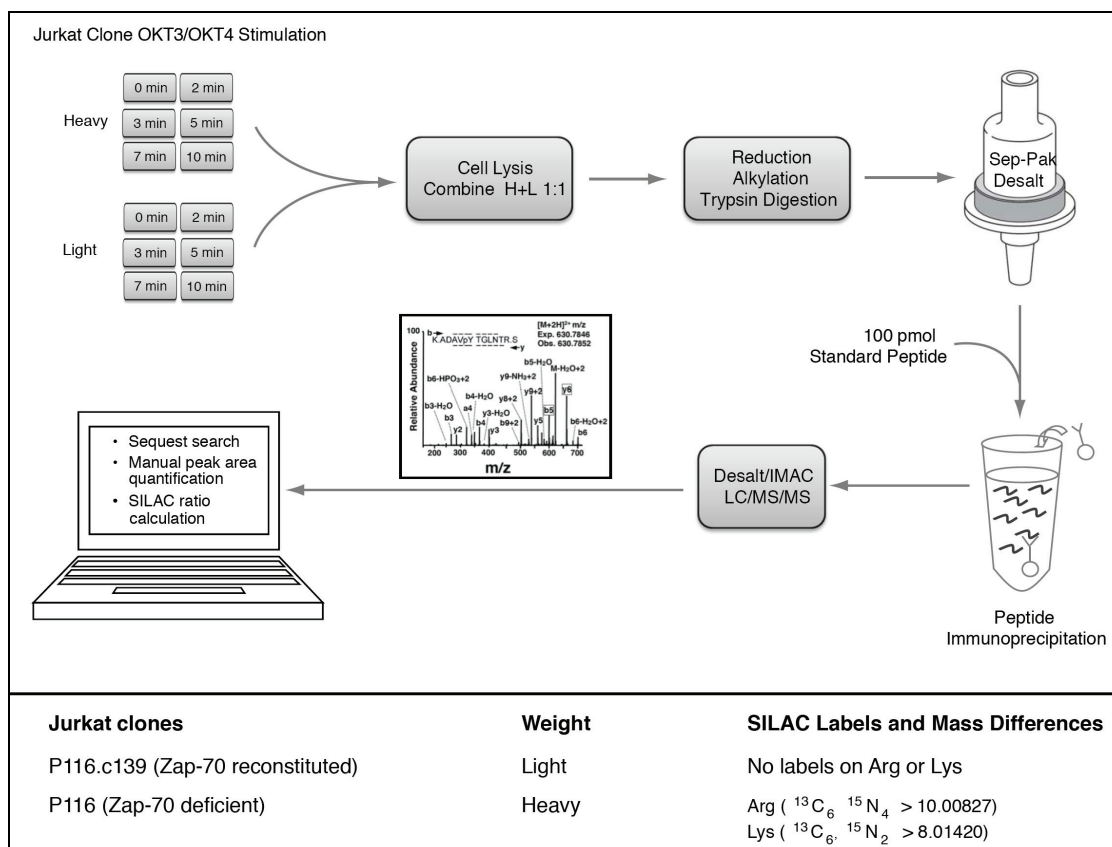


FIGURE 2.5: Experimental procedure. Two cell populations of human Jurkat T-cell clones (P116 and P116.c139) are incubated with normal or heavy isotope labeled arginine and lysine amino acids, physically differentiating the two proteomes by a shift in molecular weights. Each cell population is then pre-incubated with OKT3 and OKT4 antibodies for 10 minutes at 4 °C and then crosslinked with IgG at 37 °C for the times indicated. After cell lysis, samples are combined at an equal protein concentration ratio of 1:1. Samples are then reduced, alkylated, and trypsin-digested into peptides. Peptides are desalted by Sep-Pak cartridges and then enriched by phosphotyrosine peptide immunoprecipitation and Fe^{3+} IMAC. Peptides are then subjected to reverse-phase LC-MS/MS analysis.

2.3.2 Representation of label-free and SILAC quantitation

Two different visual representations in the form of heatmaps of quantitative data were generated for each sequenced phosphopeptide to reflect either the label-free or SILAC ratio data. These two heatmaps provide the relationship between each phosphopeptide sequenced and TCR stimulation (label-free heatmaps) or the removal of Zap-70 (SILAC ratio heatmaps).

In the label-free heatmap, the abundance of each phosphopeptide in Zap-70 reconstituted cells was compared across the T-cell receptor stimulation time course (Figure 2.6 and Figure 2.8). This type of quantitative analysis is useful for determining whether newly-discovered phosphorylation sites change in abundance after receptor stimulation, providing a list of candidate phosphorylation sites that may participate in the T-cell signaling pathway. These data are represented in the form of a heatmap in which black corresponds to the average abundance for a given peptide across all time points, yellow corresponds to phosphorylation levels above the average, and blue corresponds to phosphorylation levels below the average. As expected, for many of the phosphorylation sites already known to be involved in TCR signaling, phosphorylation levels increase after receptor stimulation followed by a steady decrease (Figure 2.6).

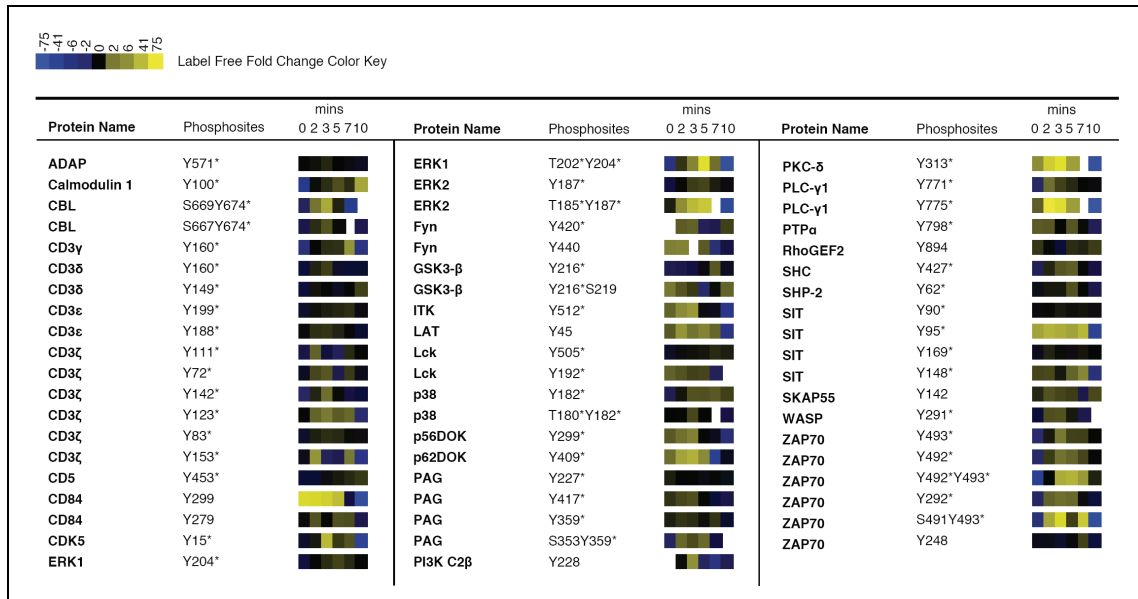


FIGURE 2.6: Quantitative phosphoproteomic analysis of known TCR signaling proteins in wild type cells. Listed above is a portion of the data collected, representing the known TCR signaling proteins that were observed in our study of human Jurkat P116.c139 (Zap-70 reconstituted) cells. Temporal quantitative changes in phosphorylation state are represented as heatmaps which represent averages of 5 replicate experiments. In the heatmap representation, yellow represents levels of phosphorylation above the average, while blue represents levels of phosphorylation below the average. Black represents average abundance for a certain peptide across all time points. Blanks in the heatmap indicate that a clearly-defined SIC peak was

not observed for that phosphopeptide in that time point. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

In the second SILAC heatmap, SILAC ratios between Zap-70 null and reconstituted cells are represented for each phosphopeptide and time point (Figure 2.1 and Figure 2.7). These data are represented in the form of a SILAC ratio heatmap representing Zap-70's removal from Jurkat T-cells for a given phosphopeptide at each time point, in which green corresponds to an increase in phosphorylation, red corresponds to a decrease in phosphorylation, and black corresponds to no difference. The intensity of the color reflects the relative magnitude of the change in phosphorylation. If phosphorylation decreases in cells lacking Zap-70 compared to wild type, the phosphorylation event can hypothetically be positioned downstream of this protein. We then assessed the utility of our isogenic mutant approach by investigating the correlation between the known structure of the T-cell signaling pathway and the changes in phosphopeptide abundance upon the removal of Zap-70. The utility of the method is validated by the large number of red heatmap bars downstream of Zap-70 in the canonical pathway (Figure 2.1), indicating Zap-70-dependent phosphorylation.

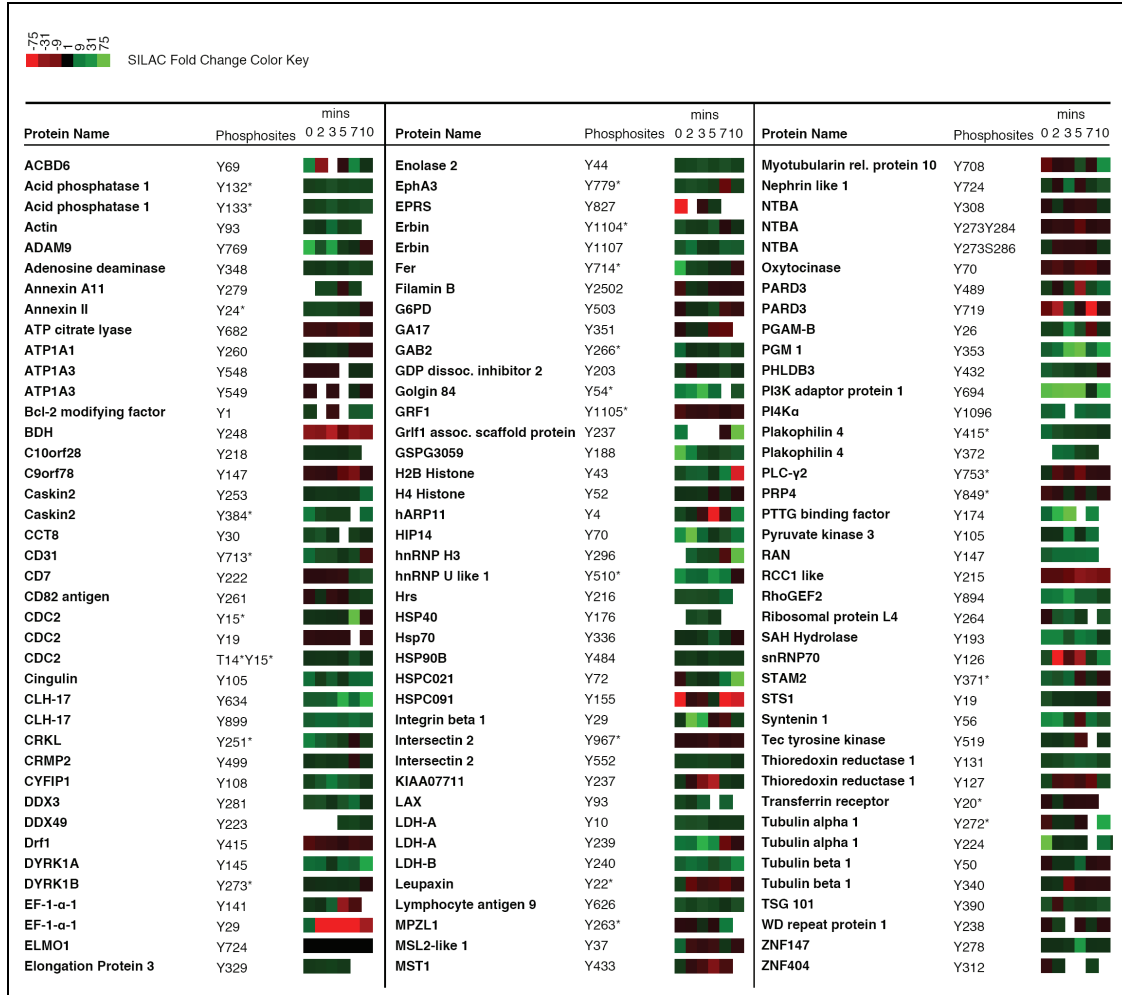


FIGURE 2.7: Zap-70 null to reconstituted SILAC ratio heatmap of proteins not known to function in TCR signaling in wild type cells. SILAC heatmap of a portion of the data collected, representing proteins that were observed in our study of human Jurkat P116 (Zap-70 mutants) clones not known to participate in the canonical T-cell signaling pathway. Zap-70 null/reconstituted SILAC ratios corresponding to the changes in phosphorylation state are represented as heatmaps. Heatmaps are calculated from the averages of 5 replicate experiments. Green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Black represents no change. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

2.3.3 Phosphoproteomic profiling of phosphorylation sites identified in receptor stimulated Zap-70 reconstituted Jurkat cells

From this analysis, we observed 168 tyrosine phosphorylation sites residing on 135 unique proteins in the Zap-70 reconstituted T-cells across 6 time points of receptor

stimulation (Figure 2.6 and Figure 2.8). Among the 135 proteins identified, 24 % of them (32 proteins) were previously functionally characterized in TCR signaling (20, 35, 36). Phosphorylation sites were observed on the earliest upstream TCR signaling components (TCR-CD3 subunits $\gamma\delta\epsilon\zeta$, tyrosine kinases LCK, FYN, Zap-70), crucial adaptor proteins (LAT), and downstream target proteins (ITK, PLC- γ -1, PI3K, ERK1, ERK2, CD5) (Figure 2.1 and 2.6).

Because phosphorylation sites that do not change in response to TCR stimulation are unlikely to be involved in the TCR pathway, we focused our analysis on sites that showed a change (greater than 3-fold change) across the TCR stimulation time course in reconstituted Jurkat T-cells as measured by label-free quantitation. These TCR-responsive sites were further classified into a variety of groups with the use of SILAC ratio data to determine the dependence of each site upon the absence or presence of Zap-70 (Figure 2.9).

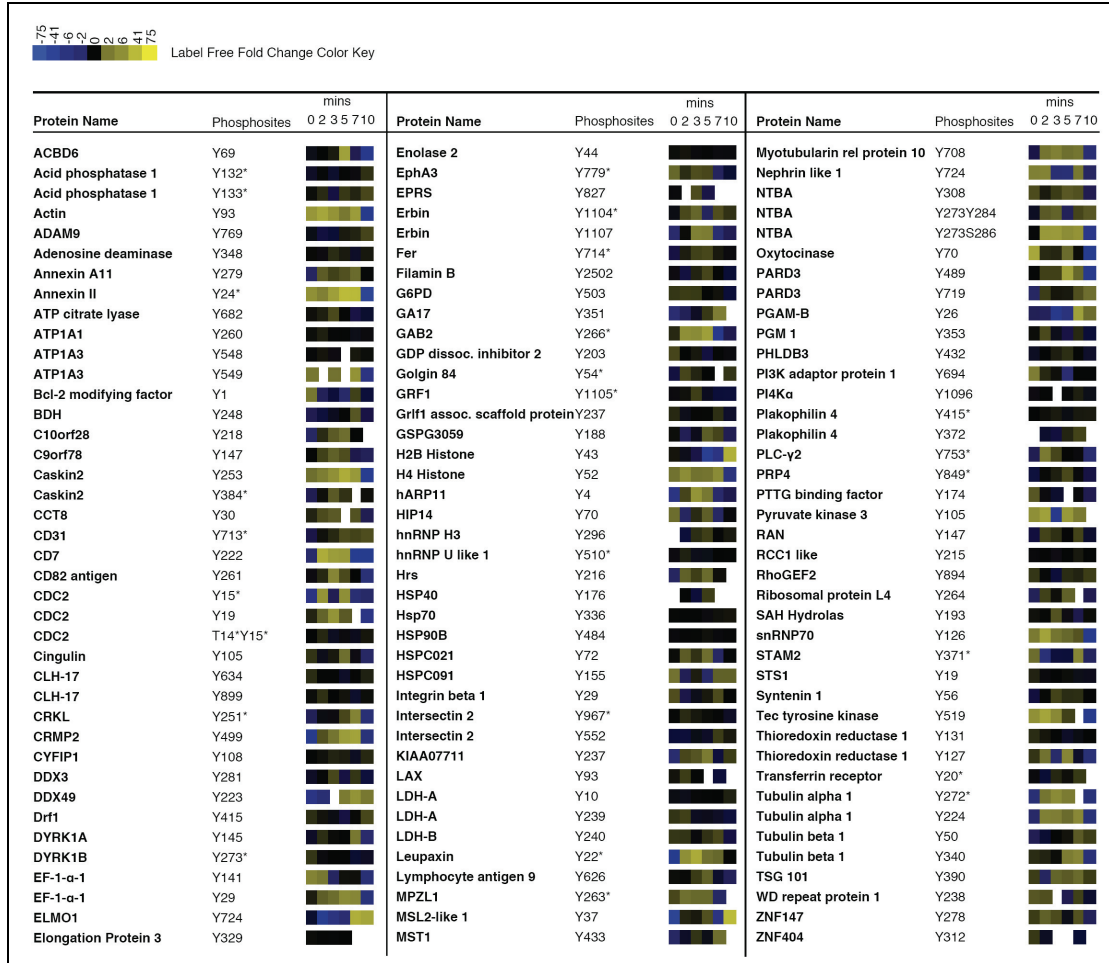


FIGURE 2.8: Quantitative phosphoproteomic analysis of proteins not known to function in TCR signaling in wild type cells. Label-free heatmap of a portion of the data collected, representing proteins that were observed in our study of human Jurkat P116.c139 (Zap-70 reconstituted) cells not known to participate in the canonical T-cell signaling pathway. Temporal quantitative changes in phosphorylation state are represented as heatmaps. Heatmaps are calculated from the averages of 5 replicate experiments. Yellow represents levels of phosphorylation above the average, while blue represents levels of phosphorylation below the average. Black represents average abundance for a certain peptide across all time points. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Note that the * next to the phosphorylation site signifies that this site has been previously described in literature.

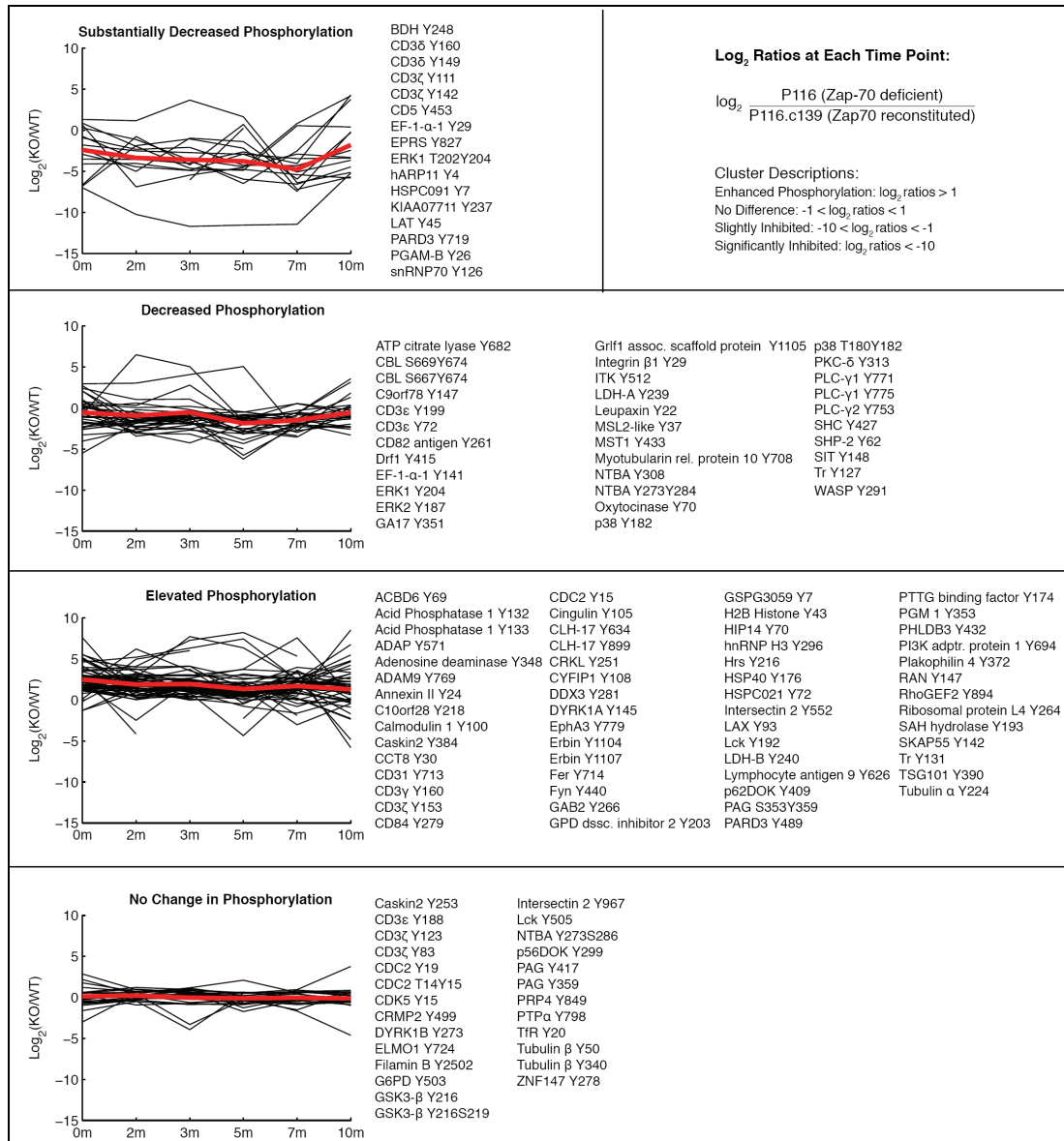


FIGURE 2.9: Classification of ZAP-70 null/reconstituted SILAC ratios. The log₂ SILAC ratios of Zap-70 null to Zap-70 reconstituted cells were classified into 4 major categories: Peptides with substantially decreased phosphorylation; Peptides with decreased phosphorylation; Peptides with elevated phosphorylation; Peptides with no change in phosphorylation. SILAC ratios are calculated from the average of 5 replicate experiments.

2.3.4 Clustering of phosphorylation sites by Zap-70 null/reconstituted SILAC ratios

Among the phosphopeptides that showed an increase in abundance with TCR stimulation in the reconstituted T-cell time course, SILAC ratios of Zap-70 null to Zap-70

reconstituted cells were classified into 4 categories (Figure 2.9): elevated phosphorylation (greater than 2-fold induction in 2 or more time points), no differences in phosphorylation (between 2-fold induction and 2-fold reduction in 4 or more time points), slightly decreased phosphorylation (between 2- and 10-fold reduction in 2 or more time points), and significantly decreased phosphorylation (greater than 10-fold reduction in 2 or more time points). From this grouping of phosphopeptide SILAC ratios, 43% showed enhanced phosphorylation, 19% showed no difference, 24% showed slight decrease, 12% showed significant decrease, and 2% represented outliers which did not fully fall into any of the 4 major categories, yet generally displayed minimal change.

Proteins with phosphorylation sites in the minimal change group included proteins known to function upstream of Zap-70 (CD3 ϵ , CD3 ζ , LCK) (20), proteins known to function in other signaling pathways in T-cells (GSK3 β , PAG) (37, 38), and ones known to not function in TCR signaling (Filamin B, CDC2, ELMO1).

In general, sites with decreased phosphorylation levels in P116 (Zap-70 null) cells suggest a position downstream of Zap-70 in the pathway. Through Western blot analysis of TCR-stimulated P116 (Zap-70 null) cells, previous studies have shown decreases in tyrosine phosphorylation on proteins such as PLC γ 1, LAT, and ERK1/2 (25, 27, 28). Our SILAC quantitation results also showed a decrease in phosphorylation on these downstream proteins in Zap-70 null cells at PLC γ 1 (Y⁷⁷¹, significantly decreased at 2 minutes, p-value < 0.05), LAT (Y⁴⁵, significantly decreased at 2 minutes, p-value < 0.05), Erk1 (Y²⁰⁴, significantly decreased at 7 and 10 minutes, p-value < 0.05), Erk2 (Y¹⁸⁷, significantly decreased at 0 and 2 minutes, p-value < 0.05).

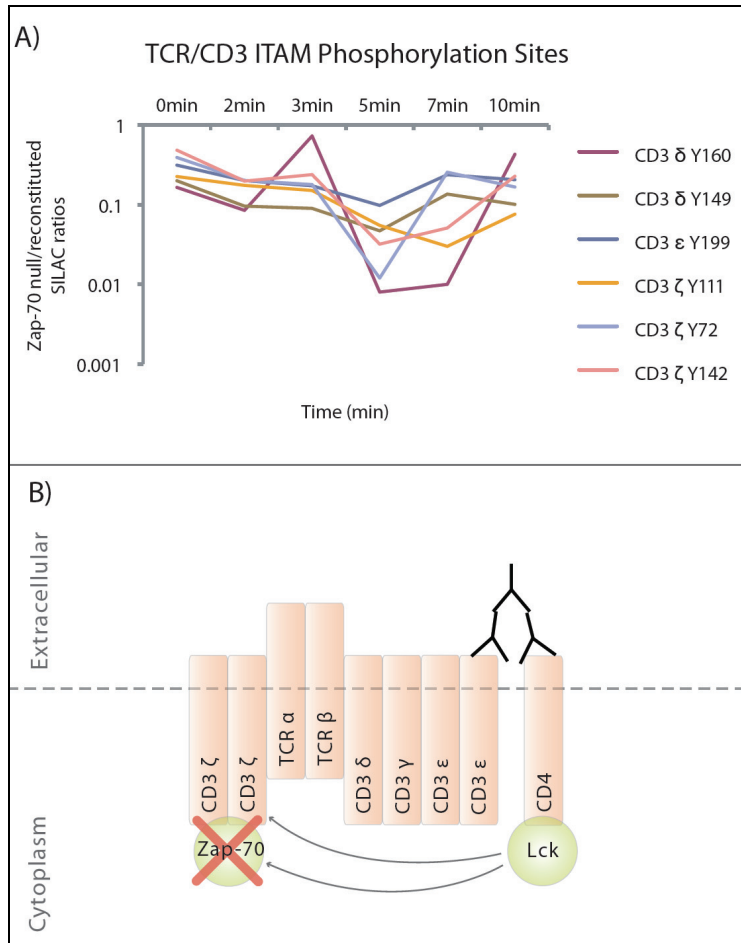


FIGURE 2.10: LCK phosphorylation of the CD3 ITAM motif requires Zap-70. A) TCR/CD3 Zap-70 null/Zap-70 reconstituted SILAC ratio profiles. Differences in phosphopeptide abundance over time were represented as SILAC ratios between P116 (Zap-70 null) and P116.c139 (Zap-70 reconstituted) cells on CD3 ζ , CD3 ϵ , and CD3 δ chains. SILAC ratios are calculated from the averages of 5 replicate experiments. B) Model of Zap-70-dependent LCK phosphorylation of the CD3 ζ ITAM motif as well as CD3/CD4 antibody co-stimulation.

Although phosphorylation sites upstream of Zap-70 would be expected to be unaffected by Zap-70 removal, three tyrosine residues within the TCR CD3 ζ (Y¹¹¹, Y⁷², Y¹⁴²) immunoreceptor tyrosine activation motif (ITAM), along with three tyrosine residues on TCR CD3 δ (Y¹⁶⁰, Y¹⁴⁹) and CD3 ϵ (Y¹⁹⁹) ITAMs, decreased significantly (p-value < 0.05 at various time points among these sites) in Zap-70 null cells (Figure 2.10). These seemingly upstream perturbations in phosphorylation are, nevertheless,

consistent with previous reports for the P116 Jurkat clone (39, 40). While LCK-mediated phosphorylation of CD3 ζ ITAMs is widely believed to precede Zap-70 recruitment and activation (41), previous studies have revealed a synergistic role of Zap-70-mediated recruitment as well as stabilization of the interaction between LCK and CD3 ζ at the ITAM regions independent of Zap-70 kinase activity (Figure 2.10A) (39, 40). Additional reports have indicated an essential role of LCK in the regulation of constitutive phosphorylation of CD3 ζ (42). From these studies, it can be proposed that the removal of Zap-70 would result in a decrease of CD3 ζ basal phosphorylation levels, which is consistent with our findings on these sites.

2.3.5 Novel phosphorylation sites identified

The use of this quantitative approach to make comparisons between Zap-70 null and reconstituted cells has also led to the generation of a broad testbed of novel, uncharacterized phosphorylation sites that can be positioned within the T-cell signaling pathway. Among the 178 total unique phosphorylation sites identified in this analysis, 105 of these sites were found to be novel, i.e. sites that were previously uncharacterized in the Human Protein Reference Database (HPRD) Version 7. Furthermore, of the 105 newly-discovered sites, 69 of these sites were changed significantly in abundance with Zap-70 removal. This data provides a wealth of information about the hypothetical placement of these sites relative to Zap-70 and the TCR (Figure 2.11).

Name	Site	0 min	2 min	3 min	5 min	7 min	10 min	Avg. SILAC Ratio
ACBD6	Y69	16.37				15.73		16.05
Actin	Y93	1.94	1.45			2.70		2.03
ADAM9	Y769	37.11						37.11
Adenosine deaminase	Y348			2.53			2.29	2.41
ATP1A1	Y260			1.76				1.76
ATP1A3	Y548	0.74						0.74
ATP1A3	Y549	0.74						0.74
BDH	Y248		0.06		0.14			0.10
C10orf28	Y218	1.48						1.48
CCT8	Y30		4.79					4.79
CD7	Y222				0.54			0.54
CD84	Y279	4.82	2.19		3.19			3.40
CLH-17	Y634	5.93		7.05		8.56		7.18
CLH-17	Y899	6.36	8.14	8.35	6.34	10.05	6.69	7.65
CRMP2	Y499		2.02			0.62		1.32
DDX3	Y281	3.49						3.49
DDX49	Y223					2.82		2.82
Drf1	Y415	0.20	0.33				0.45	0.33
DYRK1A	Y145		8.09					8.09
EF-1- α -1	Y141	1.70						1.70
Elongation Protein 3	Y329	1.86		2.49	1.97			2.11
Enolase 2	Y44					3.97		3.97
Erbin	Y1107		10.31	1.92				6.12
G6PD	Y503					0.30		0.30
GA17	Y351				0.18			0.18
GDP dissoc. inhibitor 2	Y203					3.54		3.54
Grif1 assoc. scaffold protein	Y237	12.65						12.65
GSPG3059	Y188	57.33						57.33
H2B Histone	Y43	3.43	6.34				0.02	3.26
H4 Histone	Y52	1.26	1.36					1.31
hARP11	Y4				0.01			0.01
Hrs	Y216		3.92		3.36			3.64
HSP40	Y176		3.34					3.34
HSP90B	Y484	1.81						1.81
HSPC021	Y72						145.62	145.62
Intersectin 2	Y552					2.63		2.63
LAT	Y45		0.26					0.26
LDH-A	Y239	11.41	10.11					10.76
LDH-B	Y240	6.62	9.93		3.52	6.63		6.68
MSL2-like 1	Y37		0.36		0.37			0.36
MST1	Y433					0.25		0.25
Myotubularin rel. protein 10	Y708	0.14				0.64		0.39
Nephrin like 1	Y724	2.27						2.27
NTBA	Y308					0.54		0.54
Oxytocinase	Y70				0.19			0.19
PARD3	Y489					3.18		3.18
PARD3	Y719		0.03					0.03
PGAM-B	Y26	2.49	2.28					2.39
PGM 1	Y353	6.26				7.31		6.79
PHLDB3	Y432	6.42	3.94	3.38	1.73			3.87
PI3K adaptor protein 1	Y694		69.91	100.84			38.37	69.71
PI4K α	Y1096	7.05	8.45			7.18		7.56
Plakophilin 4	Y372		9.58					9.58
PTTG binding factor	Y174		36.77	77.03		16.02		43.28
RA-GEF-2	Y1447		2.60	3.02				2.81
RAN	Y147		9.28	9.30	9.93	10.98		9.87
RCC1 like	Y215		0.19		0.05			0.12
RhoGEF2	Y894	11.73	13.16	23.77	6.29	6.40	2.89	10.71
SAH hydrolase	Y193	15.70	15.62					15.66
SKAP55	Y142			5.78			3.28	4.53
STS1	Y19	3.84	1.38	1.50				2.24
Syntenin 1	Y56		20.46					20.46
Tec tyrosine kinase	Y519		1.39					1.39
Thioredoxin reductase 1	Y131	2.74	3.32	4.95		5.66	2.98	3.93
Tubulin alpha 1	Y224	197.19				2.76	10.81	70.25
Tubulin beta 1	Y50	0.81					0.64	0.72
Tubulin beta 1	Y340						0.69	0.69
TSG 101	Y390	4.08		3.13		2.55		3.25
ZNF404	Y312	0.56				2.42		1.49

FIGURE 2.11: Dynamic effects of Zap-70 removal on novel phosphorylation sites discovered. Listed in this table is a subset of phosphorylation sites identified, representing the novel sites that displayed significant changes in response to Zap-70. Novel sites are defined as sites that were previously uncharacterized in the HPRD Version 7. Also included are the SILAC ratios (Zap-70 null/reconstituted) for the time points that showed significant changes amongst the 5 replicate experiments (p -value < 0.05) as well as the average SILAC ratio across the all time points. Squares left blank in this table represent missing data due to the sensitivity limit of the instrument, preventing the reproducible observation of SILAC ratios in at least 3 replicate experiments, or that the null hypothesis cannot be rejected.

2.4 DISCUSSION

The typical approach for elucidation of the structure of cellular signaling networks involves an iterative process of signaling protein disruptions and a large number of site-directed mutants, followed by characterization of every mutant through a battery of assays of cellular activation. One common phenotype employed to evaluate newly-created signaling protein mutants is the use of phosphorylation site-specific Western blots. The advantage of this approach is the specificity of the measurement and the ease of quantitation of the change in phosphorylation. However, localization of protein phosphorylation events within signaling pathways requires two separate types of information: the precise timing of each phosphorylation site relative to receptor stimulation, and the relationship of each phosphorylation site to canonical signaling pathway landmarks. Quantitative phosphoproteomics can provide a means to overcome some of these issues by providing a richer, unbiased site-specific view of the phosphoproteome of cells harboring altered signaling proteins. Quantitation methods, such as SILAC, can provide highly detailed information about differences between distinct populations, but the number of necessary quantitative comparisons quickly outpaces the number of isotopically-labeled amino acids available. The combination of SILAC and label-free quantitation allows for a greatly increased number of receptor

stimulation time points, while providing highly accurate comparisons between signaling protein null and reconstituted cells at each time point using SILAC.

Mutant compensation could be a complicating factor in the interpretation of differences in phosphorylation between protein null and wild type cells. Downstream targets dependent solely on the removed protein for phosphorylation should show strong decreases in phosphorylation. However, if multiple pathways exist to phosphorylate a downstream target, a pathway independent of the removed protein may mask defects in the protein-dependent pathway. For instance, in our analysis, the Zap-70 downstream target T¹⁸⁰Y¹⁸² of p38 MAPK showed only slight decreases in phosphorylation in Zap-70 null mutants, stimulating the hypothesis of alternative pathways regulating this site and compensating for the loss of Zap-70. Phosphoproteomic data from isogenic mutant analysis must be interpreted with attention and consideration for the possibility of compensation.

The interpretation of phosphoproteomic data obtained by our method could also be complicated by the existence of positive and negative feedback regulatory loops within signaling pathways. Feedback mechanisms allow upstream components to also be altered by the removal of a protein, making placement of phosphorylation sites within the pathway difficult. CSK, CD45, c-cbl, and SHP-1 are proteins known to function in negative feedback mechanisms in TCR signaling (43-47). Positive feedback mechanisms have also been observed in T-cells such as ERK phosphorylation of LCK (47-49). To face this challenge, a theoretical logical model was assembled to predict the effects of feedback inhibition or feedback activation upon SILAC ratios calculated with this

method (Figure 2.12). From this logical model, both feedback inhibition and activation would be expected to affect SILAC ratios of an upstream signaling protein phosphorylation site in a time-dependent manner, while direct downstream inhibition would be expected to decrease phosphorylation constitutively. Consistent with our feedback model, CD3 ζ Y¹²³ followed the trends predicted for Zap-70-dependent feedback activation (significantly increased at 0 minutes (p-value < 0.05) followed by significantly decreased at 7 minutes (p-value < 0.05)), stimulating the hypothesis that this site may play a role in positive feedback that has not been previously characterized (Figure 2.12D). This observation highlights the importance of studying the kinetics of the phosphoproteomes of isogenic mutants.

Additionally, for the majority of phosphorylation sites identified on the CD3 $\gamma\delta\zeta$ subunits, we observed a gradual decrease in Zap-70 null/reconstituted SILAC ratios at early time points, followed by a gradual increase in Zap-70 null/reconstituted SILAC ratios at later time points (Figure 2.12E2). These trends are consistent with the hypothesis of competing positive and negative feedback loops functioning at different stages of stimulation. At early time points, positive feedback mechanisms could be regulating these sites, leading to a gradual decrease in Zap-70 null/reconstituted SILAC ratios. At later time points, negative feedback mechanisms could be regulating these same sites, leading to an increase in Zap-70 null/reconstituted SILAC ratios when Zap-70 is removed (Figure 2.12E1). It has previously been shown that ERK positive and SHP-1 negative feedback pathways can compete to either activate or inhibit LCK function in order to allow for T-cells to discriminate between self and foreign ligands (47).

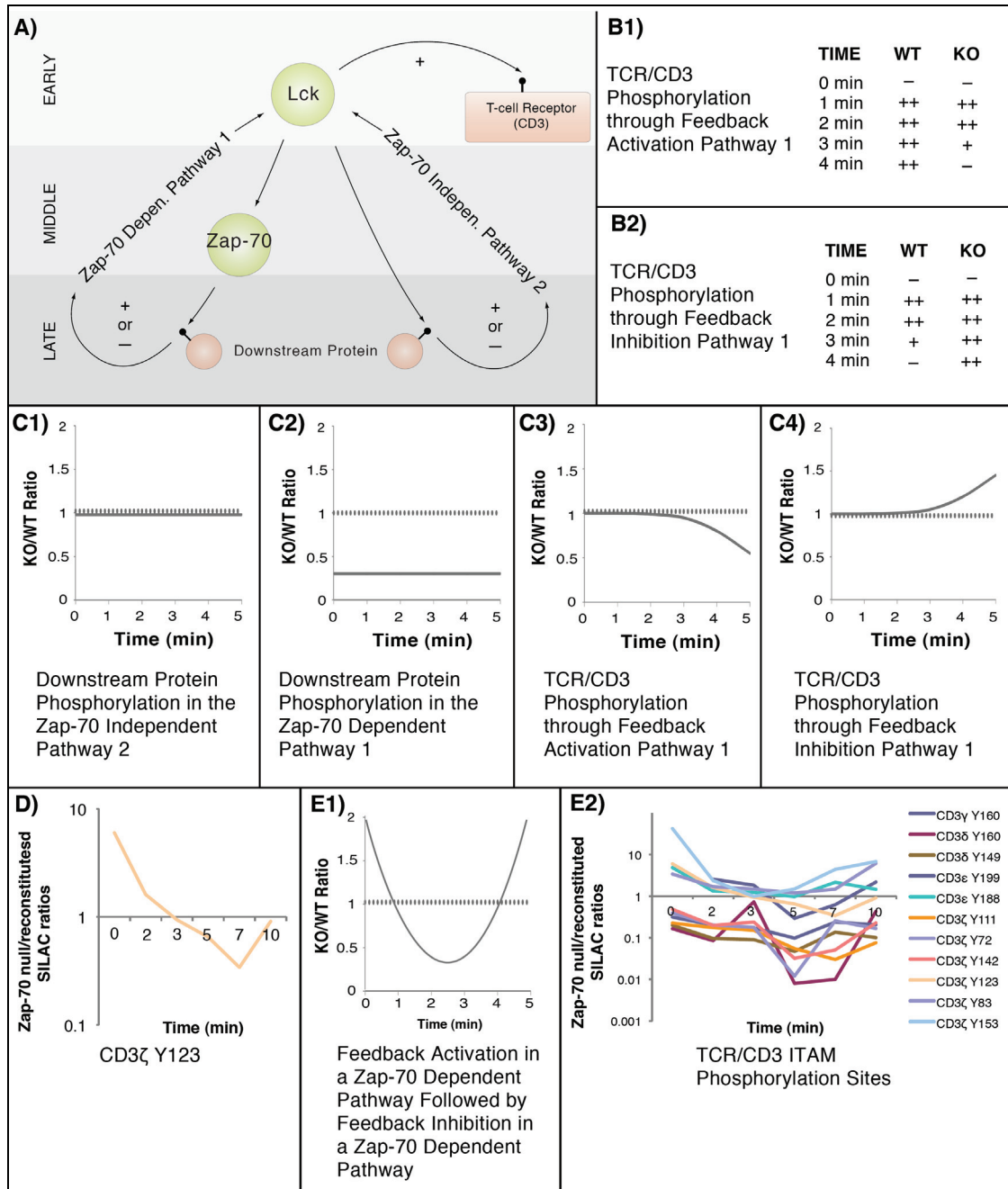


FIGURE 2.12: Theoretical model distinguishing direct inhibition from feedback regulation. A) Schematic model of pathway progression in response to T-cell activation. At later stages, TCR signaling is regulated by either positive or negative feedback loop functions. Activation is represented by “+” while inhibition is represented by “-”. Theoretical tables of expected wild type (WT) and knock-out (KO) TCR/CD3 phosphorylation temporal profiles for B1) feedback activation and B2) feedback inhibition (“-”: low level phosphorylation, “+”: moderate level phosphorylation, “++”: high level phosphorylation). B1) With positive feedback through Zap-70, phosphorylation of CD3 would fail to be reduced at later time points in wild type cells compared to Zap-70 null cells. B2) With negative feedback through Zap-70, phosphorylation of CD3 would fail to be reduced at later time points in Zap-70 null cells compared to wild type cells. Expected changes in KO/WT SILAC ratios over time for C1) TCR/CD3 in a Zap-70-independent pathway (SILAC ratio ≈ 1), C2) downstream proteins affected by direct inhibition in a Zap-70-dependent pathway (SILAC ratio < 1), C3) positive feedback on CD3, and C4) negative feedback on CD3.

Solid line (–) corresponds to expected SILAC ratio profile. D) Observed CD3 ζ Y123 Zap-70 null/Zap-70 reconstituted SILAC ratio profile. E1) Predicted changes in KO/WT SILAC ratios over time for TCR/CD3 phosphorylation with feedback activation in a Zap-70-dependent pathway followed by feedback inhibition in a Zap-70-dependent pathway. E2) Observed CD3 ζ $\gamma\delta\epsilon$ Zap-70 null/Zap-70 reconstituted SILAC ratios over time.

To determine the biological significance of the 105 novel sites discovered in this study (Figure 2.11), they must be placed within canonical pathways and networks of protein-protein interactions. Software tools that accelerate the assessment of existing protein knowledge and exploration of quantitative proteomic data in the context of protein interaction networks are essential. Although the primary literature may be investigated manually, the use of software enables enhanced efficiency. Current interactome database software is primarily web-based, and protein names must be searched one at a time. Since only proteins directly binding to the searched protein are revealed, the proteomics researcher must memorize the network to find longer-range interactions. Existing website-driven protein-protein interaction queries do not allow one to search by peptide sequence; this leads to confusion arising from protein name ambiguity. Quantitative data must also be integrated in a manual fashion with the interactome network. Therefore, the new proteomic data visual analysis tools are essential for tackling the overwhelming complexity of massive proteomic data sets and existing protein knowledge to gain insights into novel phosphorylation sites discovered in these types of studies.

We have recently developed phosphoproteomic exploration software with interactive visual integration of quantitative proteomic data, known signaling pathways, and protein-protein interaction networks, to accelerate hypothesis generation. This is

described in detail in an upcoming manuscript (<http://peptidedepot.com/viz>). Using this software, we examined the interactions between proteins observed to change in abundance in the Zap-70 null Jurkat cells. Although this analysis could have been accomplished through manual inspection of protein interactions described in hundreds of manuscripts, our newly-developed software facilitates the assimilation of this information by assembling a graphical representation of the T-cell signaling pathway scaffolded protein-protein interaction network. This depiction facilitates the rapid discovery of protein interactions amongst proteins observed in our data, while providing rapid access to the underlying manuscripts through direct hyperlinks. A user may rapidly crawl through this interaction space in a dilated view that shows just a single protein and directly interacting proteins, while simultaneously maintaining global perspective of the T-cell scaffolded network (Figure 2.13). The quantitative phosphoproteomic data generated in this manuscript is provided as an .pth input file for this software and is available for download without restriction from <http://tcellpathway.com/>.

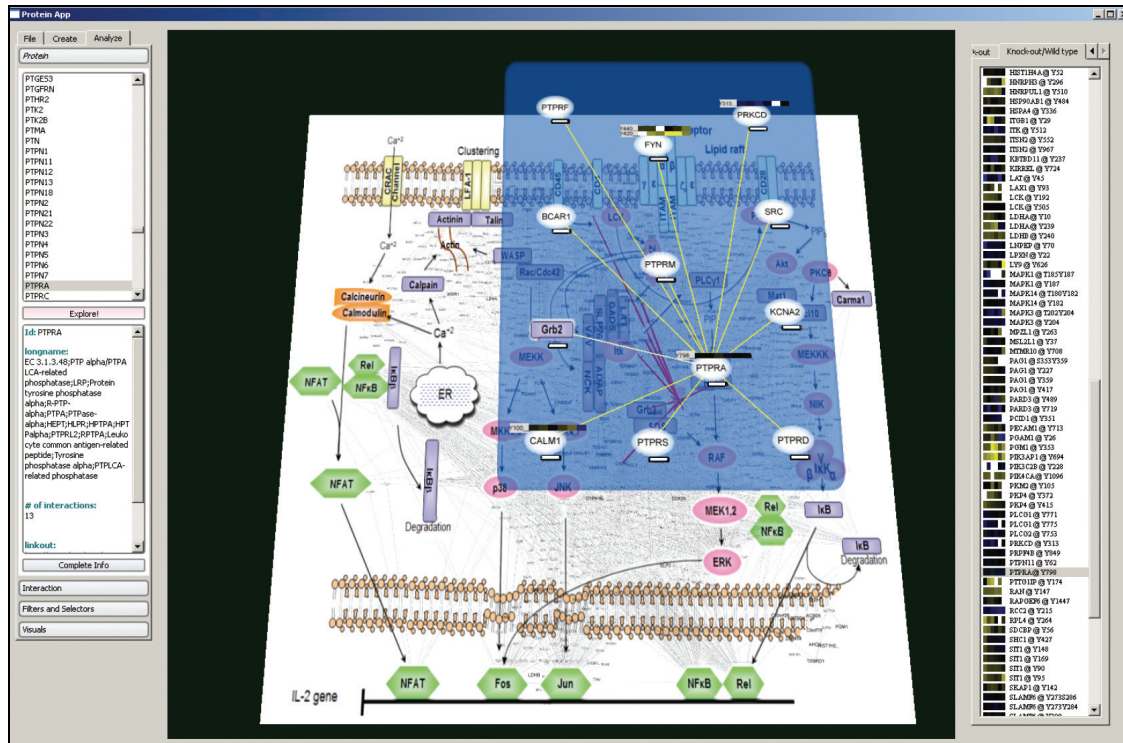


FIGURE 2.13: Custom-made exploration software for navigation through protein-protein interactions in the context of the TCR signaling pathway. This software is useful in the manual exploration of protein interaction networks, canonical signaling pathways, and quantitative proteomic data. Proteins and their interactions are imported into the software from the HPRD and structured according to the canonical TCR signaling pathway. Heatmaps representing quantitative data collected from phosphoproteomic comparative analysis experiments are shown both in the main image and in the frame on the right. Clicking on a protein in the main image or in the list on the right re-centers that protein in a semi-transparent overlay, displaying that protein on top of the main image of the TCR signaling pathway. Systematic navigation of this pathway can be accomplished by crawling through the network, revealing both a protein's local known interactions and its orientation within the global network. Meta information regarding the protein of interest, such as interactions, filters, highlighters, and other operators, are also available in a panel on the left.

The ability to visualize our data in relation to the canonical T-cell signaling pathway, focus on meaningful protein groups through the use of filters and selectors, and find possible pathways between multiple proteins with variable degrees of separation, was integral in generating the hypotheses described in detail here. For example, this software has helped us to propose an unexpected hypothesis about the potential role of the Pleckstrin homology domain (PH domain) of SKAP55 (Y^{142}). This site was observed

to have slightly enhanced phosphorylation (significantly increased at 3 and 10 minutes, p -value < 0.05) in Zap-70 null cells in response to stimulation, as compared to its reconstituted counterpart. Upon T-cell activation, FYN-associated SKAP55, in complex with the cytosolic adaptor protein ADAP, targets RAP1 GTPase to the plasma membrane to initiate cell adhesion (50-52). While the mechanisms of SKAP55's interaction with FYN and ADAP are well studied, the specific regulatory mechanisms of SKAP55's membrane targeting is still uncharacterized (50, 51). It has been established that the PH domain is involved in membrane recruitment for many cellular proteins (53). More recently, it has been shown that tyrosine phosphorylation of the PH domain of protein kinase D can serve in regulating protein function by possibly releasing the PH domain (undefined still as to whether it is from itself or the membrane), leading to its activation (54). Therefore, we hypothesize that phosphorylation at Y¹⁴² of SKAP55 may lead to the inhibition of SKAP55's ability to bind to the membrane, which would prevent RAP1 GTPase recruitment to the membrane and cell adhesion. The placement of a negatively-charged phosphate group in the PH domain could impede the ability of SKAP55 to bind to polyphosphoinositides found at the membrane (53). Further investigation in the possible function of site Y¹⁴² of SKAP55 is necessary to validate this hypothesis. The enhanced phosphorylation of Y¹⁴² on SKAP55 that we observed could be explained by the misregulation of FYN, a known regulator of SKAP55 (50, 52).

Our results also provide some insight on the possible function of NTBA in T-cells. NTBA, an ITIM containing killer Ig-like receptor (KIR), has previously been shown to be expressed in all human NK, T and B lymphocytes (55, 56). In NK cells, NTBA has been shown to display inhibitory functions by blocking the ability of NK cells

to kill Epstein-Barr Virus-infected target cells (55). Little is known about their role in T-cells. Recently, certain KIRs (KIR2DL2 and KLRG1) have been shown to disrupt late T-cell receptor-stimulated effector functions, such the production of IFN- γ and IL-2, respectively (57, 58). Furthermore, site-directed mutagenesis of a specific tyrosine residue in the ITIM motif of KLRG1 demonstrates the importance of tyrosine phosphorylation in the inhibitory process in T-cells (58). In our results, the phosphorylation of site Y³⁰⁸ located within the ITIM motif of NTBA was increased (significantly increased at 7 minutes, p-value < 0.05) in response to TCR crosslinking in Zap-70 reconstituted Jurkat cells, suggesting the involvement of NTBA in the T-cell activation pathway. It is possible that NTBA, like other KIRs, functions to inhibit late T-cell signaling events through phosphorylation of Y³⁰⁸. Such a possibility warrants additional investigation to clearly define the role of site Y³⁰⁸ of NBTA. We also observed a slightly decreased phosphorylation (significantly decreased at 7 minutes, p-value < 0.05) of Y³⁰⁸ in Zap-70 null cells, suggesting that this site may be downstream of Zap-70 activation.

The quantitative phosphoproteomic isogenic mutant approach described here can be used as a generalized approach to elucidate cell signaling pathways. By combining genetic analysis and two well-established proteomic quantitation methods with novel visual analysis tools, our approach facilitates rapid elucidation of the biological significance of high-throughput phosphoproteomic data.

2.5 REFERENCE

1. Sickmann, A., Marcus, K., Schafer, H., Butt-Dorje, E., Lehr, S., Herkner, A., Suer, S., Bahr, I., and Meyer, H. E. (2001) Identification of post-translationally modified proteins in proteome studies. *Electrophoresis* 22, 1669-1676.
2. Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U S A* 97, 9390-9395.
3. Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci U S A* 101, 12130-12135.
4. Ficarro, S. B., McClelland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20, 301-305.
5. Goshe, M. B., Conrads, T. P., Panisko, E. A., Angell, N. H., Veenstra, T. D., and Smith, R. D. (2001) Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal Chem* 73, 2578-2586.
6. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635-648.
7. Posewitz, M. C., and Tempst, P. (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal Chem* 71, 2883-2892.
8. Luo, Q., Tang, K., Yang, F., Elias, A., Shen, Y., Moore, R. J., Zhao, R., Hixson, K. K., Rossie, S. S., and Smith, R. D. (2006) More sensitive and quantitative proteomic measurements using very low flow rate porous silica monolithic LC columns with electrospray ionization-mass spectrometry. *J Proteome Res* 5, 1091-1097.
9. Brill, L. M., Salomon, A. R., Ficarro, S. B., Mukherji, M., Stettler-Gill, M., and Peters, E. C. (2004) Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T-cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal Chem* 76, 2763-2772.

10. Cao, L., Yu, K., Banh, C., Nguyen, V., Ritz, A., Raphael, B. J., Kawakami, Y., Kawakami, T., and Salomon, A. R. (2007) Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. *J Immunol* 179, 5864-5876.
11. Kruger, M., Kratchmarova, I., Blagoev, B., Tseng, Y. H., Kahn, C. R., and Mann, M. (2008) Dissection of the insulin signaling pathway via quantitative phosphoproteomics. *Proc Natl Acad Sci U S A* 105, 2451-2456.
12. Schmelzle, K., Kane, S., Gridley, S., Lienhard, G. E., and White, F. M. (2006) Temporal dynamics of tyrosine phosphorylation in insulin signaling. *Diabetes* 55, 2171-2179.
13. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A* 100, 443-448.
14. Zhang, Y., Wolf-Yadlin, A., Ross, P. L., Pappin, D. J., Rush, J., Lauffenburger, D. A., and White, F. M. (2005) Time-resolved mass spectrometry of tyrosine phosphorylation sites in the epidermal growth factor receptor signaling network reveals dynamic modules. *Mol Cell Proteomics* 4, 1240-1250.
15. Ong, S. E., and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* 1, 252-262.
16. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1, 376-386.
17. Ficarro, S. B., Salomon, A. R., Brill, L. M., Mason, D. E., Stettler-Gill, M., Brock, A., and Peters, E. C. (2005) Automated immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry platform for profiling protein phosphorylation sites. *Rapid Commun Mass Spectrom* 19, 57-71.
18. Germain, R. N., and Stefanova, I. (1999) The dynamics of T-cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu Rev Immunol* 17, 467-522.
19. Samelson, L. E. (2002) Signal transduction mediated by the T-cell antigen receptor: the role of adapter proteins. *Annu Rev Immunol* 20, 371-394.
20. Weiss, A., Kadlecsek, T., Iwashima, M., Chan, A., and Van Oers, N. (1995) Molecular and genetic insights into T-cell antigen receptor signaling. *Ann N Y Acad Sci* 766, 149-156.

21. Abraham, R. T., and Weiss, A. (2004) Jurkat T-cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* 4, 301-308.
22. Finco, T. S., Kadlecsek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity* 9, 617-626.
23. Irvin, B. J., Williams, B. L., Nilson, A. E., Maynor, H. O., and Abraham, R. T. (2000) Pleiotropic contributions of phospholipase C-gamma1 (PLC-gamma1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC-gamma1-deficient Jurkat T-cell line. *Mol Cell Biol* 20, 9149-9161.
24. Straus, D. B., and Weiss, A. (1992) Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T-cell antigen receptor. *Cell* 70, 585-593.
25. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol Cell Biol* 18, 1388-1399.
26. Yablonski, D., Kuhne, M. R., Kadlecsek, T., and Weiss, A. (1998) Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T-cell. *Science* 281, 413-416.
27. Shan, X., and Wange, R. L. (1999) Itk/Emt/Tsk activation in response to CD3 cross-linking in Jurkat T-cells requires ZAP-70 and Lat and is independent of membrane recruitment. *J Biol Chem* 274, 29323-29330.
28. Martelli, M. P., Lin, H., Zhang, W., Samelson, L. E., and Bierer, B. E. (2000) Signaling via LAT (linker for T-cell activation) and Syk/ZAP70 is required for ERK activation and NFAT transcriptional activation following CD2 stimulation. *Blood* 96, 2181-2190.
29. Griffith, C. E., Zhang, W., and Wange, R. L. (1998) ZAP-70-dependent and -independent activation of Erk in Jurkat T-cells. Differences in signaling induced by H2O2 and Cd3 cross-linking. *J Biol Chem* 273, 10771-10776.
30. Licklider, L. J., Thoreen, C. C., Peng, J., and Gygi, S. P. (2002) Automation of nanoscale microcapillary liquid chromatography-tandem mass spectrometry with a vented column. *Anal Chem* 74, 3076-3083.
31. Eng, J., A. McCormack, J. R. Yates. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. . *J. Am . Soc. Mass Spec.* 5, 976-989.

32. Yu, K., Sabelli, A., DeKeukelaere, L., Park, R., Sindi, S., Gatsonis C.A., and Salomon, A.R. (2009) Integrated platform for high-throughput statistical and manual validation of tandem mass spectra. *Proteomics* In Press.
33. Elias, J. E., and Gygi, S. P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods* 4, 207-214.
34. Beausoleil, S. A., Villen, J., Gerber, S. A., Rush, J., and Gygi, S. P. (2006) A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol* 24, 1285-1292.
35. Robey, E., and Allison, J. P. (1995) T-cell activation: integration of signals from the antigen receptor and costimulatory molecules. *Immunol Today* 16, 306-310.
36. Szamel, M., and Resch, K. (1995) T-cell antigen receptor-induced signal-transduction pathways--activation and function of protein kinases C in T lymphocytes. *Eur J Biochem* 228, 1-15.
37. Ohteki, T., Parsons, M., Zakarian, A., Jones, R. G., Nguyen, L. T., Woodgett, J. R., and Ohashi, P. S. (2000) Negative regulation of T-cell proliferation and interleukin 2 production by the serine threonine kinase GSK-3. *J Exp Med* 192, 99-104.
38. Davidson, D., Bakinowski, M., Thomas, M. L., Horejsi, V., and Veillette, A. (2003) Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor. *Mol Cell Biol* 23, 2017-2028.
39. Steinberg, M., Adjali, O., Swainson, L., Merida, P., Di Bartolo, V., Pelletier, L., Taylor, N., and Noraz, N. (2004) T-cell receptor-induced phosphorylation of the zeta chain is efficiently promoted by ZAP-70 but not Syk. *Blood* 104, 760-767.
40. Thome, M., Duplay, P., Guttinger, M., and Acuto, O. (1995) Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T-cell receptor/CD3/zeta complex. *J Exp Med* 181, 1997-2006.
41. Wange, R. L., and Samelson, L. E. (1996) Complex complexes: signaling at the TCR. *Immunity* 5, 197-205.
42. van Oers, N. S., Killeen, N., and Weiss, A. (1996) Lck regulates the tyrosine phosphorylation of the T-cell receptor subunits and ZAP-70 in murine thymocytes. *J Exp Med* 183, 1053-1062.
43. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P., and Alitalo, K. (1992) The human p50csk tyrosine kinase

phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. *EMBO J* 11, 2919-2924.

44. Mustelin, T., Williams, S., Taylor, P., Couture, C., Zenner, G., Burn, P., Ashwell, J. D., and Altman, A. (1995) Regulation of the p70zap tyrosine protein kinase in T-cells by the CD45 phosphotyrosine phosphatase. *Eur J Immunol* 25, 942-946.
45. Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thien, C. B., Langdon, W. Y., and Bowtell, D. D. (1998) Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol* 18, 4872-4882.
46. Plas, D. R., Johnson, R., Pingel, J. T., Matthews, R. J., Dalton, M., Roy, G., Chan, A. C., and Thomas, M. L. (1996) Direct regulation of ZAP-70 by SHP-1 in T-cell antigen receptor signaling. *Science* 272, 1173-1176.
47. Stefanova, I., Hemmer, B., Vergelli, M., Martin, R., Biddison, W. E., and Germain, R. N. (2003) TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol* 4, 248-254.
48. Altman, A., Kaminski, S., Busuttill, V., Droin, N., Hu, J., Tadevosyan, Y., Hipskind, R. A., and Villalba, M. (2004) Positive feedback regulation of PLCgamma1/Ca(2+) signaling by PKCtheta in restimulated T-cells via a Tec kinase-dependent pathway. *Eur J Immunol* 34, 2001-2011.
49. Mueller, D. L. (2003) Tuning the immune system: competing positive and negative feedback loops. *Nat Immunol* 4, 210-211.
50. Wu, L., Yu, Z., and Shen, S. H. (2002) SKAP55 recruits to lipid rafts and positively mediates the MAPK pathway upon T-cell receptor activation. *J Biol Chem* 277, 40420-40427.
51. Kliche, S., Breitling, D., Togni, M., Pusch, R., Heuer, K., Wang, X., Freund, C., Kasirer-Friede, A., Menasche, G., Koretzky, G. A., and Schraven, B. (2006) The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. *Mol Cell Biol* 26, 7130-7144.
52. Wang, H., and Rudd, C. E. (2008) SKAP-55, SKAP-55-related and ADAP adaptors modulate integrin-mediated immune-cell adhesion. *Trends Cell Biol* 18, 486-493.
53. Maffucci, T., and Falasca, M. (2001) Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett* 506, 173-179.

54. Storz, P., Doppler, H., Johannes, F. J., and Toker, A. (2003) Tyrosine phosphorylation of protein kinase D in the pleckstrin homology domain leads to activation. *J Biol Chem* 278, 17969-17976.
55. Bottino, C., Falco, M., Parolini, S., Marcenaro, E., Augugliaro, R., Sivori, S., Landi, E., Biassoni, R., Notarangelo, L. D., Moretta, L., and Moretta, A. (2001) NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J Exp Med* 194, 235-246.
56. Falco, M., Marcenaro, E., Romeo, E., Bellora, F., Marras, D., Vely, F., Ferracci, G., Moretta, L., Moretta, A., and Bottino, C. (2004) Homophilic interaction of NTBA, a member of the CD2 molecular family: induction of cytotoxicity and cytokine release in human NK cells. *Eur J Immunol* 34, 1663-1672.
57. Henel, G., Singh, K., Cui, D., Pryshchep, S., Lee, W. W., Weyand, C. M., and Goronzy, J. J. (2006) Uncoupling of T-cell effector functions by inhibitory killer immunoglobulin-like receptors. *Blood* 107, 4449-4457.
58. Tessmer, M. S., Fugere, C., Stevenaert, F., Naidenko, O. V., Chong, H. J., Leclercq, G., and Brossay, L. (2007) KLRG1 binds cadherins and preferentially associates with SHIP-1. *Int Immunol* 19, 391-400.

Chapter 3

**PHOSPHOPROTEOMIC AND TRADITIONAL ANALYSIS OF ERK
POSITIVE FEEDBACK AND ZAP-70-DEPENDENT REGULATION
OF FYN BY PTP ALPHA**

3.1 DISRUPTIONS IN ERK POSITIVE FEEDBACK

3.1.1 INTRODUCTION

Several proteins are known to regulate the T-cell signaling pathway through positive or negative feedback loops, which limit the extent and duration of receptor activity. For instance, tyrosine kinase CSK has been reported to negatively regulate LCK through an inhibitory phosphorylation site. Upon TCR engagement, LCK is activated to phosphorylate the CD3 ζ chains, initiating intracellular signaling events. Eventually, CSK is recruited to phosphorylate LCK at the inhibitory C-terminal site Y⁵⁰⁵. Inactivation of LCK in this manner results in reduced phosphorylation on CD3 ζ chains, causing inhibition of intracellular signaling by TCR (1). Other proteins known to function in negative feedback mechanisms include: CD45 (2), c-cbl (3), and SHP-1 (4, 5).

Positive feedback mechanisms have also been observed in T-cells, but are less defined (5-8). In particular, it has been reported that in response to high affinity agonists, ERK is activated to positively regulate TCR signaling through LCK (Figure 3.1) (5). Upon TCR engagement, LCK becomes phosphorylated at S⁵⁹ by ERK (9, 10), leading to the modification of LCK's SH2 domain, thereby effectively decreasing the accessibility or affinity for phosphoproteins to bind (11). Modification of the SH2 domain of LCK interferes with the recruitment of phosphorylated SHP-1, an inhibitory phosphatase, thus preventing LCK inactivation and allowing for a longer-lasting TCR-induced stimulatory signal (5). Here we examine the ERK positive feedback pathway in further detail with the use of a highly selective MEK1/2 inhibitor, U0126 (12) (Figure 3.2). A

phosphoproteomic analysis designed to capture the ERK positive feedback pathway was performed. The results of this analysis, as well as follow-up studies, will be discussed.

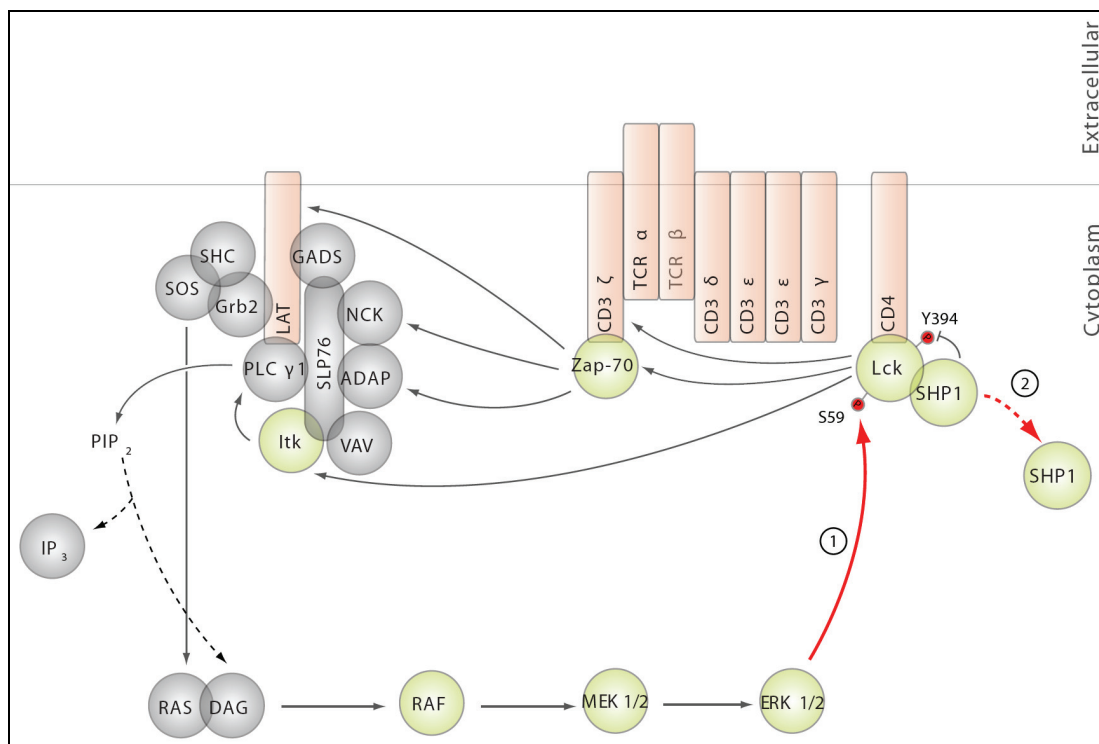


FIGURE 3.1: ERK positive feedback. 1) ERK phosphorylation at S⁵⁹ of LCK leads to modifications of LCK's SH2 domain. 2) Modification of LCK's SH2 domain prevents SHP-1's association with LCK.

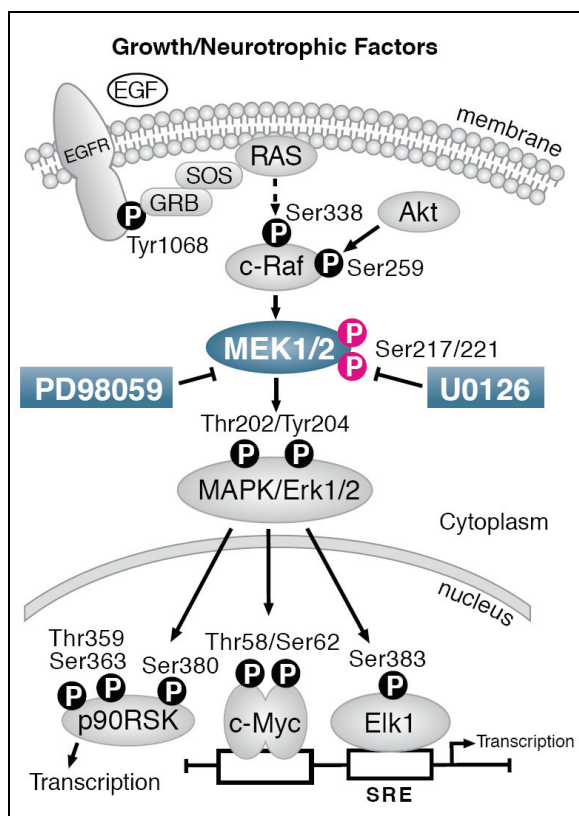


FIGURE 3.2: Schematic representation of MEK 1/2 inhibition by U0126. (Figure adopted from <http://cellsignal.com>)

3.1.2 MATERIAL AND METHODS

3.1.2.1 U0126 MEK 1/2 inhibition

For the initial titration experiment, prior to stimulation, Jurkat E6-1 cells were treated with U0126 (Cell Signaling Technology) at various concentrations (0 μ M, 10 μ M or 20 μ M) from 10 mM concentrated stocks (dissolved in DMSO) for 2 or 2.5 hours at 37 $^{\circ}$ C. Cells were then washed twice with 4 $^{\circ}$ C phosphate buffer saline (PBS).

For the phosphoproteomics experiments, prior to stimulation, Jurkat E6-1 cells were treated with 20 μ M of U0126 for 2.5 hours at 37 $^{\circ}$ C. Cells were then washed twice with 4 $^{\circ}$ C PBS.

3.1.2.2 Cell culture, SILAC labeling, T-cell OKT3/OKT4 stimulation, and cell lysis

Jurkat E6-1 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated undialyzed fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. SILAC labeling was performed as described (14).

Anti-CD3 and anti-CD4 (clones OKT3 and OKT4; eBioscience) stimulation was performed as described (13). Briefly, cells were washed once with 4 °C phosphate buffer saline (PBS), and reconstituted at a concentration of 1×10^8 cells/ml in PBS. For each time point, 1×10^8 cells were treated with OKT3 and OKT4 primary antibodies at a concentration of 2.5 µg/ml of each antibody for 10 minutes at 4 °C. Cells were then crosslinked with 22 µg/ml of goat anti-mouse IgG (Jackson ImmunoResearch) and incubated at 37 °C for 0, 1, 2, 3, 5, 7, or 10 minutes. To halt the stimulation, cells were placed in lysis buffer (9 M urea, 1 mM sodium orthovanadate, and 20 mM HEPES, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, pH 8.0) and incubated for 20 minutes at 4 °C. Lysates were then sonicated at 30 W output with 2 bursts of 30 seconds each and cleared at 12,000 xg for 15 minutes at 4 °C.

3.1.2.3 Protein reduction, alkylation, digestion, and peptide immunoprecipitation

Protein concentrations were measured by the DC Protein Assay (Bio-Rad). Once protein concentrations were determined, cell lysates from U0126-treated and -untreated cells were combined in a 1:1 protein concentration ratio and reduced with 45 mM DTT for 20 minutes at 60 °C, followed by alkylation with 100 mM iodoacetamide for 15 minutes at room temperature. Cell lysates were then diluted 4-fold with 20 mM HEPES

buffer (pH 8.0) and digested with sequencing grade modified trypsin (Promega) in a 1:100 (w/w) trypsin:protein ratio overnight at room temperature. Tryptic peptides were acidified to pH 2 with concentrated HCl, cleared at 1800 xg for 5 minutes at 22 °C, and desalted using C18 Sep-Pak plus cartridges (Waters) as described (14), with the exception that trifluoroacetic acid (TFA) was used instead of acetic acid (although at the same required concentrations) for our solutions. Eluents containing peptides were lyophilized for 48 hours to dryness.

A 3 pmol fraction of synthetic phosphopeptide LIEDAEpYTAK was added to each time point sample prior to peptide immunoprecipitation. Dry peptides from each time point were reconstituted in ice cold IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and further dissolved through gentle shaking for 30 minutes at room temperature and brief sonication in a sonicator waterbath. Peptide solutions were then cleared at 1800 xg for 5 minutes and at 22 °C, and combined with pre-conjugated pTyr100 phosphotyrosine antibody beads (40 µl; 80 µl slurry) (Cell Signaling), and then incubated overnight at 4 °C. Beads were washed 3 times with IAP buffer and twice with cold ddH₂O, and eluted with 0.15% TFA. Samples were then desalted using a home-made stage tip purification procedure as described (16).

Results presented throughout this chapter are collected from the average of five total replicate analyses for each time point. Two biological replicates from the same frozen stock of JE6-1 cells were cultured in RPMI-containing isotopic heavy or light amino acids. These biological replicates were performed three weeks apart. The first biological replicate contained three technical replicates while the second biological

replicate contained two technical replicates. Cells were SILAC-labeled heavy (U0126 treated) and light (untreated), stimulated, and lysed. Lysates were combined and digested with trypsin. For technical replicates, digested peptides from each time point were then divided into three or two equal fractions before desalting by C18 Sep-Pak plus cartridges and then combined and divided again prior peptide immunoprecipitation. Samples were kept separated throughout the rest of the experiment.

3.1.2.4 Automated nano-LC/ESI-MS, database analysis, and quantitation of relative phosphopeptide abundance

These procedures were performed as described (14).

3.1.2.5 Western blotting for phospho-ERK 1/2 and GAPDH

Total cellular protein from 9 M urea cell lysates was diluted 1:1 with gel loading buffer containing 4% SDS, 125 mM Tris-HCl (pH 6.8), 20% v/v glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8 from each proteomic sample. Equal amounts of protein (as measured by Lowry DC assay, Bio-Rad) were separated by 4-20% gradient SDS-polyacrylamide gel electrophoresis (Item 25204, Thermo Fisher Scientific), and electroblotted to an Immobilon membrane (Millipore). The membrane was blocked for 30 minutes in Odyssey blocking buffer at 22 °C (Li-Cor, Lincoln, NE) and then incubated for 12 hours at 4 °C with 1:1000 of rabbit anti-human phospho-p44/p42 MAPK (Thr202/Tyr204) (Cell Signaling Technology) and mouse anti-human GAPDH (Sigma). The membrane was washed 4x for 5 minutes at 22 °C in PBS/Tween-20. The membrane was then stained with 1:10000 of anti-rabbit IgG and anti-mouse IgG directly conjugated to an IRDye (Li-Cor) for 1 hour in Odyssey blocking buffer at 22 °C and

washed 3x for 5 minutes with PBS/Tween-20. Bands were visualized using an Odyssey Imaging System (Li-Cor).

3.1.3 RESULTS AND DISCUSSION

As proposed in the logical model in Figure 2.10 (14), feedback activation would be expected to affect KO/WT SILAC ratios of an upstream signaling protein phosphorylation site in a time-dependent manner. Therefore, disruptions in positive feedback mechanisms would result in a decrease in KO/WT SILAC ratios over time (Figure 2.10 B1 and C3). In accordance with this model, our group has recently observed phosphoproteomic SILAC patterns consistent with defects in positive feedback mechanisms in SLP-76 null compared to reconstituted Jurkat clones. Comparative phosphoproteomic profiling of KO/WT cells revealed decreases in phosphorylation over time for many upstream signaling proteins (LCK, Zap-70, CD3 $\delta\epsilon\zeta$ chains) (Cao and Salomon, unpublished Figure 3.3). Furthermore, ERK phosphorylation upon TCR stimulation was disrupted at the same time points in the SLP-76 null cells as compared to their reconstituted counterparts (data not shown), suggesting that SLP76-dependent inhibition of the ERK positive feedback pathway may be responsible for the decrease in phosphorylation of these upstream signaling proteins. Given the previously described role of the MAPK pathway in regulating TCR signaling, we hypothesized that treatment of a highly specific MEK1/2 inhibitor, U0126, on wild type Jurkat cells could display similar phosphorylation profiles of upstream signaling proteins as seen in the SLP-76 mutant clones.

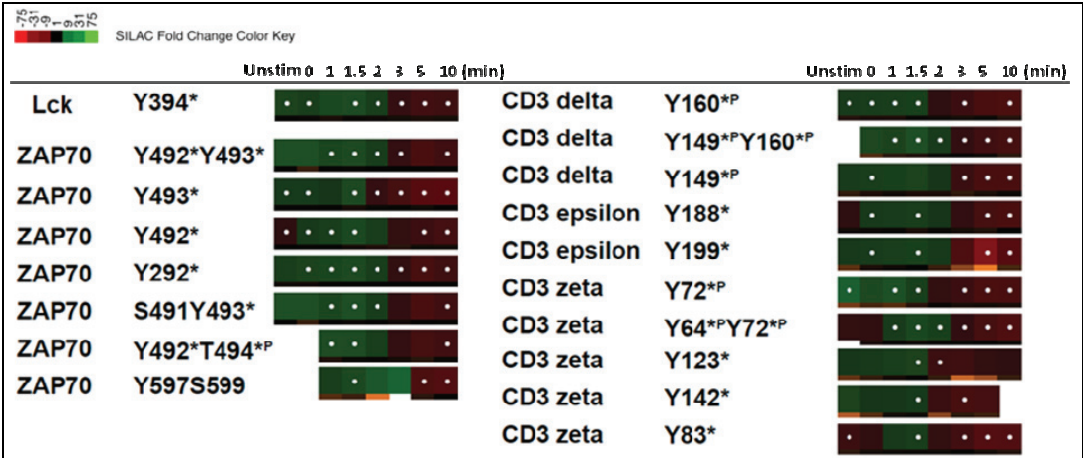


FIGURE 3.3: SLP-76 null to reconstituted SILAC ratio heatmap of known upstream T-cell signaling proteins. SILAC heatmap of a portion of the data collected, representing proteins that were observed in our study of human Jurkat J14 (SLP-76 mutants) clones known to participate in the upstream canonical T-cell signaling pathway. SLP-76 null/reconstituted SILAC ratios corresponding to the changes in phosphorylation state are represented as heatmaps. Heatmaps are calculated from the averages of 3 replicate experiments. Green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Black represents no change. White dots within the heatmap indicate a statistically significant ($p < 0.05$) difference in KO/WT SILAC ratios for that time point. Below each heatmap time point is a separate heatmap representing the coefficient of variation (CV) for that time point. Orange represents a high degree of variation, while black represents a low degree of variation. Blanks indicate a lack of replicate data required to accurately determine the CV. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

To analyze the effects of U0126 on wild type Jurkat cells, we first performed a titration, preincubation time course experiment to determine the optimal concentration and incubation time for inhibition of ERK activation by MEK1/2. Treatment of U0126 at a concentration of 20 μ M for 2.5 hours led to almost complete inhibition of ERK1/2 phosphorylation at their active sites in both the 0 and 5 minutes stimulated conditions (Figure 3.4).

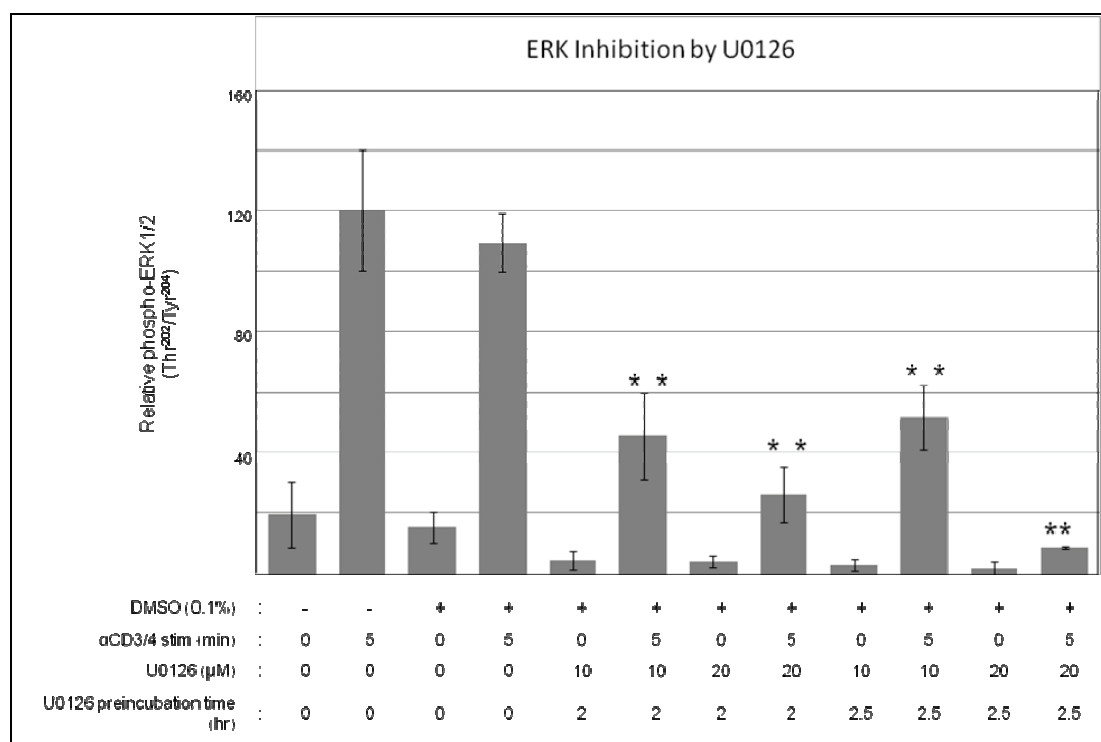


FIGURE 3.4: Titration to determine the optimal conditions for U0126 MEK1/2 inhibition. Jurkat JE6-1 cells were treated with various concentrations (0, 10, 20 μ M) of U0126 MEK1/2 inhibitor for multiple incubation periods (0, 2, 2.5 hours). 0 μ M samples were treated with 0.1% DMSO, as this was the background control. Inhibition is determined by ERK immunoblots. Protein lysates after U0126 treatment were separated by SDS-Page and immunoblotted with phospho-p44/p42 MAPK- (ERK1 and ERK2) specific antibodies. Relative inhibition was calculated by normalizing to a GAPDH loading control. The data shown is an average of 3 replicates. Note that the (**) above the bar graph indicate statistical significance ($p < 0.01$).

Using the quantitative phosphoproteomic approach developed in our laboratory (14), we assessed the effects of U0126 on the global phosphorylation network of wild type Jurkat cells (Figure 3.5). We hoped to observe disruptions in phosphorylation on upstream T-cell signaling proteins, consistent with the role of ERK in positive feedback, as putatively displayed in the SLP-76 mutant clones. For the phosphoproteomic profiling, we hypothesized that treatment of U0126 prior to stimulation would incur significant losses in phosphorylation at later time points on known upstream signaling proteins (LCK, Zap-70, CD3 $\delta\epsilon\zeta$ chains). However, phosphorylation at early time points should remain

unaffected, similar to what was expected for the untreated cells. We also predicted that these disruptions in phosphorylation on upstream signaling proteins could be followed by decreases in phosphorylation on many adaptor and downstream signaling proteins (LAT, SLP-76, ITK, etc.) at similar time points. This is supported by previous phosphoproteomic studies showing complete inhibition of the majority of phosphorylation sites upon the removal of LCK (15).

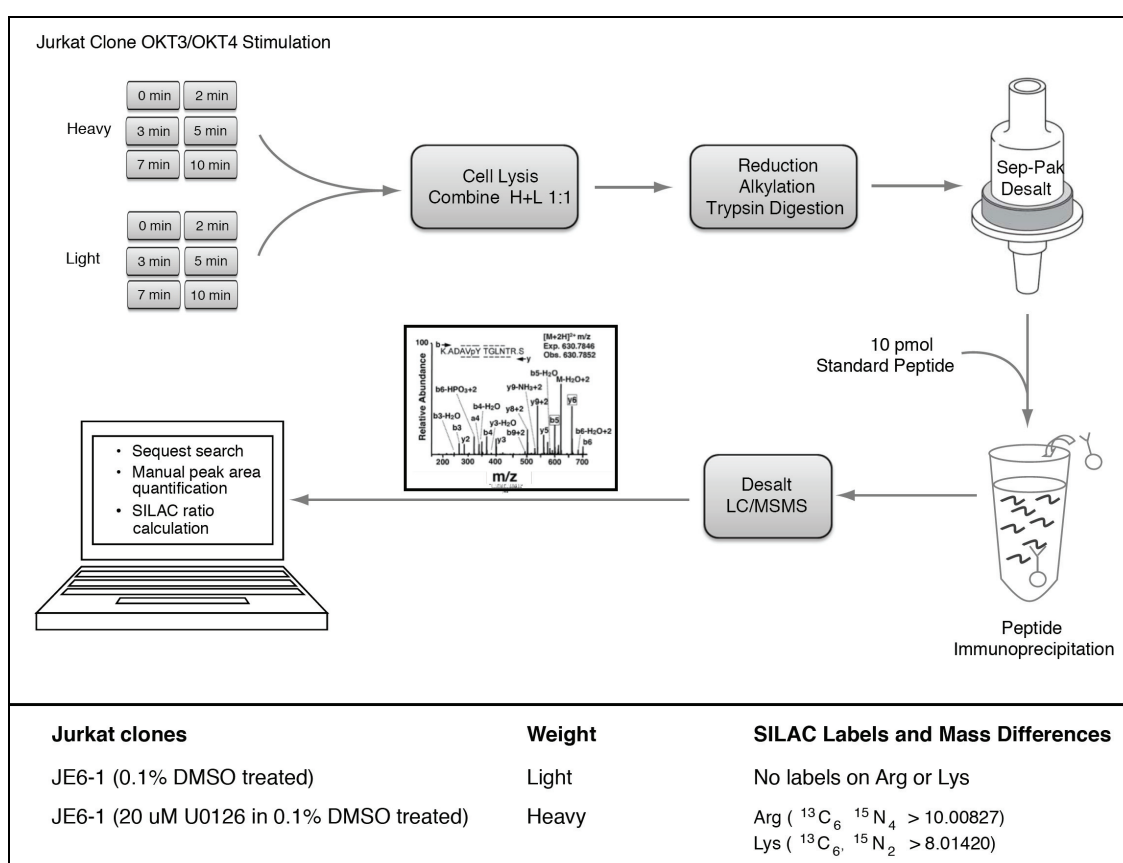


FIGURE 3.5: Experimental procedure. Human Jurkat E6-1 (wild type) cells are incubated with normal or heavy isotope labeled arginine and lysine amino acids, physically differentiating the two proteomes by a shift in molecular weights. Cells are treated with either U0126 (heavy labeled cells) or DMSO (light labeled cells) for 2.5 hours prior to stimulation. Each cell population is then pre-incubated with OKT3 and OKT4 antibodies for 10 minutes at 4 °C and then crosslinked with IgG at 37 °C for the times indicated. After cell lysis, samples are combined at an equal protein concentration ratio of 1:1. Samples are then reduced, alkylated, and trypsin-digested into peptides. Peptides are desalted by Sep-Pak cartridges and then enriched by phosphotyrosine peptide immunoprecipitation. Peptides are then subjected to reverse-phase LC-MS/MS analysis.

To our surprise, we found that U0126 treatment of wild type Jurkat cells led to constitutive decreases in phosphorylation on many tyrosine residues found on upstream signaling proteins (CD3 $\delta\epsilon\zeta$ chains, LCK, Zap-70), as well as adaptor and downstream signaling proteins (LAT, SLP-76, VAV-1, ITK, and PLC γ -1) (Figure 3.6). A possible explanation for this could be that the constitutive decreases in phosphorylation seen are the result of basal ERK inhibition. It has been well-established that ERK is active at low levels in resting Jurkat cells, and it is possible that inhibiting basal ERK activity prior to stimulation may have resulted in the suppression of basal phosphorylation on upstream signaling proteins. These results are, however, consistent with the hypothesis that ERK is a positive feedback regulator in TCR proximal signaling.

In the future, decreasing the concentration of U0126 treatment and/or preincubation time could reveal a more subtle differential inhibition of upstream protein phosphorylation in CD3/4-stimulated Jurkat T-cells compared to unstimulated basal phosphorylation. These changes in U0126 treatment conditions may result in more of a time-dependent ERK inhibition of upstream signaling during a time course of TCR stimulation, rather than constitutive inhibition, as seen in our initial studies. Phosphoproteomic follow-up studies using various conditions are required to validate this interpretation. In addition, re-performing this analysis using primary T-cells may display more of a time-dependent inhibition of upstream phosphorylation in response to ERK inhibition. It is possible that the levels of basal ERK activity are so high in the Jurkat T-cell line that the expected pattern was not seen, thus making it an imperfect system to study feedback. However, the largest obstacle preventing us from performing this analysis is the fact that primary T-cells do not culture very well in media. This makes

utilizing them even more difficult when applying our quantitative approach, considering that SILAC labeling requires multiple passages and long incubation times (8 days or more) in order for the isotopic amino acids to become fully incorporated. To overcome this issue, we can modify our quantitative approach by substituting iTRAQ labeling instead of SILAC labeling to measure the differences in phosphorylation between U0126-treated and -untreated cells. iTRAQ quantitation requires covalent attachment of isobaric mass tags to peptide-amine groups after cell lysis and trypsin digestion (17), whereby bypassing the need to culture cells for extended periods of time.

It is worth mentioning that we observed constitutively elevated phosphorylation on CD3 ζ Y¹²³ and Zap-70 Y⁵⁹⁸ in response to U0126 treatment (Figure 3.6). While many of the tyrosine sites identified on CD3 ζ and Zap-70 showed decreases in phosphorylation, these sites showed an increase. Although the molecular basis for this pattern remains unclear, our results suggest that the regulation for these sites may differ from that of their neighboring sites. For example, it may be possible that ERK activation is upstream of a tyrosine phosphatase that dephosphorylates these sites.

The existence of ERK positive feedback loops in TCR signaling is still under debate, and some groups argue against the model (5, 8), as reported by the Germain group (NIH, Bethesda, MD). Our phosphoproteomic results described here further support the existence of ERK positive feedback in TCR signaling. Using our unbiased quantitative phosphoproteomic approach with different levels of U0126 treatment could provide additional insights on ERK positive feedback.

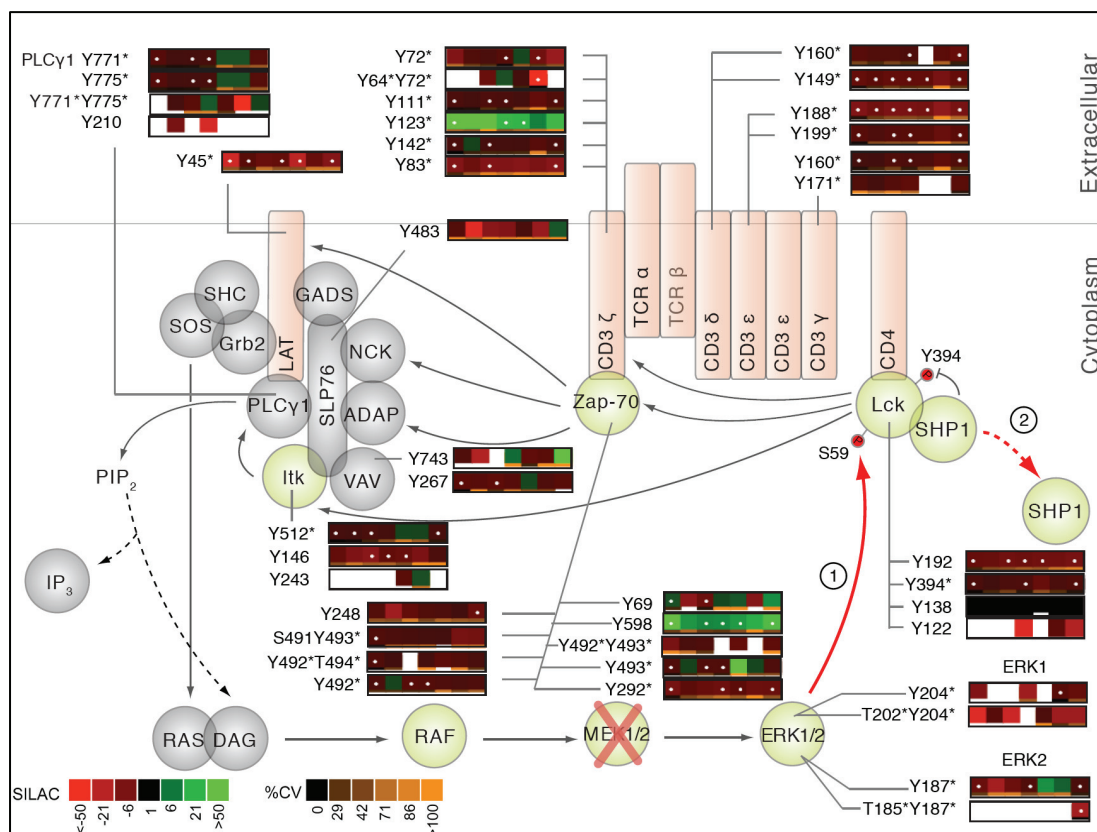


FIGURE 3.6: MEK1/2 inhibition by U0126 treatment. Model of ERK positive feedback with quantitative JE6-1 U0126 treated/untreated SILAC ratio heatmaps beside individual proteins, corresponding to the changes in phosphorylation. Heatmaps are calculated from the averages of 5 replicate experiments. Green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Black represents no change. White dots within the heatmap indicate a statistically significant ($p < 0.05$) difference in KO/WT SILAC ratios for that time point. Below each heatmap time point is a separate heatmap representing the coefficient of variation (CV) for that time point. Orange represents a high degree of variation, while black represents a low degree of variation. Blanks indicate a lack of replicate data required to accurately determine the CV. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

3.1.4 REFERENCE

1. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P., and Alitalo, K. (1992) The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. *EMBO J* 11, 2919-2924.
2. Mustelin, T., Williams, S., Tailor, P., Couture, C., Zenner, G., Burn, P., Ashwell, J. D., and Altman, A. (1995) Regulation of the p70zap tyrosine protein kinase in T-cells by the CD45 phosphotyrosine phosphatase. *Eur J Immunol* 25, 942-946.

3. Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thien, C. B., Langdon, W. Y., and Bowtell, D. D. (1998) Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol* 18, 4872-4882.
4. Plas, D. R., Johnson, R., Pingel, J. T., Matthews, R. J., Dalton, M., Roy, G., Chan, A. C., and Thomas, M. L. (1996) Direct regulation of ZAP-70 by SHP-1 in T-cell antigen receptor signaling. *Science* 272, 1173-1176.
5. Stefanova, I., Hemmer, B., Vergelli, M., Martin, R., Biddison, W. E., and Germain, R. N. (2003) TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol* 4, 248-254.
6. Altman, A., Kaminski, S., Busuttil, V., Droin, N., Hu, J., Tadevosyan, Y., Hipskind, R. A., and Villalba, M. (2004) Positive feedback regulation of PLCgamma1/Ca(2+) signaling by PKCtheta in restimulated T-cells via a Tec kinase-dependent pathway. *Eur J Immunol* 34, 2001-2011.
7. Mueller, D. L. (2003) Tuning the immune system: competing positive and negative feedback loops. *Nat Immunol* 4, 210-211.
8. Altan-Bonnet, G., and Germain, R. N. (2005) Modeling T-cell antigen discrimination based on feedback control of digital ERK responses. *PLoS Biol* 3, e356.
9. Winkler, D. G., Park, I., Kim, T., Payne, N. S., Walsh, C. T., Strominger, J. L., and Shin, J. (1993) Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase p56lck. *Proc Natl Acad Sci U S A* 90, 5176-5180.
10. Watts, J. D., Sanghera, J. S., Pelech, S. L., and Aebersold, R. (1993) Phosphorylation of serine 59 of p56lck in activated T-cells. *J Biol Chem* 268, 23275-23282.
11. Joung, I., Kim, T., Stolz, L. A., Payne, G., Winkler, D. G., Walsh, C. T., Strominger, J. L., and Shin, J. (1995) Modification of Ser59 in the unique N-terminal region of tyrosine kinase p56lck regulates specificity of its Src homology 2 domain. *Proc Natl Acad Sci U S A* 92, 5778-5782.
12. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feese, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 273, 18623-18632.
13. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003)

- Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A* 100, 443-448.
14. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T-cell receptor activation. *Mol Cell Proteomics* 8, 2418-2431.
 15. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A* 100, 443-448.
 16. Rappsilber J., Ishihama Y., Mann M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75, 663–670.
 17. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3, 1154-1169.

3.2 ENHANCED PHOSPHORYLATION OF FYN

3.2.1 INTRODUCTION

The regulation of SRC-family kinases (SFK) by protein tyrosine phosphatases in T-cells is an active area of research. Although the roles of many of the SFK have been resolved over time, the redundant and independent roles of LCK and FYN in TCR signaling remain unclear (1). Upon TCR engagement, LCK tethers to either the CD4 or CD8 co-receptors directly, positioning LCK in close proximity to the CD3 ITAMs (2). Some reports indicate that FYN acts in a parallel manner by directly binding to the CD3 subunits themselves, but it is still unknown how FYN becomes activated in this context (3). Uncovering the pathways regulating FYN activity may lead to a greater understanding of how T-cells function.

While the precise mechanism of FYN's regulation is unresolved, it is clear that the protein tyrosine phosphatases PTP α and PTEN are important in this process (4, 5). Recently, it has been shown that cross-linking of primary T-cells with antibodies against CD3 and CD4 has led to sequential phosphorylation and activation of LCK and then FYN, suggesting the possibility that FYN activation was dependent on prior activation of LCK (6). In support of this hypothesis, FYN activation is absent in LCK-deficient primary T-cells (6). On the contrary, other reports indicate that FYN can partially compensate for LCK deficiency, suggesting that FYN activation can occur independent of LCK (7).

Comparative phosphoproteomic profiling of Zap-70 null and reconstituted Jurkat clones have revealed elevated FYN phosphorylation in response to Zap-70 removal. Using the pathway exploration software (Figure 2.13) developed in our laboratory (8), a

hypothetical pathway between Zap-70, PTP α , and FYN was uncovered. This software revealed the interaction between PTP α and FYN, as well as provided a direct connection to previously published reports describing their interaction in detail. In general, elevation of phosphorylation in response to Zap-70 removal could be caused by misregulation of a kinase or phosphatase, or by disruption of protein turnover mechanisms. Misregulation of PTP α could explain the elevated phosphorylation seen in response to Zap-70 removal on FYN Y⁴⁴⁰ and known FYN targets such as Y⁹⁰ (significantly increased at 0 and 7 minutes, p-value < 0.05) on SIT (Figure 3.7) (9). Previous studies revealed that FYN activity is elevated in the absence of PTP α in primary thymocytes (4), and that the activity of PTP α is regulated by 3 phosphorylation sites (S¹⁸⁰, S²⁰⁴, and Y⁷⁹⁸) (10, 11). Along with showing the interactions between FYN and PTP α , this software also provided clues on how PTP α may be misregulated by identifying interactions between PTP α , PI3K, and PKC δ . It has been previously described that PTP α activation occurs through formation of a complex with PI3K and PKC δ , allowing for PKC δ to subsequently phosphorylate S¹⁸⁰ and S²⁰⁴ independently of Y⁷⁹⁸ phosphorylation (10-12). Our data is consistent with the hypothesis that the removal of Zap-70 leads to a decrease in phosphorylation on the activation site Y³¹³ of PKC δ as well as Y²²⁸ of PI3K, causing a reduction in PTP α activity (through the lack of phosphorylation on S¹⁸⁰ and S²⁰⁴). This will then lead to an increase in FYN Y⁴⁴⁰ phosphorylation and phosphorylation of the FYN substrate SIT at Y⁹⁰. Furthermore, PTP α selectively regulates FYN over other Src-family kinases such as LCK, dephosphorylating FYN at both Y⁴²⁰ in the activation loop and the C-terminal inhibitory site Y⁵³¹ (4, 13). Although we observed the phosphorylation on site Y⁷⁹⁸ of PTP α to be unaffected in response to Zap-70 removal,

defects in phosphorylation on S¹⁸⁰ and S²⁰⁴ of PTPα may be the cause of selective FYN misregulation. To test the hypothesis that the removal of Zap-70 led to the enhanced phosphorylation of FYN through the misregulation of PTPα, we initiated a series of biochemical experiments to test the interactions between FYN and PTPα. The results as well as follow-up studies using both biochemical and proteomic approaches will be discussed.

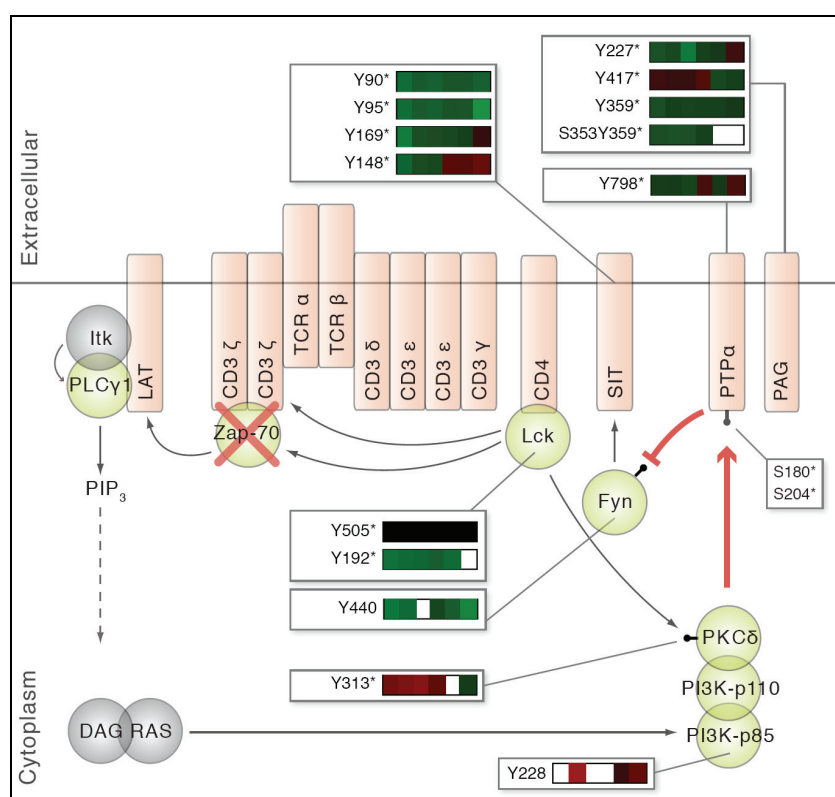


FIGURE 3.7: Regulation of FYN signaling. Model of Zap-70-dependent FYN inhibition with quantitative Zap-70 null/Zap-70 reconstituted SILAC ratio data represented as heatmaps beside individual proteins. Heatmaps are calculated from the average of 5 replicate experiments. Green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Black represents no change. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Note that the * next to the phosphorylation site signifies that this site has been previously described in literature.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Protein immunoprecipitation

Human Recombinant Proteins (SRC, LYN, FYN, YES, LCK (Cell Signaling Technology) were initially resuspended in PBS at the desired protein concentration. Recombinant proteins were then solubilized in cold lysis buffer (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and complete mini protease inhibitors cocktail (Roche) and incubated for 1 hour at 4 °C. Recombinant proteins were cleared at 12,000 xg for 20 minutes at 4 °C.

For each sample, 5 µg of a mixture of 3 monoclonal mouse anti-FYN antibodies, provided by J. Rivera at the National Institutes of Health, was diluted in PBS to a final volume of 500 µl, and conjugated to 75 µl of Protein G bound agarose bead slurry (Roche). Antibodies and beads were incubated for 1 hour at 4 °C. Antibody-bound beads were then washed twice with lysis buffer before use.

Recombinant proteins were then combined with antibody-bound beads, and incubated overnight at 4 °C. Beads were washed 4x with cold lysis buffer. To elute, immunoprecipitates were diluted 1:1 volume with gel loading buffer (4% SDS, 125 mM Tris-HCl (pH 6.8), 20% v/v glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and boiled for 5 minutes at 96 °C. Immunoprecipitates were then cleared at 1,600 xg for 10 minutes and stored -20 °C.

3.2.2.2 Colloidal gold membrane protein stain

Protein-bound membranes were washed 3 times for 20 minutes each with TTBS solution and at 22 °C. Membranes were then rinsed 3 times for 2 minutes each with water at 22 °C. Colloidal gold protein stain solution (Bio-Rad) was then added to the

membranes and incubated for 1-2 hours, or until bands become visible. When staining was satisfactory, the membrane was rinsed 3 times for 1 minute each with water at 22 °C.

3.2.2.3 Western blotting for FYN

Recombinant protein samples eluted in gel loading buffer were separated by 4-20% gradient SDS-polyacrylamide gel electrophoresis (Item 25204, Thermo Fisher Scientific), and electroblotted to an Immobilon membrane (Millipore). The membrane was blocked for 30 minutes in Odyssey blocking buffer at 22 °C (Li-Cor, Lincoln, NE) and then incubated for 12 hours at 4 °C with 1:1000 of mouse anti-mouse FYN (provided by J. Rivera lab). The membrane was washed 4x for 5 minutes at 22 °C in PBS/Tween-20. The membrane was then stained with 1:10000 of anti-mouse IgG directly conjugated to an IRDye (Li-Cor) for 1 hour in Odyssey blocking buffer at 22 °C and washed 3x for 5 minutes with PBS/Tween-20. Bands were visualized using an Odyssey Imaging System (Li-Cor).

3.2.3 RESULTS AND DISCUSSION

To test whether the removal of Zap-70 led to the enhanced phosphorylation of FYN through the misregulation of PTP α , we plan to perform co-immunoprecipitation studies of FYN and PTP α , as well as verify whether PTP α activation occurs through PKC δ and PI3K. Since antibody specificity is critical for these experiments, we first accessed a variety of commercially available FYN-specific antibodies, and found that many of the antibodies that targeted FYN also cross-reacted with other Src-family kinases (data not shown). These Src-family kinases (FYN, LCK, LYN, YES, SRC) share the same molecular weight (60 kDa), thus making it very difficult to differentiate FYN

through immunoblots. To overcome this challenge, we were fortunate to obtain a mixture of 3 highly selective monoclonal mouse anti-FYN antibodies made in the laboratory of J. Rivera (NIH, Bethesda, MD; unpublished). FYN immunoprecipitation and immunoblotting studies using these mouse antibodies showed very selective enrichment and recognition of human FYN proteins (Figure 3.8).

CD3 and CD4 co-stimulated P116 Jurkat clones (Zap-70 null and reconstituted) will be immunoprecipitated against FYN using these antibodies, and immunoblotted for PTP α , PI3K and PKC δ to verify their interactions. We expect to see defects in FYN and PTP α interactions in the Zap-70 null cells as compared to the Zap-70 reconstituted cells, suggesting FYN's association with its regulatory proteins is dependent upon the presence of Zap-70. Moreover, immunoblotting for phosphorylated-S¹⁸⁰ and -S²⁰⁴ of PTP α will allow us to determine whether these known activation sites are important for Zap-70-dependent regulation. If an interaction between Fyn and PTP α is not observed in either the Zap-70 null or reconstituted Jurkat clones, the interaction between these proteins may be too transient to detect by immunoprecipitation or the proteins may not interact. To capture transient interactions, reversible cross-linking with formaldehyde prior to Fyn immunoprecipitation followed by blotting for PTP α will be performed as described (14). To establish whether PI3K and PKC δ act in a Zap-70-dependent manner to regulate PTP α , shRNA constructs for each of these proteins will be produced and transduced into Zap-70 Jurkat T-cells (null and reconstituted). We expect that the suppression of PI3K and PKC δ expression will cause the enhanced phosphorylation of Fyn and its substrates in Zap-70 reconstituted T-cells, thus providing further support for our hypothesis.

Alternatively, a proteomic approach will also be utilized to detect Fyn binding partners. CD3 and CD4 co-stimulated P116 Jurkat clones (Zap-70 null and reconstituted) will be immunoprecipitated against FYN, and eluents (containing Fyn-interacting proteins) will be separated through SCX fractionation and sequenced using LC/MS/MS methodology. We expect to identify peptides corresponding to PTP α , PKC δ and PI3K in our stimulated Zap-70 reconstituted fractions, while observing an absence of these peptides in our Zap-70 null fractions. These results would provide us with further support that Fyn is regulated by PTP α in a Zap-70-dependent manner.

We are aware that other phosphatases, such as PTEN, can regulate FYN activity. Although this mechanism has yet to be reported in T-cells, PTEN has been shown to suppress FYN activation in glioma cells to regulate cell migration (5). In addition, multiple phosphatases could simultaneously regulate FYN activity that requires the presence of Zap-70 to function properly. Further studies on Zap-70-dependent phosphatases and their roles in regulating FYN in primary T-cells could provide novel insights on how T-cells are regulated.

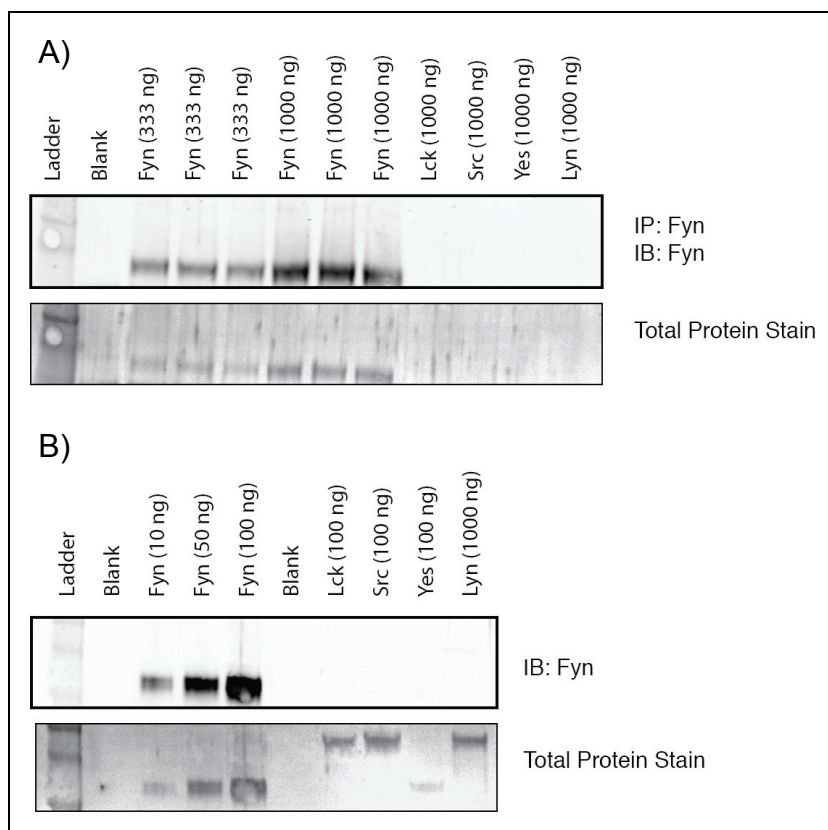


FIGURE 3.8: Determining FYN antibody specificity for use in protein immunoprecipitation and immunoblotting. A) FYN protein immunoprecipitation. Human recombinant proteins were immunoprecipitated with FYN-specific antibodies bound to Protein G agarose beads, separated by SDS-Page and immunoblotted with FYN-specific antibodies. Total proteins were visualized using a Colloidal gold protein stain solution. B) FYN immunoblot. Human recombinant proteins separated by SDS-Page and immunoblotted with FYN-specific antibodies. Total proteins were visualized using a Colloidal gold protein stain solution.

3.2.4 REFERENCE

1. Salmond, R. J., Filby A., Qureshi I., Caserta S., Rose Zamoyska R. (2009) T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunological Reviews* 228, 9-22.
2. Kim, P.W., Sun, Z. Y., Blacklow, S. C., Wagner G., Eck M. J. (2003) A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. *Science* 201, 1725-1828.
3. Timson Gauen, L. K., Kong, A. N., Samelson, L. E., Shaw, A. S. (1992) p59^{fyn} tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. *Mol. Cell. Biol.* 12, 5438-5446.

4. Maksumova, L., Le, H. T., Muratkhodjaev, F., Davidson, D., Veillette, A., and Pallen, C. J. (2005) Protein tyrosine phosphatase alpha regulates FYN activity and Cbp/PAG phosphorylation in thymocyte lipid rafts. *J Immunol* 175, 7947-56.
5. Dey, N., Crosswell, H. E., De, P., Parsons, R., Peng, Q., Su, J. D., and Durden, D. L. (2008) The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration. *Cancer Res* 68, 1862-1871.
6. Filipp, D., Zhang, J., Leung, B.L., Shaw, A., Levin, S.D., Velillette, A., Julius, M. (2003) Regulation of Fyn through translocation of activated Lck into lipid rafts. *JEM* 197, 1221-1227.
7. Lovatt, M., Filby, A., Parravicini, V., Werlen, G., Palmer, E., Zamoyska, R. (2006) Lck regulates the threshold of activation in primary T cells, while both Lck and Fyn contribute to the magnitude of the extracellular signal-related kinase response. *Molecular and Cellular Biology* 26, 8655-8665.
8. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T-cell receptor activation. *Mol Cell Proteomics* 8, 2418-2431.
9. Marie-Cardine, A., Kirchgessner, H., Bruyns, E., Shevchenko, A., Mann, M., Autschbach, F., Ratnofsky, S., Meuer, S., and Schraven, B. (1999) SHP2-interacting transmembrane adaptor protein (SIT), a novel disulfide-linked dimer regulating human T-cell activation. *J Exp Med* 189, 1181-94.
10. Zheng, X. M., Resnick, R. J., and Shalloway, D. (2002) Mitotic activation of protein-tyrosine phosphatase alpha and regulation of its Src-mediated transforming activity by its sites of protein kinase C phosphorylation. *J Biol Chem* 277, 21922-9.
11. den Hertog, J., Tracy, S., and Hunter, T. (1994) Phosphorylation of receptor protein-tyrosine phosphatase alpha on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo. *EMBO J* 13, 3020-32.
12. Stetak, A., Csermely, P., Ullrich, A., and Keri, G. (2001) Physical and functional interactions between protein tyrosine phosphatase alpha, PI 3-kinase, and PKCdelta. *Biochem Biophys Res Commun* 288, Page 564-72.
13. Maksumova, L., Wang, Y., Wong, N. K., Le, H. T., Pallen, C. J., and Johnson, P. (2007) Differential function of PTPalpha and PTPalpha Y789F in T-cells and regulation of PTPalpha phosphorylation at Tyr-789 by CD45. *J Biol Chem* 282, 20925-32.

14. Gong, F., Fahy, D., Smerdon, M .J. (2006) Rad4–Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair. *Nature Structural & Molecular Biology*. 13, 902-907.

Chapter 4

METHOD DEVELOPMENT WITH THE AIM OF IMPROVING THE SAMPLE PREPARATION PROCEDURE

4.1 INTRODUCTION

Phosphoproteomic analysis is a method widely used to study many cellular signaling pathways. Studies in phosphoproteomics have led to the discovery of hundreds to thousands of phosphorylation sites, while also measuring their changes in abundance in response to stimulation (2, 5, 6). Experiments in phosphoproteomics typically follow the following schematic: cell stimulation and lysis, protein digestion, enrichment of phosphotyrosine peptides through peptide immunoprecipitation and Fe^{3+} -IMAC, and LC/MS/MS sequencing. Although this workflow has proven successful in its studies of signaling pathways, the optimization of each step in the process has the potential to significantly increase the selectivity and the yield of the phosphopeptides identified.

Our group's desire to improve our sample preparation procedure for LC/MS analysis stems from observed inconsistencies between samples. We noted variable enrichment of phosphotyrosine peptides during peptide immunoprecipitation, clogging and contamination during Fe^{3+} -IMAC phosphopeptide enrichment, and less-than-optimal MS/MS ion detention settings during LC/MS sequencing. Together, these issues resulted in a low yield of identified phosphopeptides. In order to resolve the issues, modifications needed to be made to our sample preparation procedure before processing further samples.

Recently, our group has worked in collaboration with Dr. Al Mortiz at Cell Signaling Technology to try to correct some of the problems that we have experienced in the past. Using the Cell Signaling Technology sample preparation protocol as a template (<http://www.cellsignal.com/pdf/7900.pdf>), our group has engaged in our own method development, with the aim of improving our sample preparation procedure to provide higher

and more constant yields of phosphopeptides identified while eliminating the use of Fe³⁺-IMAC enrichment. A battery of experiments was performed on pervanadate-stimulated Jurkat T-cells in order to make the experimental conditions as ideal as possible. These experiments included titering the pTyr100 phosphotyrosine antibody to determine the optimal antibody concentration for peptide immunoprecipitation, testing other anti-phosphotyrosine antibodies to determine their selectivity and efficiency of enrichment for phosphotyrosine peptides, examining milder elution conditions during peptide immunoprecipitation, comparing trypsin enzymes for protein digestion, and determining the optimal MS/MS ion detection settings during LC/MS analysis. The results of these experiments, as well as modifications to our sample preparation procedure, will be discussed.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture, T-cell pervanadate stimulation, and cell lysis

Jurkat E6-1 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated undialyzed fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. Pervanadate stimulation was performed as described (1). A 50 mM pervanadate stock was freshly prepared before each use: 100 mM sodium orthovanadate (Sigma) was added to an equal volume of 10 mM hydrogen peroxide (Sigma), incubated at 22 °C for 20 minutes, and then stored on ice until use. Cells were washed once with 37 °C RPMI 1640 medium, reconstituted at a concentration of 1x10⁷ cells/ml in RPMI 1640 medium, and incubated for 10 minutes at 37 °C. Cells were then stimulated with 500 µM of

pervanadate for 20 minutes at 37 °C. To halt the stimulation, cells were washed once with cold RPMI 1640 medium, placed in lysis buffer (9 M urea, 1 mM sodium orthovanadate, and 20 mM HEPES, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, pH 8.0), and incubated for 20 minutes at 4 °C. Lysates were then sonicated at 30 W output with 2 bursts of 30 seconds each and cleared at 12,000 xg for 15 minutes at 4 °C.

4.2.2 Protein reduction, alkylation, digestion, and peptide immunoprecipitation

Cell lysates were reduced with 45 mM DTT for 20 minutes at 60 °C, followed by alkylation with 100 mM iodoacetamide for 15 minutes at room temperature and a 4-fold dilution with 20 mM HEPES buffer (pH 8.0). Proteins were digested with either sequencing grade modified trypsin (Promega) in a 1:100 (w/w) trypsin:protein ratio, or with TRTPCK-TPCK trypsin (Worthington, Lakewood, NJ) in a 1:10 (w/w) trypsin:protein ratio, overnight and at room temperature. Tryptic peptides were acidified to pH 2 with concentrated HCl, cleared at 1800 xg for 5 minutes at 22 °C, and desalted using C18 Sep-Pak plus cartridges (Waters) as described (2), with the exception that trifluoroacetic acid (TFA) was used instead of acetic acid (although at the same required concentrations) for our solutions. Eluents containing peptides were lyophilized for 48 hours to dryness.

Dry peptides were reconstituted in ice cold IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and further dissolved through gentle shaking for 30 minutes at room temperature and brief sonication in a sonicator waterbath. Peptide solutions were then cleared at 1800 xg for 5 minutes and at 22 °C, followed by combination with one of the following: pre-conjugated pTyr100 phosphotyrosine antibody beads (40 μ l; 80 μ l slurry) (Cell Signaling) at the desired protein concentration, pTyr100 antibody (Cell

Signaling) non-covalently conjugated to agarose beads (Roche) at the desired protein concentration, or other anti-phosphotyrosine antibodies that were tested for their selectivity and efficiency for enrichment of phosphotyrosine peptides. These antibodies included 4G10 (Santa Cruz Biotechnology, San Cruz, CA) which was non-covalently conjugated to agarose beads (Roche), 1G2 (obtained from Dr. A. Raymond Frackelton, Brown University) which was pre-conjugated to sepharose beads, pre-conjugated PT66 antibody beads (Sigma), pre-conjugated PY20 antibody beads (Santa Cruz Biotechnology), and pre-conjugated pTyr99 antibody beads (Santa Cruz Biotechnology). 250 µg of each antibody was used per sample. Peptides solutions were incubated with phosphotyrosine antibody-bound beads overnight at 4 °C. Beads were washed 3 times with IAP buffer and twice with cold ddH₂O, and eluted with 0.15% TFA (or with 1 mM phenyl phosphate for the antibody regeneration experiments). Samples were then desalted using a home-made stage tip purification procedure as described (3).

4.2.3 Automated nano-LC/ESI-MS, database analysis, and quantitation of relative phosphopeptide abundance

These procedures were performed as described (2).

4.3 RESULTS AND DISCUSSION

Our initial efforts in improving our sample preparation procedure focused on optimizing the amount of pTyr100 antibodies used during peptide immunoprecipitation. Our original sample preparation procedure constantly produced lower yields of phosphopeptide

identification than to be expected. We hypothesized that the amount of antibody (27 μ g of pTyr100 antibody per sample) being used was directly affecting yield size, given that it was only a tenth of the amount recommended by Cell Signaling (manufacturers of pTyr100) for peptide immunoprecipitation. Consistent with our hypothesis, we observed that increasing the antibody concentration by 10 times the original amount led to a 4-fold increase in the number of phosphotyrosine peptides identified (1X pTyr100: 116 phosphotyrosine peptides IDs, 10X pTyr100: 424 phosphotyrosine peptides IDs; $n = 1$) (Figure 4.1). In addition, we found that substituting pTyr100-non-covalently conjugated to agarose beads with pre-conjugated pTyr100 antibody beads produced similar yields at the same antibody concentration (10X pTyr100: 424 phosphotyrosine peptides IDs, 1 tube pTyr100 bead bound: 455 phosphotyrosine peptides IDs; $n = 1$) (Figure 4.1). From these results, we have modified our sample preparation procedure (Appendix C) to include the use of 250 μ g of pre-conjugated pTyr100 beads during peptide immunoprecipitation.

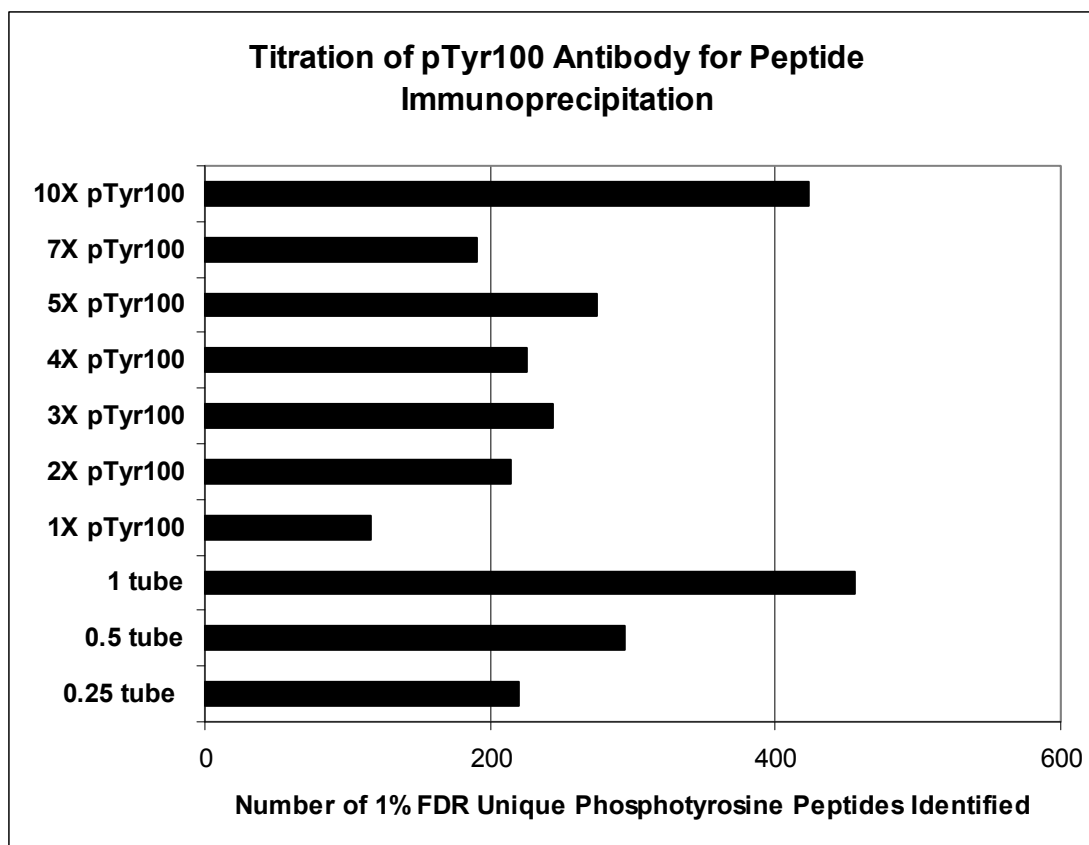


FIGURE 4.1: Titration of pTyr100 antibody to determine optimal antibody concentration for peptide immunoprecipitation. Peptide immunoprecipitation experiments using pTyr100 antibodies were performed on pervanadate-stimulated Jurkat E6-1 cell lysates. Peptide immunoprecipitation experiments tested various antibody concentrations of pTyr100 to determine how this would affect the yield of phosphotyrosine peptides identified. 1 tube corresponds to 250 μ g of pTyr100 antibodies that are pre-conjugated to 40 μ l agarose bead volume. 1X pTyr100 corresponds to 25 μ g of pTyr100 antibodies that are non-covalently conjugated to 40 μ l agarose bead volume.

An issue that arises in our updated sample preparation procedure is financial: increasing the antibody concentration by 10 times during peptide immunoprecipitation raises the cost that considerably. In order to drive cost down, our group has tested a variety of amino acid-independent anti-phosphotyrosine specific antibodies (both commercially available antibodies and those produced in the laboratory) to seek out which antibodies will offer the highest selectivity and enrichment at the lowest cost. Of the antibodies tested (pTyr100, pTyr99, 4G10, PT66, PY20, and 1G2) on pervanadate-stimulated Jurkat E6-1 cell

lysates, PT66 and 1G2 were highly selective in enriching for phosphotyrosine peptides (PT66: 88.5 % phosphotyrosine IDs, 1G2: 83 % phosphotyrosine IDs; $n = 2$) (Figure 4.2), and produced similar yields of enrichment (PT66: 407.5 phosphotyrosine peptide IDs, 1G2: 246.5 phosphotyrosine peptide IDs; $n = 2$) to that of pTyr100 (pTyr100: 228.5 phosphotyrosine peptides IDs; $n = 2$) (Figure 4.3). PT66 is one-third the cost of pTyr100 per sample, while the 1G2 antibody is essentially free-- our group is currently in the process of producing 1G2 antibodies from mouse ascites, which will allow us unlimited access to 1G2 at a negligible price (considering the cost of production). These antibodies may be viable and economically favorable options in place of pTyr100 for our phosphotyrosine peptide enrichment strategy. A side-by-side comparison of the phosphorylation sites enriched by the various antibodies is included in Appendix A. It is worth noting that each of the antibodies tested enriched for a different set of phosphotyrosine peptides, suggesting that each antibody has a different specificity of binding. Combining these antibodies in our peptide immunoprecipitation procedure may provide a higher dynamic range of enrichment.

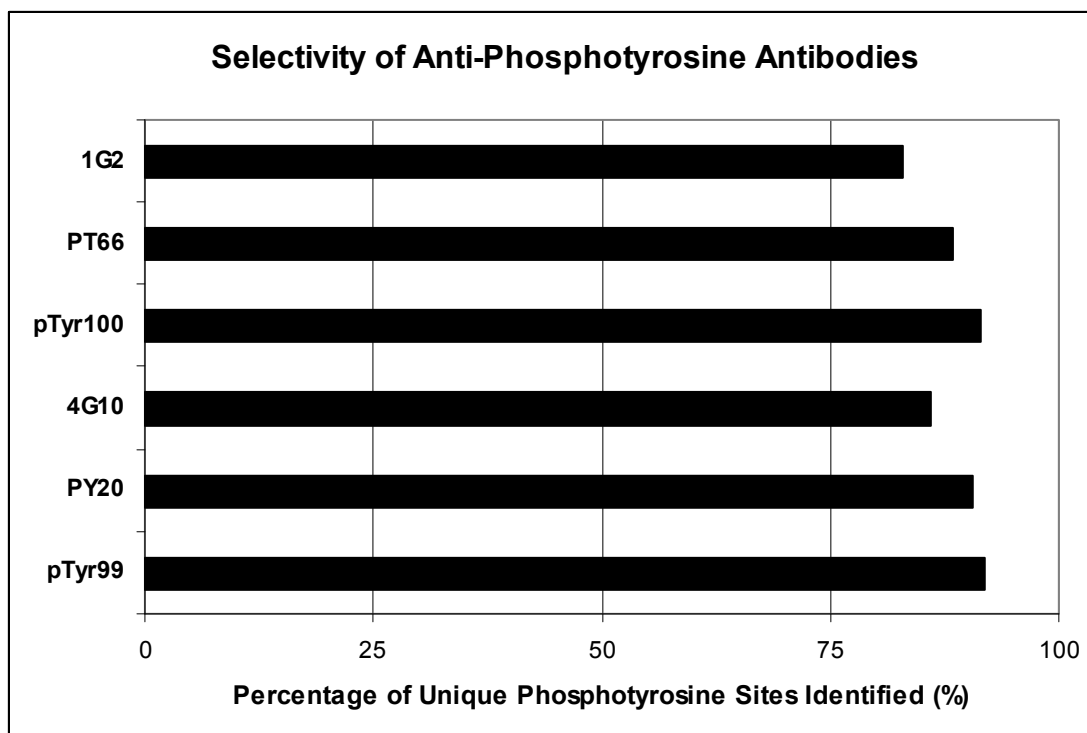


FIGURE 4.2: Selectivity of various amino acid-independent phosphotyrosine specific antibodies. Peptide immunoprecipitation experiments using various anti-phosphotyrosine antibodies were performed on pervanadate-stimulated Jurkat E6-1 cell lysates. 250 μ g of each antibody was non-covalently conjugated (pTyr100, 4G10) or pre-conjugated (pTyr99, 1G2, PY20, PT66) to 40 μ l bead volume. Data represents an average of 2 replicate experiments.

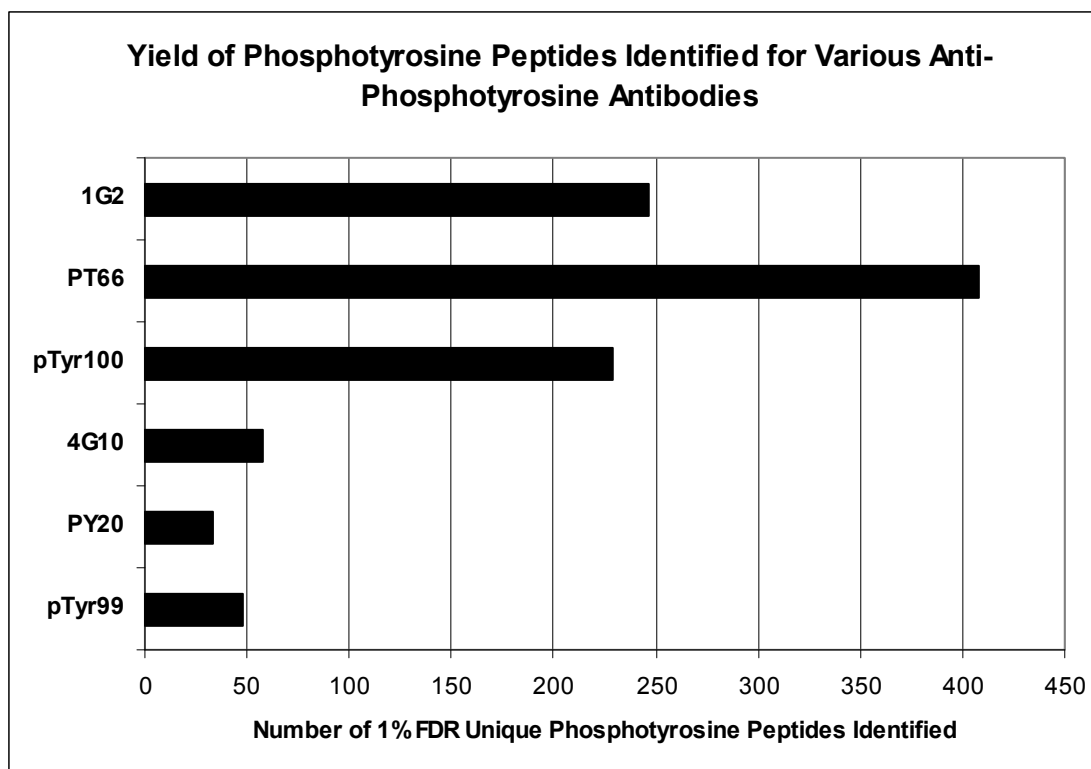


FIGURE 4.3: Phosphotyrosine peptide yields of various amino acid-independent phosphotyrosine specific antibodies. Peptide immunoprecipitation experiments using various anti-phosphotyrosine antibodies were performed on pervanadate-stimulated Jurkat E6-1 cell lysates. 250 μ g of each antibody was non-covalently conjugated (pTyr100, 4G10) or pre-conjugated (pTyr99, 1G2, PY20, PT66) to 40 μ l bead volume. Data represents an average of 2 replicate experiments.

In addition, studies have been performed to examine the effects of a milder peptide elution procedure on peptide immunoprecipitation, with the hope that the anti-phosphotyrosine antibodies may possibly be reused future experiments. A detailed protocol of this procedure can be found in Appendix D. Using 1 mM phenyl phosphate elution instead of 0.15 % TFA elution, we have successfully eluted phosphopeptides from the 1G2 antibodies-bound beads (pre-generation: 500.5 phosphotyrosine peptide IDs; $n = 2$), and regenerated them for future use (post-generation: 332 phosphotyrosine peptide IDs; $n = 2$) (Figure 4.4). However, it is worth noting that we also observed some carryover of

phosphopeptides in our no-lysate blank control sample (19 phosphotyrosine peptide IDs; $n = 2$). This has the potential to alter the quantitative abundance of phosphopeptides in future experiments. Before this elution approach can be incorporated into our sample preparation procedure, ideal elution conditions must be characterized.

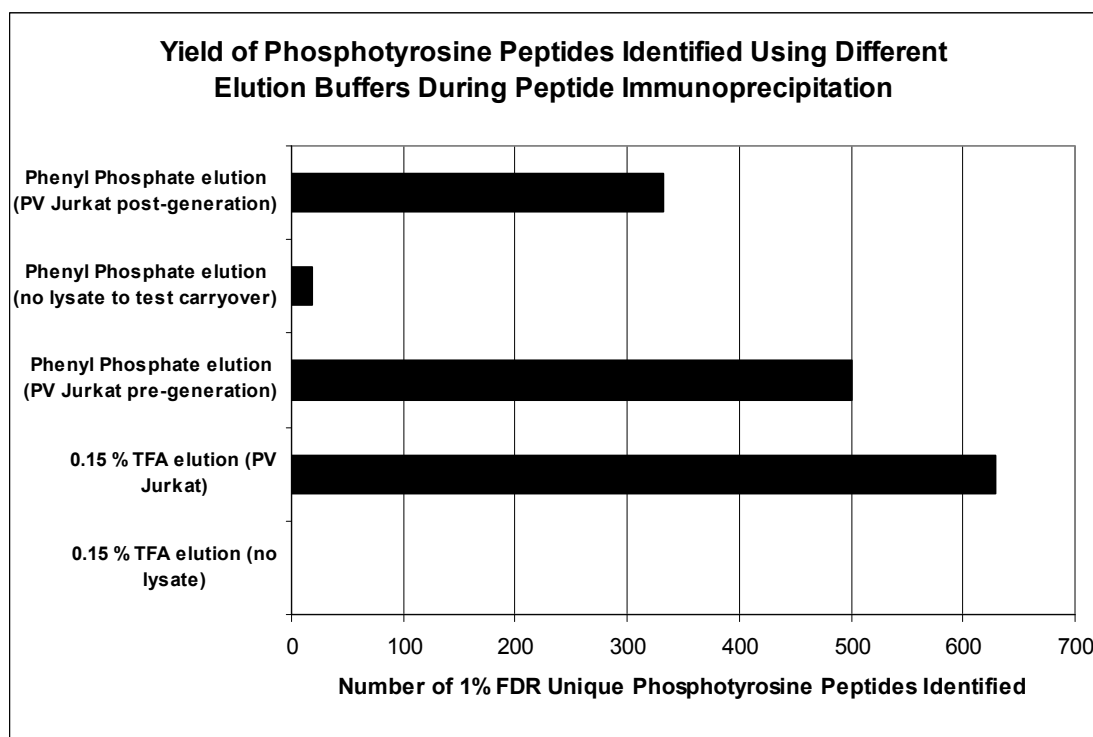


FIGURE 4.4: Phosphotyrosine peptide yields of 0.15 % TFA elution vs. 1 mM phenyl phosphate elution during peptide immunoprecipitation. Experiments using different peptide elution conditions were performed on pervanadate (pv)-stimulated Jurkat E6-1 T-cells. Peptide immunoprecipitation was then performed to enrich for phosphotyrosine peptides using 250 μg of IG2 antibodies pre-conjugated to 40 μl bead volume. For the phenyl phosphate elution conditions, the first peptide immunoprecipitation (PV Jurkat pre-generation) was followed by washing the antibodies-bound to beads with regeneration buffer, combining with no lysates, and processing through peptide immunoprecipitation/phenyl phosphate elution to test for carryover. Antibodies-bound to beads were then washed with regeneration buffer a second time, combined with pv-stimulated Jurkat cell lysates (PV Jurkat post-generation), and processed through peptide immunoprecipitation/phenyl phosphate elution. As a control, phosphopeptides from pv-stimulated Jurkat cell lysates (positive) or no-lysate blanks (negative) were eluted with 0.15% TFA. Data represents an average of 2 replicate experiments.

In further efforts to alleviate cost, we are exploring the use of different commercially available trypsin enzymes for protein digestion. Currently, we are utilizing Promega trypsin

(cat. V511C) at a protein concentration ratio of 1:100 (enzyme:protein), but hope to replace this with Worthington trypsin (cat. 3740) at a protein concentration ratio of 1:10 (enzyme:protein), which is far less expensive. In a preliminary study, we have observed similar yields (Promega: 332 phosphotyrosine peptide IDs, Worthington: 261 phosphotyrosine peptide IDs; $n = 2$) of phosphotyrosine peptides identified when comparing these enzymes (Figure 4.5). Further experiments are still required to titer the optimal enzyme-to-protein concentration ratio so as to reduce the amount of peptide miscleavages; however, we expect that Worthington trypsin will serve as a feasible alternative.

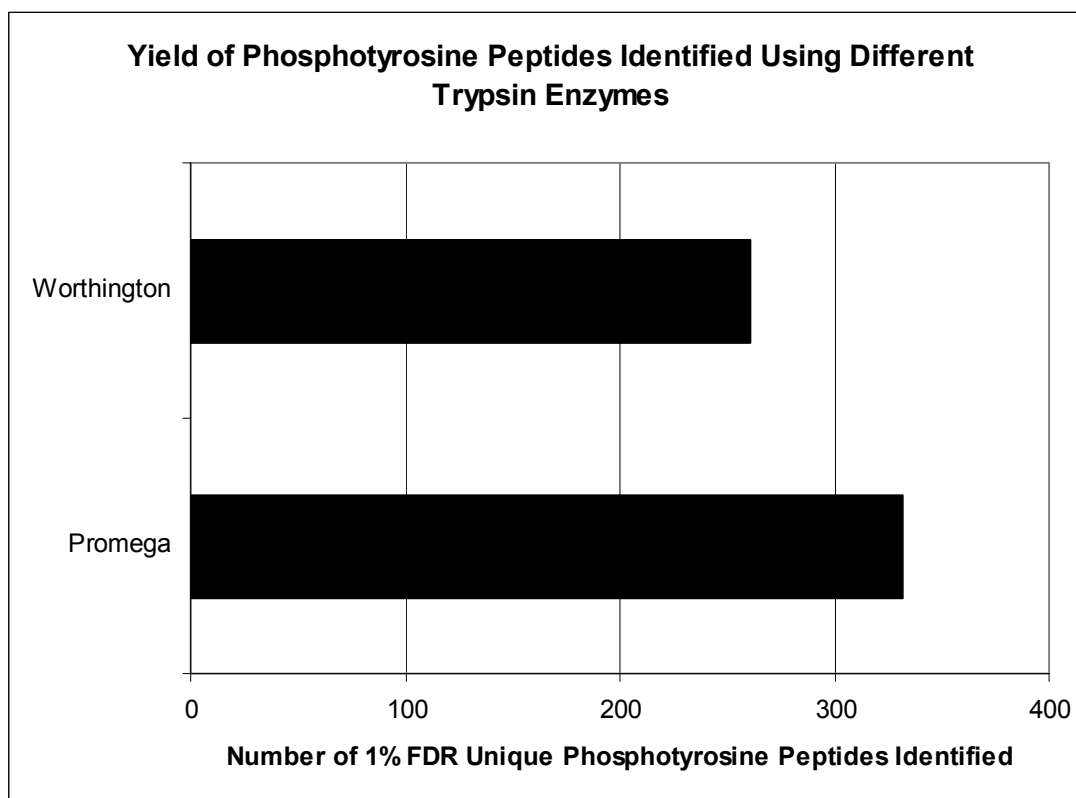


FIGURE 4.5: Phosphotyrosine peptide yields of Promega vs. Worthington trypsin. Experiments using different trypsin enzymes for protein digestion were performed on pervanadate-stimulated Jurkat E6-1 T-cells. Proteins were digested with either Promega trypsin (1:100 enzyme/protein; concentration ratio currently being used) or Worthington trypsin (1:10 enzyme/protein) overnight at room temperature. Peptide immunoprecipitation was then performed to enrich for phosphotyrosine peptides using 250 μ g of pTyr100 antibodies non-covalently conjugated to 40 μ l agarose bead volume. Data represents an average of 2 replicate experiments.

In order to maximize the yield of identified phosphotyrosine peptides, it is important to consider optimizing the ion detection settings in the mass spectrometer. In particular, two of these parameters hold considerable influence. These include the number of unique ions being selected by the instrument at a given time, and the size exclusion window of the ion that has been selected. Both of these parameters can be readily controlled by the user. Prior to running the sample, the user can select the number of unique ions that the instrument can detect at a given time. The instrument, in turn, will decide which ions to select based on their relative abundances to one another. At present, our group uses an ion detection setting that selects the 5 most abundant ions at a given time (Top 5). These ions are then excluded from MS/MS fragmentation and sequencing for a certain period of time (30 seconds for our LTQ-FT instrument) to minimize the chance of detecting the same ions more than once. In addition, there must be a balance between the number of ions being selected and the MS cycle time (the time required to collect an MS and corresponding MS/MS scans all of the selected ions at a given time). If the number of ions selected at a given time is too large, the length of the cycle will increase, while the instrument's efficiency detecting other ions will decrease.

The user must also determine the size exclusion window for the ions that have been detected. Because a given ion has a range of masses (composed of a monoisotopic peak and non-monoisotopic peaks), defining an exclusion width is critical in rejecting that ion from subsequent selections of the next Top ion groupings. A monoisotopic peak is entirely composed of the most abundant isotopes (^1H and ^{12}C), while non-monoisotopic peaks are

molecules with one (or more) heavy isotope(s) incorporated (^2H or ^{13}C). It is important that the exclusion width be large enough to reject these non-monoisotopic peaks from subsequent selections of the next Top ion groupings. However, if the mass width is too large, one runs the risk of possibly excluding other ions whose masses also fall within an overlapping range. We optimized the number of unique ions being selected and size exclusion windows to provide us with the highest phosphotyrosine peptide yields possible. We observed that changing the MS/MS detection method from Top 5 to Top 9 (selecting the 9 most abundant ions at a given time), along with increasing the size exclusion window (from a width of 0.1 - 0.5 Daltons to a width of 0.10 - 2.52 Daltons) resulted in an increased yield of identified phosphotyrosine peptides (ddTop5: 423 phosphotyrosine peptides IDs, ddTop9 wide exclusion: 507 phosphotyrosine peptides IDs; $n = 1$) (Figure 4.6). In addition, with this increase in exclusion width, we now have the capacity to reject non-monoisotopic peaks that have incorporated up to seven heavy isotopes for a +3 ion. Presently, all samples analyzed on the LTQ-FT are utilizing the ddTop9 wide exclusion MS/MS ion detection setting.

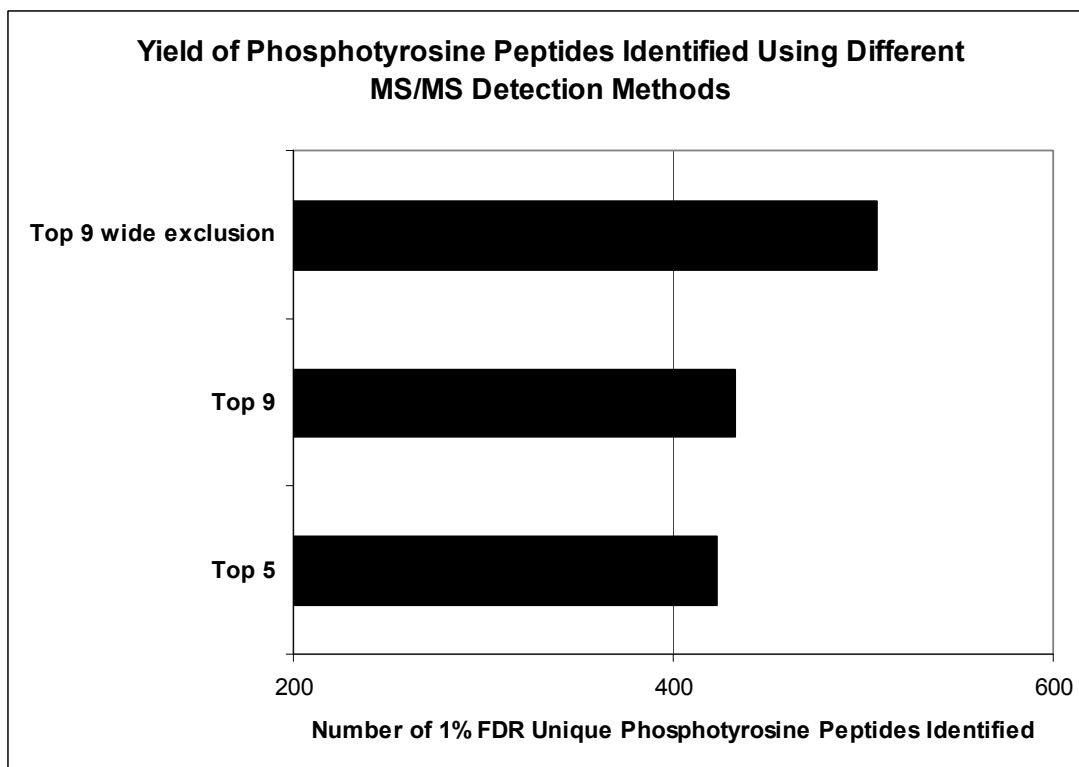


FIGURE 4.6: Phosphotyrosine peptide yields of Top 5 vs. Top 9 ion detection methods. Experiments using different MS/MS ion detection methods were performed on pervanadate-stimulated Jurkat E6-1 T-cells. Proteins were digested with Promega trypsin (1:100 enzyme/protein) overnight at room temperature. Peptide immunoprecipitation was then performed to enrich for phosphotyrosine peptides using 250 μ g of pTyr100 antibodies non-covalently bound to 40 μ l agarose bead volume. Phosphopeptides were sequenced using the LTQ-FT with different modifications of the ion detection that define which ions are isolated and sequenced through MS/MS. The modifications include the top 5 most abundant ions in the detector at a given time (Top 5), the top 9 most abundant ions in the detector at a given time (Top 9), and the top 9 most abundant with increased size exclusion (from a width of 0.1 - 0.5 Daltons to a width of 0.10 - 2.52 Daltons) (Top 9 wide exclusion).

In the near future, our group will have full access to a LTQ-Orbitrap instrument to analyze our samples. The main advantage of using an Orbitrap, as opposed to the FTMS currently being used is that the Orbitrap possesses a considerably faster MS/MS scan speed, allowing for more acquisitions of MS/MS spectra in a given amount of time. Previous reports have demonstrated increased yields of peptide identifications by the LTQ-Orbitrap compared to the LTQ-FT (4). Consistent with these findings, our group has observed similar results when comparing both instruments in the analysis of the global phosphotyrosine

network of pervanadate-stimulated Jurkat E6-1 cells (LTQ-Orbitrap: 819 phosphotyrosine peptide IDs, LTQ-FT: 190 phosphotyrosine peptide IDs) (Figure 4.7). Future access to the LTQ-Orbitrap will enable our group to identify and study more phosphorylation sites than previously possible.

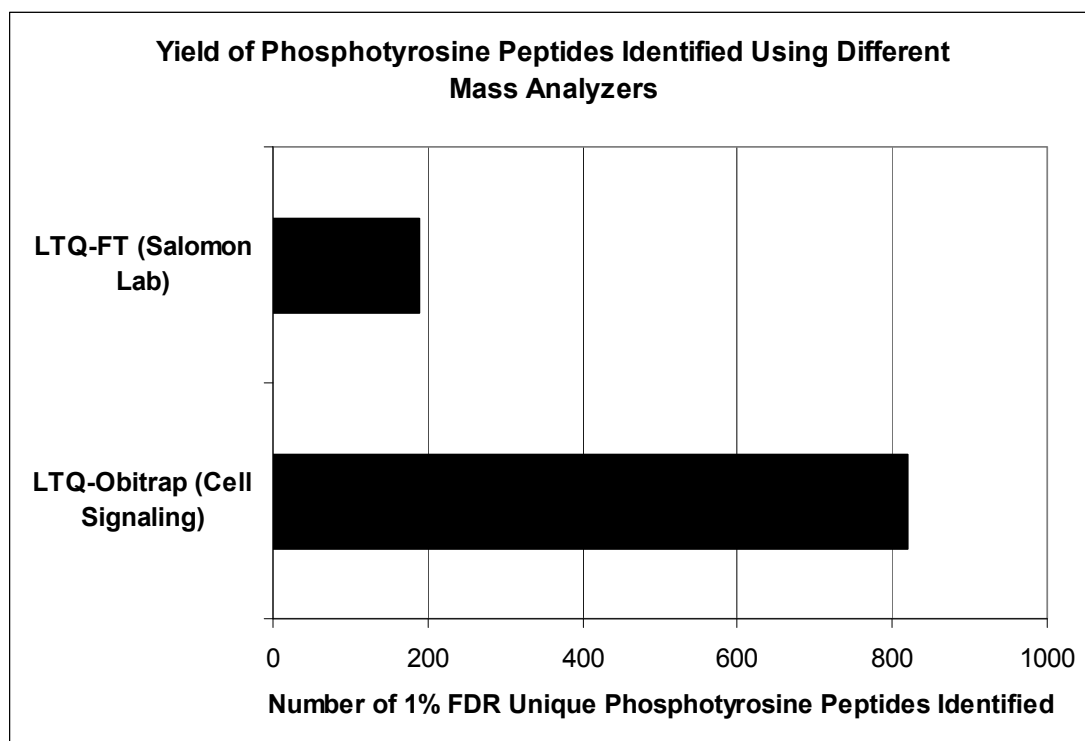


FIGURE 4.7: Phosphotyrosine peptide yields of LTQ-FT vs. LTQ-Orbitrap for peptide sequence analysis. Experiments using different mass analyzers were performed on pervanadate-stimulated Jurkat E6-1 T-cells. Proteins were digested with Worthington trypsin (1:10 enzyme/protein) overnight at room temperature. Peptide immunoprecipitation was then performed to enrich for phosphotyrosine peptides using 250 μ g of pTyr100 antibodies non-covalently bound to 40 μ l agarose bead volume. Phosphopeptides were sequenced using either the LTQ-FT in the Salomon Laboratory (Brown University, Providence, RI) or the LTQ-Orbitrap at Cell Signaling Technology (Danvers, MA).

It is worth noting that the data presented here was generated from pervanadate-stimulated Jurkat E6-1 T-cells. This type of stimulation is non-physiological, and leads to robust levels of phosphorylation in the cell. When applying our updated sample preparation procedure (Appendix C) to samples generated from cells stimulated with more physiological

conditions (and hence exhibiting lower levels of phosphorylation), one should gauge his/her expectations of phosphopeptide yields accordingly.

4.4 REFERENCE

1. Brill, L. M., Salomon, A. R., Ficarro, S. B., Mukherji, M., Stettler-Gill, M., and Peters, E. C. (2004) Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T-cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal Chem* 76, 2763-2772.
2. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T-cell receptor activation. *Mol Cell Proteomics* 8, 2418-2431.
3. Rappsilber J., Ishihama Y., Mann M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 75, 663–670.
4. Olsen, J. V., Schwartz, J. C., Griep-Raming, J., Nielsen, M. L., Damoc, E., Denisov, E., Lange, O., Remes, P., Taylor, D., Splendore, M., Wouters, E. R., Senko, M., Makarov, A., Mann, M., and Horning, S. (2009) A dual pressure linear ion trap Orbitrap instrument with very high sequencing speed. *Mol Cell Proteomics* 8, 2759-2769.
5. Schmelzle, K., Kane, S., Gridley, S., Lienhard, G. E., and White, F. M. (2006) Temporal dynamics of tyrosine phosphorylation in insulin signaling. *Diabetes* 55, 2171-2179.
6. Kim, J. E., and White, F. M. (2006) Quantitative analysis of phosphotyrosine signaling networks triggered by CD3 and CD28 costimulation in Jurkat cells. *J Immunol* 176, 2833-2843.

Chapter 5

CONCLUSIONS

5.1 SIGNIFICANCE AND IMPLICATIONS

The goal of the research presented here was to gain a comprehensive, global understanding of coordinated phosphorylation networks regulating the TCR-mediated signaling pathway using novel proteomic approaches. Initial investigation of the TCR signaling pathway focused on globally determining the tyrosine phosphorylation sites involved and their changes in abundance in response to TCR-CD3 and CD4 co-stimulation (2). Although these studies provided us with great insight on which phosphorylation sites may have a role in the signaling pathway, they alone did not give enough information to make accurate predictions about the structure of the pathway. To overcome this challenge, we proposed a phosphoproteomic comparative analysis of well-established Jurkat deficient T-cell clones to approximate within which branch of the signaling pathway newly-discovered phosphoproteins may reside. Currently, our group has obtained a variety of protein-deficient Jurkat T-cells (LCK (J.CaM.1) (3), LAT (J.CaM.2) (4), PLC- γ -1 (J.gamma.1) (5), SLP-76 (J14) (6), and is in the process of performing this analysis. Comparison of data from the removal of multiple signaling proteins at different positions within a single pathway (upstream, middle, and downstream) will provide a means of organizing hundreds to thousands of phosphorylation sites relative to canonical pathway signaling landmarks.

Before this analysis could be performed, the development of a novel hybrid quantitative approach capable of multi-dimensional comparison of mutant and wild type cells through a time course of receptor stimulation was critically important. By combining genetic analysis and two well-established proteomic quantitation methods

(SILAC and label-free), as well as novel visual analysis tools, our approach facilitates rapid elucidation of the biological significance of high-throughput phosphoproteomic data (1). Although our hybrid quantitative approach was applied to the analysis of wide-scale tyrosine phosphorylation in TCR-mediated signaling, it is generic in design and adaptable to a wide range of phosphopeptide enrichment strategies. Our goal is to provide the scientific community with a generalized approach to elucidate cell signaling pathways using phosphoproteomics. Quantitative phosphoproteomic phenotyping of signaling protein mutants will be an ideal complement to traditional signaling approaches, accelerating our understanding of the architecture of phosphorylation networks involved in a wide range of biological processes.

The phosphoproteomic phenotyping of Jurkat T-cells could also provide insights into other signaling pathways. In particular, we have identified phosphorylation sites on many signaling proteins that are known to function in well-conserved pathways. Some examples include PLC and PKC (Ca^{2+} signaling), PI3K (ITK and AKT pathway), and ERK and p38 (MAPK pathway). A better understanding of the roles of these proteins in TCR signaling could eventually lead to a more thorough understanding of how these proteins act in nature. Moreover, although we studied ERK positive feedback loops in T-cells, this mechanism is known to regulate other signaling pathways in a conserved manner, such as with ERK's feedback activation of proliferation and migration in breast cancer cells (21). We have also identified phosphorylation sites on proteins known to share peptide sequence homology with proteins in other signaling pathways. For instance, Zap-70 (which functions in T-cell signaling) and SYK (which functions in B-cell signaling) share ~50% peptide sequence homology. Novel phosphorylation sites

identified in one protein could suggest that homologous proteins may be phosphorylated at the same or similar position.

Ultimately, studies in TCR signaling are aimed towards the development of improved treatments for human diseases. Zap-70 is an attractive therapeutic target due to its critical role in TCR signaling. Moreover, correlations between the overexpression of Zap-70 and chronic lymphocytic leukemia have been reported (19). To date, the development of a cell-permeable inhibitor that specifically targets Zap-70 has been difficult, and of the potential Zap-70 inhibitors currently available (20), none have been shown to be highly effective *in vivo*. The creation of such an inhibitor would also prove beneficial in situations of allograft rejection or T-cell-mediated autoimmunity, where blocking of T-cell activation may be a viable therapy. Further characterizing the pathways in which Zap-70 regulates within T-cells will aid in the development of a more effective treatment, as well as improve our understanding of the consequences of Zap-70's inhibition or overexpression.

5.2 SUMMARY OF RESULTS

Phosphoproteomic profiling of isogenic Zap-70 null and reconstituted Jurkat T-cells was utilized here to validate our hybrid quantitative approach. This analysis led to the identification of 168 tryosine phosphorylation sites found on 135 unique proteins. Using label-free quantitation, we were able to measure the changes in relative abundance of phosphorylation in response to TCR stimulation, thus providing clues regarding which

unknown phosphorylation sites may be involved in the pathway. Because sites that do not change in response to TCR stimulation are unlikely to be involved in the TCR pathway, we focused our analysis on sites that showed a change across the TCR stimulation time course in Zap-70 reconstituted Jurkat T-cells. These TCR-responsive sites were further categorized into groups with the use of SILAC ratio data to determine the dependence of each site upon the absence or presence of Zap-70. From this grouping of SILAC ratios, 43% showed enhanced phosphorylation, 19% showed no difference, 24% showed a slight decrease, and 12% showed a significant decrease. More importantly, we observed many of the perturbations in phosphorylation that would be expected in response to Zap-70 removal, whereby providing further confidence for our approach. The use of this quantitative approach has also led to the discovery of novel, uncharacterized phosphorylation sites that can be positioned within the TCR signaling pathway. Among the 105 novel sites identified, 69 of these sites were changed significantly in abundance with Zap-70 removal. This data offers a wealth of information about the hypothetical placement of these sites relative to Zap-70.

Although the use of the Jurkat T-cell line as the model system for our analysis may raise concerns of physiological relevance (due to the lack of expression of PTEN and SHIP (14-16)), this was an appropriate model to validate our quantitative approach. We are aware that this cell line has higher basal activity levels than primary T-cells due to the constitutive activation of the PI3K pathway (17). However, the purpose of these studies was to identify newly-discovered phosphorylation sites that change in abundance in response to both TCR stimulation and Zap-70 removal. The un-stimulated 0 minute time point and the use of Zap-70 reconstituted Jurkat T-cells are critical controls that

provide a benchmark against which to compare basal activity levels. In addition, the availability of highly characterized Zap-70-deficient Jurkat T-cells was essential in providing us with previously described defects in TCR signaling, against which we were able to compare our phosphoproteomic data.

For our analysis, we chose to compare Zap-70 null to Zap-70 reconstituted Jurkat cells, as opposed to comparing Zap-70 null to wild type Jurkat cells. Our justification for this was that we wanted the cell populations to be as closely matched as possible. Zap-70-deficient Jurkat clones were isolated through genetic screens in which wild type Jurkat cells were mutagenized with the frameshifting mutagen ICR-191, followed by an isolation of clones that failed to increase calcium in response to pervanadate stimulation (22). Although this cell line has no detectable ZAP-70 mRNA or protein expression (and also fails to express SYK), we hypothesize that ICR-191 treatment could have also induced mutations on other genes that have yet to be identified. If this were the case, basal activity of these cells would be divergent from that of wild-type cells. Consistent with this hypothesis, we observed differences in phosphorylation profiles for many of the phosphopeptides identified when comparing Zap-70 reconstituted to wild type Jurkat cells in response to TCR stimulation (Appendix B). Therefore, comparing Zap-70 null to reconstituted Jurkat cells is a more appropriate control. The only difference between these cells is the reintroduction of Zap-70, which has been shown to fully reverse TCR signaling defects (22).

A logical model to visualize feedback loops using this hybrid approach was discussed in Chapter 2. Consistent with our model, when comparing SLP-76 null and

reconstituted Jurkat T-cells, we observed perturbations in phosphorylation of upstream signaling proteins that are indicative of disruptions in positive feedback (Cao and Salomon, unpublished). We hypothesized that the misregulation of the ERK positive feedback pathway was the cause for this disruption. By utilizing the MEK1/2 inhibitor, U0126, we designed a phosphoproteomic follow-up strategy to possibly mimic this defect. Unexpectedly, we observed that many of the phosphorylation sites on upstream, adaptor, and downstream signaling proteins were constitutively inhibited in response to MEK1/2 inhibition. We predict that this may be due to the suppression of basal ERK activity that was also observed in preliminary experiments using phosphorylation site-specific western blots for ERK after treatment with U0126. Further phosphoproteomic experiments with various amounts of U0126 may result in the observation of more time-variable patterns of upstream protein phosphorylation increase or decrease. For example, it is plausible that with a lower concentration of U0126, the extent of inhibition of basal ERK activity will be less dramatic, thereby giving rise to different phosphorylation profiles for the CD3 subunits, LCK, and Zap70. The results described here, which are for the first time at high resolution on many phosphorylation sites, consistent with the hypothesis that ERK activity can contribute to upstream TCR signaling.

Surprisingly, the removal of Zap-70 surprisingly enhanced the phosphorylation of FYN and its substrates. With the assistance of novel visual analysis tools, we have identified known FYN interacting proteins, which aided in the formulation of a hypothetical model that suggests PTP α 's misregulation as the possible cause for FYN's enhanced phosphorylation. We hypothesized that upon Zap-70 removal, PKC δ and PI3K (regulators of PTP α) activation would be disrupted, causing the reduction in PTP α

activity. This, in turn, would lead to the enhanced phosphorylation of FYN. Highly selective mouse anti-FYN antibodies that were validated against recombinant human FYN proteins will be utilized in co-immunoprecipitation studies between PTP α and FYN. Further biochemical and proteomic follow-up studies to test our hypothesis were discussed.

Recently, our group has experienced major breakthroughs in method development of sample preparation that has led to the increased sensitivity to, and selectivity for, the enrichment of phosphotyrosine peptides. The modifications in our sample preparation procedure include increasing the antibody concentration of pTyr100 by 10 times the original amount during peptide immunoprecipitation, substituting pTyr100-non-covalently conjugated to agarose beads with pre-conjugated pTyr100 antibody beads at the same antibody concentration, changing the MS/MS detection method from Top 5 to Top 9 with an increased size exclusion width, and eliminating Fe³⁺ IMAC enrichment. Detailed protocols of the improved sample preparation procedure have been included in Appendix section C and D.

It is important to note that the quantitative phosphoproteomic approach applied in the studies presented here is limited to identifying phosphorylation sites and measuring their changes in relative abundance in response to receptor stimulation. This approach, however, it is not capable of measuring the absolute abundance of the phosphoproteins identified. Therefore, it is possible that any increases or decreases in phosphorylation abundance that we observed could be due to a change in protein expression levels, and not necessarily to a change in phosphorylation levels. For the phosphorylation sites that

elicit follow-up studies, it is critical that quantitative immunoblots first be performed to verify protein expression levels.

5.3 FUTURE DIRECTIONS

5.3.1 Applying the hybrid quantitative approach towards further studies of TCR signaling

Along with performing a comparative analysis to study the effects of removing multiple signaling proteins at different positions within the TCR signaling pathway, we plan on utilizing our quantitative hybrid approach to examine the consequences of removing multiple domains and site-directed mutations within a single protein--specifically, the adaptor protein SLP-76. Performing such an analysis will provide greater insights on the various domains and phosphorylation sites of SLP-76 in TCR signaling. We have obtained SLP-76-deficient Jurkat T-cells that have been reconstituted with SLP-76 constructs that contain domain region deletions or point mutations (Figure 5.1) (7). These domain regions and phosphorylation sites in SLP-76 have previously been shown to have distinct and essential roles in TCR signaling (6-9). We will perform a quantitative phosphoproteomic analysis similar to the one previously described (2), in which we will be comparing the various SLP-76 domain deletion or point mutation Jurkat T-cells with full length, wild type SLP-76 reconstituted Jurkat T-cells to examine the effects of these mutations on phosphorylation networks in response to TCR stimulation. We assume that we will observe disruptions in tyrosine phosphorylation on known TCR signaling proteins corresponding to the appropriate mutations that have been previously

reported (6-9). For example, we expect to observe the SLP-76 N-terminus deletion mutant to show inhibition in tyrosine phosphorylation for PLC- γ -1 (7). We also expect to observe novel perturbations in phosphorylation on many uncharacterized TCR signaling proteins.

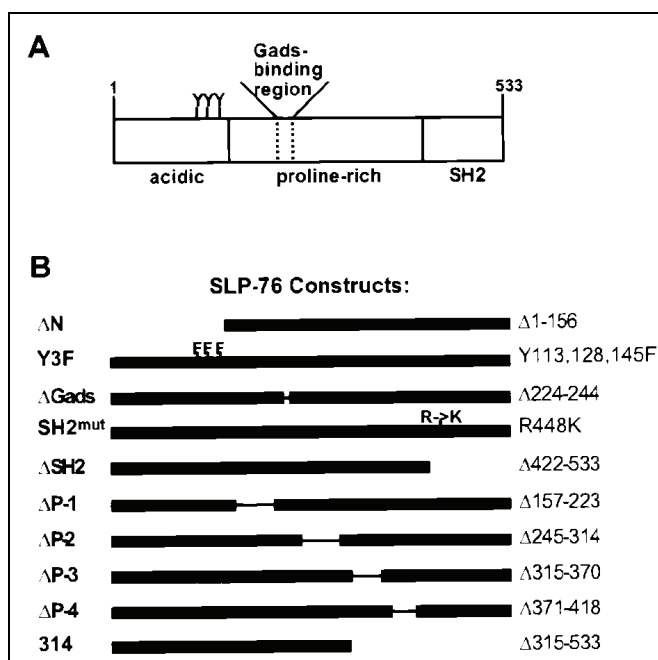


FIGURE 5.1: SLP-76 Expression constructs. A) The major functional domains of SLP-76. B) Diagrammatic depiction of the SLP-76 mutant constructs that will be used in our quantitative phosphoproteomic study. (Figure adopted from Yablonski et al., 2001) (7)

An alternative approach to utilizing the Jurkat T-cell line for phosphoproteomic studies in TCR signaling is to utilize primary T-cells obtained from transgenic knock out and wild type mice. Although we are aware that this study would be more physiologically relevant, the reason we did not initially perform this analysis was due to difficulty in obtaining enough cells to have multiple time points and replicates for our study. Since then, our group has applied our improved sample preparation procedure (Appendix C) in the analysis of drug-stimulated primary T-cells, and has been successful

in the identification and quantitation of 135 unique tyrosine phosphorylation sites on 122 proteins (Yu and Tilahun, unpublished; data not shown). In the future, we plan to survey a variety of knock out mutants that are currently available in the mouse model.

5.3.2 ERK positive feedback in TCR signaling

Although the results presented here are consistent with the primary literature describing the existence of ERK positive feedback (10), re-performing this analysis using primary T-cells may display more of a time-dependent inhibition of upstream phosphorylation in response to ERK inhibition. It is possible that the levels of basal ERK activity are so high in the Jurkat T-cell line that the expected pattern was not seen, thus making it an imperfect system to study feedback. The fact that primary T-cells do not culture very well in media makes utilizing them even more difficult in our quantitative analysis, considering that SILAC labeling requires multiple passages and long incubation times (8 days or more) in order for the isotopic amino acids to become fully incorporated. The use of iTRAQ labeling instead of SILAC labeling to measure differences in phosphorylation between U0126-treated and -untreated cells may allow us to overcome this challenge. iTRAQ quantitation requires covalent attachment of isobaric mass tags to peptide-amine groups after cell lysis and trypsin digestion (18), whereby bypassing the need to culture cells for extended periods of time.

A possible solution to using Jurkat T-cells as our model for these studies is to decrease the concentration of U0126 and/or the preincubation time, which may produce more of a time-dependent manner of inhibition, rather than constitutive inhibition on

upstream phosphorylation, as seen in these initial studies. These conditions still need to be optimized.

Another reagent, PD98059, has also been shown to act *in vivo* as a highly selective inhibitor of MEK1/2 and the MAP kinase cascades (11, 12). PD98059 binds to inactive forms of MEK1 and prevents its activation from upstream activators such as c-Raf (13). It would be interesting to observe whether or not utilizing this reagent in a quantitative phosphoproteomic analysis of wild type Jurkat T-cells would provide similar results to those collected for U0126. Performing such an analysis could add further support for the existence of ERK positive feedback in TCR signaling.

5.3.3 Zap-70-dependent FYN regulation through PTP α

We plan on utilizing both traditional biochemical and proteomic approaches to test our Zap-70-dependent Fyn regulation hypothesis. Highly selective anti-FYN antibodies will be used in co-immunoprecipitation studies to verify whether or not disruptions in FYN's interaction with PTP α occur in response to Zap-70 removal. Once this hypothesis has been tested, shRNA constructs for PI3K and PKC δ will be produced and transduced into Zap-70 deficient Jurkat T-cells (null and reconstituted) to determine whether these proteins act in a Zap-70-dependent manner to regulate PTP α . We hypothesize that knocking-down PI3K and PKC δ expression will cause enhanced phosphorylation of FYN in Zap-70 reconstituted T-cells. Immunoblots for phosphorylated-S¹⁸⁰ and -S²⁰⁴ of PTP α from TCR-stimulated Zap-70 null and reconstituted cell lysates will also be performed to establish whether these known activation sites are important for Zap-70-dependent regulation.

Alternatively, a proteomic approach will also be utilized to detect Fyn binding partners. TCR-stimulated Zap-70 null and reconstituted T-cells will be immunoprecipitated against FYN, and eluents will be separated through SCX fractionation and sequenced using LC/MS/MS methodology. We expect to identify peptides corresponding to PTP α , PKC δ and PI3K in our stimulated Zap-70 reconstituted fractions, while observing an absence of these peptides in our Zap-70 null fractions. These results, in addition to our biochemical studies, would provide strong evidence that Fyn is regulated by PTP α in a Zap-70-dependent manner.

5.4 REFERENCE

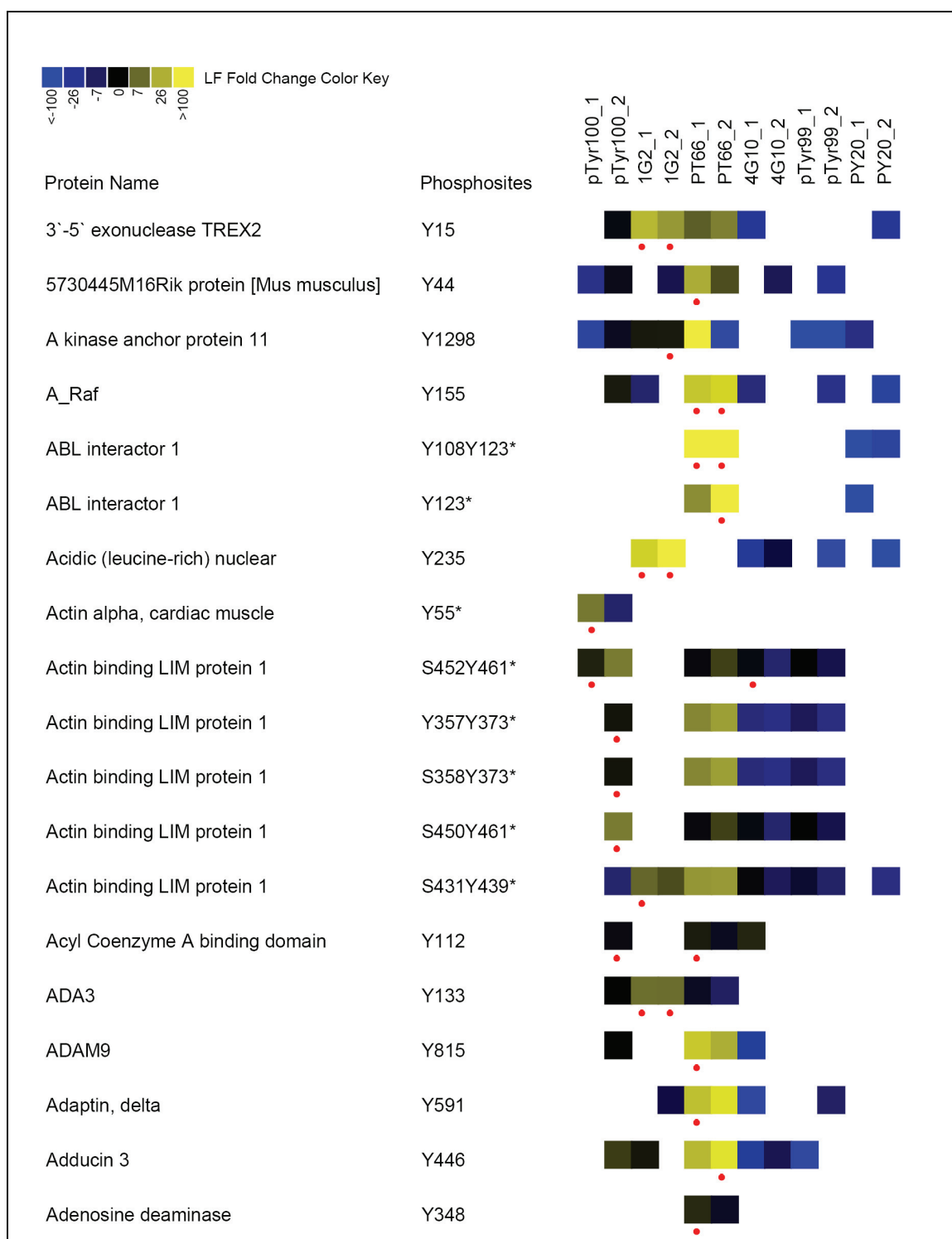
1. Salomon, A. R., Ficarro, S. B., Brill, L. M., Brinker, A., Phung, Q. T., Ericson, C., Sauer, K., Brock, A., Horn, D. M., Schultz, P. G., and Peters, E. C. (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proc Natl Acad Sci U S A* 100, 443-448.
2. Nguyen, V., Cao, L., Lin, J. T., Hung, N., Ritz, A., Yu, K., Jianu, R., Ulin, S. P., Raphael, B. J., Laidlaw, D. H., Brossay, L., and Salomon, A. R. (2009) A new approach for quantitative phosphoproteomic dissection of signaling pathways applied to T-cell receptor activation. *Mol Cell Proteomics* 8, 2418-2431.
3. Straus, D. B., and Weiss, A. (1992) Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T-cell antigen receptor. *Cell* 70, 585-593.
4. Finco, T. S., Kadlecsek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity* 9, 617-626.
5. Irvin, B. J., Williams, B. L., Nilson, A. E., Maynor, H. O., and Abraham, R. T. (2000) Pleiotropic contributions of phospholipase C-gamma1 (PLC-gamma1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC-gamma1-deficient Jurkat T-cell line. *Mol Cell Biol* 20, 9149-9161.

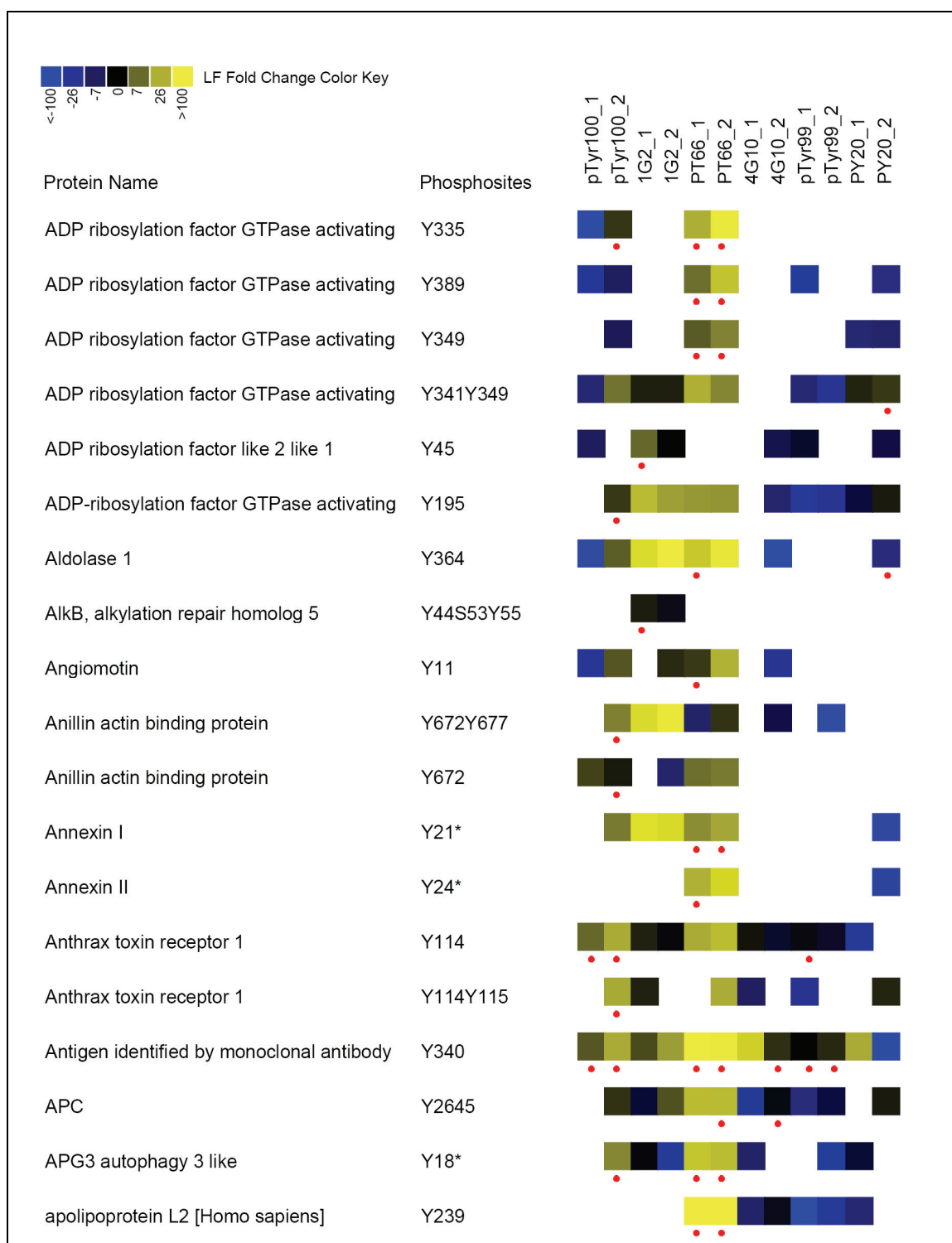
6. Yablonski, D., Kuhne, M. R., Kadlecsek, T., and Weiss, A. (1998) Uncoupling of nonreceptor tyrosine kinases from PLC- γ 1 in an SLP-76-deficient T-cell. *Science* 281, 413-416.
7. Yablonski, D., Kadlecsek, T., and Weiss, A. (2001) Identification of a Phospholipase C- γ 1 (PLC- γ 1) SH3 Domain-Binding Site in SLP-76 Required for T-Cell Receptor-Mediated Activation of PLC- γ 1 and NFAT. *Mol Cell Biol* 21(13), 4208-4218.
8. Bogin, Y., Ainey, C., Beach, D., and Yablonski, D. (2007) SLP-76 mediates and maintains activation of the Tec family kinase ITK via the T cell antigen receptor-induced association between SLP-76 and ITK. *Proc Natl Acad Sci U S A* 104(16), 6638-6643.
9. Kuhné, M. R., Joseph Lin, J., Deborah Yablonski, D., Mollenauer, M. N., Richie Ehrlich, L. I., Huppa, J., Mark M. Davis M. M., and Weiss, A. (2003) Linker for Activation of T Cells, ζ -Associated Protein-70, and Src Homology 2 Domain-Containing Leukocyte Protein-76 are Required for TCR-Induced Microtubule-Organizing Center Polarization. *J. Immunol* 171, 860-866.
10. Stefanova, I., Hemmer, B., Vergelli, M., Martin, R., Biddison, W. E., and Germain, R. N. (2003) TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol* 4, 248-254.
11. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* 258, 478-480.
12. Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) Identification of sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J.* 13(7), 1610-1619.
13. Rosen, L. B., Ginty, D. D., Weber, M. J., and Greenburg, M. E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* 12(6), 1207-1221.
14. Astoul, E., Edmunds, C., Cantrell, D. A., and Ward, S. G. (2001) PI3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol* 22, 490-496.
15. Shan, X., Czar, M. J., Bunnell, S. C., Liu, P., Liu, Y., Schwartzberg, P. L., and Wange, R. L. (2000) Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol. Cell. Biol.* 20, 6945-6957.

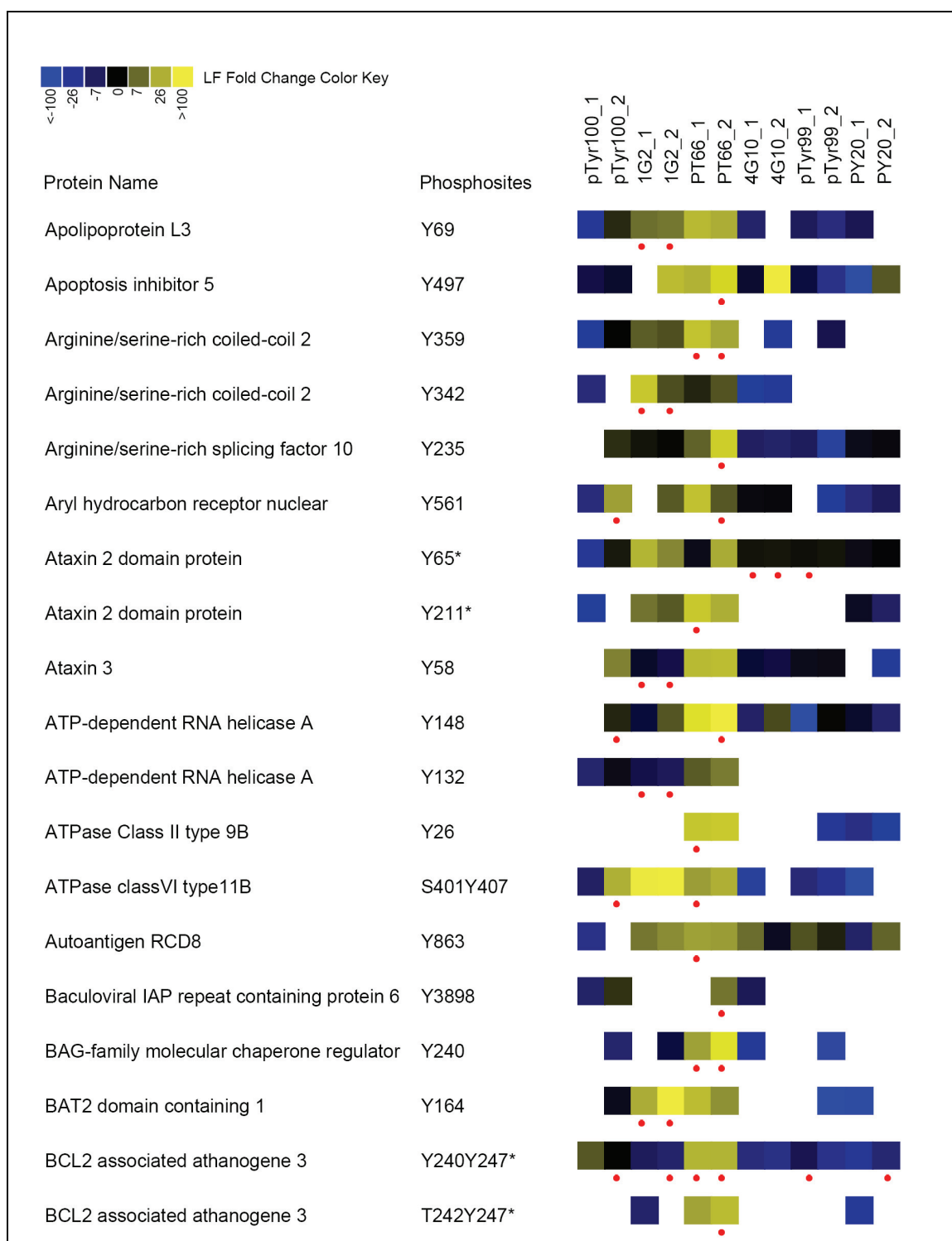
16. Wang, X., Gyorloff-Wingren, A., Saxena, M., Pathan, N., Reed, J. C., and Mustelin, T. (2000) The tumor suppressor PTEN regulates T cell survival and antigen receptor signaling by acting as a Phosphatidylinositol 3-Phosphatase. *J. Immunol.* 164, 1934-1939.
17. Seminario, M. C. and Wange, R. L. (2002) Signaling pathways of D3-phosphoinositide-binding kinases in T cells and their regulation by PTEN. *Semin. Immunol.* 14, 27-36.
18. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 3, 1154-1169.
19. Herishanu, Y., Kay, S., Rogowski, O., Pick, M., Naparstek, E., Deutsch, V. R., and Polliack, A. (2005) T-cell ZAP-70 overexpression in chronic lymphocytic leukemia (CLL) correlates with CLL cell ZAP-70 levels, clinical stage and disease progression. *Leukemia* 19, 1289-1291.
20. Hirabayashi, A., Mukaiyama, H., Kobayashi, H., Shiohara, H., Nakayama, S., Ozawa, M., Miyazawa, K., Misawa, K., Ohnata, H., Isaji, M.. (2009) Structure-activity relationship studies of 5-benzylaminoimidazo[1,2-c]pyrimidine-8-carboxamide derivatives as potent, highly selective ZAP-70 kinase inhibitors. *Bioorg Med Chem* 17: 284-294.
21. You, J., Mi, D., Zhou, X., Qiao, L., Zhang, H., Zhang, X., and Ye, L. (2009) A positive feedback between activated extracellularly regulated kinase and cyclooxygenase/lipoxygenase maintains proliferation and migration of breast cancer cells. *Endocrinology* 150, 1607-1617.
22. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol Cell Biol* 18, 1388-1399.

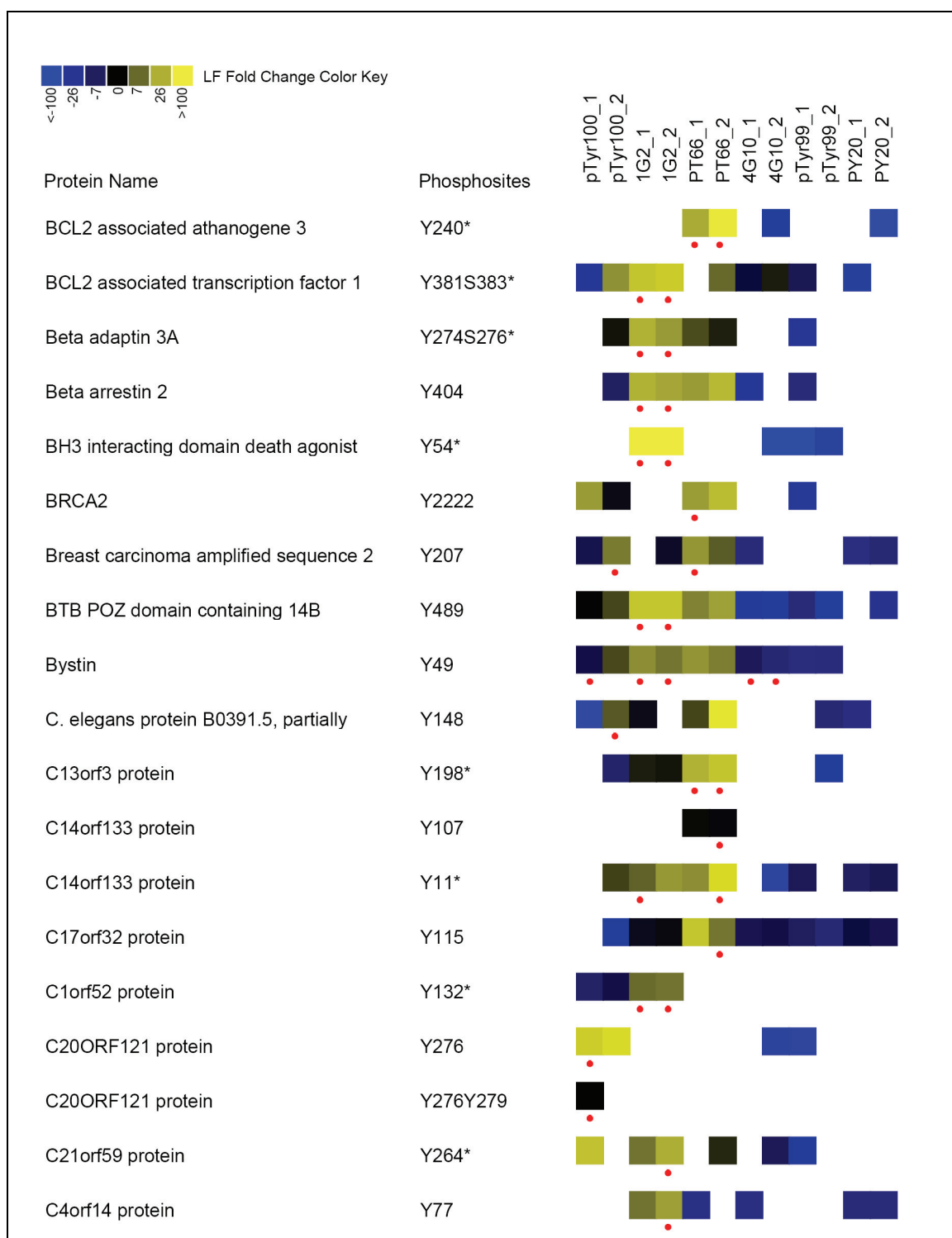
Appendix

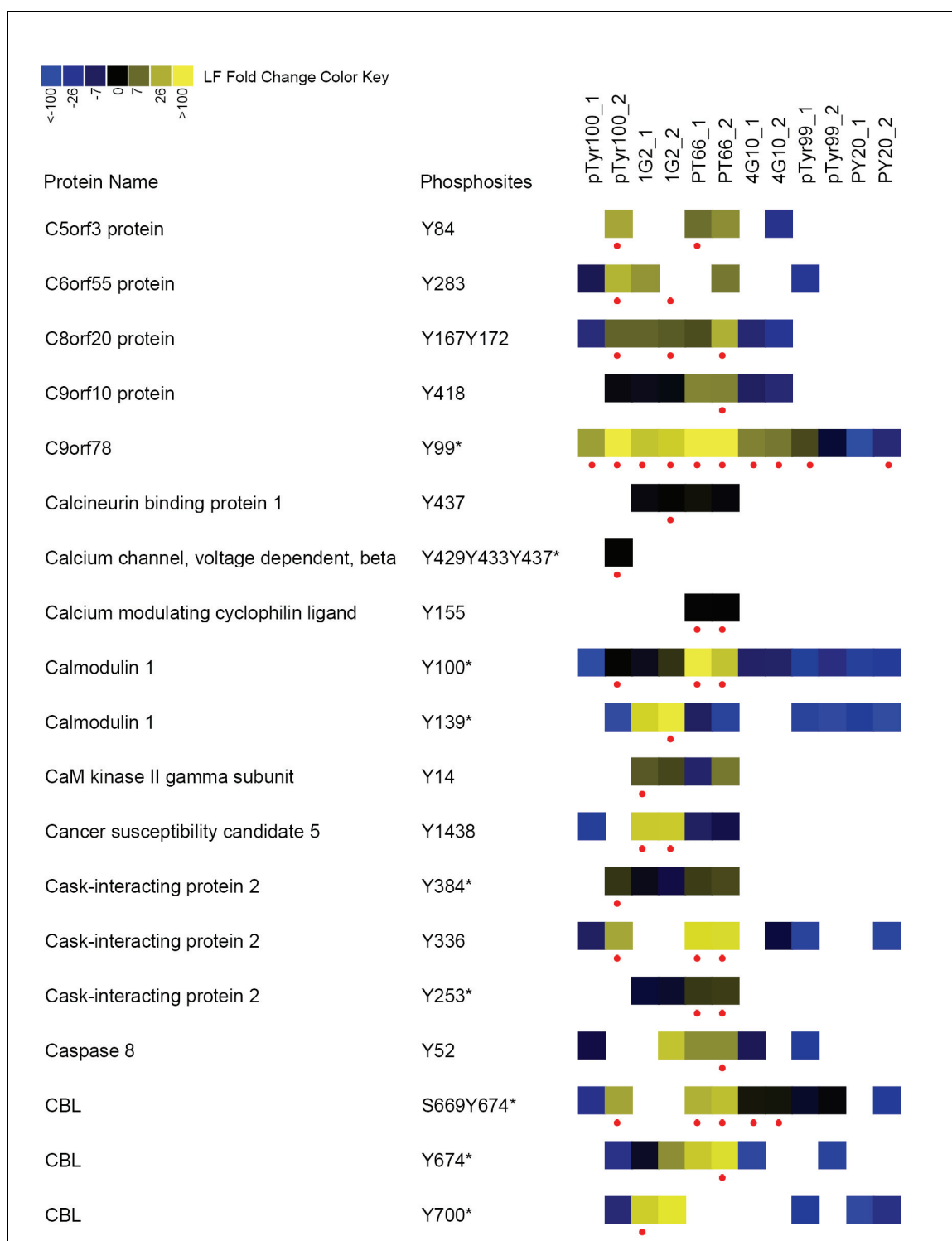
A) Comparison of the phosphorylation sites enriched by the various anti-phosphotyrosine antibodies.

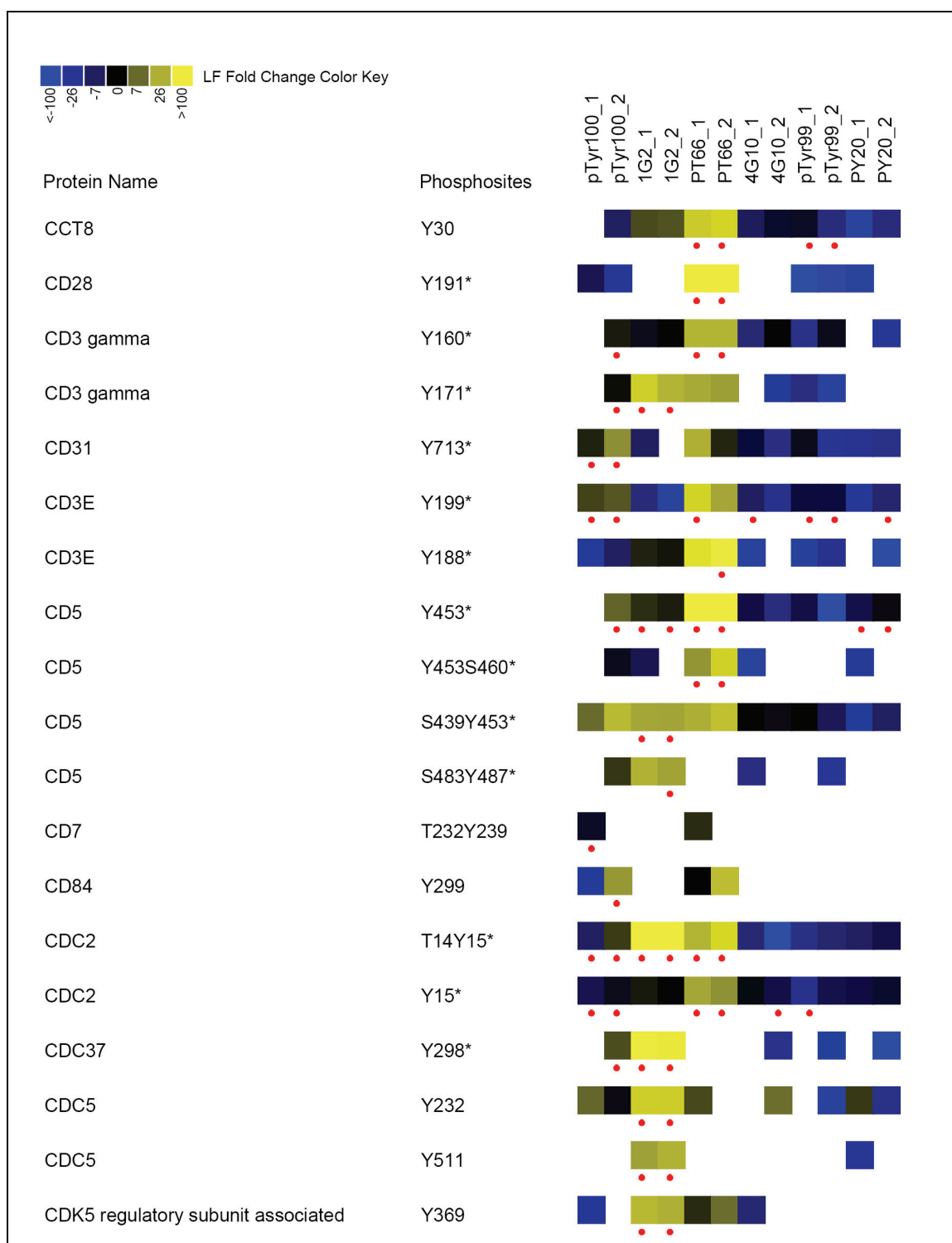


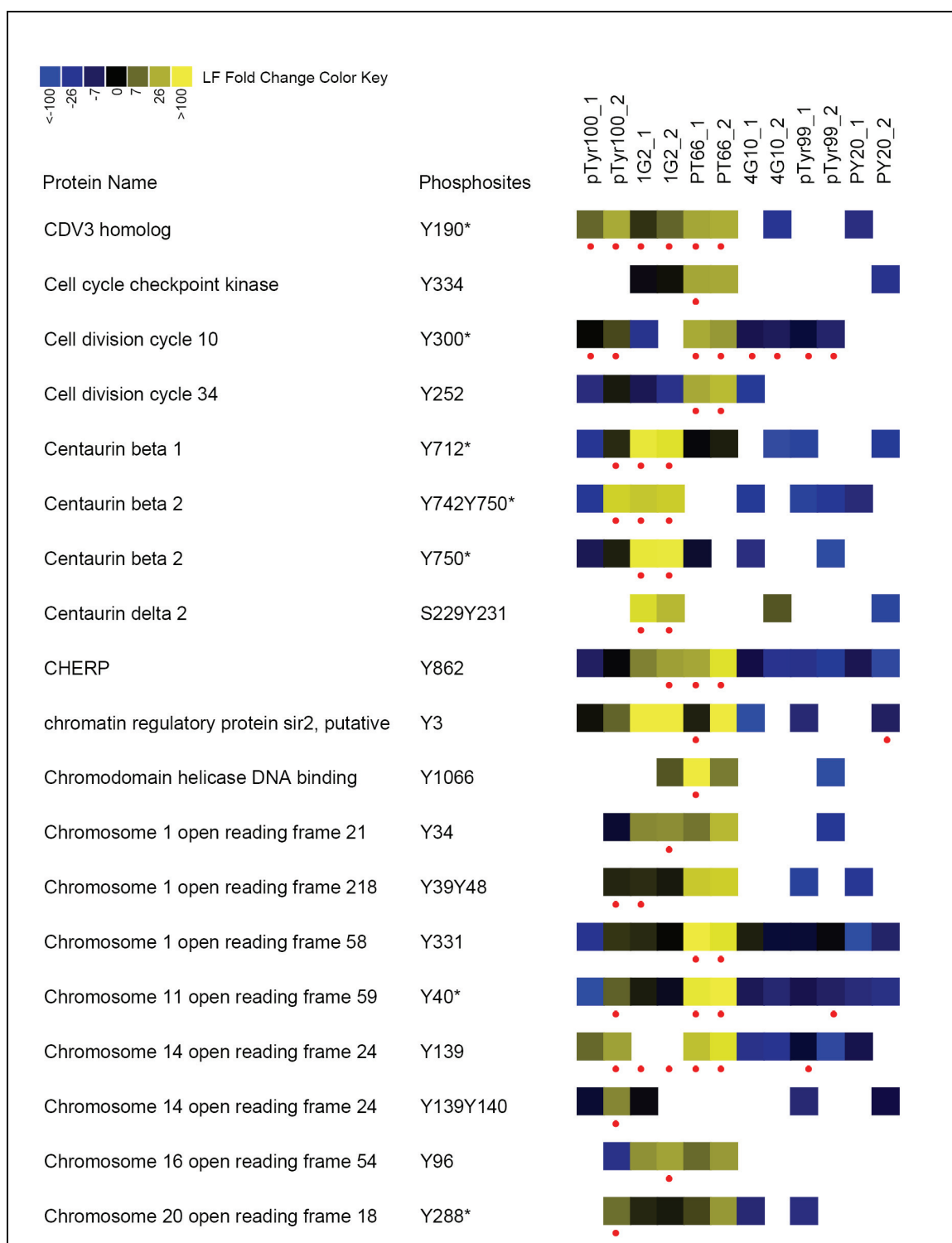


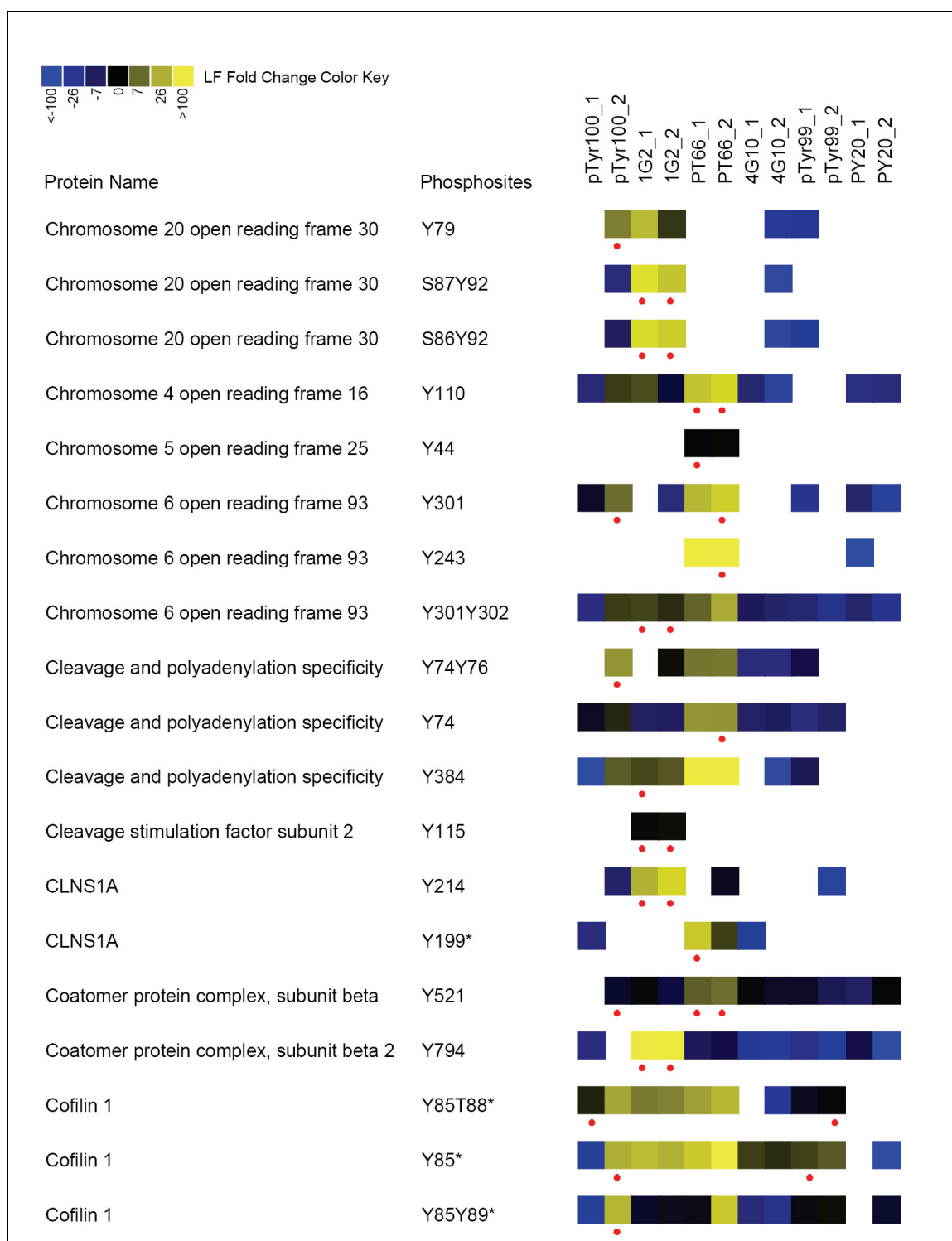


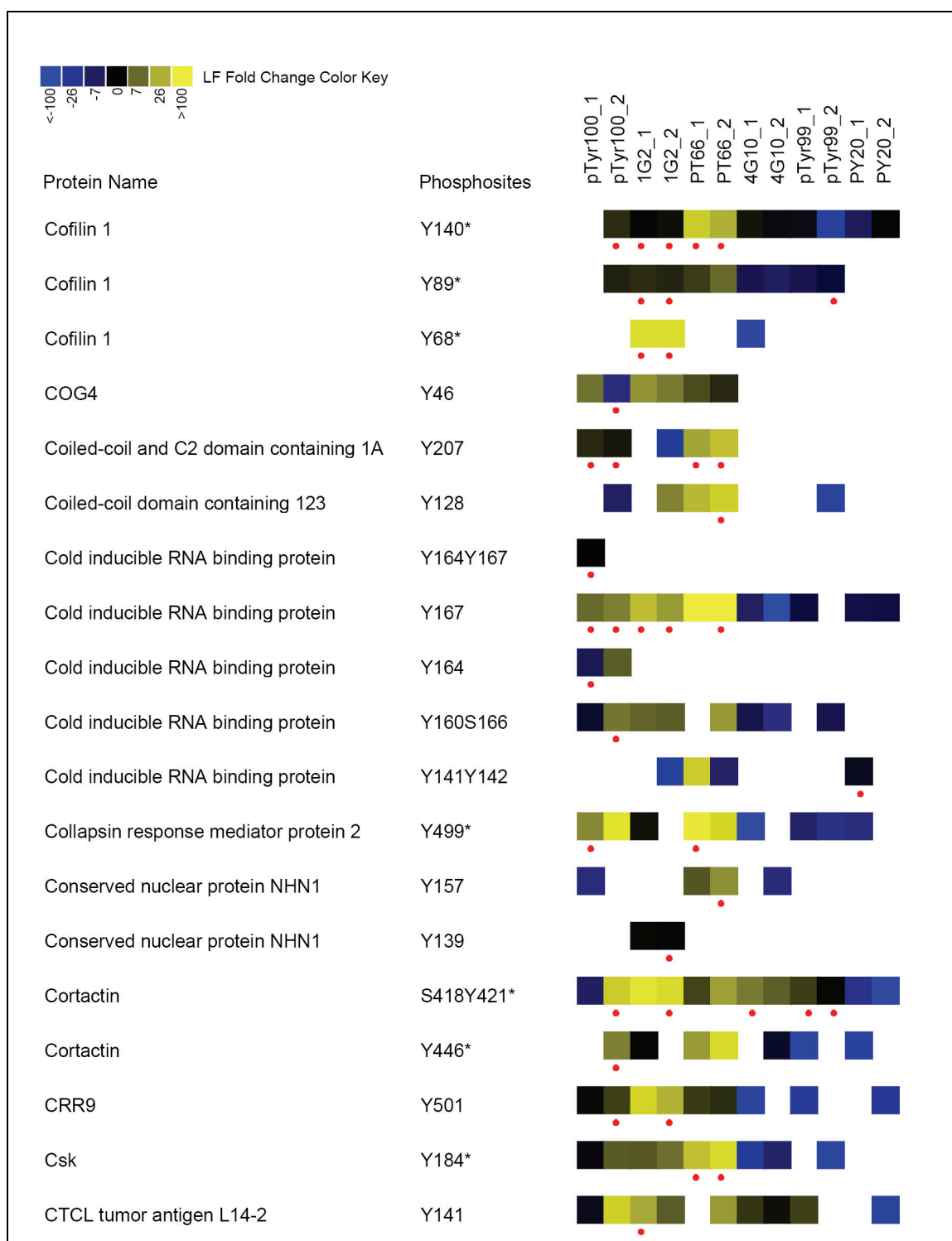


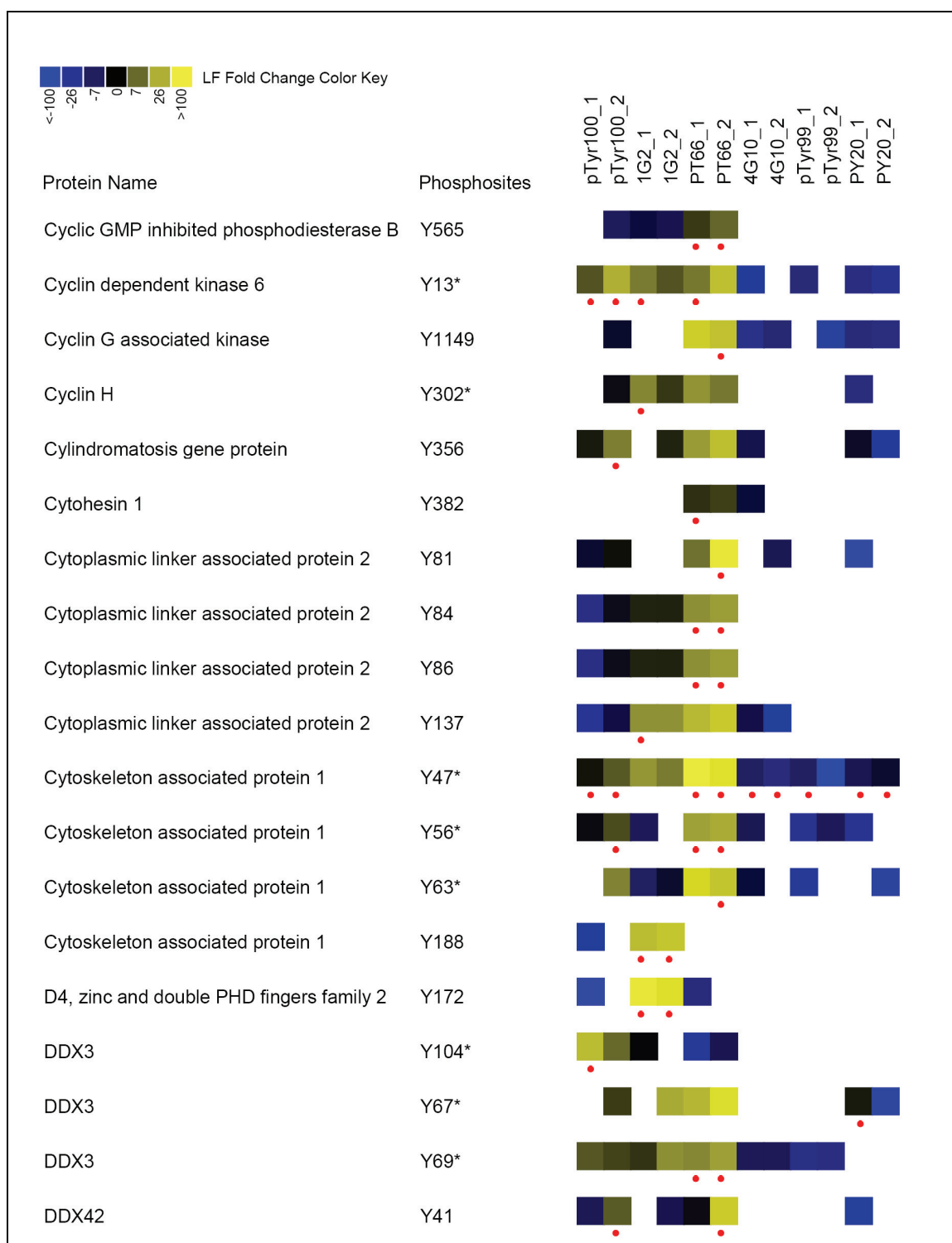


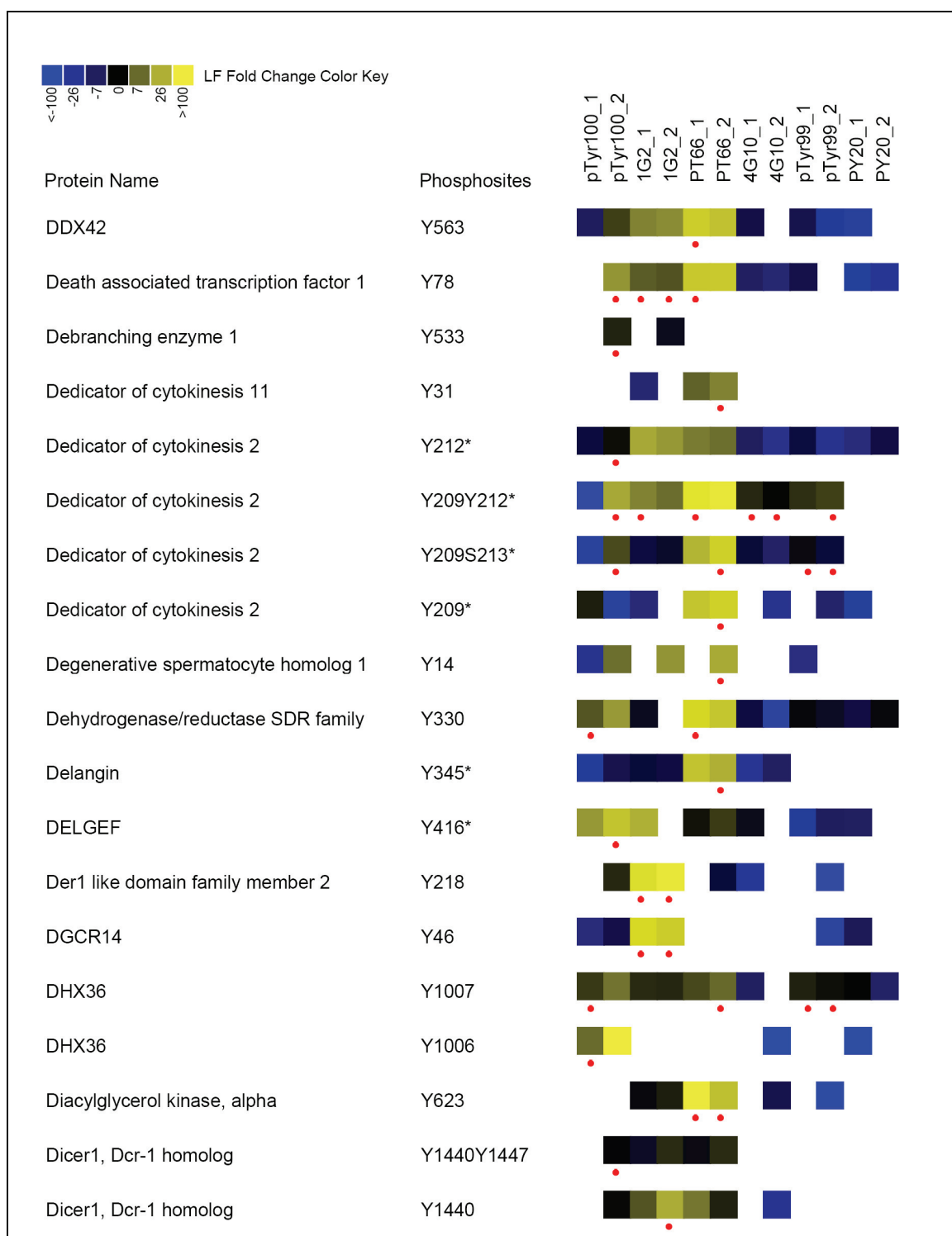


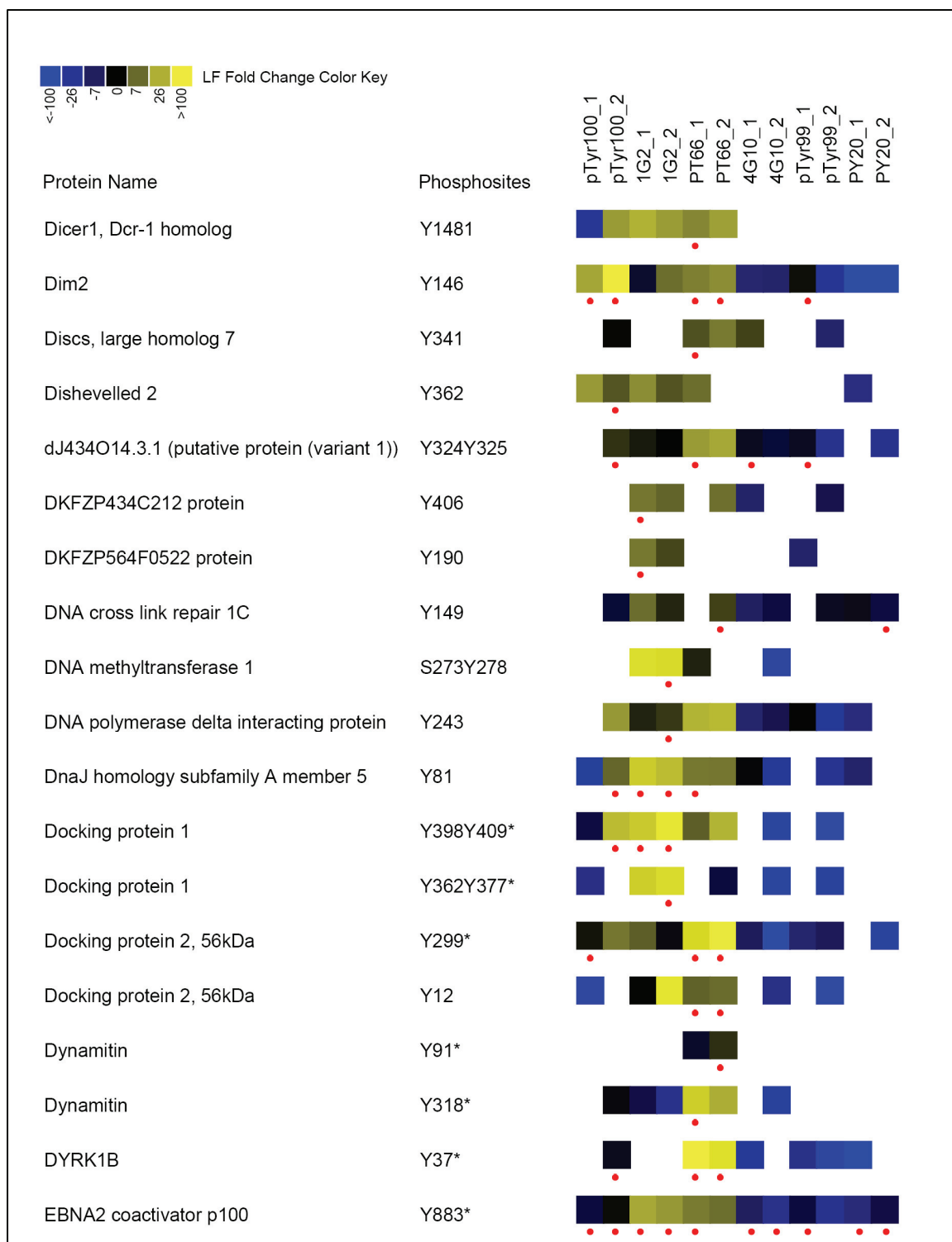


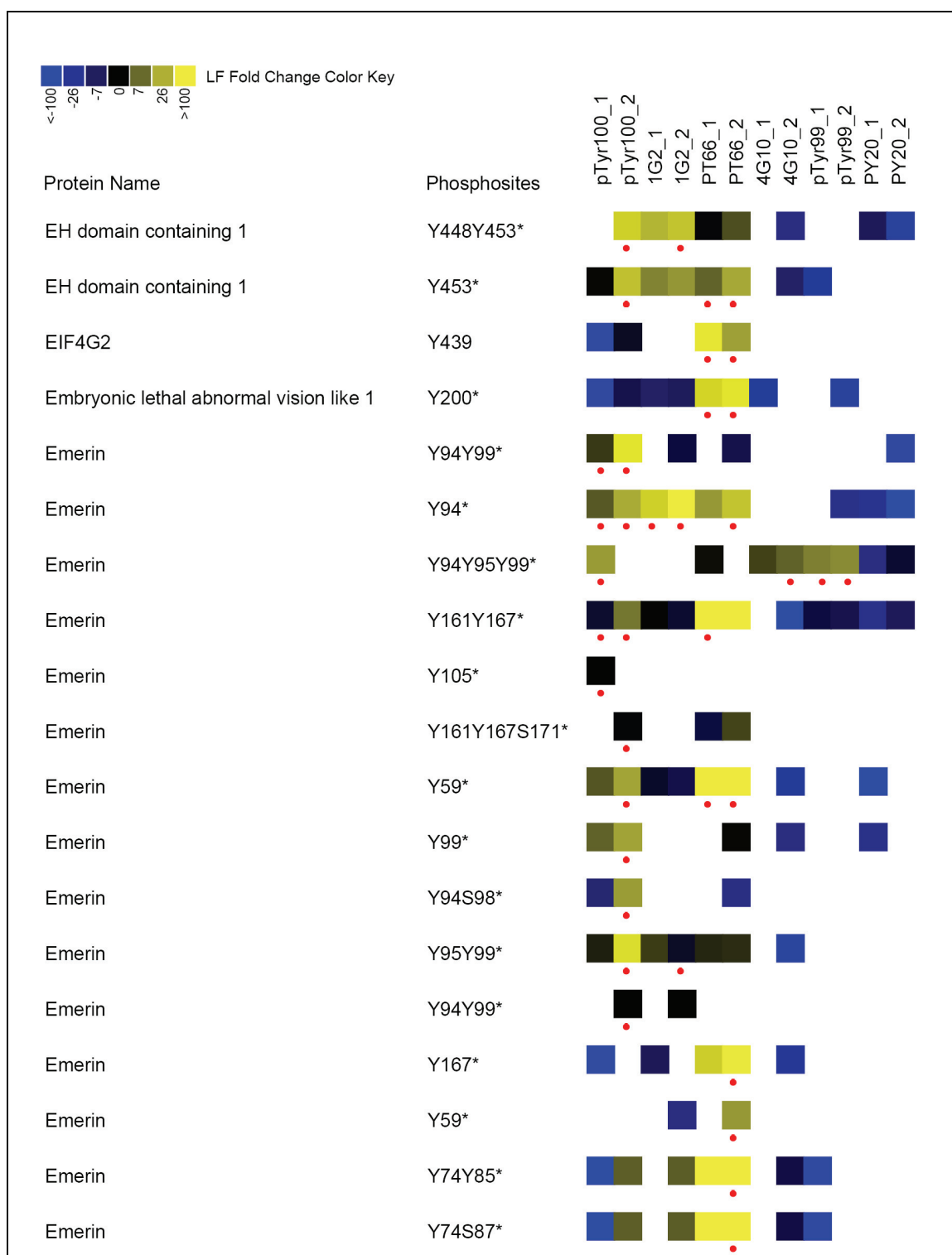


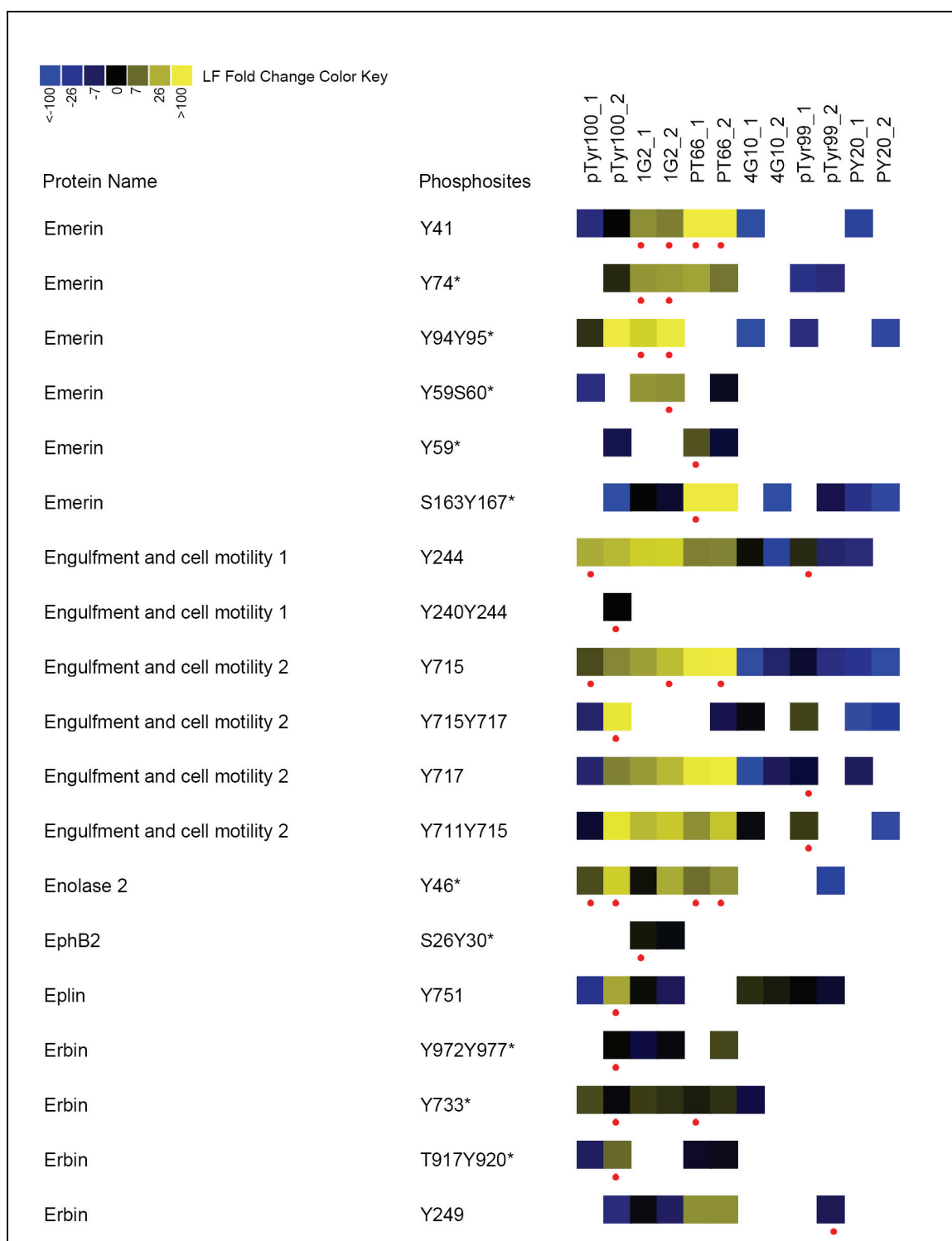


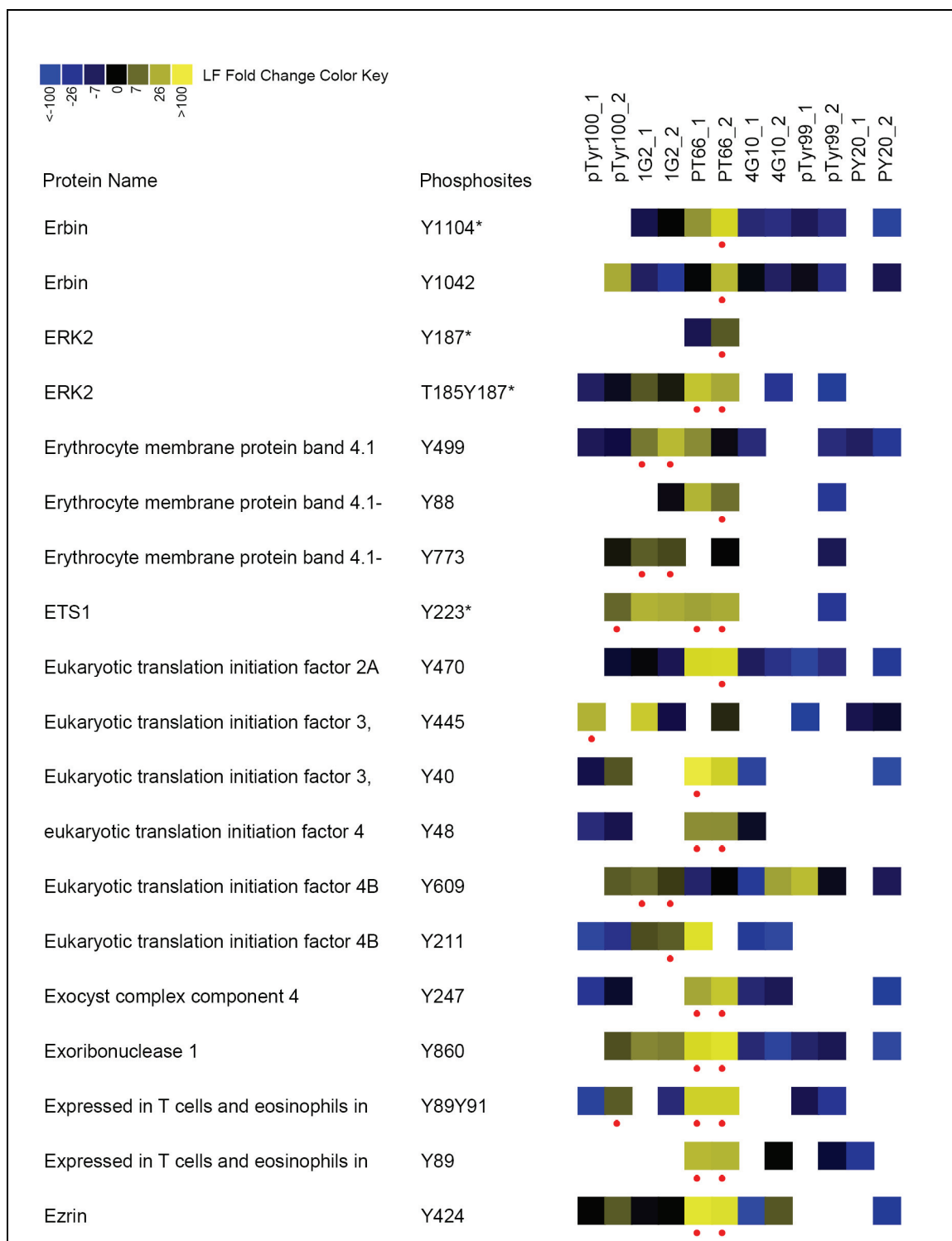


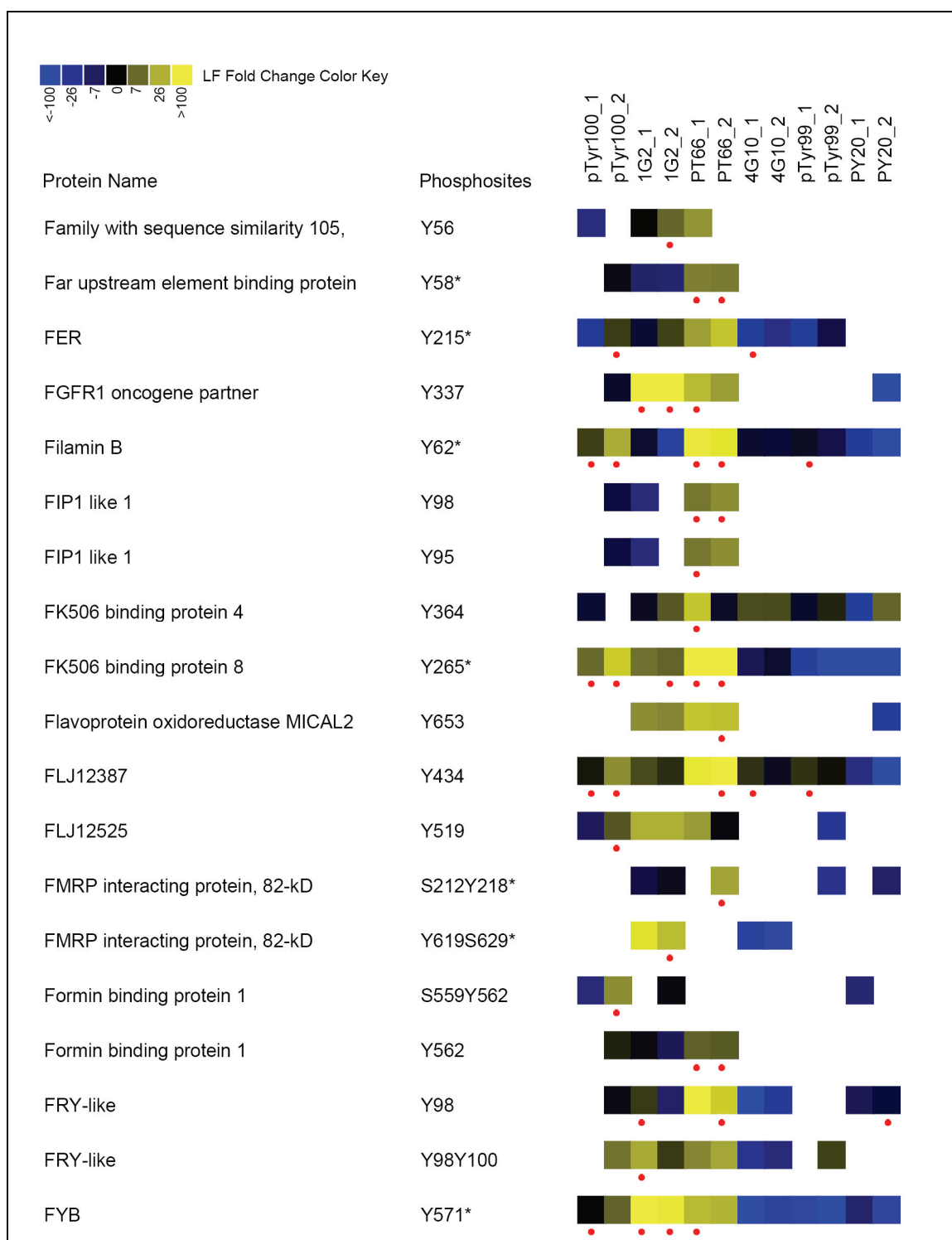


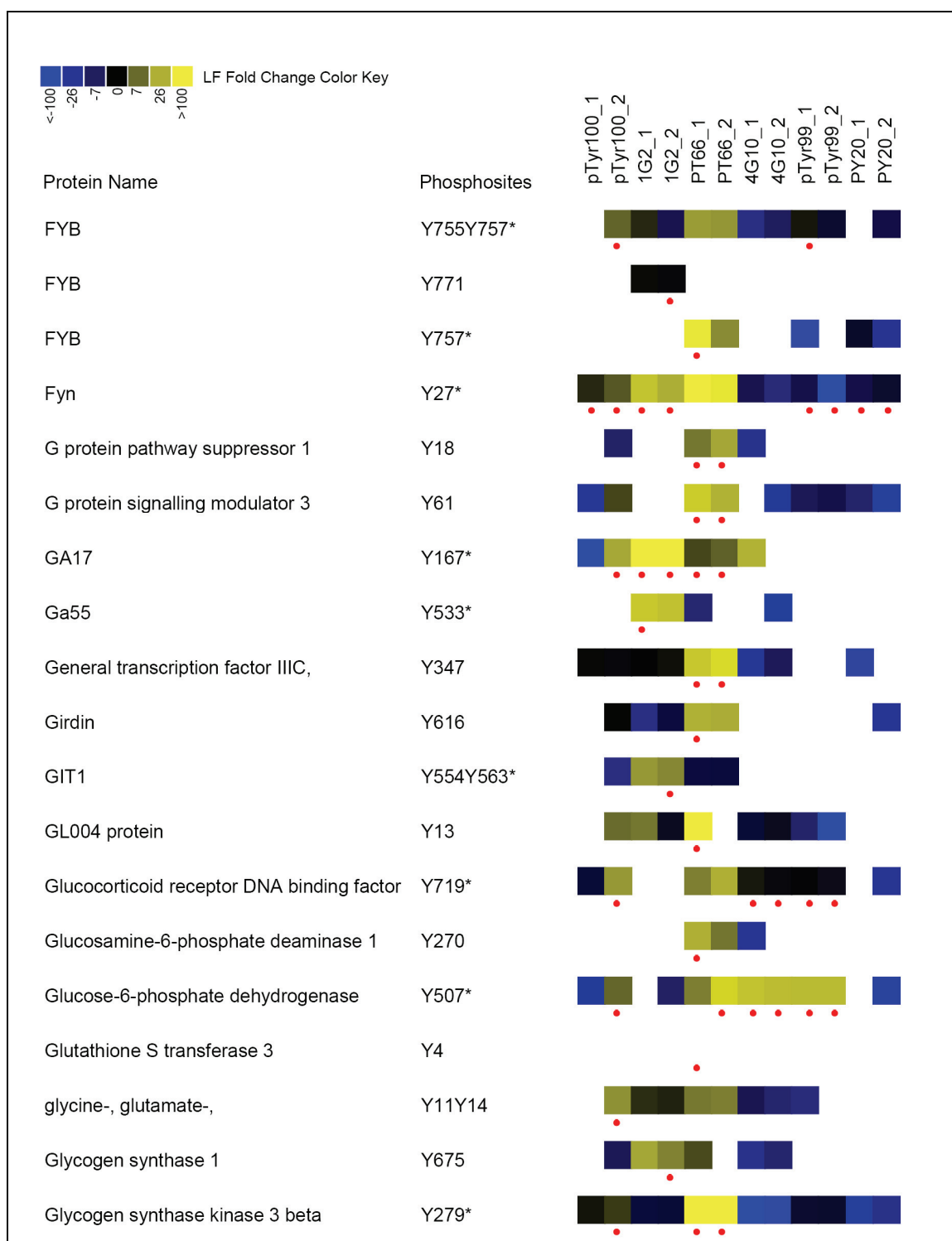


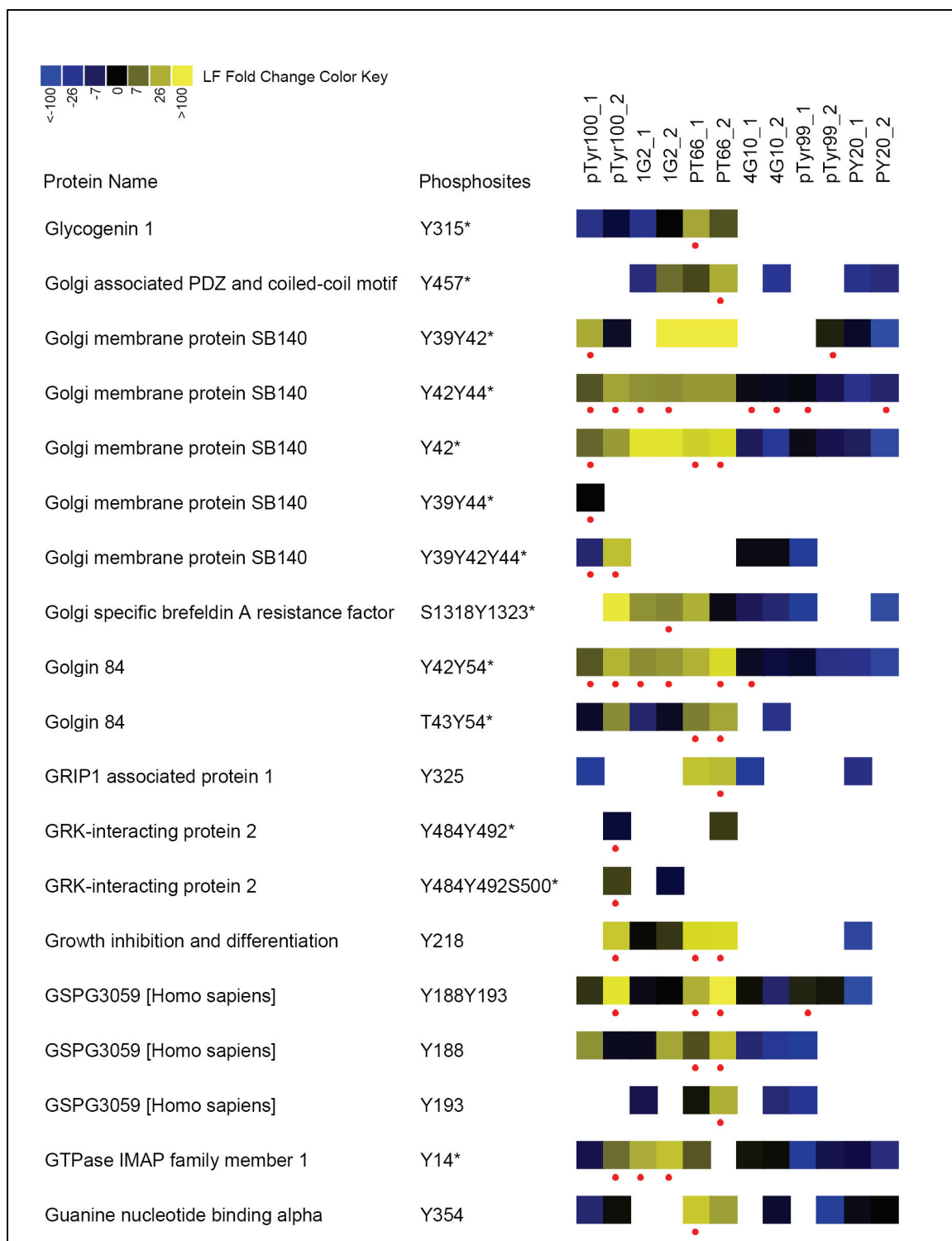


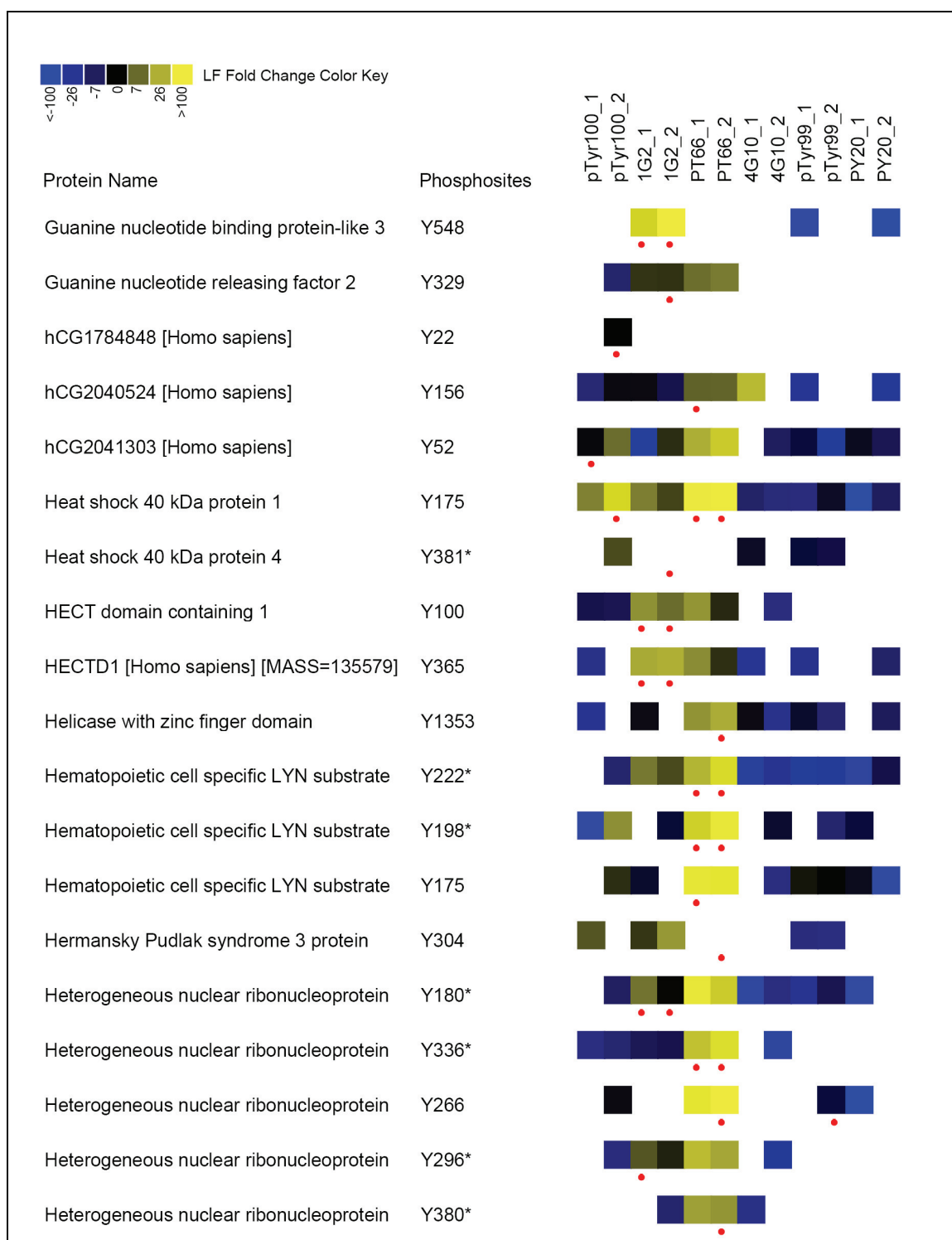


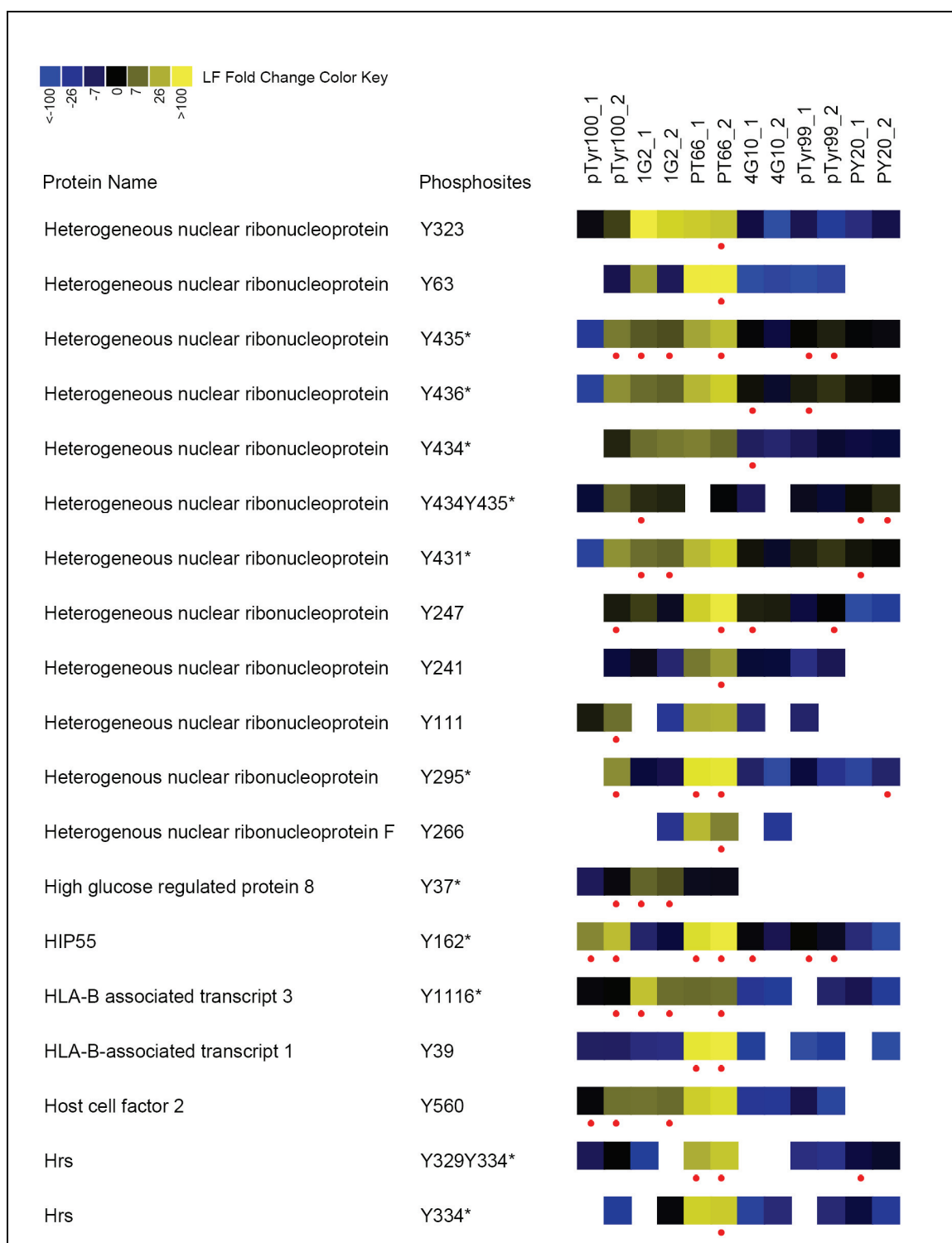


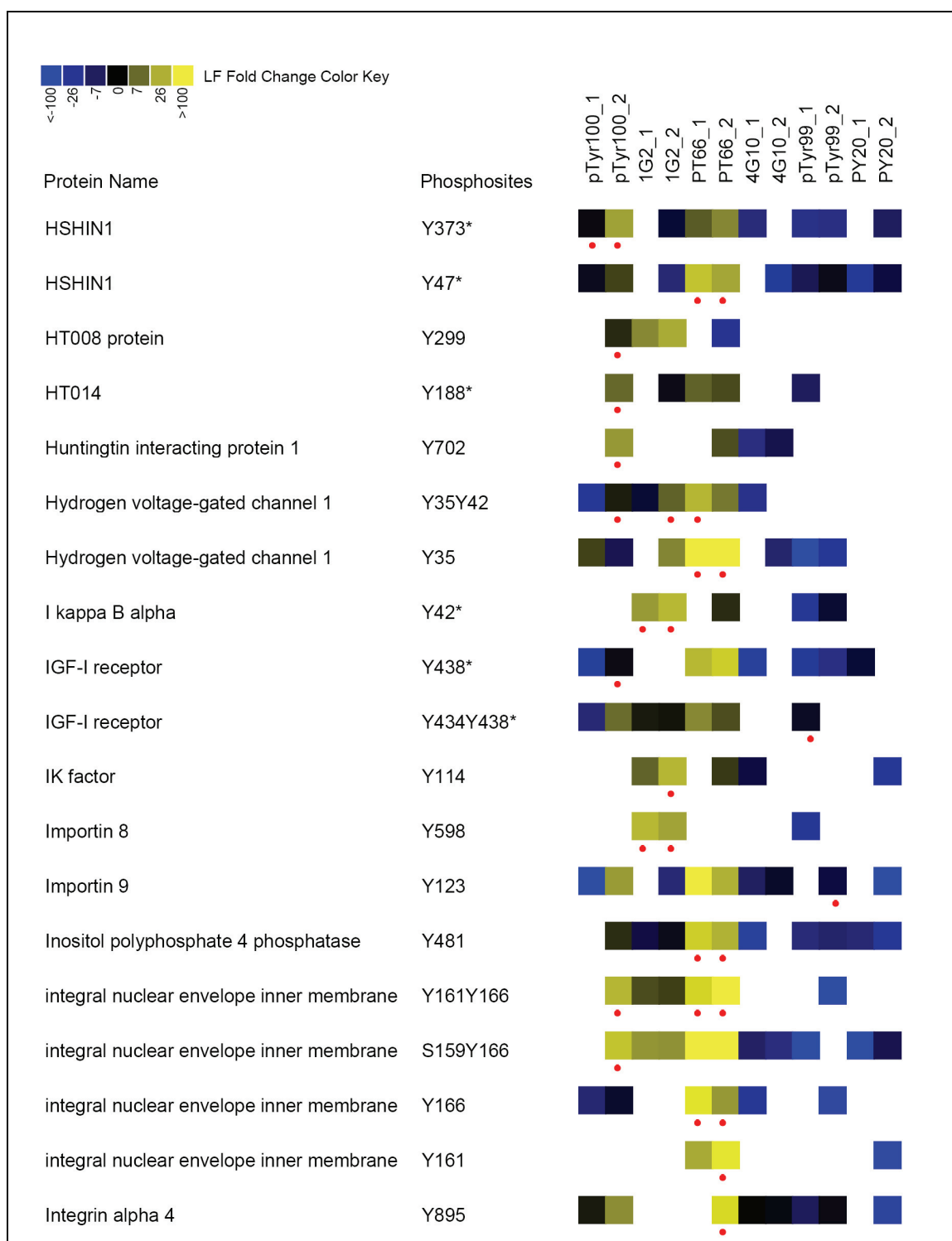


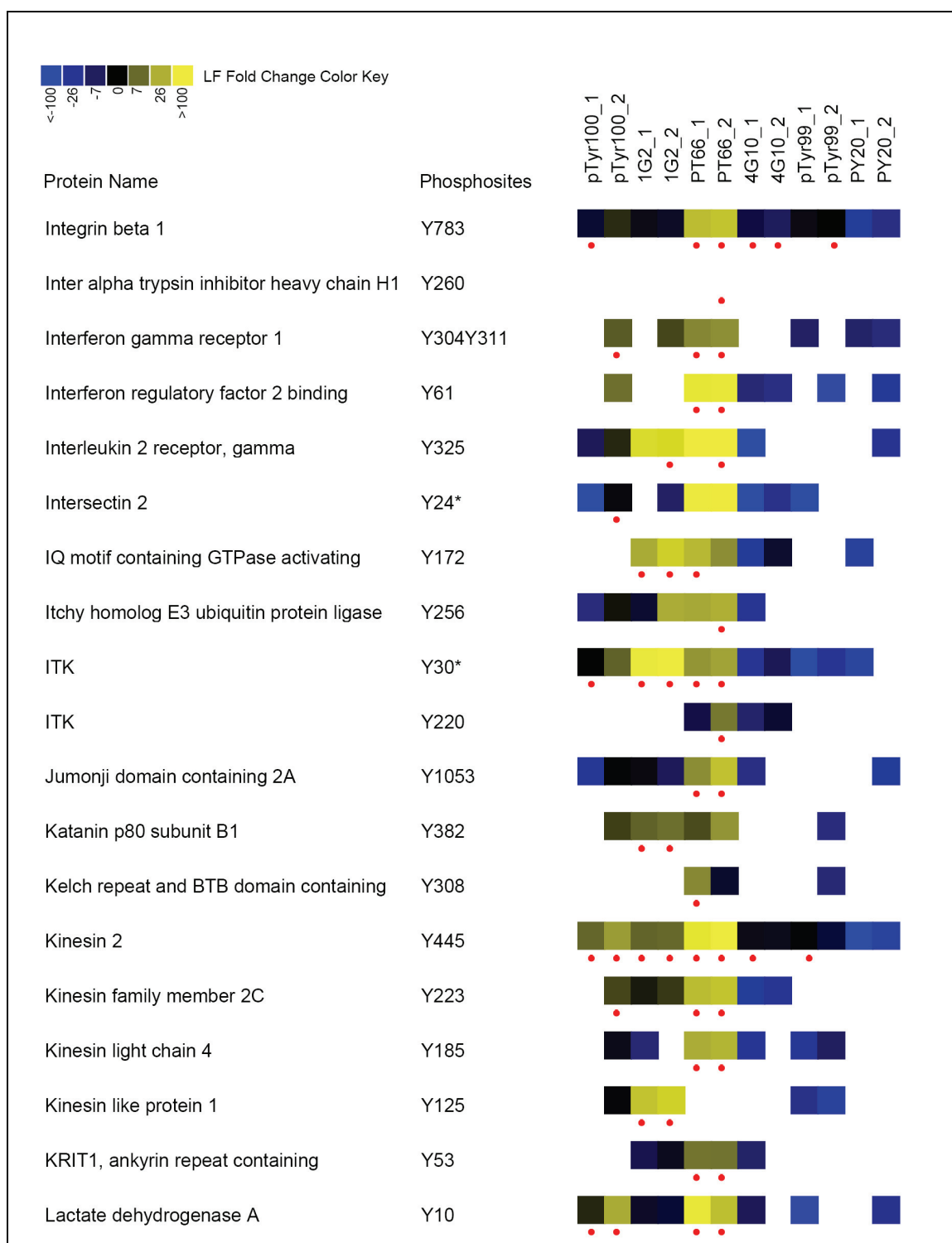


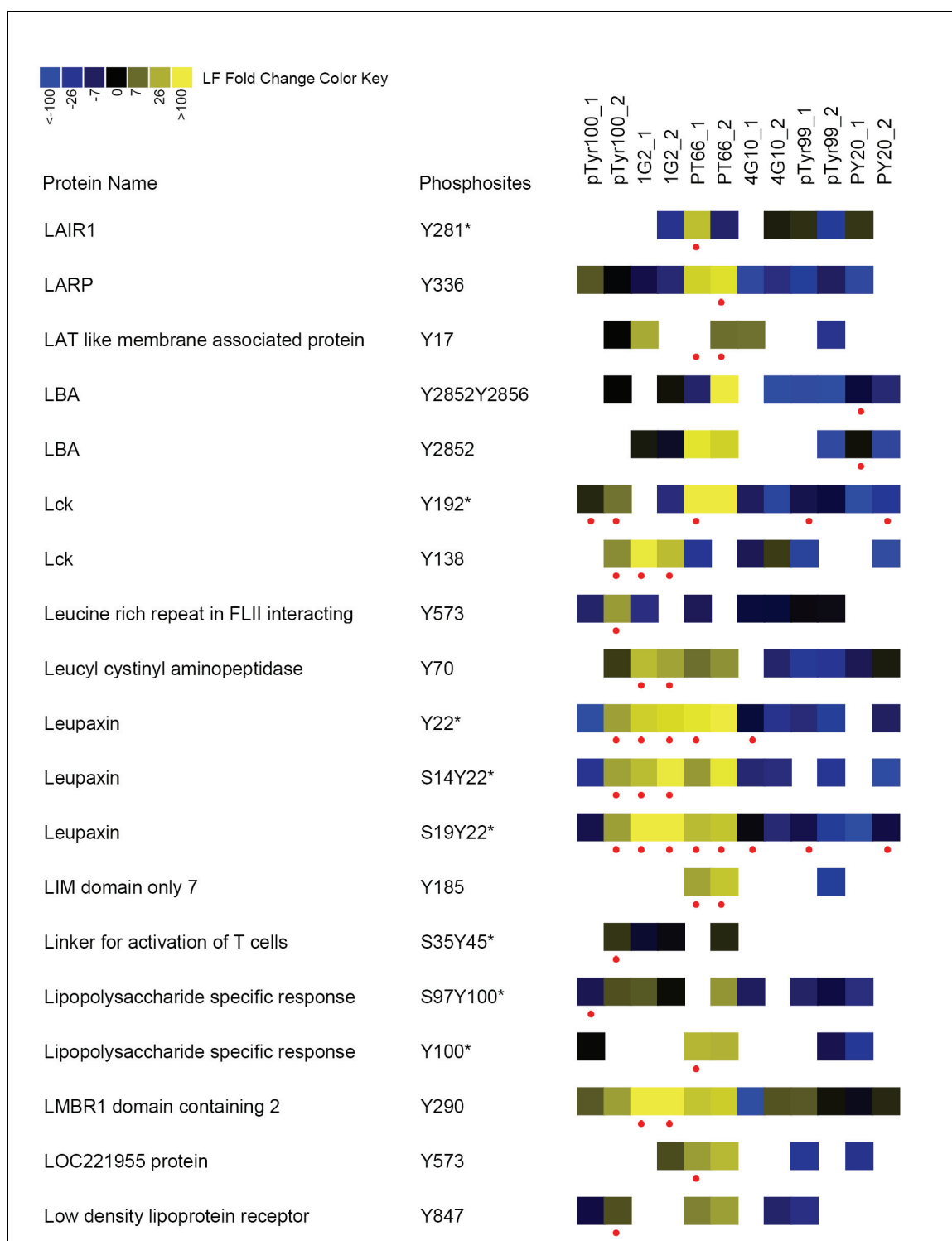


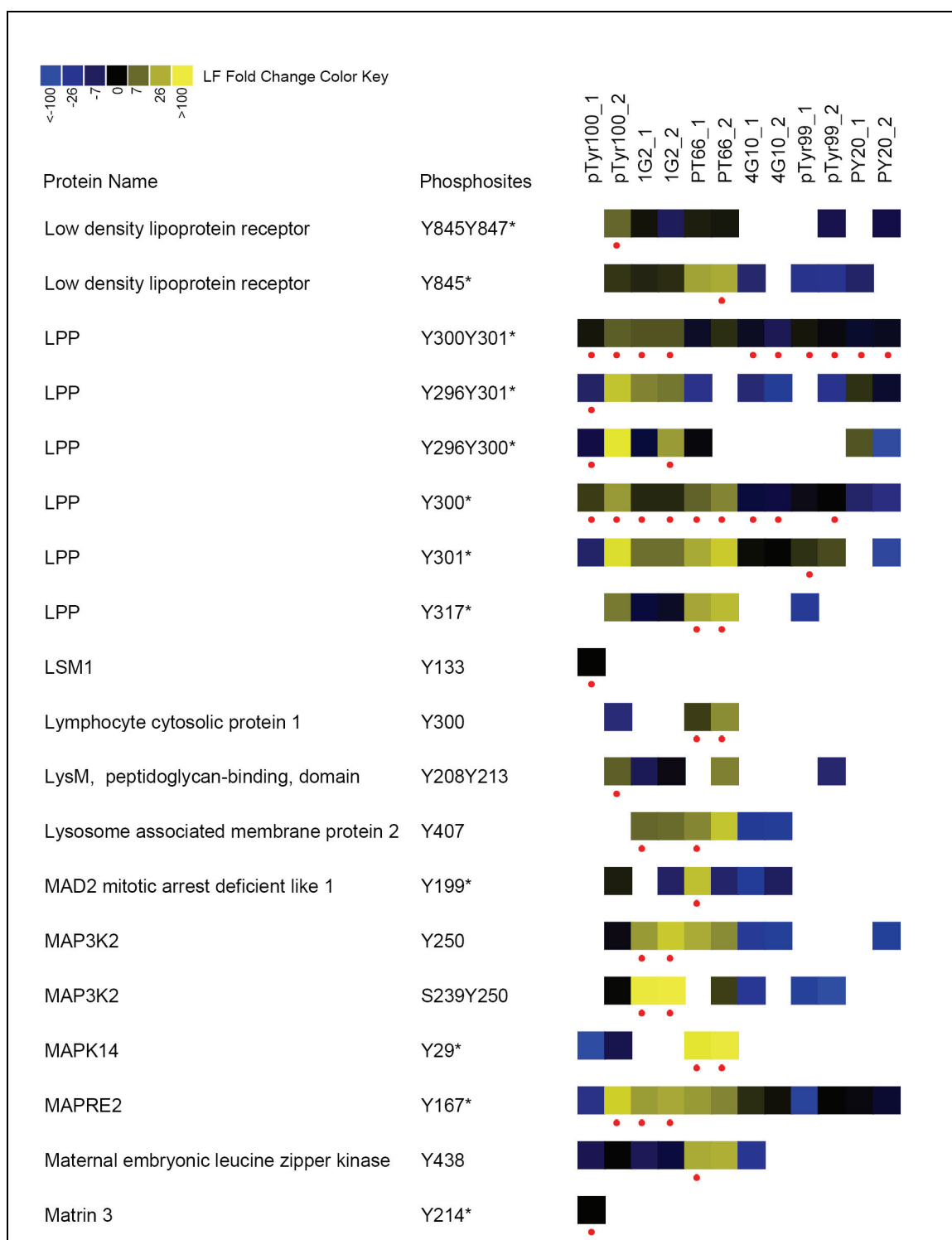


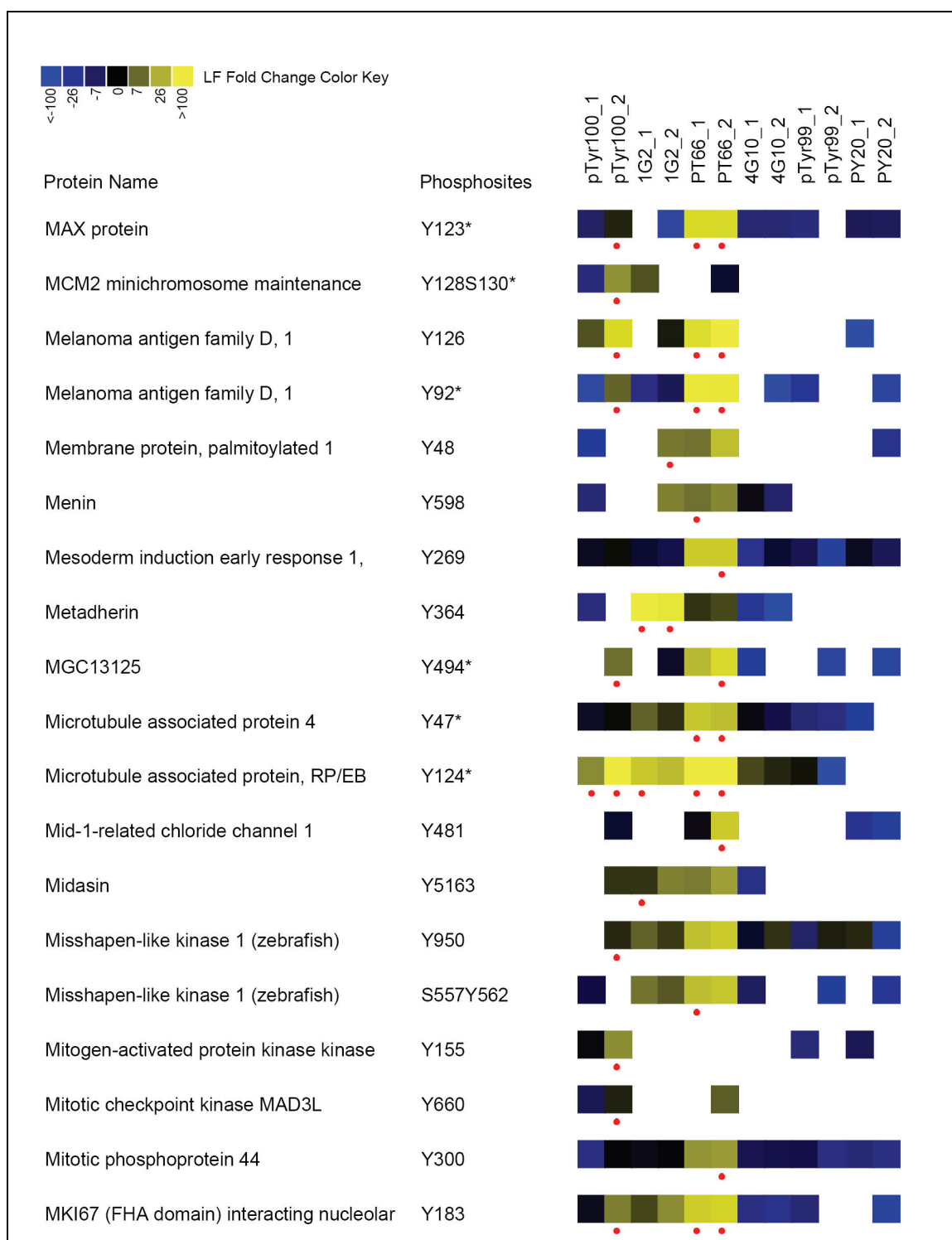


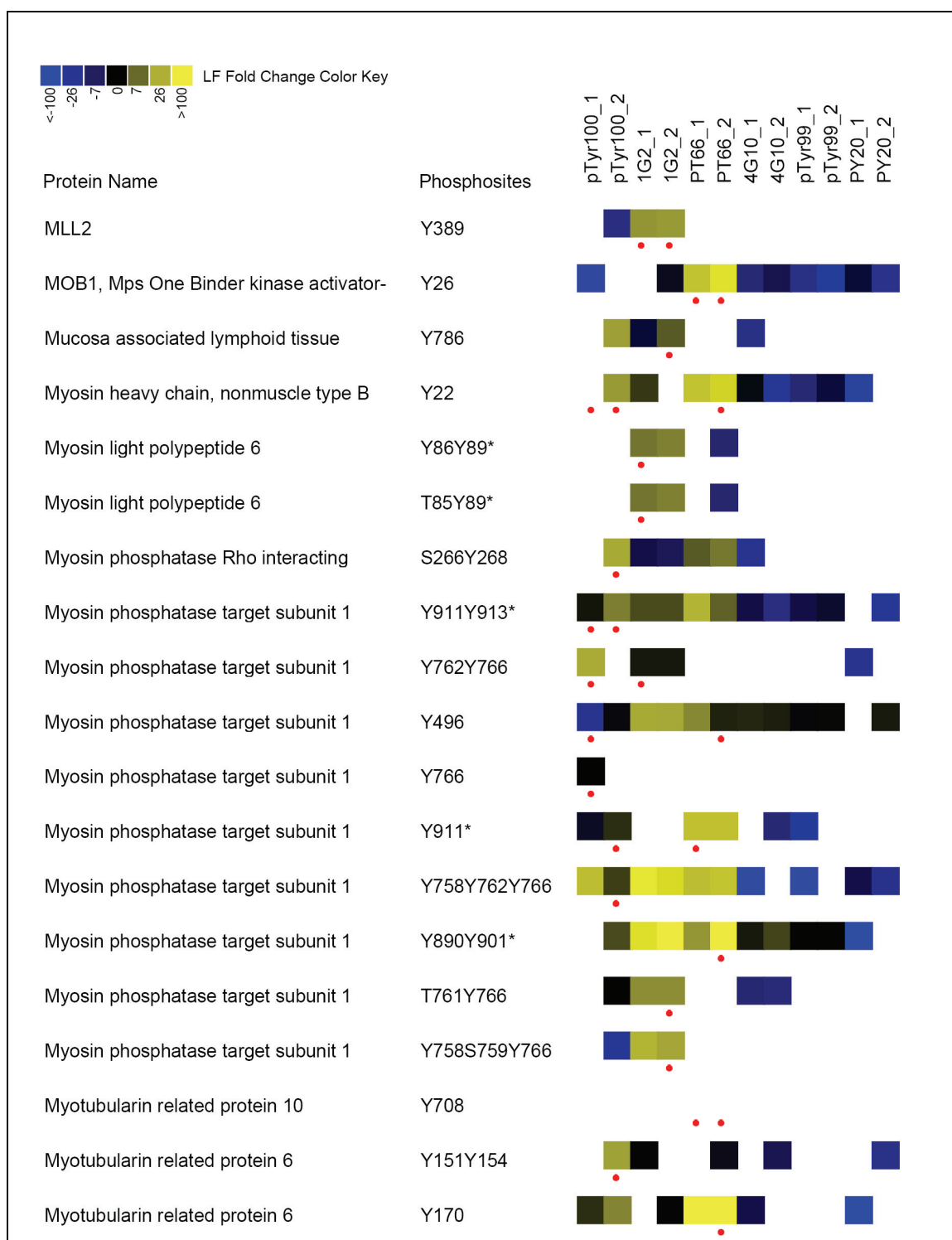


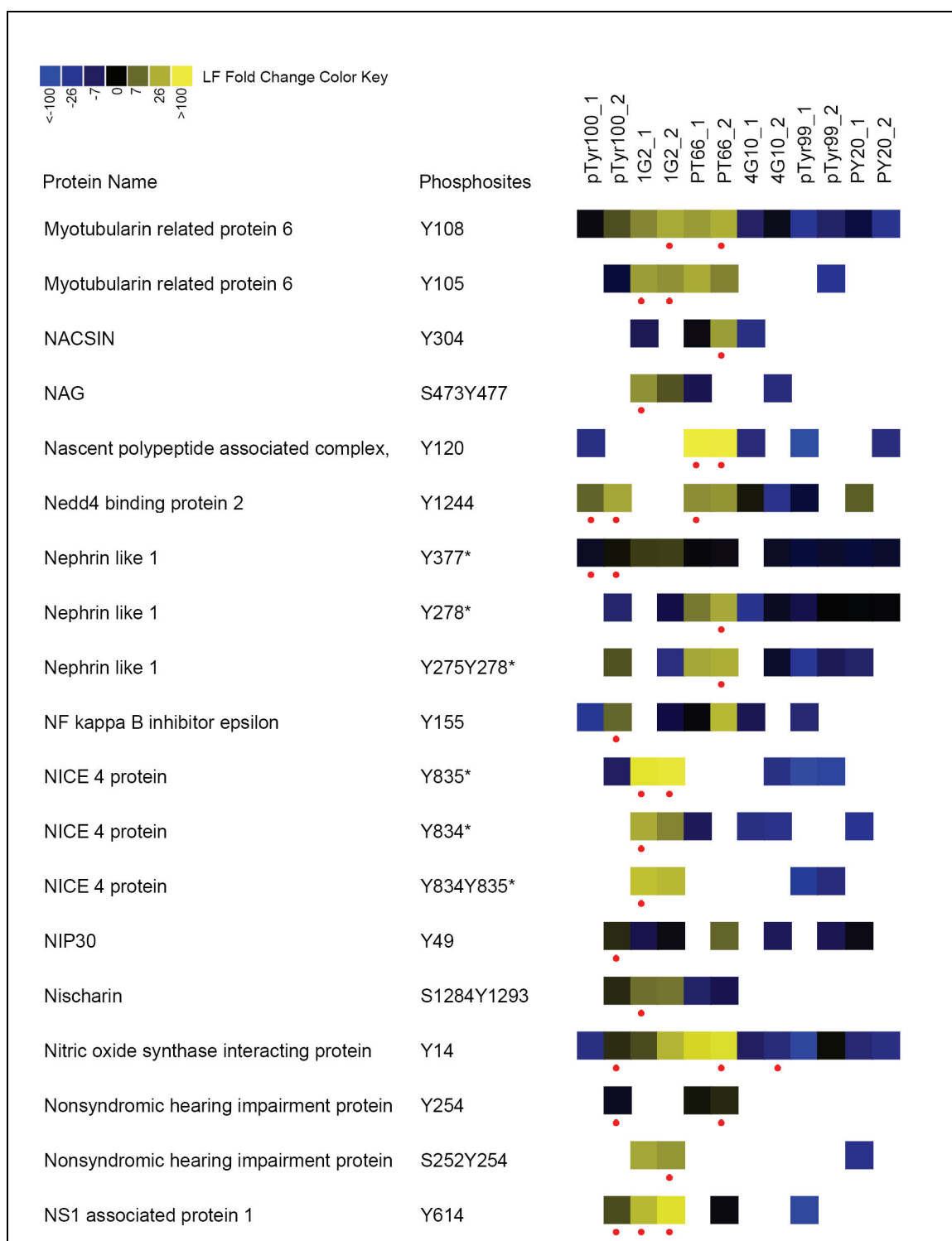


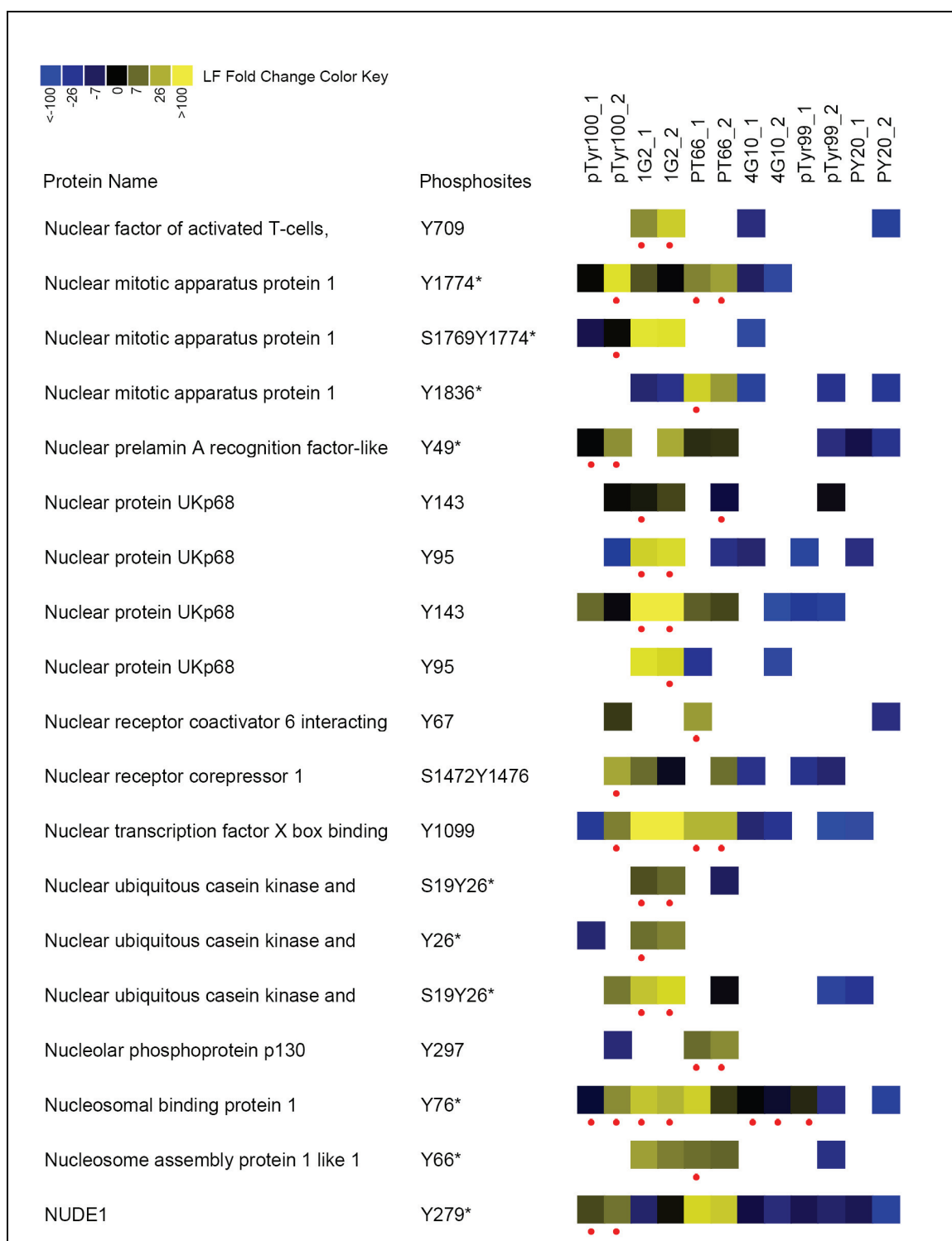


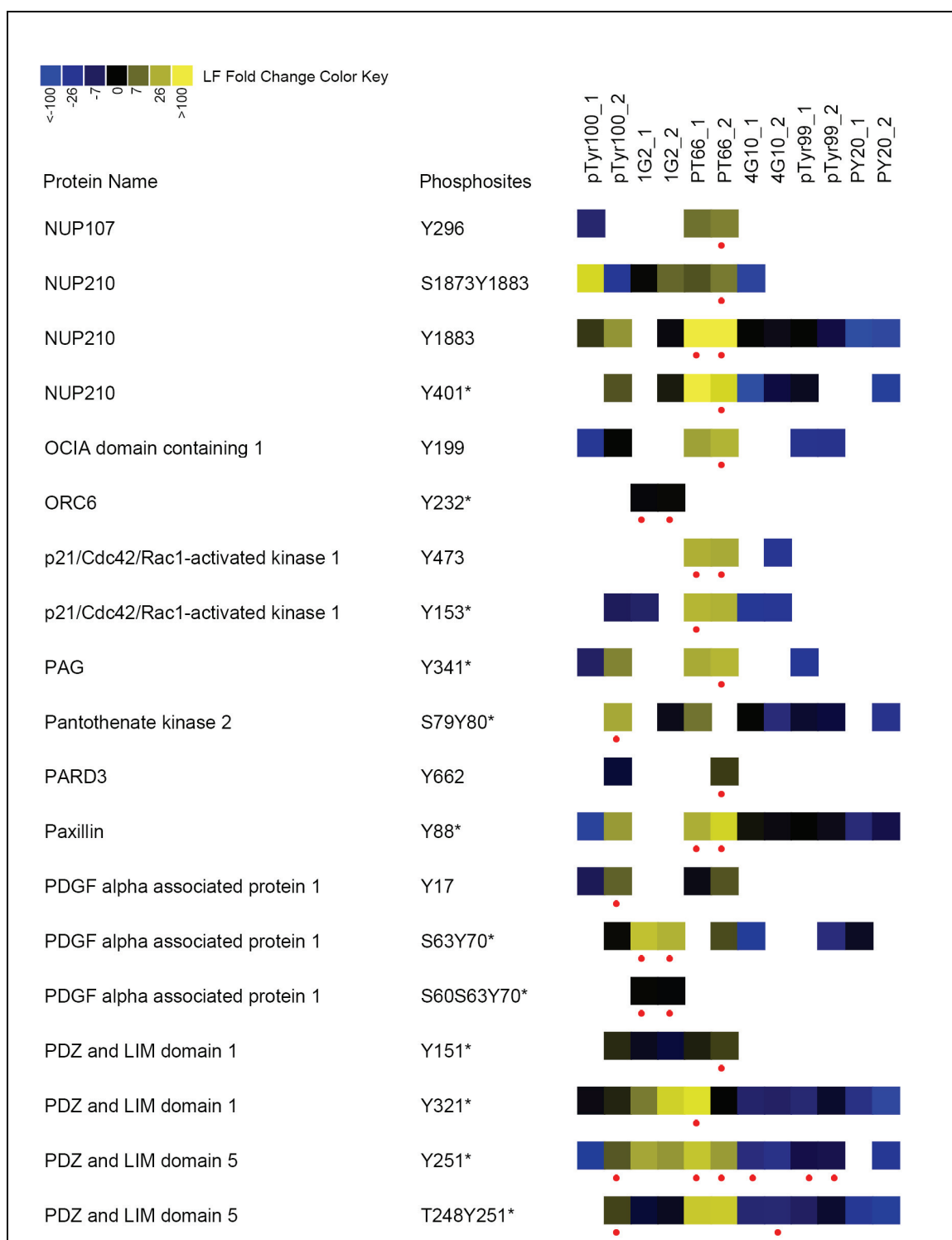


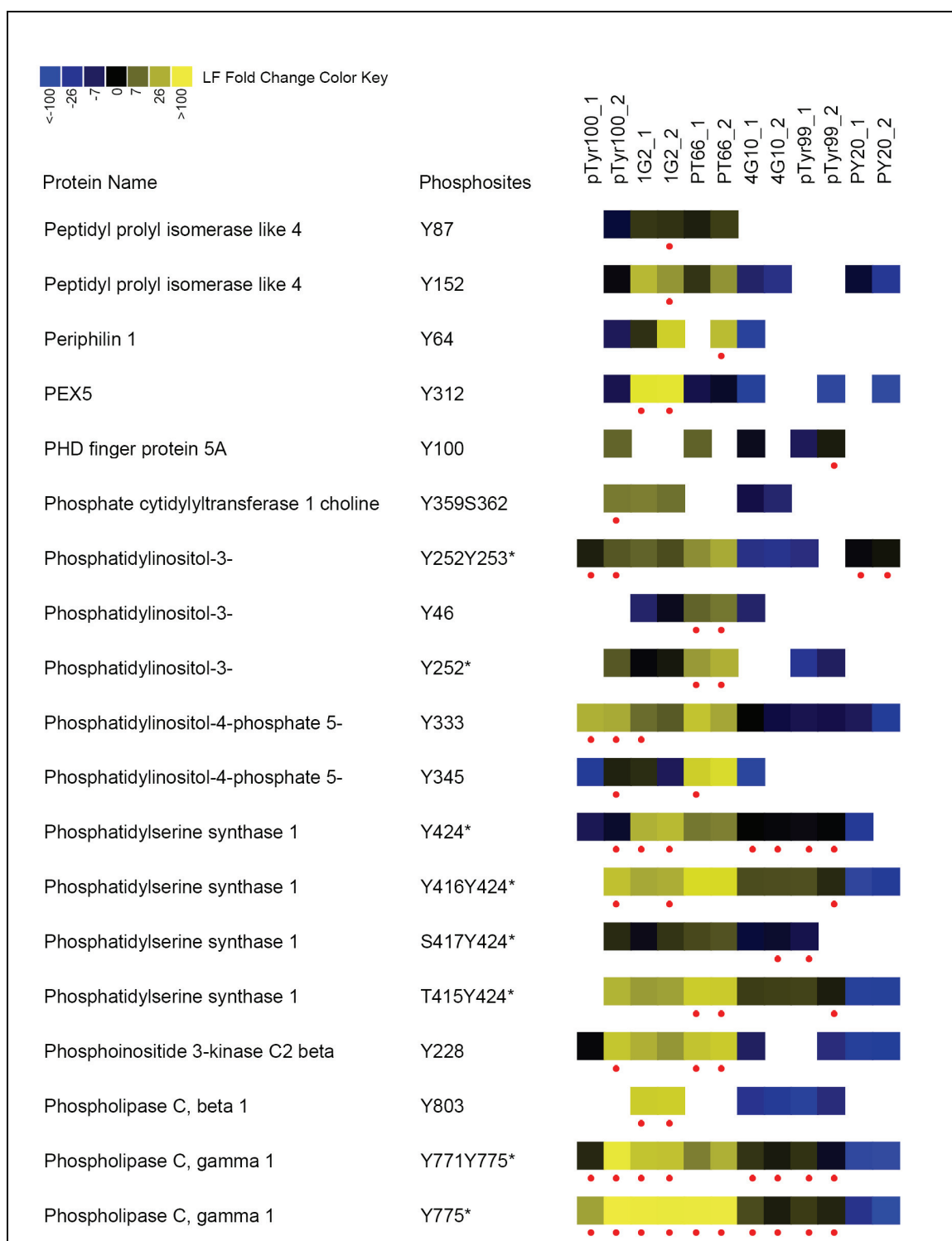


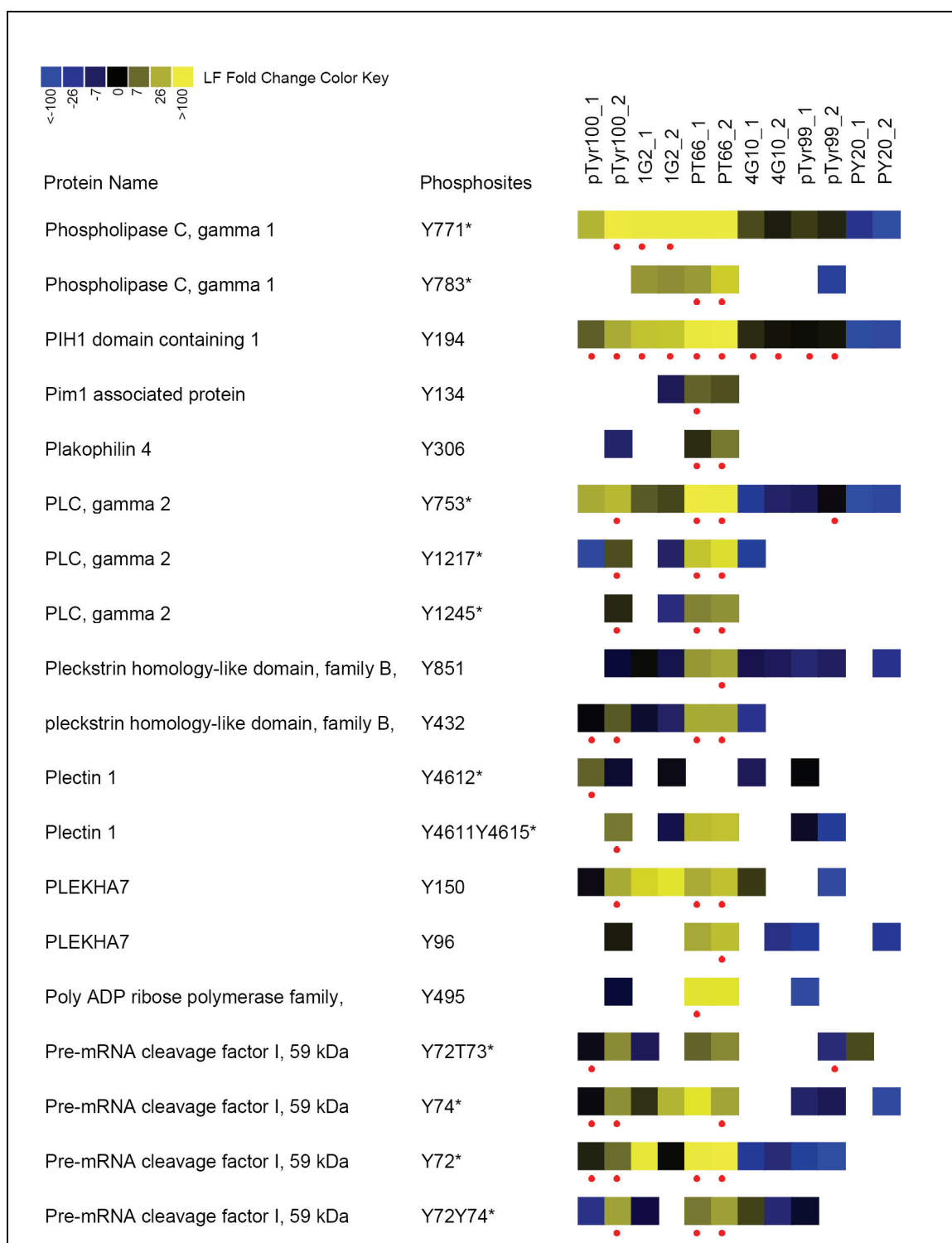


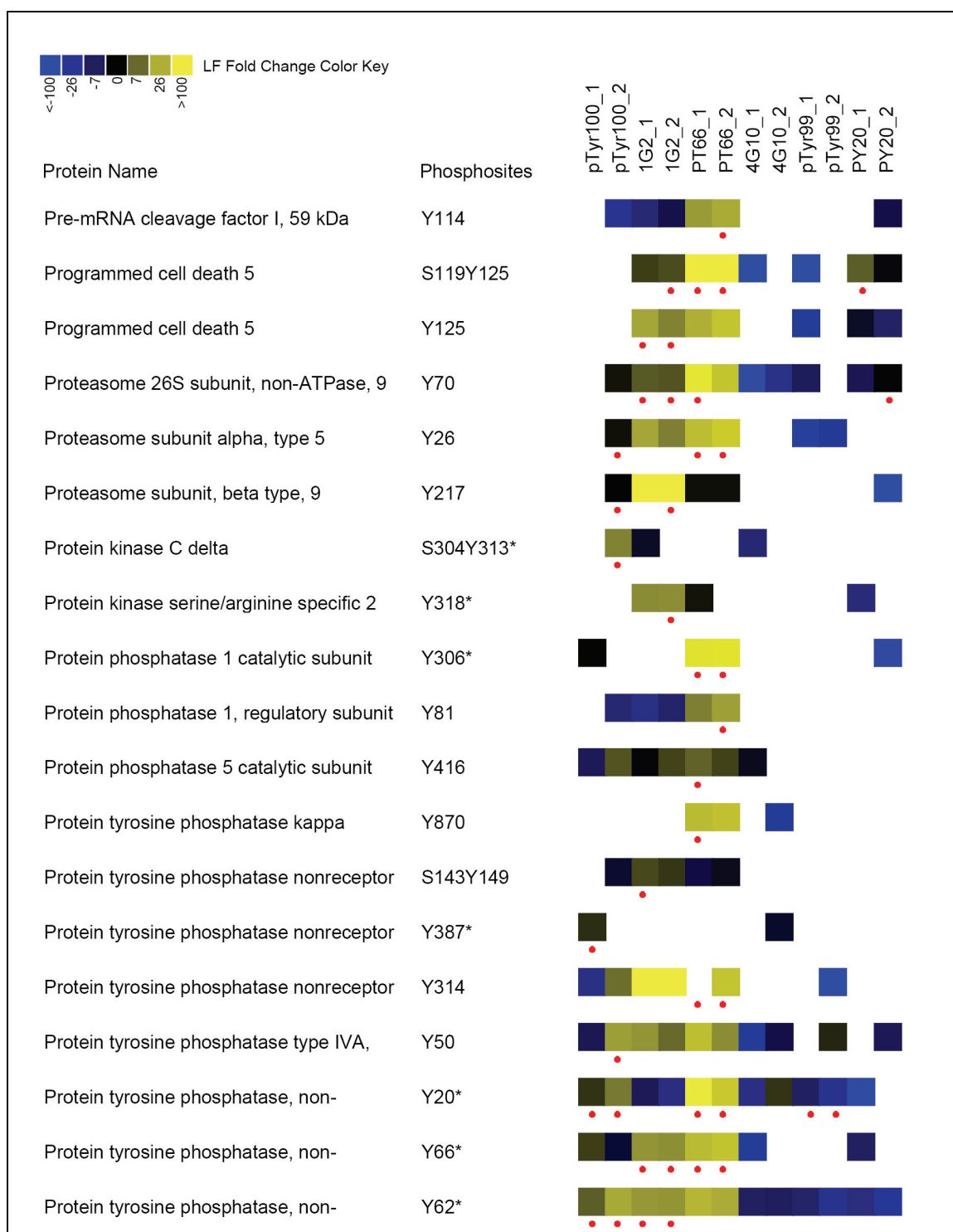


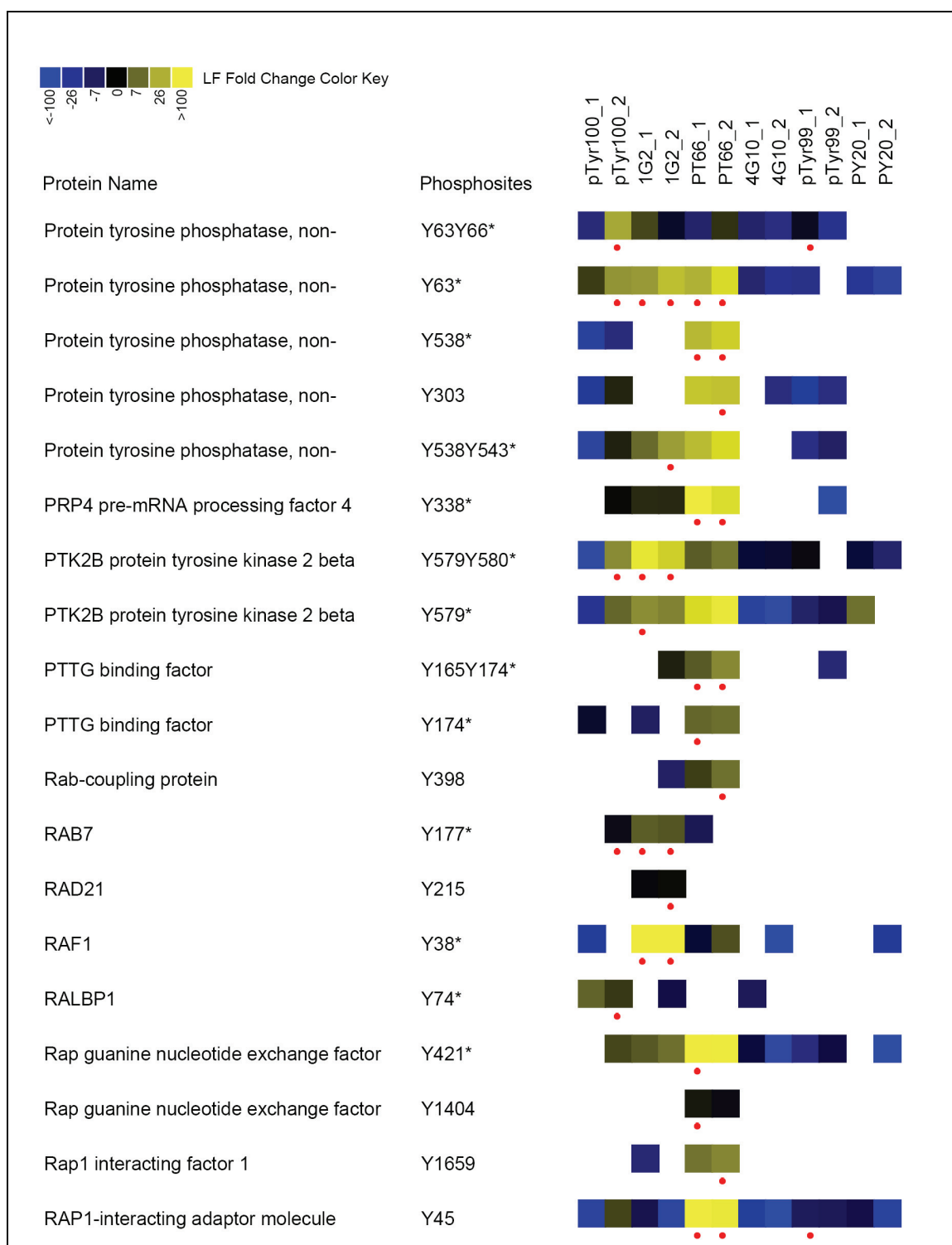


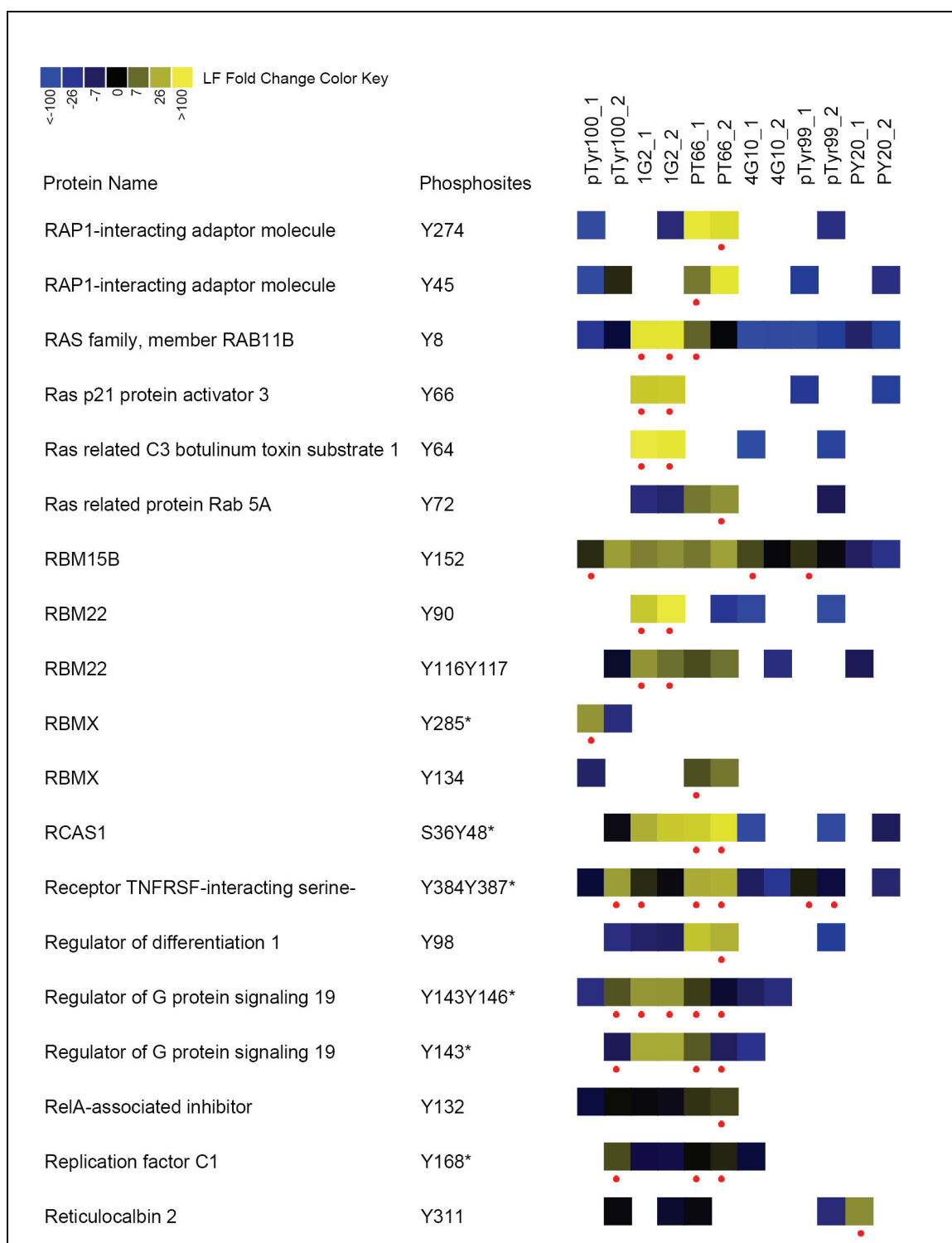


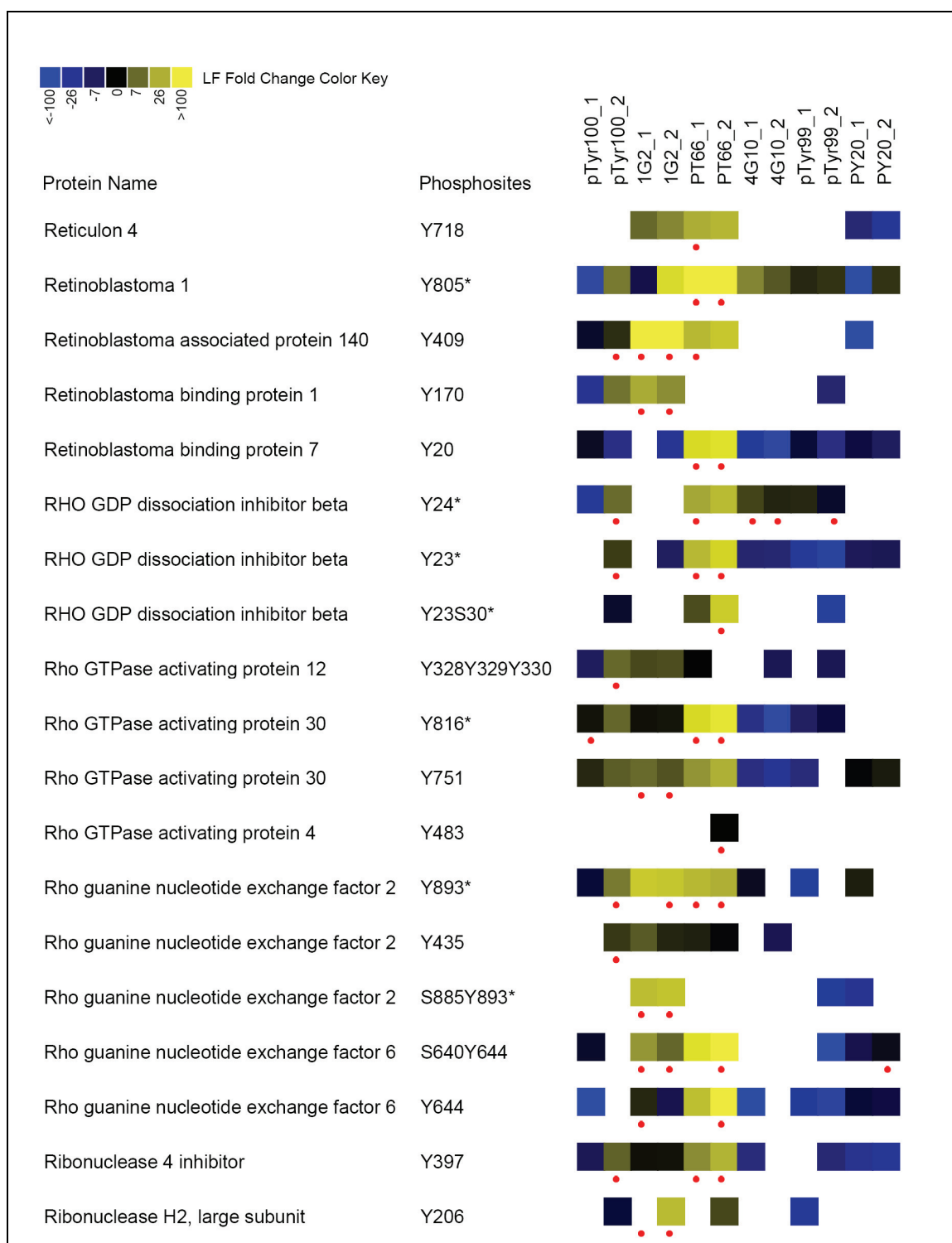


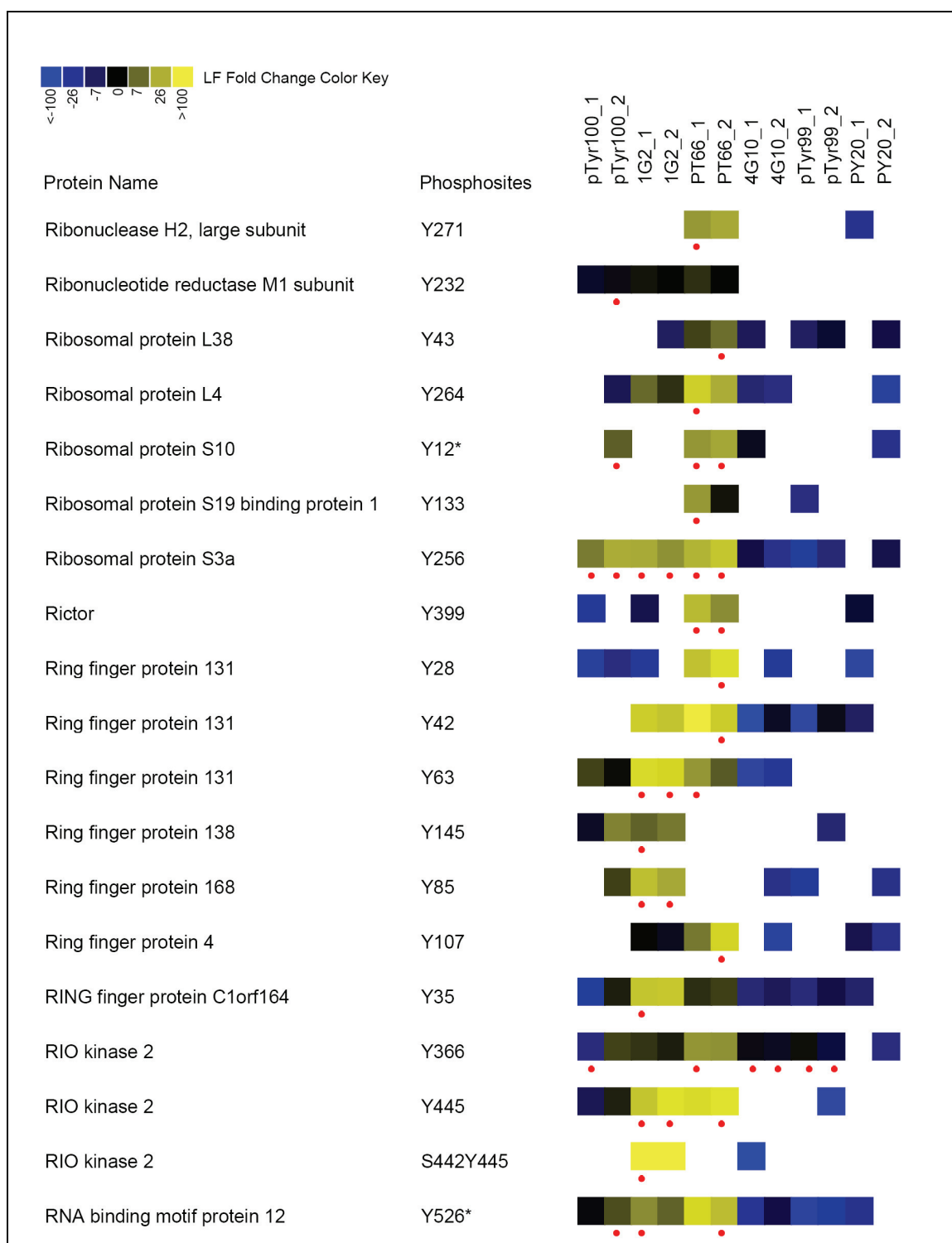


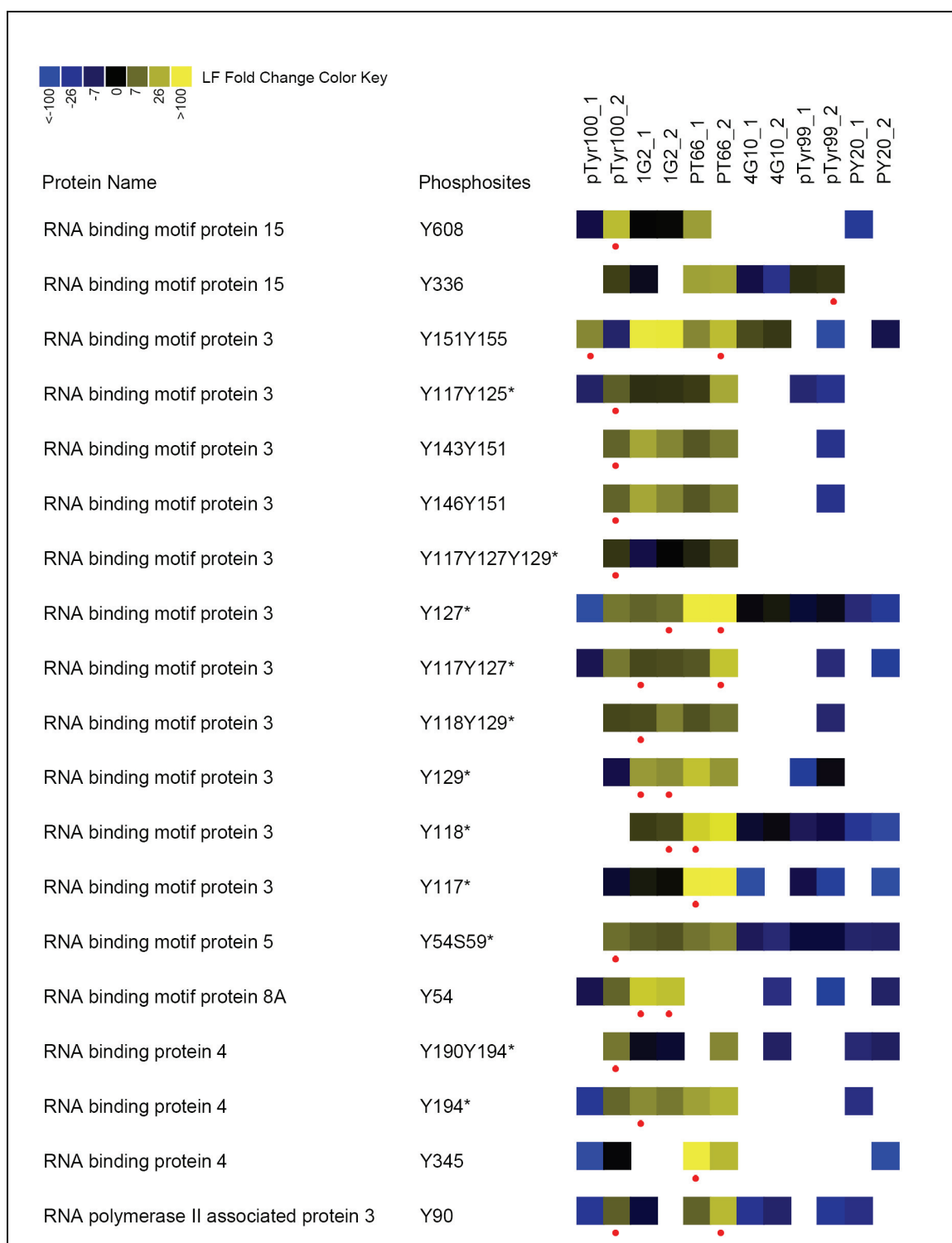


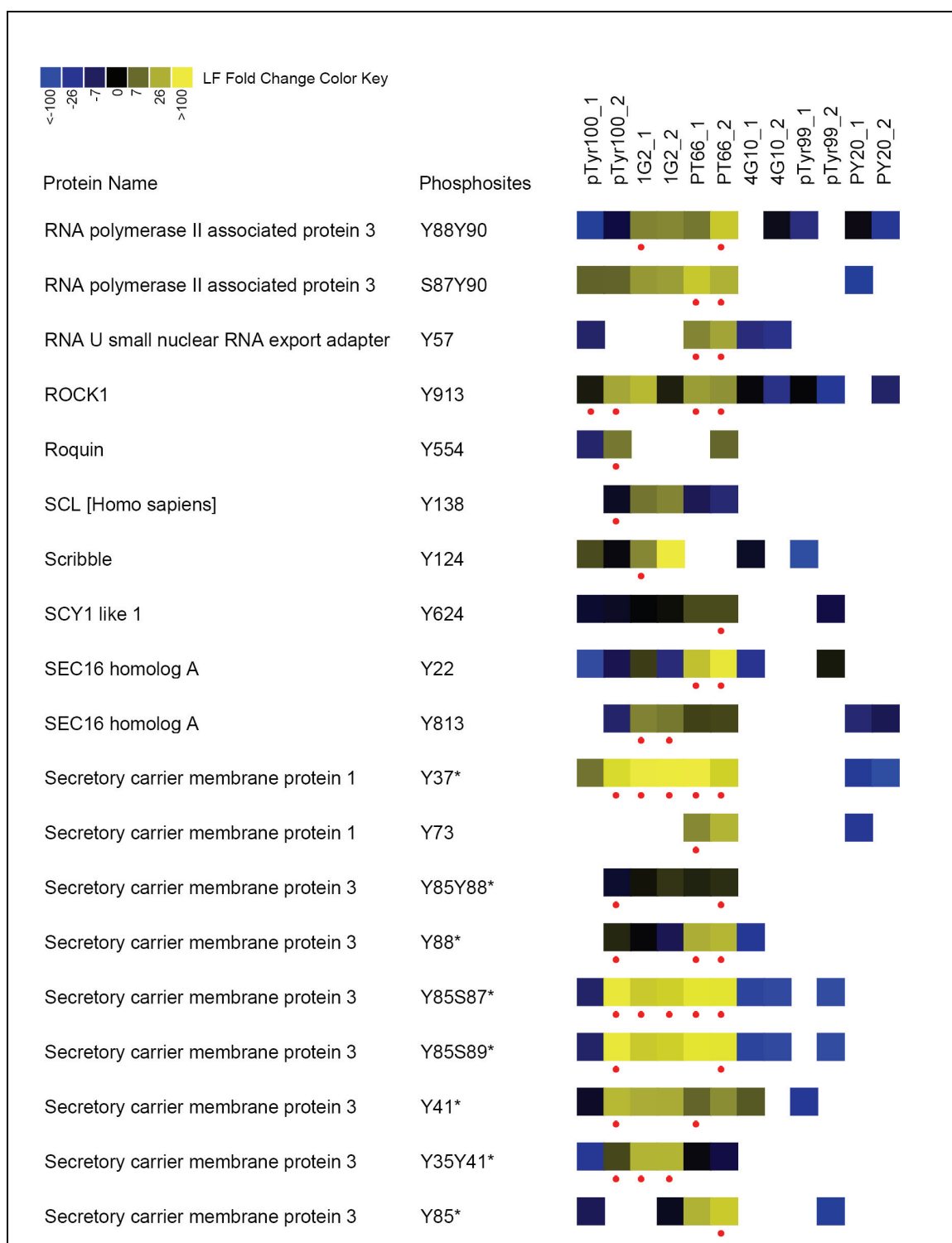


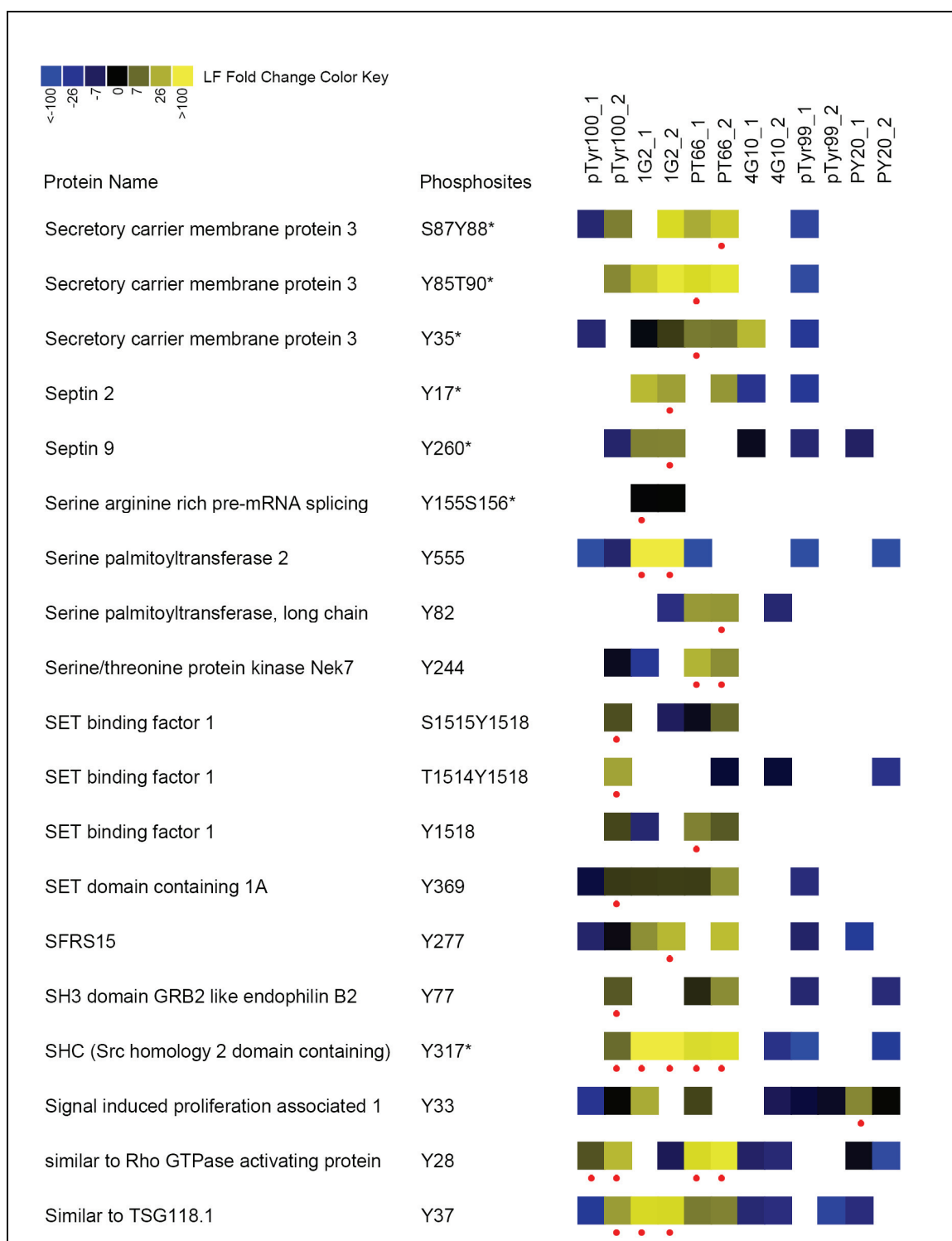


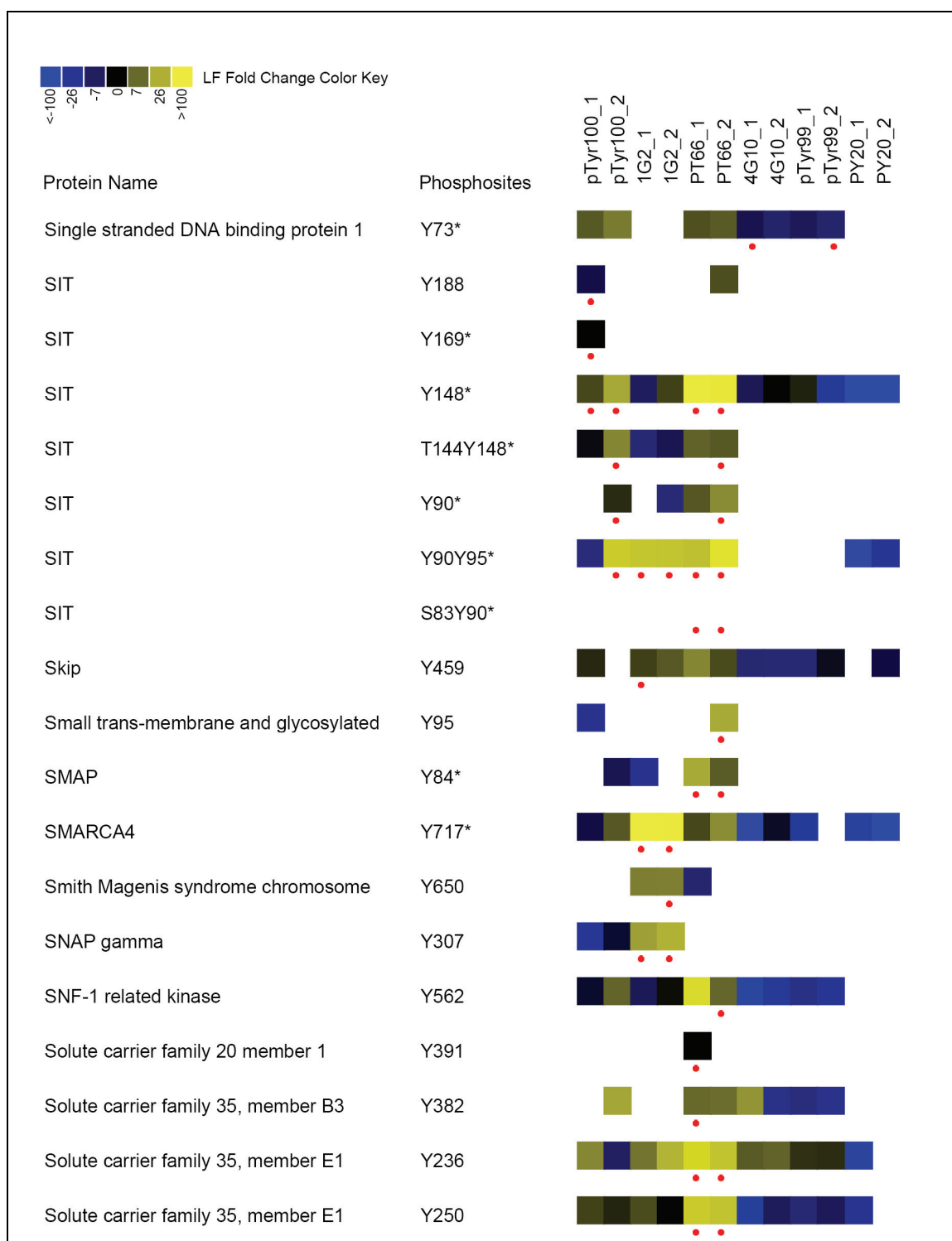


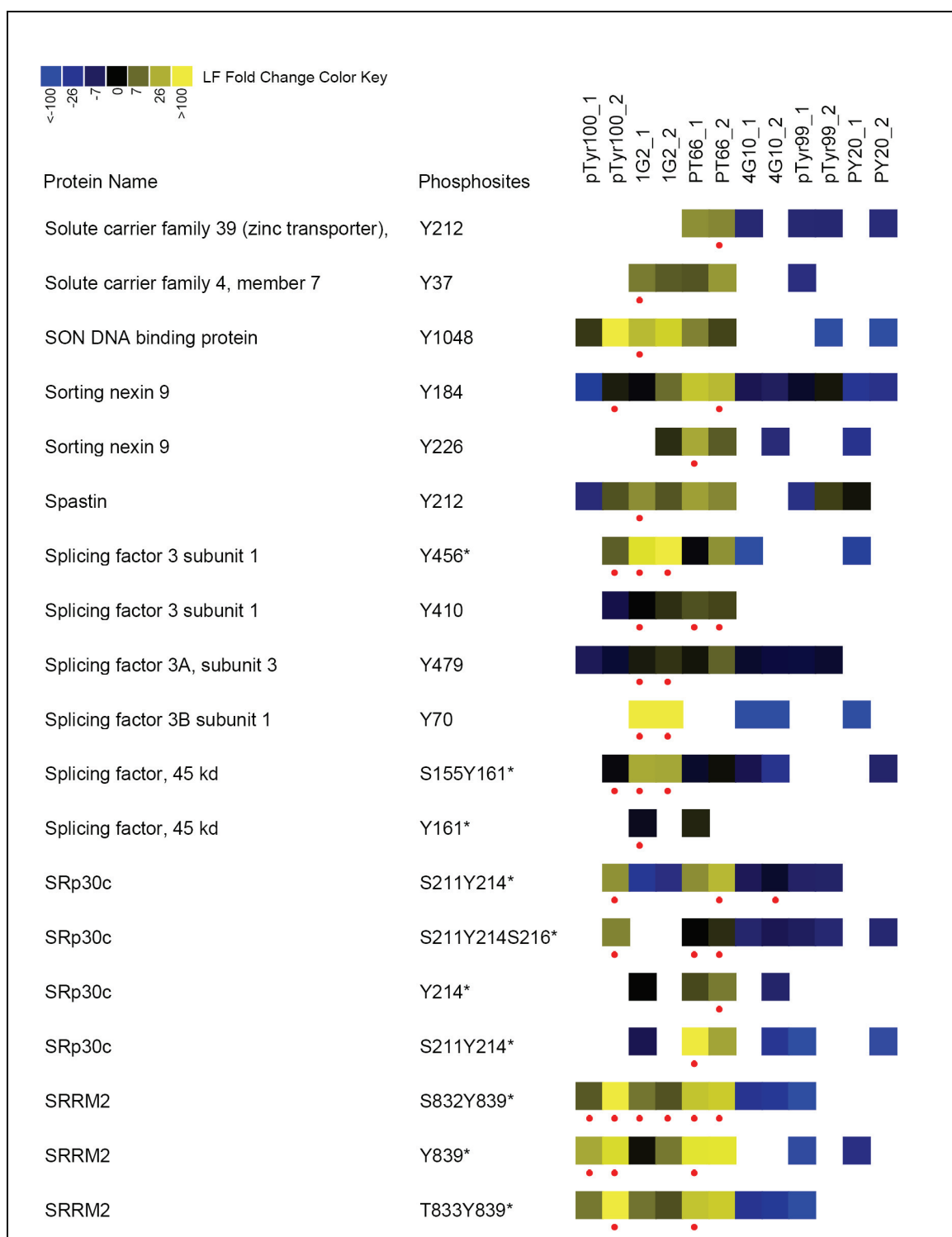


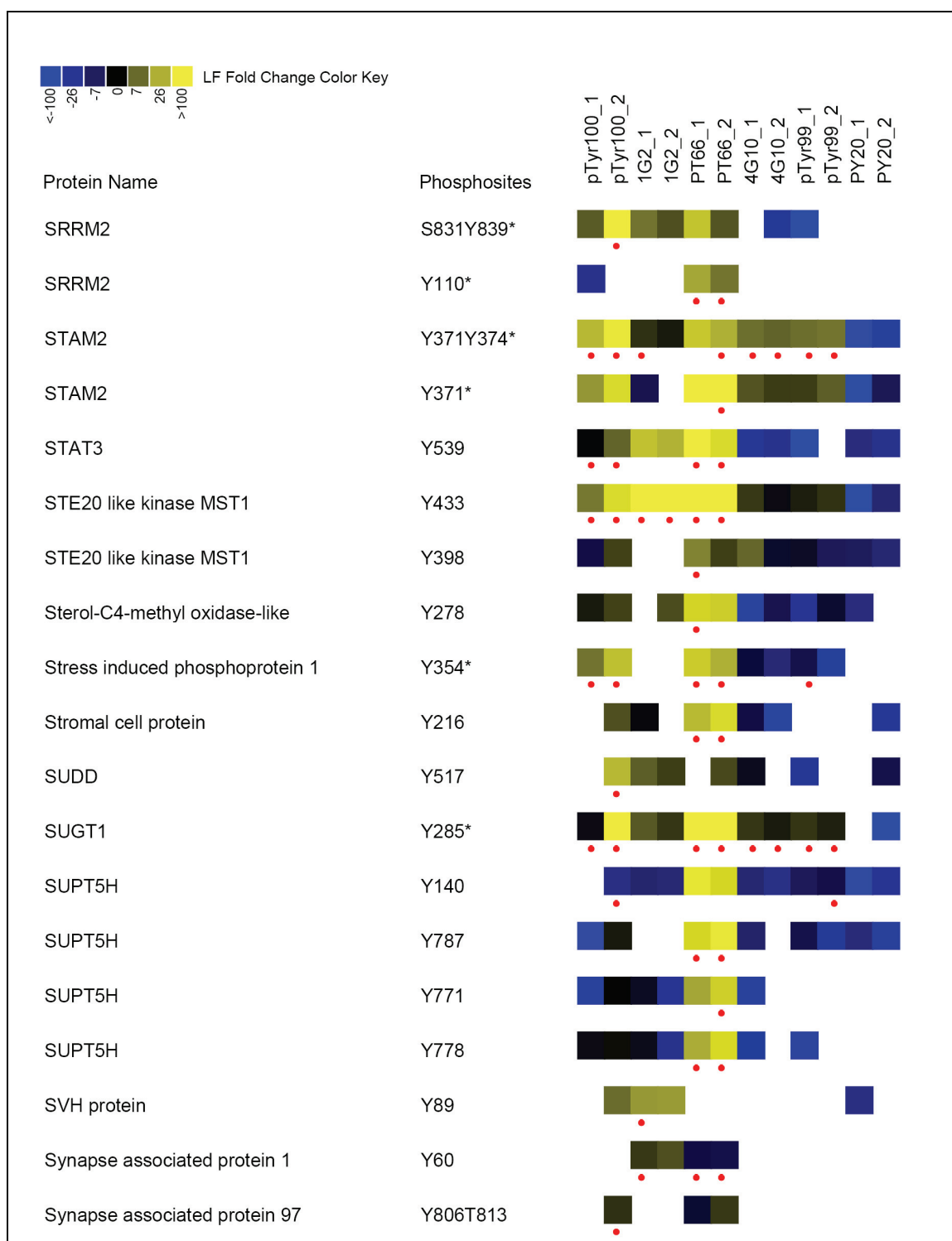


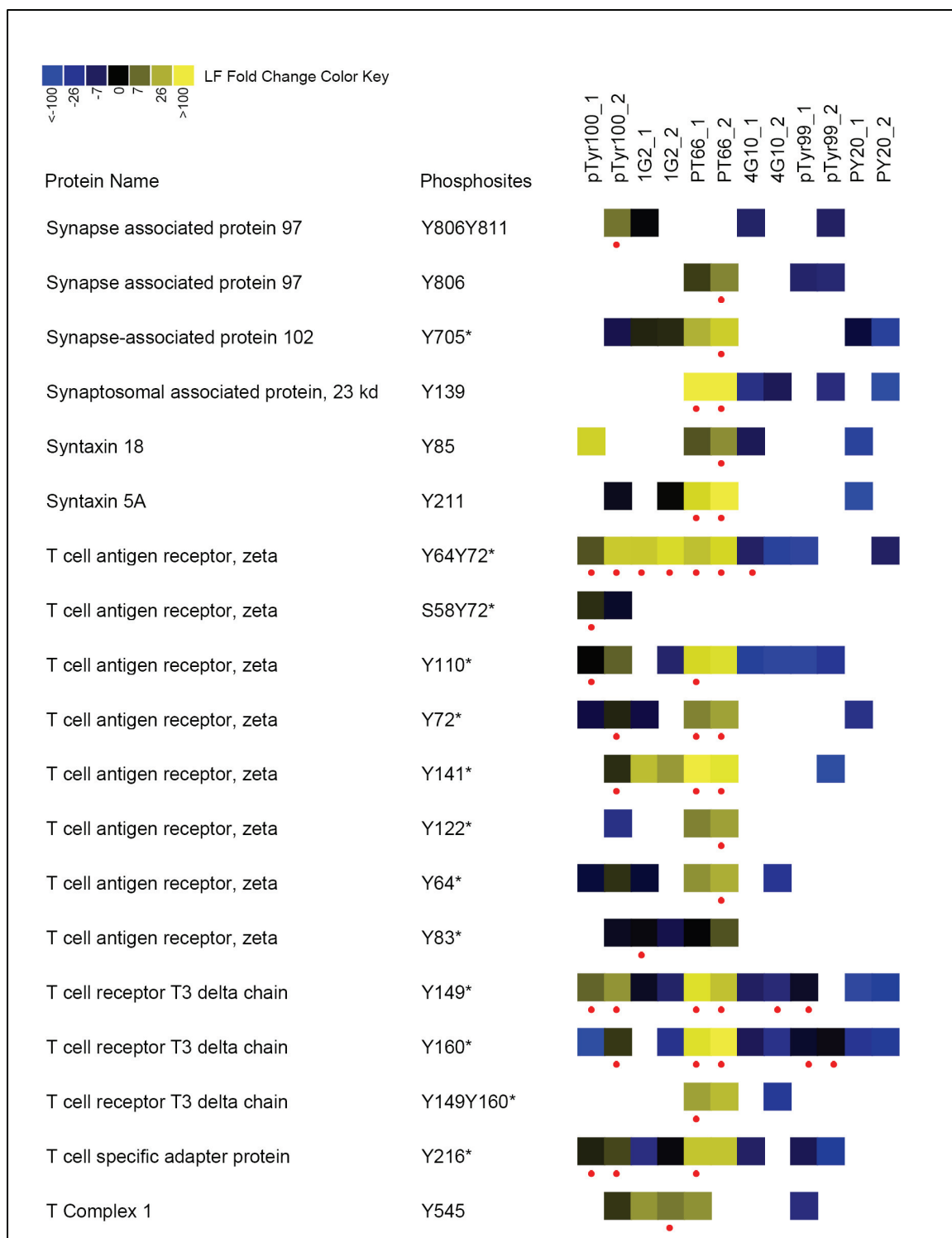


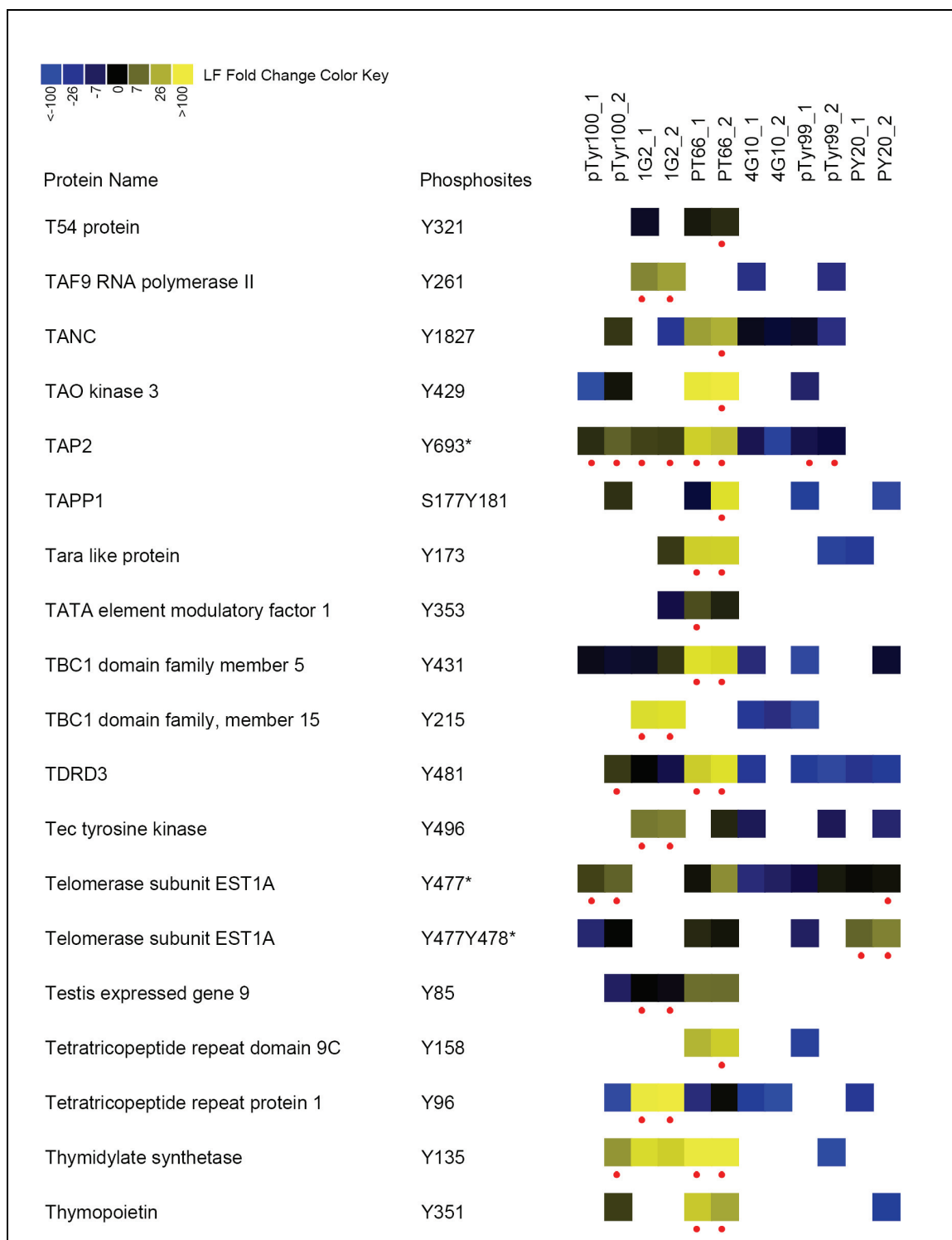


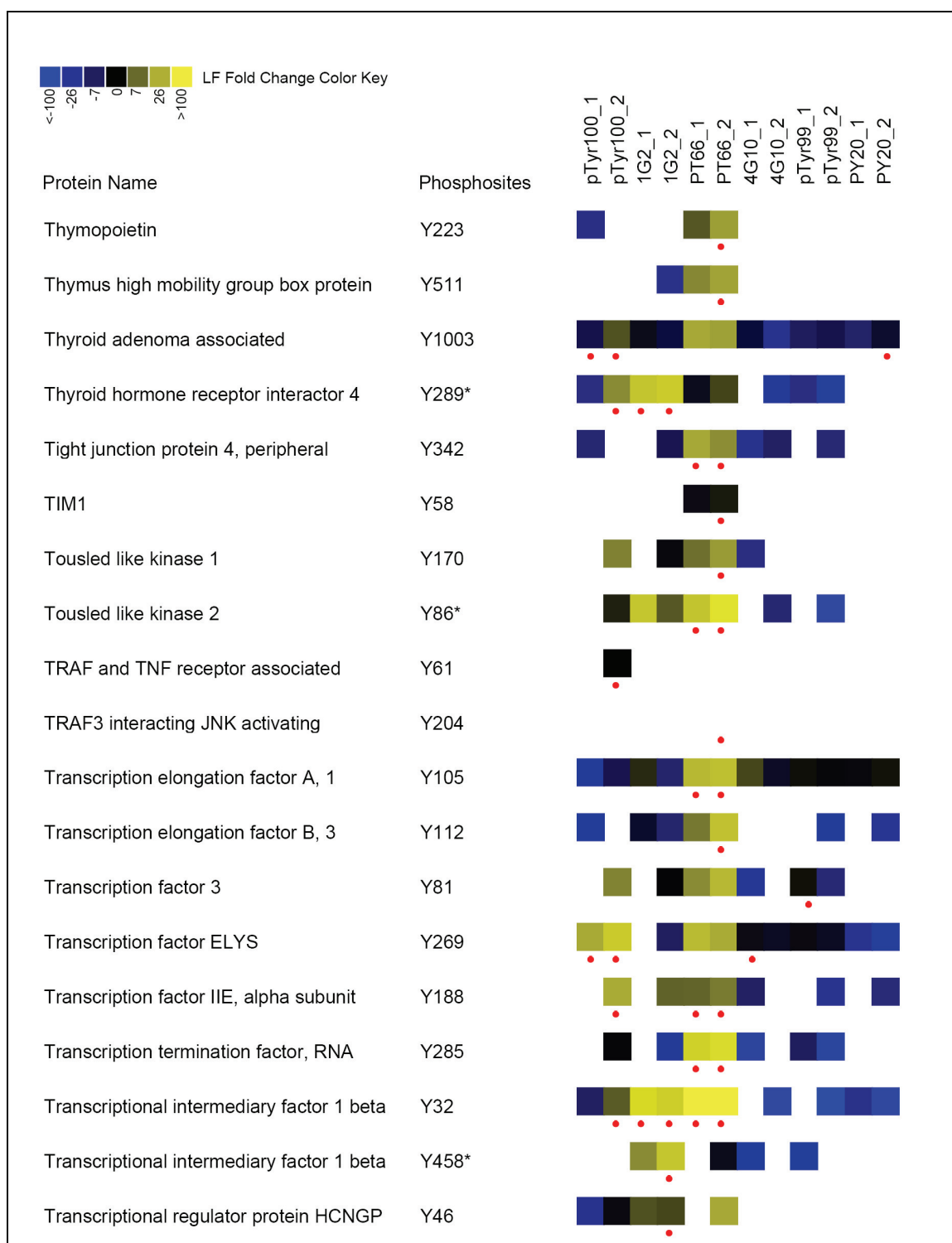


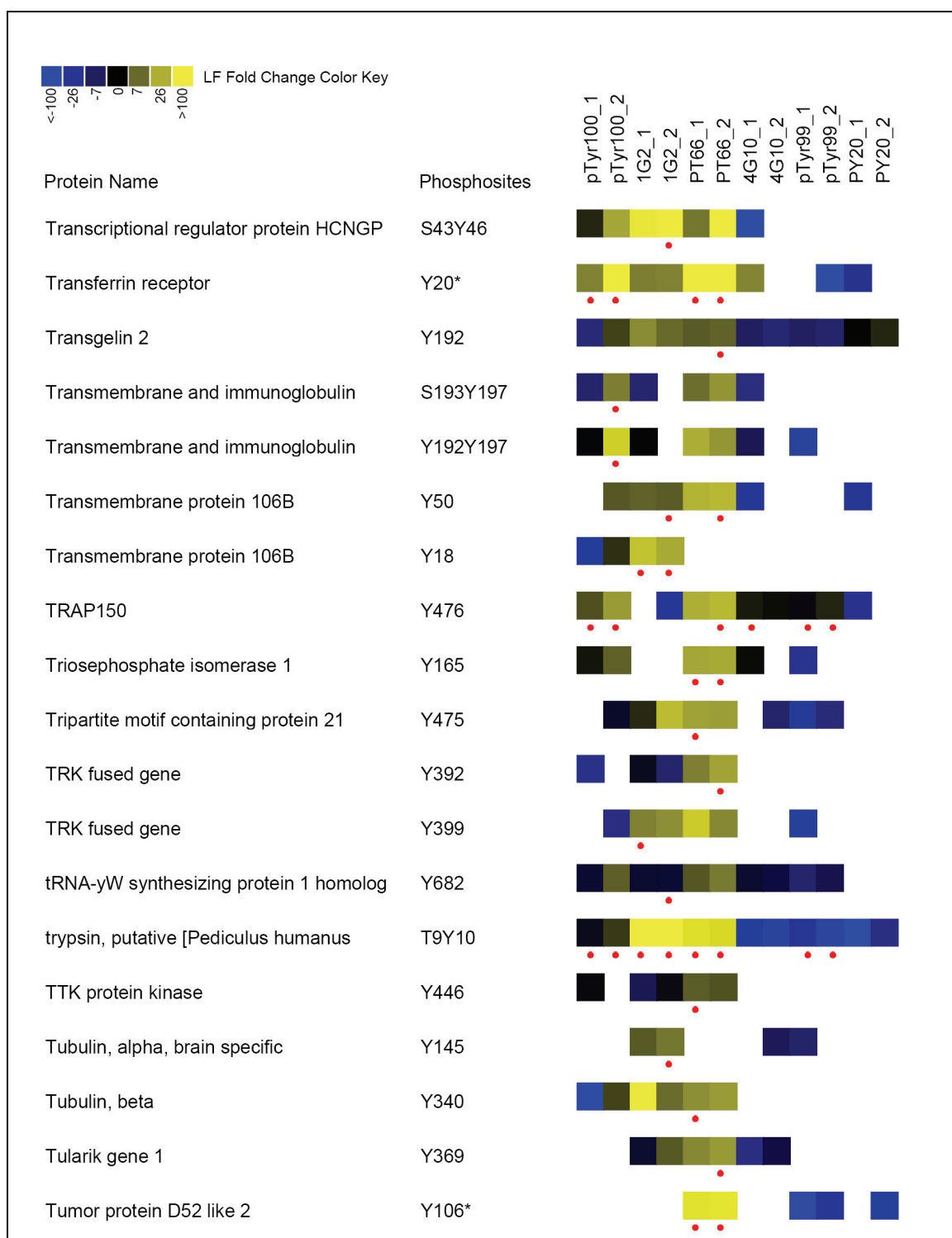


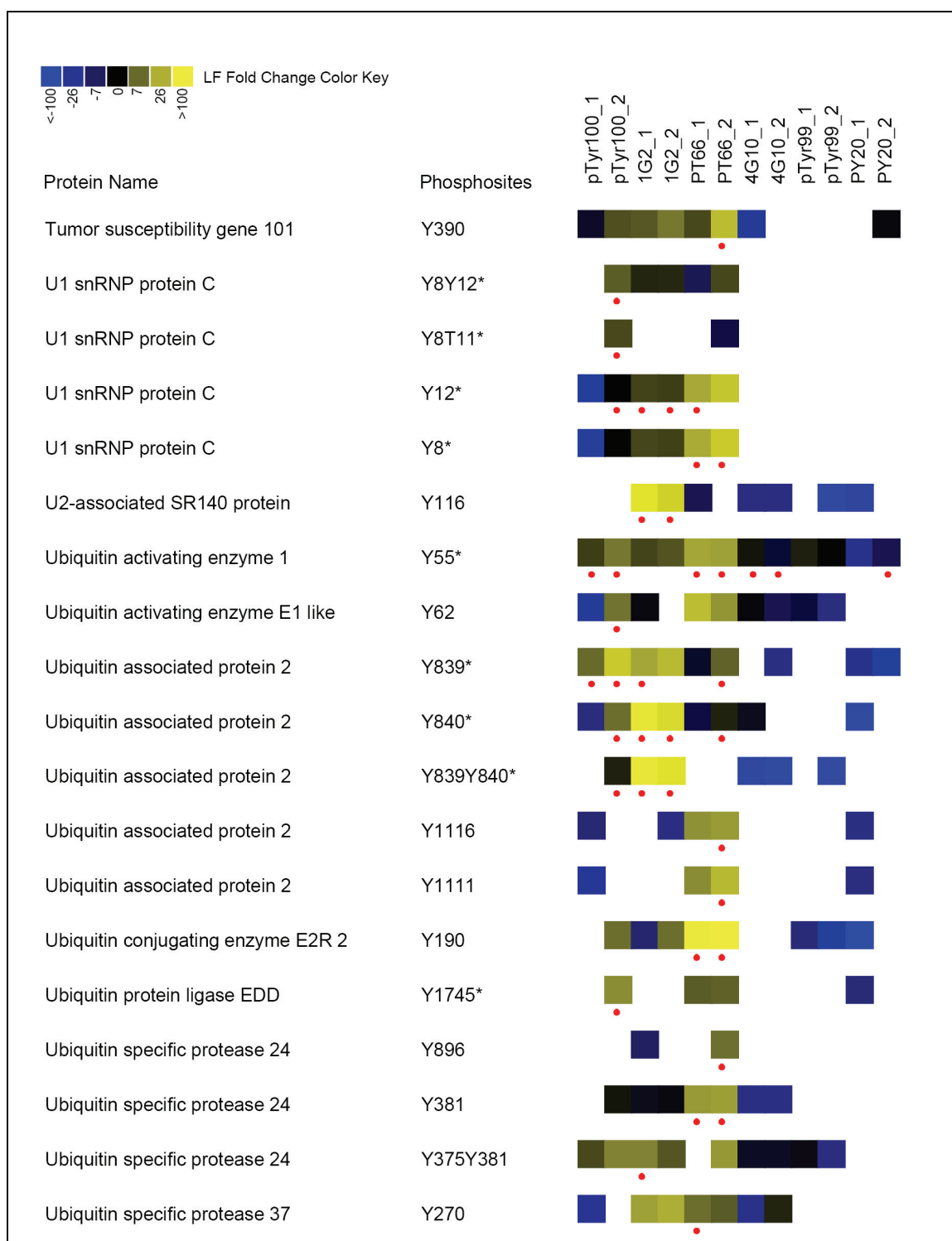


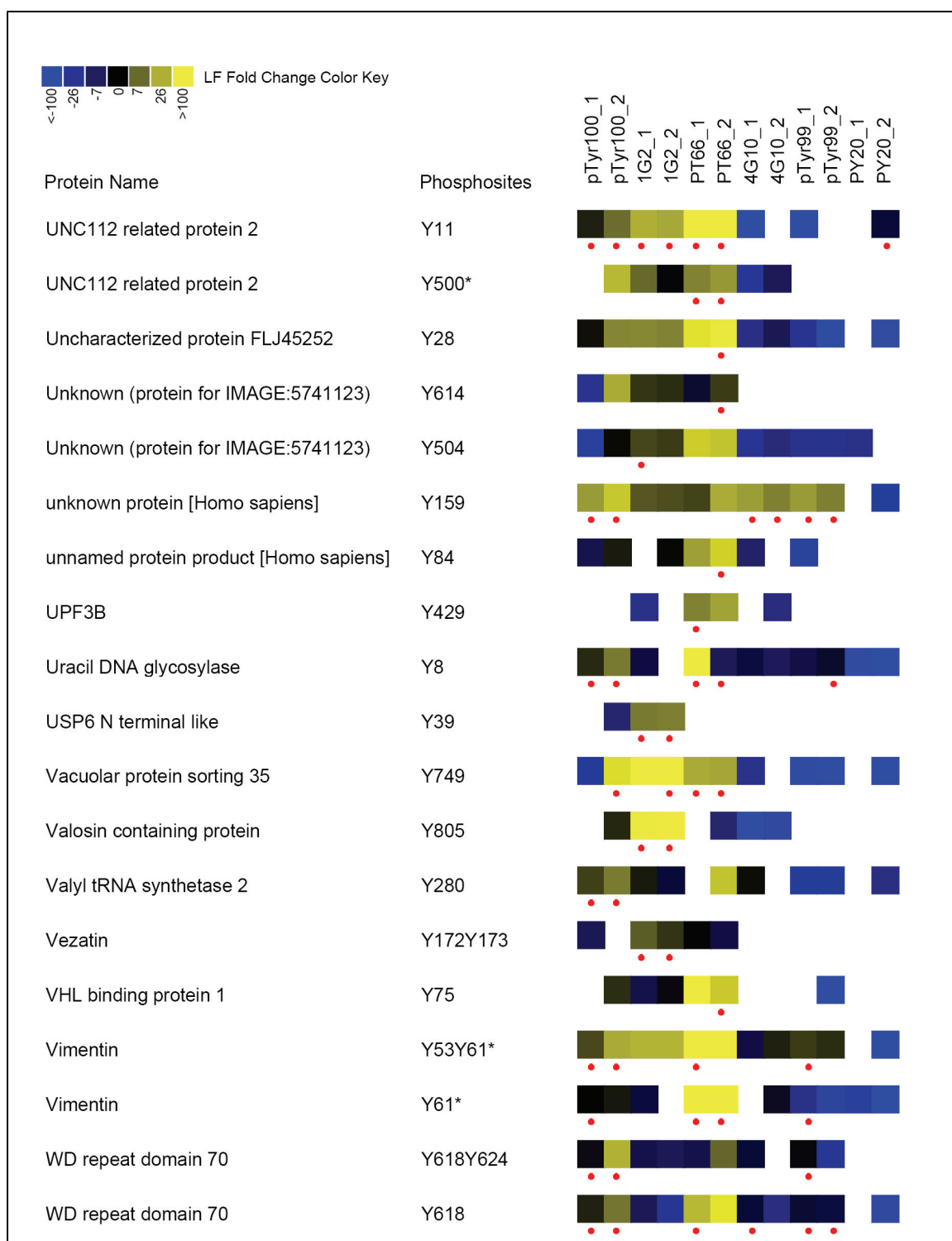


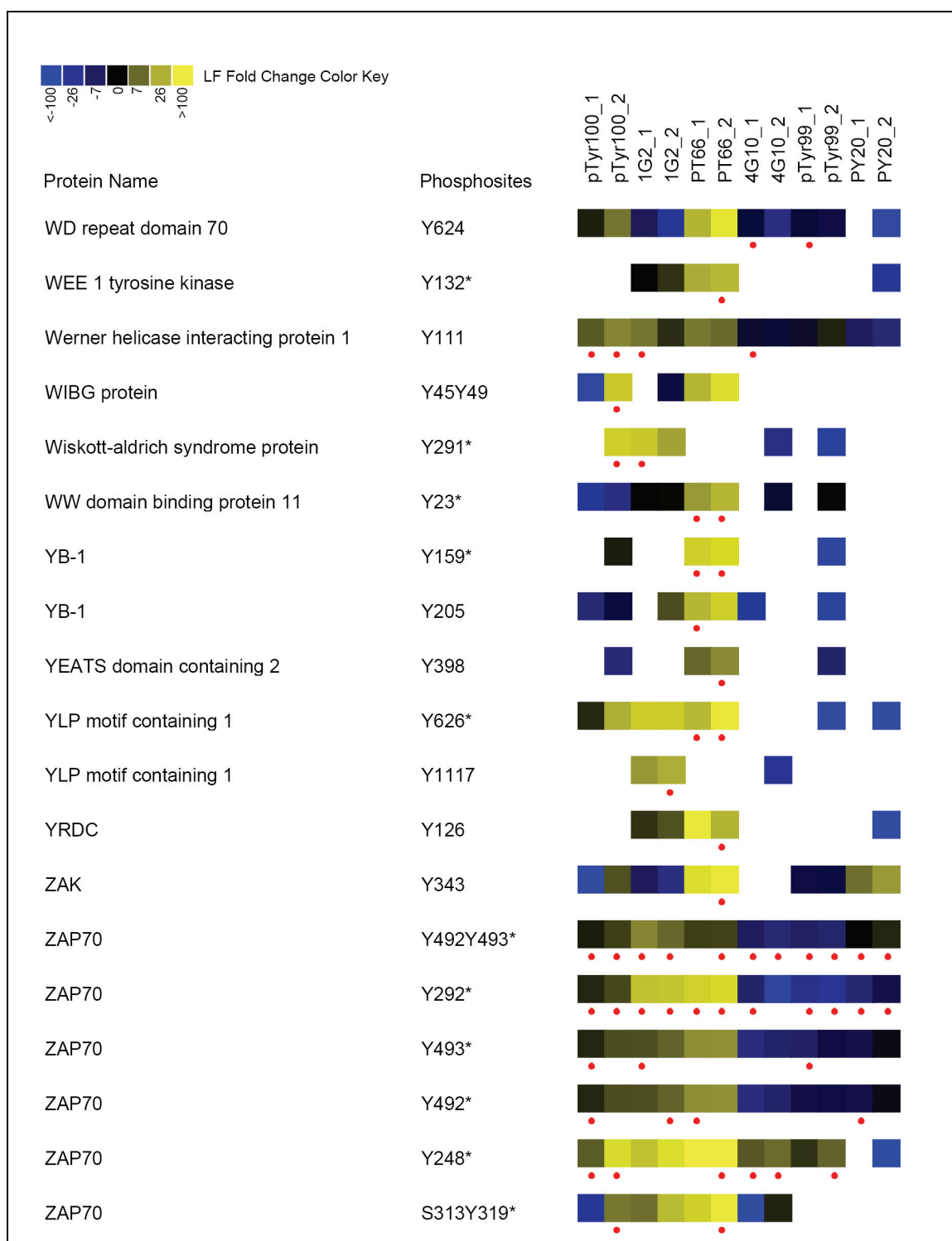


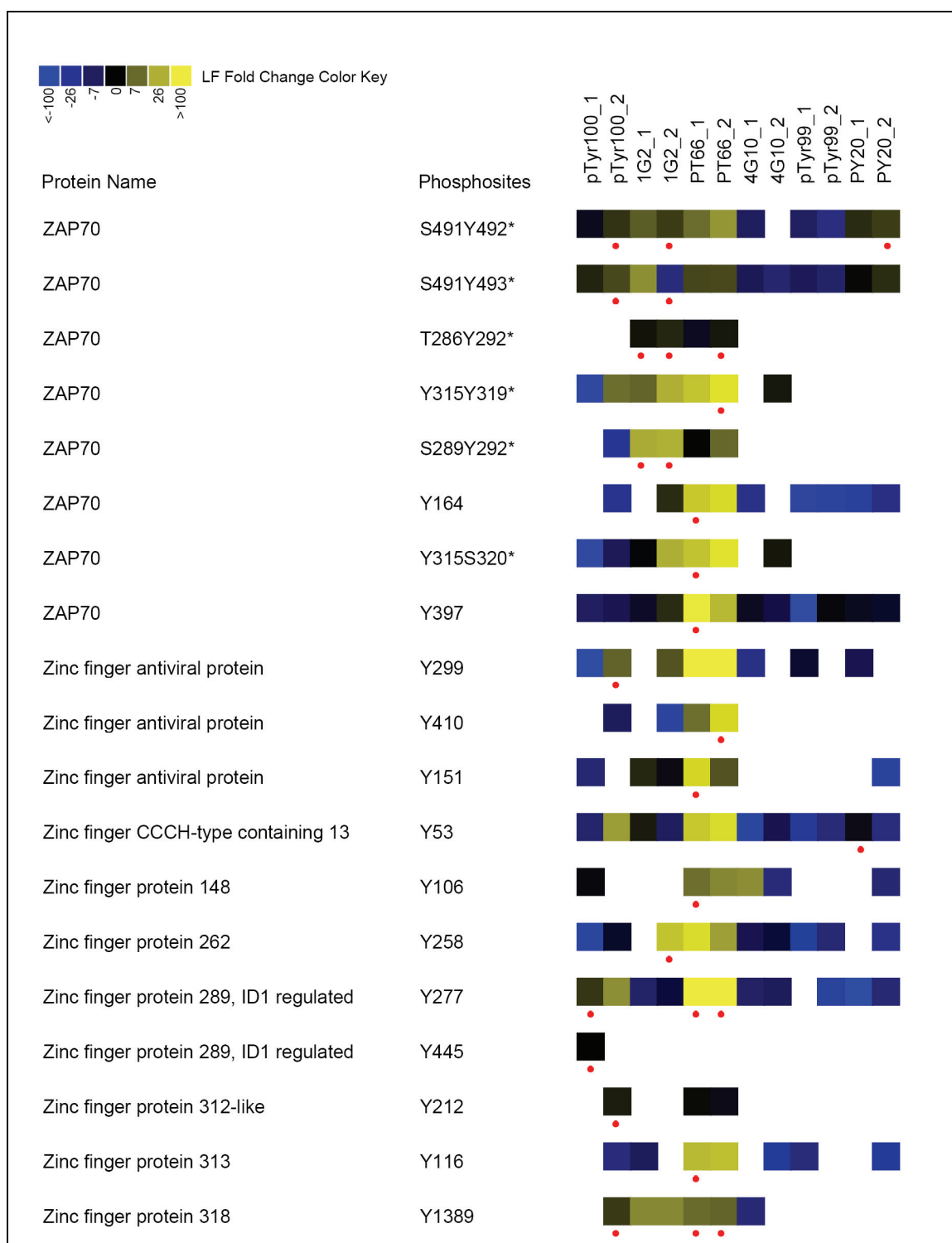


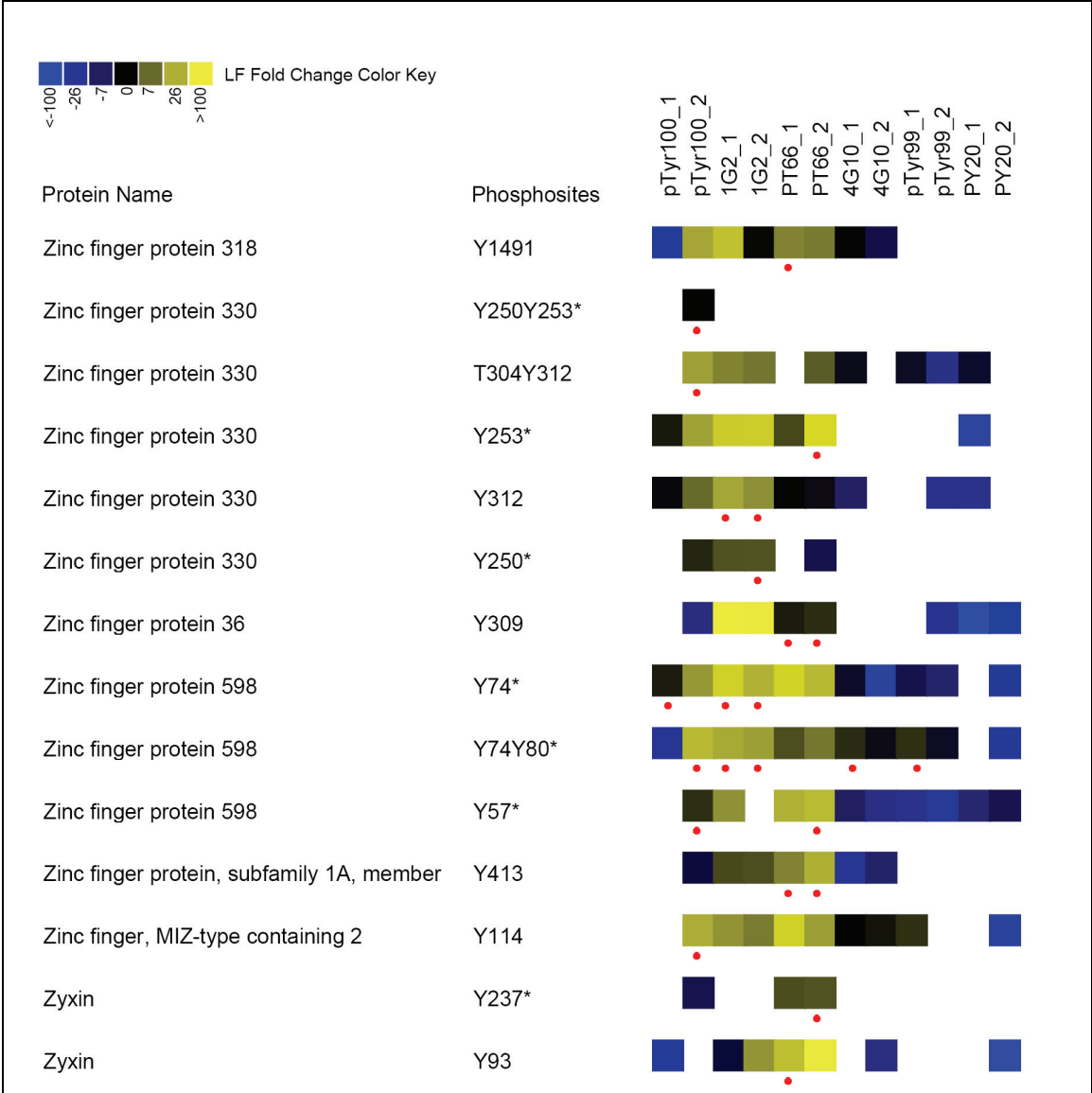






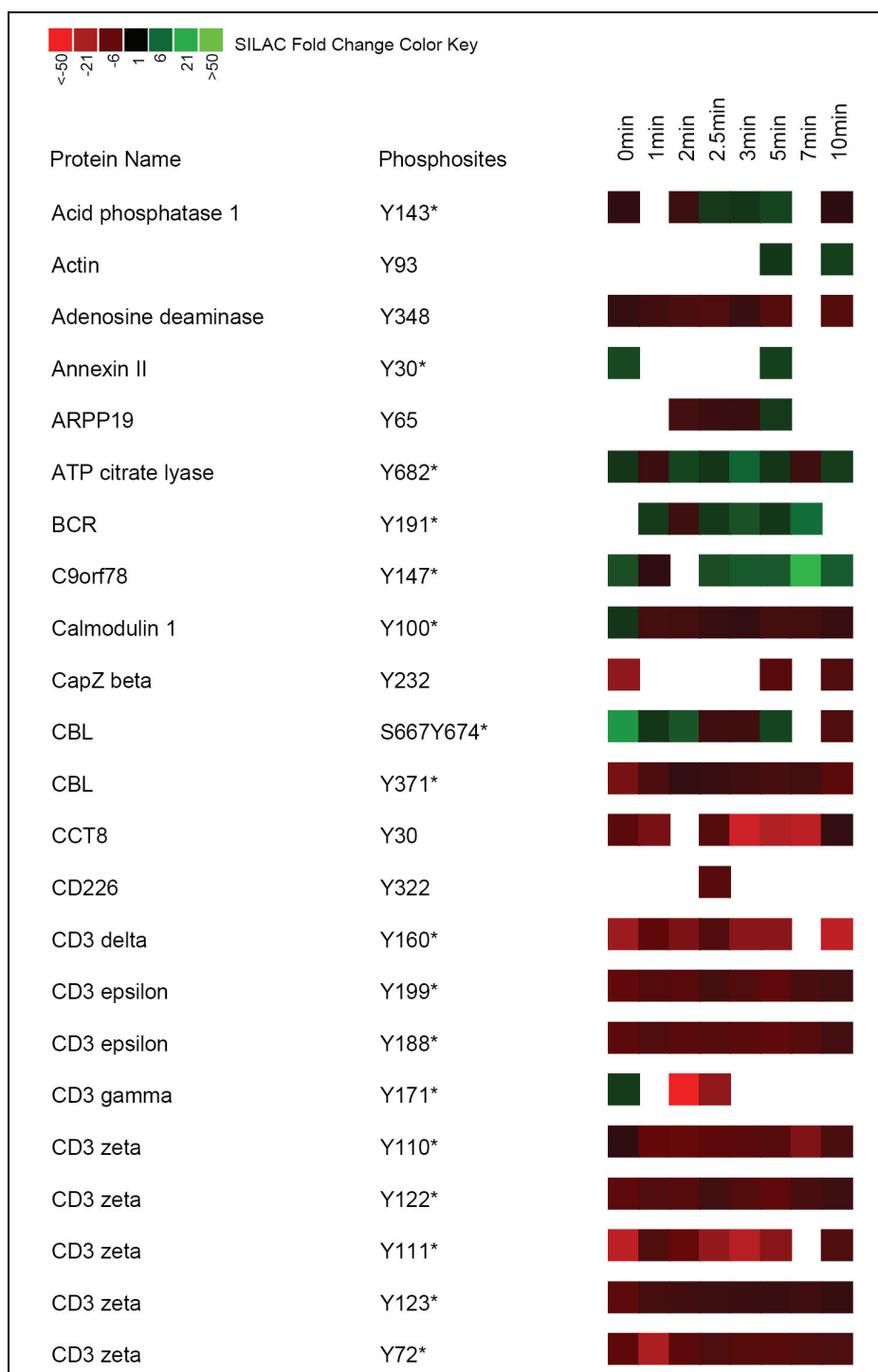


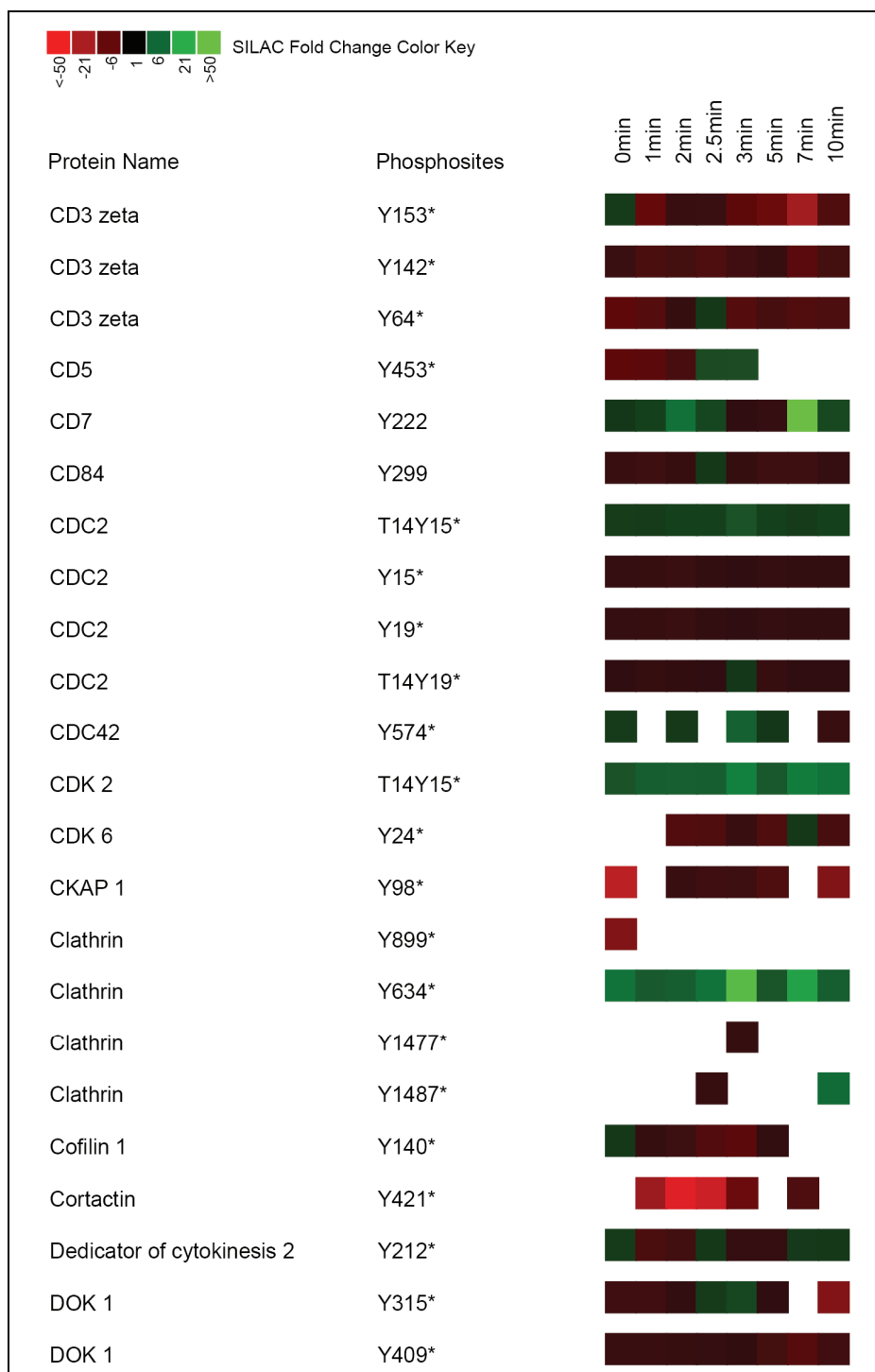


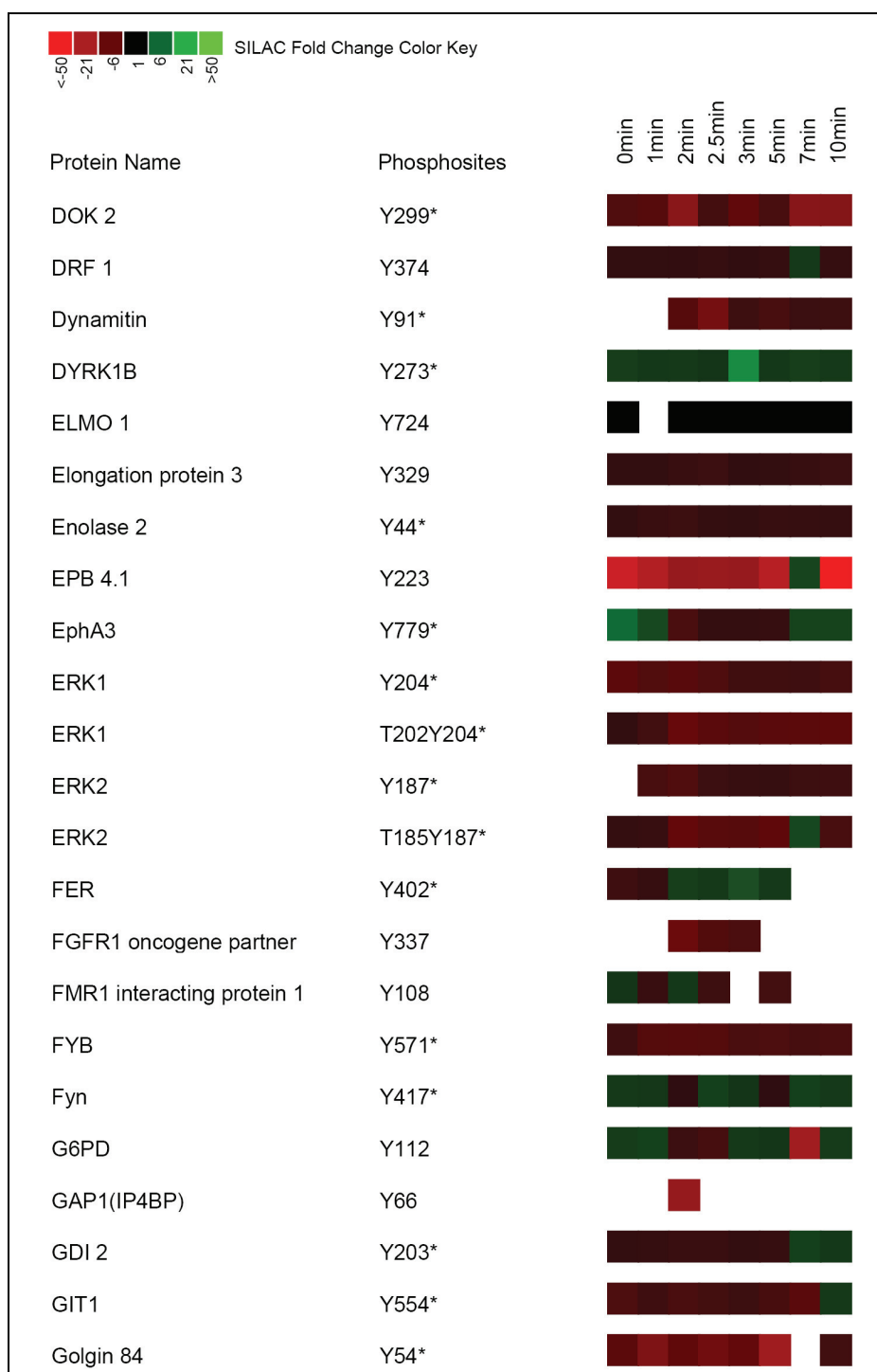


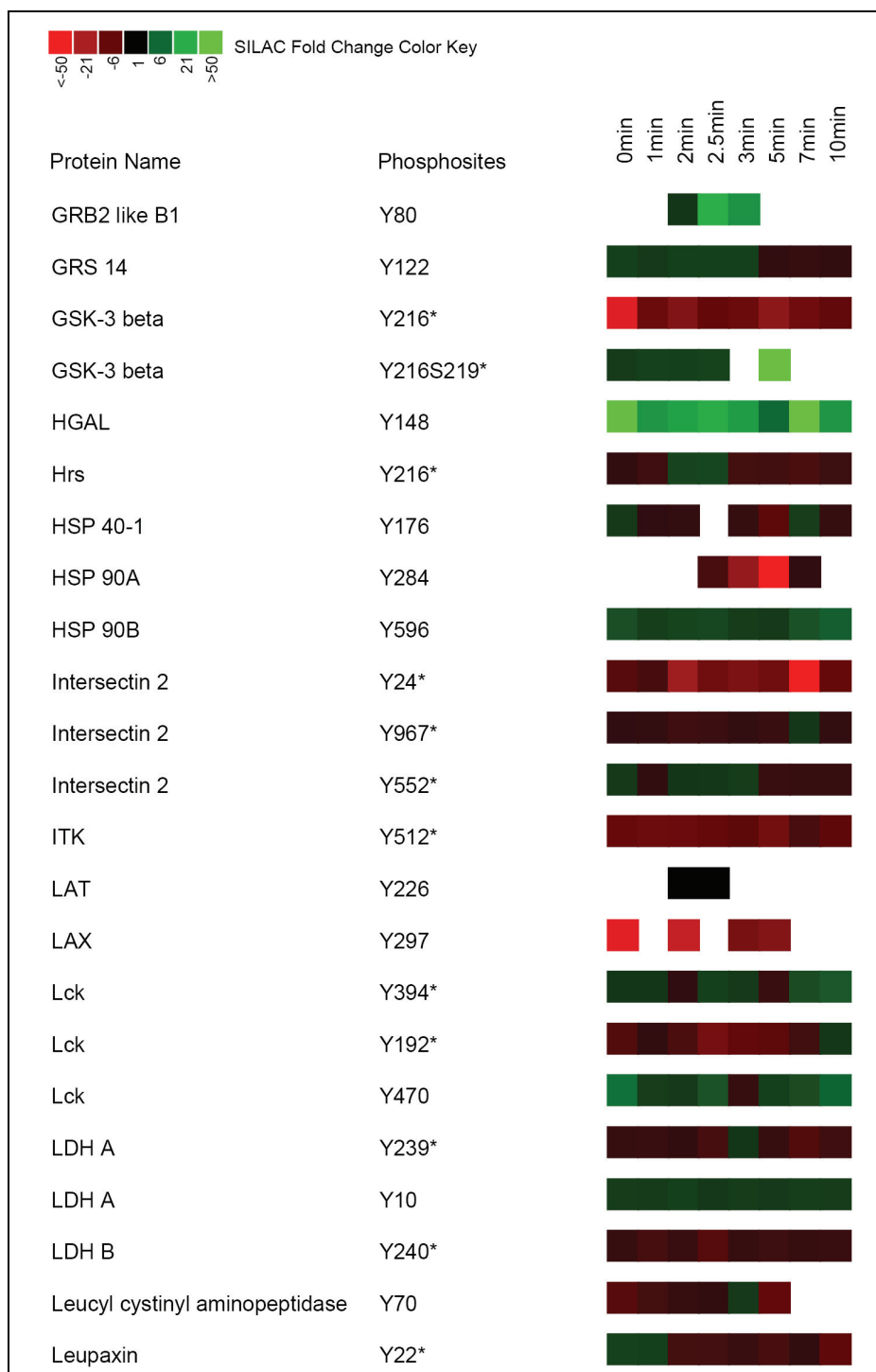
Listed above is all data collected, representing the phosphorylation sites enriched by various anti-phosphotyrosine antibodies, and their relative abundance. Peptide immunoprecipitation experiments using the various anti-phosphotyrosine antibodies were performed on pervanadate-stimulated Jurkat E6-1 cell lysates. Replicates are listed side-by-side to display the variations in phosphopeptide enrichment within the same antibody. Temporal quantitative changes in phosphorylation state are represented as heatmaps. In the heatmap representation, yellow represents levels of phosphorylation above the average, while blue represents levels of phosphorylation below the average. Black represents average abundance for a certain peptide across all time points. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. A red dot below the heatmap square indicates that a MS/MS scan was collected for that condition. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

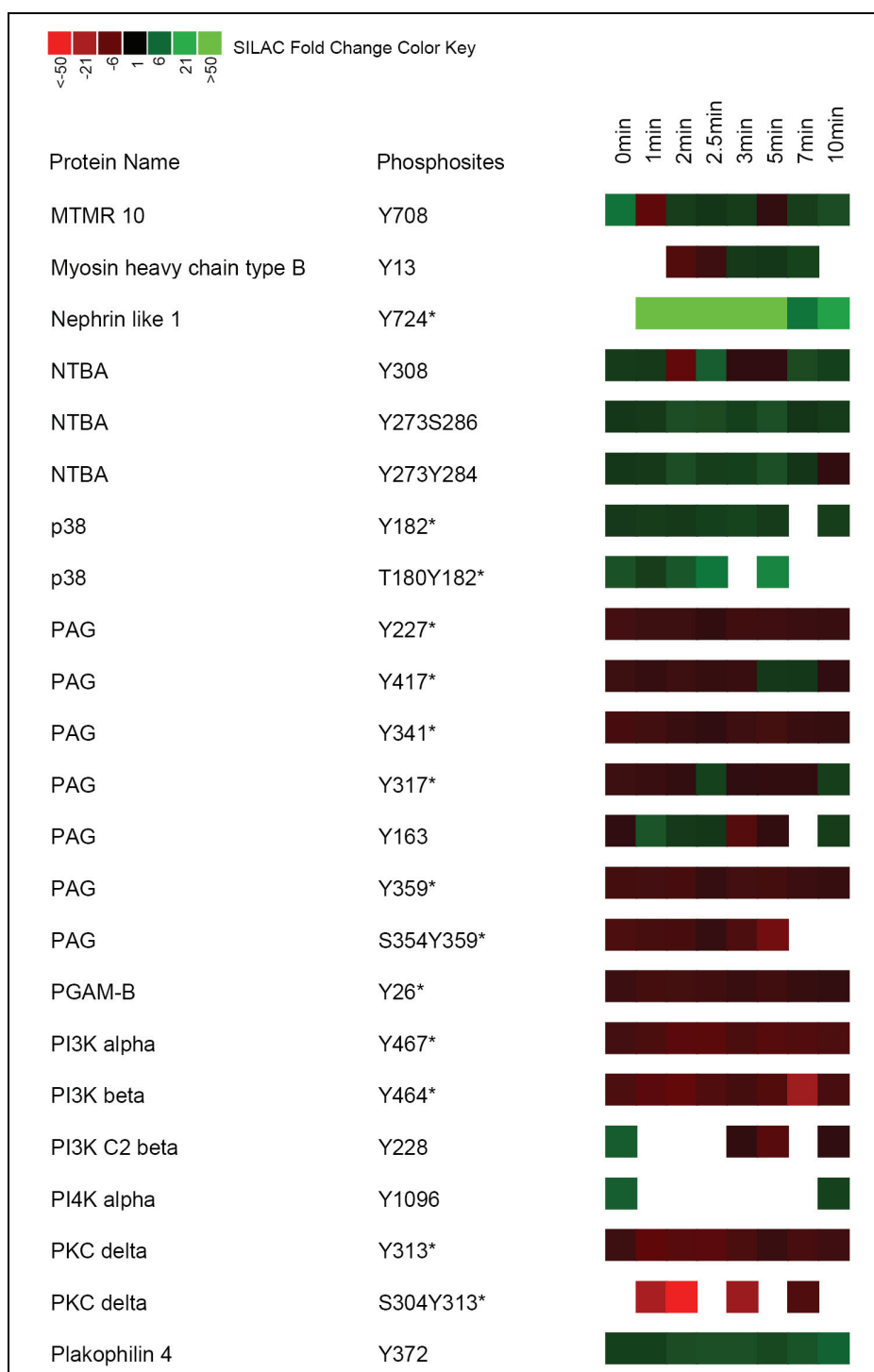
B) SILAC ratio heatmap comparing the differences in phosphorylation profiling of Zap-70 reconstituted to wild type Jurkat cells in response to TCR stimulation.

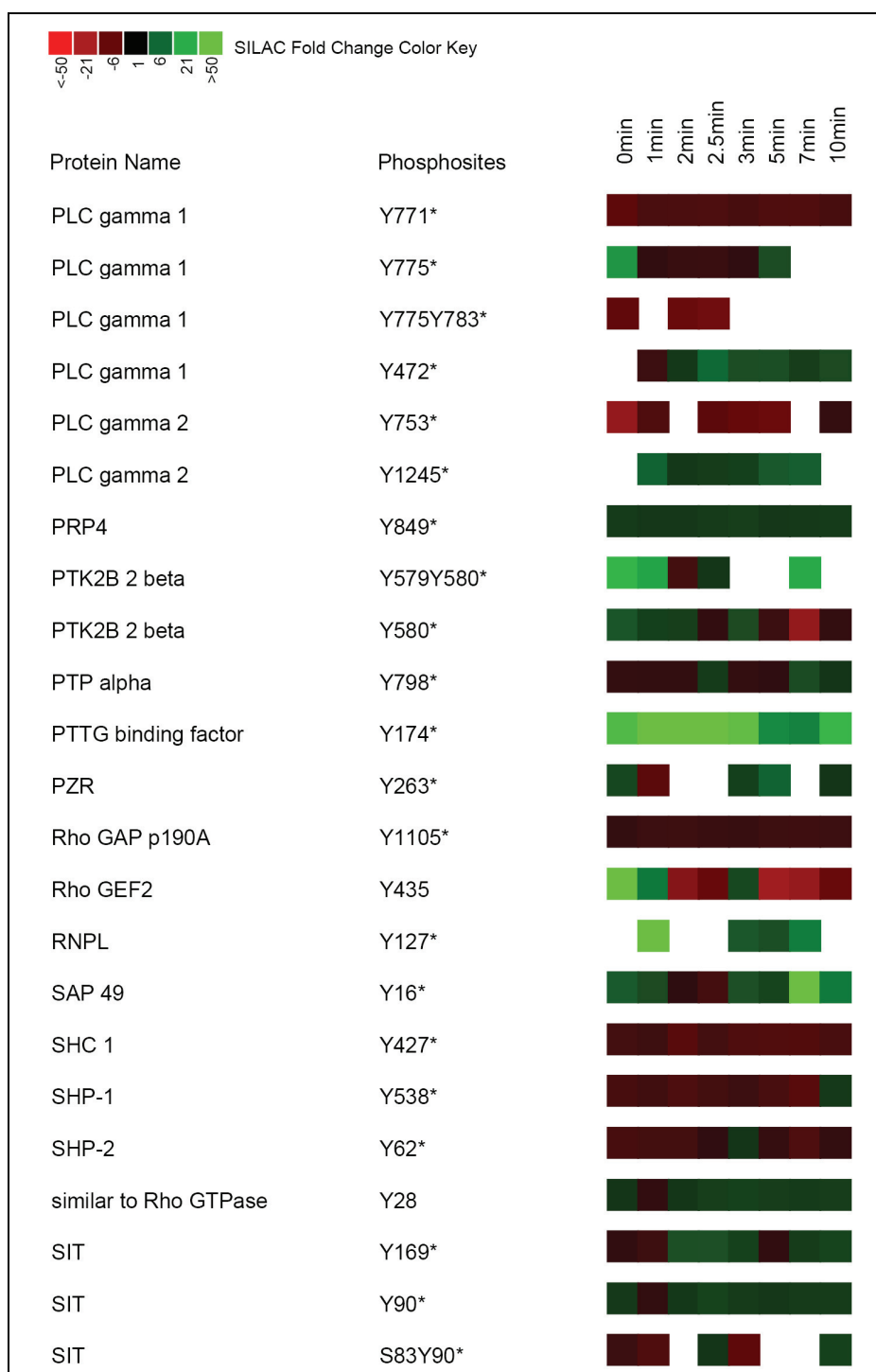


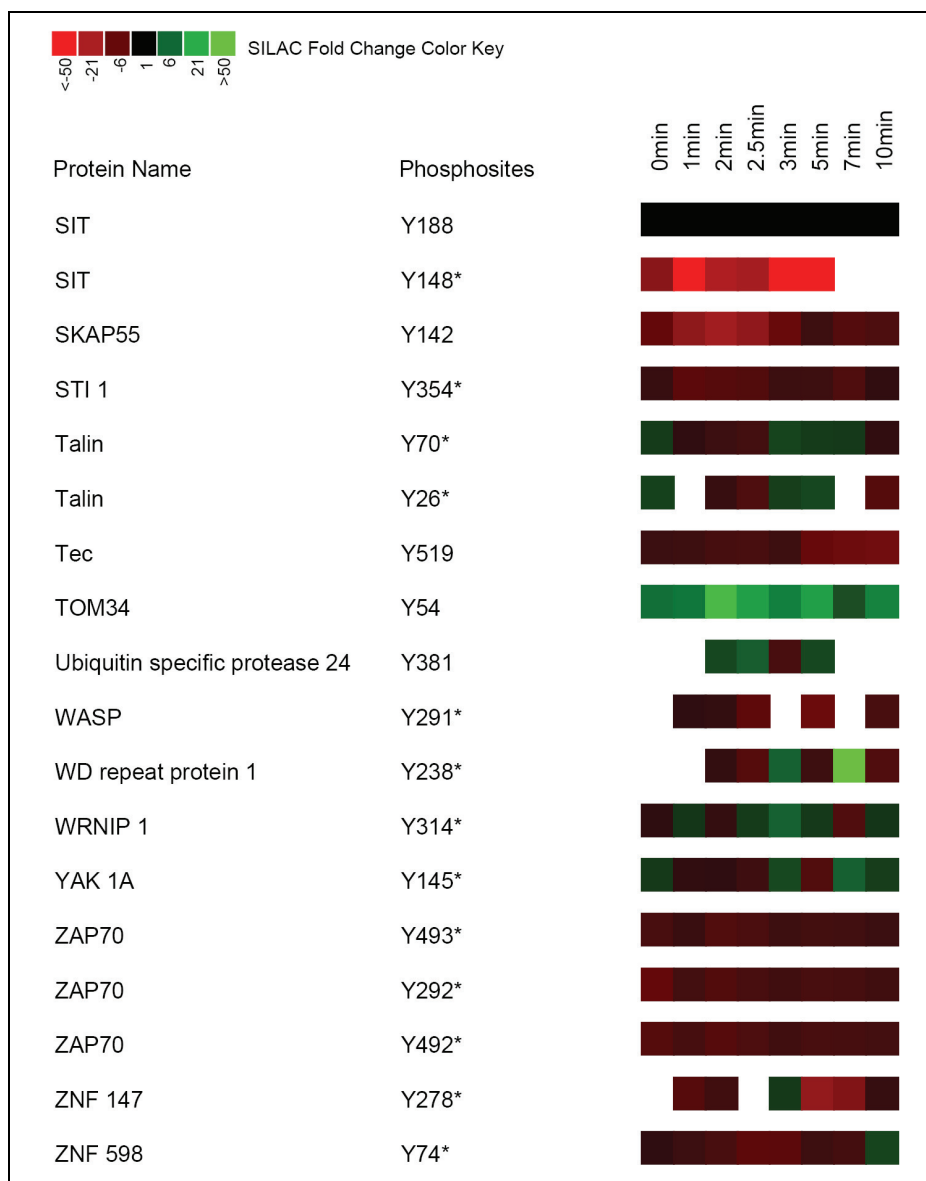












SILAC heatmap of all of the data collected, representing proteins that were observed in our comparison of human Jurkat P116.c139 (Zap-70 reconstituted) and wild type Jurkat JE6-1 cells. Zap-70 reconstituted/wild type SILAC ratios corresponding to the changes in phosphorylation state are represented as heatmaps. Green represents elevated phosphorylation in response to Zap-70 removal, while red represents a decrease in phosphorylation in response to Zap-70 removal. Blanks in the heatmap indicate that a clearly-defined SIC peak was not observed for that phosphopeptide in that time point. Black represents no change. Note that the * next to the phosphorylation site signifies that this site has been previously described in the literature.

C) Updated protocol for SILAC labeling, Jurkat T-cell OKT3/OKT4 co-stimulation, and sample preparation for LC/MS/MS analysis.

I) SILAC labeling

A) Jurkat clones and corresponding isotopic labels.

Jurkat clones	Labels and Mass Differences
Jurkat clone 1 (Heavy)	Arg ($^{13}\text{C}_6$, $^{15}\text{N}_4$ => 10.00827); Lys ($^{13}\text{C}_6$, $^{15}\text{N}_2$ => 8.01420)
Jurkat clone 2 (Light)	No labels on Arg ($^{12}\text{C}_6$, $^{14}\text{N}_4$) or Lys ($^{13}\text{C}_6$, $^{15}\text{N}_2$)

B) Concentration of Arg and Lys and Isotopic stocks:

Amino acids	Molarity (mM)	Concentration (mg/L) Heavy	Concentration (mg/L) Medium	Concentration (mg/L) Light
Arg (1X)	1.15	200	196.5	158
Lys (1X)	0.22	40	34.4	25.7

Note: Media for all conditions will contain **1/3X Arg and 1X Lys**. All other amino will be equal to endogenous RPMI 1640 medium levels.

Amino Acid Isotope Stocks (all stocks were dissolved in deficient RPMI 1640 medium):

- 1) Arg (Heavy): 500X stock 100mg/ml
- 2) Arg (Light): 500X stock 79mg/ml
- 3) Lys (Heavy): 1000X stock 40mg/ml
- 4) Lys (Light): 1000X stock 25.7 mg/ml

Each sample will be prepared as follows:

1. Thawed Jurkat cells and first seed them in normal RPMI 1640 medium supplemented with 10% heat-inactivated undialyzed fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin.
2. Three days later, wash cells twice with deficient RPMI 1640 medium (w/o Arg, Lys).
3. After 3rd wash, resuspend cells in RPMI 1640 medium containing either $^{12}\text{C}_6$, $^{14}\text{N}_4$ Arg and $^{12}\text{C}_6$, $^{14}\text{N}_2$ Lys (Light labeled) or $^{13}\text{C}_6$, $^{15}\text{N}_4$ Arg and $^{13}\text{C}_6$, $^{15}\text{N}_2$ Lys (Heavy labeled) amino acids. All medium was supplemented with 10% heat-inactivated dialyzed fetal bovine serum (Sigma), 2mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin.

4. All cell populations must be grown in this medium for ~7 to 8 doublings before the experiment/stimulation can be performed.

4 Doublings:

$1e^5$ cells/ml $\Rightarrow$ $2e^5$ cells/ml $\Rightarrow$ $4e^5$ cells/ml $\Rightarrow$ $8e^5$ cells/ml $\Rightarrow$ $1.6e^6$ cells/ml (passage/expand cells in fresh RPMI medium and reseed them at a concentration of $1e^5$ cells/ml).

4 Doublings:

$1e^5$ cells/ml $\Rightarrow$ $2e^5$ cells/ml $\Rightarrow$ $4e^5$ cells/ml $\Rightarrow$ $8e^5$ cells/ml $\Rightarrow$ $1.6e^6$ cells/ml (First day of experiment)

Note: Cells are grown in a humidified incubator with 5% CO₂ at 37C.

II) Jurkat T-cell OKT3/OKT4 co-stimulation and Cell Lysis

1. Grow cells to a concentration of $\sim 1e^6$ cells/ml. (at ~7 to 8 doublings)
2. Centrifuge cells down at 400 xg for 5 minutes at 22 °C.
3. Wash cells one time 4 °C PBS and resuspend them at the concentration of $1e^8$ cells/ml of cold PBS (1 ml total per sample).
4. Add in 2.5 ug/ml each of OKT3 and OKT4 primary antibody (eBioscience). Incubate for 10 minutes at 4 °C.
5. To co-stimulate the cells, add in 22ug/ml of goat anti-mouse IgG crosslinker (Jackson Immuno Research) at 37 °C quickly using the waterbath for the desired time of stimulation.
6. To stop the stimulation, remove cells from waterbath, gently pipet the cells out of the epi tube you stimulated them in and add into 5 ml lysis buffer (9 M urea, 20 mM HEPES, pH 8.0, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate) and vortex for 30 seconds.

Note: Prepare lysis buffer the same day as use.

7. Using a microtip, sonicate at 15 W output with 2 bursts of 30 sec each. Cool on ice for 1 minute between the bursts. Clear the lysate by centrifugation at 20,000 x g for 15 minutes and collect the supernatant.

III) Sample preparation for LC/MS/MS analysis

1. Add 1/10 vol. of 45 mM DTT to the cleared cell supernatant (e.g. 1 ml if the

supernatant is 10 ml), mix well, place the tube into a 60 °C water bath for 20 minutes.

2. Cool the solution in ice/water for approximately 10 minutes until it has reached room temperature (tube should feel neither warm nor cold).
3. Add 100 mM iodoacetamide at an equal volume as the DTT solution, mix well, incubate for 15 minutes at room temperature in the dark.
4. Dilute lysates 4-fold to a final concentration of 2 M urea with 20 mM HEPES buffer, pH 8.0 and digest with trypsin (promega) (1:100 w/w) overnight at room temperature. Determine the amount of trypsin by protein lowry assay (Bio-rad).

Note: 10 mg/ml of protein will require 5 tubes of trypsin.

5. The following day, tryptic peptides were acidified by adding 1/20 volume of 20 % TFA to the digest for a final concentration of 1 % TFA (let stand for 10 minutes), centrifuged (1800 xg for 5min at 22C), and desalted by C18 sep-pak plus (Waters) cartridge and lyophilized for 48 hours to dryness.
 1. Pre-wet the column with 5ml 100% MeCN.
 2. Wash with 7ml (10vol, applied as 2 x 3.5ml) 0.1% TFA.
 3. Load acidified and cleared digest.
 4. Wash column with 10ml of 0.1% TFA.
 5. Elute column with 6 ml of 40% MeCN with 0.1% TFA into a 15ml polypropylene tube.
 6. Lyophilize frozen peptide solution (freeze peptides using isopropanol/dry ice bath) for 2 days and store at -80 °C until peptide immunoprecipitation.

Note: Use the flow rates around 2 ml/min.

6. Spin tube with lyophilized peptide at 1800 xg for 5min at 22C to collect all peptide at the bottom.
7. Add 1.3 ml of ice cold IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) to the lyophilized peptide. Further dissolve peptides under gentle shaking for 30 minutes at room temperature and by brief sonication in a sonicator waterbath. Clear solution by centrifugation for 5 minutes at 1,800 x g (the pellet of insoluble matter may at times seem considerable, but this will not pose a problem since most of the peptide will be dissolved nonetheless). Cool on ice.
8. Add in 10 pmol LIEDAEpY standard peptide sample before you add the antibody beads.

9. Transfer the peptide solution into the microfuge tube containing pre-conjugated pTyr100 phosphotyrosine antibody beads (40 μ l; 80 μ l slurry) (Cell Signaling). Incubate overnight on a rotator at 4°C.
10. Beads were washed 3 times with 1 ml IAP buffer and 2 times with 1 ml cold ddH₂O. Between washes, centrifuge at 1,500 x g for 1 minute. On the last wash, use a 1/2cc insulin syringe (20G) to remove all fluid from the beads.

Note: The following washes are all done on ice (4 °C)

11. To elute peptides, add 55 μ l of 0.15% TFA to the beads, tap the bottom of the tube several times (do not vortex) and let stand at room temperature for 10 minutes.
12. Tap the bottom of the tube again, centrifuge at 1,500 x g for 1 minute (room temperature), remove the supernatant and add it to a fresh epi tube.
13. Add 45 μ l of 0.15% TFA to the beads, tap the bottom of the tube a few times, centrifuge at 1,500 x g for 1 minute (room temperature) and combine the supernatant with the first eluate, mix.
14. Centrifuge eluate at 1,500 x g for 5 min and remove 90 μ l of supernatant and add it to a fresh epi tube. Try not to suck up any beads that were spun to the bottom. Samples are now ready for home made stage tip purification.

D) Protocol for 1G2 antibody immunoprecipitation with 1.0 mM phenyl phosphate elution and antibody regeneration.

- 1) Prepare 5 equivalents of $1e^8$ pervanadate (pv)-stimulated Jurkat sep-paked and lyophilized peptides (prepared as described in Appendix B). 2 will be used as pre-1G2 bead regeneration samples and 2 will be used as post-1G2 bead regeneration samples and 1 will be used as a positive control with 0.15% TFA elution.
- 2) Dissolve 2 of the $1e^8$ pv-stimulated Jurkat sep-paked and lyophilized samples with 1 ml IP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) to each sample and briefly sonicate in a sonicator waterbath to dissolve pellet.
- 3) Before using 1G2 bead bound antibodies, regenerate anti-phosphotyrosine beads: Wash the beads alternating with wash buffer 1 (0.1 M NaHCO_3 , 0.5 M NaCl, pH 8.3) and then with wash buffer 2 (0.1 M sodium acetate buffer, 0.5 M NaCl, pH 4.0) 4 times. Then wash the beads with regeneration buffer (5 mM Tris Base, 1 M NaCl, pH 7.6) 4 times.
- 4) Pre wash the beads with (1X PBS, 3X IP buffer, 1X PBS).
- 5) Transfer dissolved pv-stimulated Jurkat samples to regenerated/prewashed 50 μl bead volume (100 μl slurry). Incubate for 30 minutes on a rotator at 4 °C.
- 6) Centrifuge at 1500 x g for 1 minute and remove supernatant (unretained fraction).
- 7) Wash beads 5 times (3X IP buffer, 2X with ddH_2O).
- 8) Elute with 2x 55 μl Elution buffer (10 mM Tris Base, 50 mM NaCl, 1.0 mM phenyl phosphate, pH 7.6). For each elution, resuspend the beads by gently mixing them by tipping the tube. Incubate for 10 minutes on room temperature, occasionally resuspending the beads by.
- 9) Centrifuge eluent at 1500 x g for 1 minute and remove supernatant to completely remove any residual beads carried over.
- 10) Add 0.15 % TFA to lower eluent to pH 2 (for optimal binding to C^{18} resin). Store samples at 4 °C until time to purify sample using homemade Zip-tip (C^{18} purification of peptides in pipette tip) procedure (Cell signaling protocol).
- 11) To regenerate used anti-phosphotyrosine beads: Wash the beads with regeneration buffer 4X. Then further wash the 1G2 bead bound antibodies (1X PBS, 3X IP buffer, 1X PBS) before next immunoprecipitation.

- 12) To determine whether there is carry-over after elution and regeneration, we will now immunoprecipitate using the 1G2 bead bound antibodies with just the IP buffer (no sample lysate). Add 1 ml IP buffer to beads for 30 minutes on a rotator at 4 °C.
- 13) Wash beads 5 times (3X IP buffer, 2X with ddH₂O)
- 14) Elute with 2x 55 µl Elution buffer. Resuspend the beads by gently mixing them by tipping the tube. Incubate for 10min at room temperature, occasionally resuspending the beads.
- 15) Centrifuge eluent at 1500 x g for 1 minute and remove supernatant to completely remove any residual beads carried over.
- 16) Add 0.15 % TFA to lower eluent to pH 2 (for optimal binding to C¹⁸ resin). Store samples at 4 °C until time to purify sample using homemade Zip-tip (C¹⁸ purification of peptides in pipette tip) procedure (Cell signaling protocol).
- 17) To determine whether the beads can be reused, dissolve 2 more 1e⁸ pv-stimulated Jurkat sep-paked and lyophilized samples with 1 ml IP buffer (50mM MOPS, 10mM sodium phosphate, 50mM NaCl, pH 7.2) and briefly sonicate in a sonicator waterbath to dissolve pellet.
- 18) IP and elute pv-stimulated jurkat sample by repeating steps 5-10.
- 19) Regenerate used anti-phosphotyrosine beads and store them separately at 4C. (step 11).
- 20) Prepare control samples with a positive control of TFA IP elution of cell sample and negative control of TFA elution of beads without cell sample.
- 21) **Positive control:** 1e⁸ pv-stimulated Jurkat samples IPed with 50 µl bed volume (100ul slurry) of prewashed 1G2 bead bound antibodies (1X PBS, 3X IP buffer, 1X PBS).
- 22) Incubate for 30 minutes on a rotator at 4 °C and elute using 0.15 % TFA and purify sample using homemade Zip-tip procedure (Cell signaling protocol).
- 23) **Negative control:** Same as positive control but no lysate added to beads. This control will be used to determine the background binding of the beads.