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Ubiquitination of Zeta-associated Protein of 70 kDa Regulates the Activation of T Cell Receptor-proximal Signaling Pathways

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Abstract of the Dissertation

Ubiquitination of Zeta-associated Protein of 70 kDa Regulates the Activation of T Cell Receptor-proximal Signaling Pathways

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The tyrosine kinase Zap-70 is a key regulator of T cell receptor (TCR) signaling downstream of antigen presentation. It plays an essential role in signaling pathways involved in T cell development and function. Loss of Zap-70 results in severe developmental and functional defects in the T cell compartment. Lack of Zap-70 or a complete loss of its function leads to the development of rare severe primary immunodeficiencies, while more common polymorphisms that lead to subtle defects in TCR signaling are paradoxically associated with autoimmunity.

The coordinated regulation of Zap-70 kinase activity is critical for proper T cell proliferation, differentiation, and effector function during an immune response. Zap-70 is cytosolic in unstimulated T cells, but is rapidly recruited to the TCR complex following receptor stimulation. Its activity is regulated both by binding to subunits of the TCR and

by phosphorylation on multiple tyrosine residues. We and others have previously reported that Zap-70 is also ubiquitinated following TCR stimulation. Herein, we report the identification and functional characterization of novel Zap-70 ubiquitination sites. Three sites, including Lys-193, Lys-217, and Lys-376, displayed greater than 20-fold increase in modification levels following TCR stimulation. Abrogation of Lys-217 ubiquitination results in increased kinase activation and enhanced activation of downstream signaling pathways. Increased activation of TCR signaling pathways is accompanied by elevated IL-2 production following TCR stimulation. These data suggest that Zap-70 ubiquitination contributes to the regulation of Zap-70 signaling following TCR stimulation. Importantly, we also demonstrate the appearance of ubiquitinated Zap-70 in primary human T cells, indicating the likelihood that Zap-70 ubiquitination plays a widespread and critical role in regulating Zap-70 signaling functions as part of a mechanism that controls the extent of T cell activation following antigen stimulation.

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AP-1	Activating protein 1
APC	Antigen-presenting cell
Arp2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
B-CLL	B cell chronic lymphocytic leukemia
BCR	B cell receptor
ConA	Concanavalin A
CRAC	Ca^{2+} release-activated Ca^{2+} (channel)
cSMAC	Central supramolecular activation cluster
DAG	Diacylglycerol
DN	Double negative
DP	Double positive
DUB	Deubiquitinating (enzyme)
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FRAP	Fluorescence recovery after photobleaching
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate

HECT	Homologous to the E6-associated protein carboxyl terminus
HP	Histidine phosphatase
IFN	Interferon
IL	Interleukin
IP	Immunoprecipitation
IP3	Inositol 1,4,5-trisphosphate
IRES	Internal ribosome entry site
ITAM	Immunoreceptor tyrosine-based activation motif
IκB	Inhibitor of κB
LAT	Linker for activation of T cells
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MS	Mass spectrometry
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NK	Natural killer (cell)
NLR	NOD-like receptor
NOD	Nucleotide oligomerization domain
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PEI	Polyethylenimine
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
pSMAC	Peripheral supramolecular activation cluster
PTB	Phosphotyrosine-binding
PV	Pervanadate
RING	Really interesting new gene
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SED	Staphylococcal enterotoxin D
SH2	Src homology 2 domain
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SP	Single positive
Sts	Suppressor of TCR signaling
Syk	Spleen tyrosine kinase
TBS	Tris-buffered saline
Tc	T cytotoxic (cell)
TCR	T cell receptor
Th	T helper (cell)
TLR	Toll-like receptor
Treg	T regulatory (cell)
Ub	Ubiquitin

UBA	Ubiquitin-association (domain)
WASP	Wiskott–Aldrich Syndrome protein
WT	Wild-type
Zap-70	Zeta-chain associated protein of 70 kDa

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Chapter 1: Introduction

1.1 The immune system

The immune system is a complex network of interconnected systems, which functions to confer protection to the host against harmful pathogens. It is divided into two main branches, innate and adaptive immunity, based on the speed and specificity of the response. The innate and adaptive immune systems work together to protect the host from disease.

A. Innate immune system

Innate immunity encompasses the elements of the immune system (neutrophils, macrophages, dendritic cells, and complement) which provide immediate host defenses. The innate immune response is non-specific and is triggered by pathogen-associated molecular patterns (PAMPs). PAMPs are evolutionarily conserved molecules that are essential for the life-cycle of the pathogen (peptidoglycan, lipopolysaccharides, dsRNA, CpG-DNA, etc.) (126). These molecular patterns are detected by germline-encoded pattern recognition receptors, such as Toll-like receptors (TLRs) and Nucleotide oligomerization domain (NOD)-like receptors (NLRs), found on the cell surface or within distinct intracellular compartments (41, 99, 126). PAMP detection induces the production of proinflammatory mediators, such as cytokines and interferons, which activate innate immune responses and play a role in activating and directing the adaptive immune response (126).

Cells involved in the innate immune response include phagocytes (such as macrophages and neutrophils), dendritic cells and natural killer (NK) cells (Figure 1.1). Neutrophils are recruited to the site of infection where they phagocytose and destroy invading microbes. NK cells have the morphology of lymphocytes but lack a specific antigen receptor. NK cells recognize and destroy infected and abnormal cells by releasing granules containing perforin and granzymes onto the surface of the cell to which they have adhered. Pore-forming protein perforin makes holes in the cell membrane and serine protease granzymes enter the cell and induce apoptosis (152, 191).

Cells of the innate immune system are important mediators in activation of the adaptive immune response. Macrophages and dendritic cells act as professional antigen-presenting cells (APCs). These cells phagocytose invading microbes and process their proteins, generating pathogen-derived peptides (Figure 1.1). These peptides are bound to major histocompatibility complex (MHC) molecules and presented on the cell surface where they are recognized by cells of the adaptive immune system (8, 165). Antigen presentation by APCs is crucial for initiating a cell-mediated adaptive immune response (27).

B. Adaptive immune system

The adaptive immune response is highly specific to the particular pathogen that induced it. The hallmark of the adaptive immune system is immunological memory, which confers long-lasting protection to the host against specific pathogens (41, 42).

The two major cell types of the adaptive immune system are B and T lymphocytes. B cells are involved in humoral immunity and secrete antigen-specific antibodies. B cell activation is triggered by binding of an antigen to the B cell receptor (BCR). BCR engagement results in rapid phosphorylation and activation of tyrosine kinase Syk, and leads to activation of signaling pathways leading to expression of genes that allow the B cell to proliferate and differentiate (71, 196). Receptor engagement also initiates signaling events leading to internalization of the antigen for processing and presentation to T cells (59). Antigen presentation to T cells provides the B cell with stimulatory signals which are required for an effective B cell response (9, 27, 42). When B cells undergo differentiation into plasma cells, they acquire the ability to produce and secrete antibodies (Figure 1.1). These antibodies aid other components of the immune system in defending against the invading pathogen by neutralizing toxins, opsonizing bacteria for phagocytosis, and sensitizing infected cells for destruction by cytotoxic cells (27, 42, 152).

T cells are non-antibody producing lymphocytes and constitute the basis of cell-mediated immunity. T cell activation is initiated by the engagement of the T cell receptor (TCR) with peptide–MHC complex presented on the surface of an APC (12, 13, 182). When activated, T cells differentiate into effector cells: $CD4^+$ T helper (Th) cells and $CD8^+$ T cytotoxic (Tc) cells (Figure 1.1). $CD4^+$ Th cells release cytokines that regulate and assist in the active immune response. They are further divided functionally into several subsets with distinct cytokine secretion profiles (114, 152).

$CD8^+$ Tc cells have a role in destroying infected and abnormal cells. $CD8^+$ Tc cells are cytotoxic to cells bearing their specific antigen. Infected cells display peptides derived from intracellular viral proteins on their surface bound to MHC molecules. Cytotoxic T cells bind to this antigen–MHC complex on the cell surface and kill the infected cell (42, 98, 152). In addition to direct killing of infected cells, $CD8^+$ Tc cells also produce a number of cytokines, including IFNγ which activates antiviral mechanisms in adjacent cells (42, 86).

Following encounter with an antigen, B and T cells clonally expand and differentiate. The clonal expansion generates a large number of antigen-specific effector cells. Following pathogen clearance, some B and T cells are maintained as antigen-specific memory cells (42, 152). The adaptive immune response provides the immune system with the ability to recognize and remember specific pathogens, and mount a stronger response to them if they are subsequently encountered. The anamnestic response mediated by memory B and T cells is more rapid and aggressive than the primary response and can control secondary infections quickly and reduce the severity of disease or prevent it entirely (86).

1.2 T cell development and function

T cells are key mediators of adaptive immune responses. T cells play a critical role in recognition and elimination of harmful pathogens and mediate immune surveillance against tumor cells. They produce cytokines to stimulate and mobilize cells of the immune system, attack and destroy infected and abnormal cells, and play a vital role in immunological memory (1, 13, 42, 152).

T cell development

T cells develop from bone marrow-derived precursors in the thymus through a series of coordinated developmental stages. Generation of mature T cells results from a strictly regulated selection process involving TCR rearrangement and a series of phenotypic changes (18, 54, 55, 92). Several checkpoints during T cell development ensure the development of functional but non-self-reactive T cells.

The earliest T cell progenitors in the thymus are double negative (DN) thymocytes, which do not express CD4 or CD8. DN cells are separated into four sequential phenotypic stages (DN1 to DN4) on the basis of CD25 and CD44 surface expression (18). T cell lineage commitment is a gradual process that occurs in DN thymocytes. A crucial step in T cell development, the rearrangement of variable gene segments to produce a functional TCR chain, occurs in T-committed DN3 cells (18, 92).

Two developmental checkpoints that depend on signal transduction by the TCR control progress to the next developmental stage. The first checkpoint occurs when a DN precursor progresses to the $CD4^+$ $CD8^+$ double positive (DP) stage. This checkpoint,

known as β-selection, requires thymocytes to signal through a pre-TCR consisting of a successfully rearranged TCRβ chain, a pre-TCRα chain, and CD3 subunits. Effective pre-TCR-mediated signaling assures successful β-chain rearrangement and allows passage of the cell beyond the first checkpoint. Thymocytes that pass β-selection initiate expression of CD4 and CD8, becoming DP thymocytes, and initiate α-chain rearrangement, which results in surface expression of the mature αβ TCR complex (18, 55, 189). The specificity and binding strength of the correctly assembled αβ TCR for self-peptide–MHC complexes determines cell survival and differentiation (55).

Passage beyond the second checkpoint depends on signals through the mature TCR. The TCRαβ interacts with endogenous peptides presented by MHC expressed by the thymic epithelium (55, 91). Positive and negative selection at this checkpoint depend on the strength of the interaction of the TCR with the peptide–MHC complex. Cells with TCRs which interact productively with self-peptide–MHC complexes (positive selection) but not so strongly to be autoreactive (negative selection) are rescued from programmed cell death and progress to the more mature single positive (SP) $CD4^+$ and $CD8^+$ stage (18, 54, 55, 91). Functional CD4-CD8 lineage differentiation includes the initiation of gene expression programs characteristic of helper (CD4) or cytotoxic (CD8) cells. Single positive thymocytes are the immediate precursors to the mature peripheral αβ T cells (18, 54, 55, 91, 189).

Major T cell subsets

Several T cell subsets can be distinguished on the basis of the composition of their T cell antigen receptor (αβ or γδ) and their effector potential. αβ T cells constitute the bulk of mature T cells and generally recognize peptides presented by MHC class I or MHC class II molecules, whereas γδ T cells are generally not MHC-restricted and seem to be involved in a lymphoid stress-surveillance response to microbial and nonmicrobial tissue perturbation (18, 61).

$CD4^+$ Th cells are the main orchestrators of the adaptive immune system and are essential in achieving an effective immune response to pathogens (82, 114). They secrete cytokines to regulate and assist other cells of the immune system. $CD4^+$ T cells differentiate into distinct effector lineages following activation depending on the cytokines present in the microenvironment. These subtypes are defined by the pattern of cytokines they produce (9, 114). Th1 cells are involved in elimination of intracellular pathogens and mainly secrete interleukin 2 (IL-2), which promotes $CD8^+$ T cell proliferation and cytotoxicity, and interferon γ (IFNγ), which activates macrophages and enhances phagocytic activity (114, 152). Th2 cells are involved in immune responses to extracellular pathogens. The key effector cytokines secreted by Th2 cells are interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), and interleukin 10 (IL-10), which favor antibody production (114, 152). Besides the classical Th1 and Th2, other subsets have been identified, including T-helper 17 (Th17), T-helper 9 (Th9), T-helper 22 (Th22), and follicular helper T (Tfh) cells with characteristic cytokine profiles (26, 58, 106, 114, 150, 152, 192).

$CD8^+$ Tc lymphocytes are very important for defense against intracellular pathogens, such as viruses or bacteria, and for tumor surveillance (27, 152). Cytotoxic T cells recognize and kill the infected cell by perforin/granzyme-induced apoptosis or by Fas-mediated apoptosis (9, 42, 98, 152). Tc cells also produce a number of cytokines which activate antiviral mechanisms in adjacent cells rendering them resistant to infection (42).

Regulatory T (Treg) cells are a developmentally and functionally distinct T cell subpopulation that is engaged in modulating immune responses. Tregs play an indispensable role in maintenance of immunological tolerance to self-antigens and in suppressing excessive and deleterious immune responses (142, 173).

Memory T cells are a subset of antigen-specific T cells that persist for a long time after an infection has been cleared, providing protection against reinfection by the same pathogen (41, 152). Memory T cells consist of both $CD4^+$ and $CD8^+$ T cells that can rapidly acquire effector functions to kill infected cells or secrete inflammatory cytokines (48, 86).

1.3 T cell activation

The activation of T cells during an immune response is mediated by the T cell receptor (TCR). The TCR is a multisubunit complex of integral membrane proteins, which consist of a clonotypic αβ dimer coupled to a signal transduction complex of six subunits (CD3γε, CD3δε and CD3ζζ pairs). The cytoplasmic segments of the CD3 subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) (a single copy on CD3γ, CD3δ and CD3ε chains, and three copies on CD3ζ chain), which are essential for the signaling capacity of the TCR (70, 113). Signaling through the TCR determines the functional outcome of T cell activation (1, 13, 104, 182).

A. TCR signaling pathways

The TCR has no intrinsic catalytic activity and depends on recruitment of protein kinases to transmit the activating signal (76, 87, 113). TCR engagement by peptide-bearing APC results in activation of Src family kinase Lck, which phosphorylates tyrosine residues within the ITAMs of the CD3ζ chain (33, 76, 113). ITAM phosphorylation leads to recruitment of protein tyrosine kinase Zap-70 to the activated receptor complex and its subsequent activation. Activated Zap-70 phosphorylates signaling adaptor proteins, which serve as scaffolds to recruit other signaling molecules involved in calcium mobilization, Ras activation, and cytoskeletal rearrangement (Figure 1.2) (1, 13, 76, 95, 102, 118, 168, 174, 182, 190). The functional outcome of these early signaling events is the transcription of gene products which allow the T cell to proliferate, differentiate, and secrete cytokines (69, 182). T cell activation and acquisition of appropriate effector functions requires integration of multiple signaling pathways that

originate from the engagement of the TCR by antigen-MHC at the site of T cell–APC contact (104, 178, 182, 213).

TCR proximal signaling

The earliest detectable event following TCR engagement is phosphorylation of ITAMs within the CD3ζ chain by Src family kinase Lck (76, 113). Tyrosine kinase Zap-70 is recruited to phosphorylated ITAMs where it is activated by Lck-mediated phosphorylation (60, 102, 146). The downstream targets of Zap-70 include signaling adaptor proteins LAT and SLP-76 (1, 14, 76, 182). Phosphorylation of adaptor proteins by Zap-70 results in recruitment of downstream signaling molecules, leading to activation of signaling pathways.

PLCγ activation

One molecule recruited to phosphorylated adaptor proteins is phospholipase Cγ1 (PLCγ1). Its localization to the proximal signaling complex, and subsequent activation, is dependent on LAT and SLP-76 phosphorylation (118). Activated PLCγ1 cleaves Phosphatidylinositol 4,5-bisphosphate (PIP2) located in the inner leaflet of the plasma membrane, producing second messengers Inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These second messengers are essential for initiating signaling pathways leading to T cell activation (76, 104, 118, 130).

Ras activation

Ras plays a critical role in cytokine gene expression and T cell proliferation (132). Ras is a Guanosine triphosphate (GTP)-binding protein which cycles between a

Guanosine diphosphate (GDP)-bound (inactive) and GTP-bound (active) state. Ras activity is controlled by the opposing effects of guanine nucleotide exchange factors (GEFs), which stimulate formation of the GTP-bound (active) state, and GTPase-activating proteins, which promote the intrinsic GTPase activity of Ras, leading to GTP hydrolysis and inactivation (132, 187). In GTP-bound form, Ras can interact with downstream effector proteins, leading to the activation of a number of kinases which are responsible for the eventual activation of mitogen activated protein kinases (MAPK) Erk1/2 (13, 76, 104). These kinases translocate to the nucleus and directly phosphorylate transcription factors involved in the formation of heterodimeric transcription factor Activator protein 1 (AP-1), which is essential for IL-2 expression in activated T cells (117, 132, 216).

Ca^{2+} mobilization

IP3 is a soluble molecule produced by the hydrolysis of PIP2 by PLCγ1. It induces the release of Ca^{2+} from the Endoplasmic Reticulum (ER). IP3 binds to IP3 receptors on ligand-gated Ca^{2+} channels on the surface of the ER. This results in release of Ca^{2+} stores from the ER lumen into the cytoplasm. Depletion of intraluminal Ca^{2+} leads to activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the plasma membrane, which allows influx of extracellular Ca^{2+} into the cell (179, 182). In response to increased Ca^{2+} levels, calcium sensor Calmodulin binds to protein phosphatase Calcineurin, resulting in activation of the phosphatase (169, 182). Calcineurin dephosphorylates Nuclear factor of activated T cells (NFAT), which allows it to enter the nucleus where it cooperates with

other transcription factors to bind to promoter elements and induce transcription of several cytokine genes, including IL-2 and IFNγ (37, 67, 104, 117).

NF-κB

Engagement of the TCR by an antigen also leads to activation of Nuclear factor κB (NF-κB) transcription factor (194). Activation of PLCγ1 results in production of second messengers IP3 and DAG. DAG in turn stimulates Protein kinase C θ (PKCθ) (104, 182). PKCθ phosphorylates scaffold protein CARMA1, which facilitates the recruitment of adaptor protein Bcl10 and paracaspase MALT1, leading to formation of a stable CARMA1/Bcl10/MALT1 (CBM) complex (35, 182, 194). Assembly of the CBM signalosome leads to activation of IκB kinase (IKK) allowing for phosphorylation and degradation of IκB, resulting in NF-κB nuclear localization (2, 104, 182, 194).

Cytoskeletal Rearrangement

Engagement of the TCR leads to dynamic rearrangement of the cortical cytoskeleton at the T cell–APC contact area, which accompanies and regulates T cell activation (100, 113). TCR stimulation together with CD28 co-stimulation leads to activation of Westcott-Aldrich syndrome protein (WASP), which allows it to bind to the actin regulatory complex, Arp2/3. Recruitment and activation of Arp2/3 results in actin polymerization and cytoskeletal reorganization, which ultimately leads to formation of the immunological synapse (1, 12, 13, 76, 174).

Immunological synapse

The immunological synapse forms at the site of T cell–APC contact and is characterized by segregation of key molecules into central and peripheral supramolecular activation clusters (cSMAC and pSMAC, respectively). The immunological synapse is thought to mediate sustained signaling required for T cell activation (12, 133, 145).

Although the formation of well-defined cSMAC often predicts effective T cell activation, the initial activation of TCR signaling occurs much earlier than immunological synapse formation (56, 78, 135, 178). Within seconds of TCR engagement by antigen, TCR-rich microclusters assemble at the site of T cell–APC contact and converge to form the cSMAC. After the cSMAC forms, sustained signaling required for T cell activation is driven by the continuous formation of TCR microclusters generated in the periphery of the mature immunological synapse. These microclusters, which contain TCR, Zap-70, and adaptor proteins, are the sites for generating the initial and sustained activation signals (15, 53, 178, 197, 213). Peripheral microclusters persist for only a short time (2-3 min), and then merge with the cSMAC (213). Centralization of TCR containing microclusters is believed to play a role in signal termination, as Zap-70, SLP-76 and other signaling molecules dissociate from the microclusters during their translocation into the cSMAC (178, 197).

B. Models for initiation of TCR signaling

The TCR recognizes pathogen-derived and self-peptides bound to MHC molecules displayed on diverse cell types. In order to prevent deleterious autoimmune responses,

T cells must discriminate between endogenous and foreign-peptide–MHC complexes. Early events in TCR signaling must be able to not only distinguish between self and foreign ligands with high selectivity, but also have high sensitivity required to respond to small numbers of agonist peptides. There are several models of initiation of TCR signaling that attempt to explain the ability of T cells to detect low number of foreign peptide–MHC ligands in the presence of high levels of self-peptide–MHC molecules.

Conformational change

Some models propose that TCR binding to peptide–MHC causes conformational changes that make the CD3ζ chain accessible to phosphorylation. According to one model, the TCR could act as a mechanosensor. When a T cell crawls on an antigen-presenting cell, a force associated with the binding event could cause deformations in the TCR complex that result in reorientation of the TCR component chains to help facilitate signal initiation. This model is supported by the observation that agonist CD3 antibodies bind the TCR in a different orientation that antibodies that do not trigger signaling (21, 89). Another possibility is that the TCR acts as an allosteric receptor and that binding to peptide–MHC induces a conformation change in the CD3 cytoplasmic domains which enable ITAM phosphorylation and signal initiation (21, 32, 195).

Aggregation

Some models of TCR triggering propose that aggregation of TCR complexes following TCR engagement could lead to enhanced phosphorylation (21, 195). Aggregation could increase proximity of associated Lck molecules to a second receptor

and facilitate trans-phosphorylation. The co-receptor heterodimerization model postulates that CD4 and CD8 co-receptors bound to the same peptide–MHC complex would recruit the co-receptor associated Lck into close proximity to the TCR complex and facilitate ITAM phosphorylation (34, 195). Another model, the pseudodimer model, postulates a role for self-peptide–MHC complexes. TCRs interact weakly with endogenous peptide–MHC complexes which are present at much higher density on the APC surface. According to this model, one TCR binds to antigen–MHC while a nearby second TCR interacts with self-peptide–MHC, promoting clustering of receptors and co-receptors and facilitating signal initiation (21, 97, 195).

Kinetic segregation

Another model for TCR signal initiation is the kinetic segregation model. Normally, TCR complexes are embedded in the plasma membrane along with molecules that promote signaling and molecules that inhibit signaling. TCR triggering occurs when the local balance of these molecules is altered in a way that promotes signal initiation by the formation of close contact zones (38, 195). In this model, TCR binding to an agonist peptide–MHC results in apposition of the T cell and APC membranes, leading to exclusion of molecules with large ectodomains (such as the inhibitory phosphatases CD45 and CD148) (21, 32, 195). This model is supported by the observation that CD45 and CD148 are excluded from the TCR–APC contact area (105, 197).

How a TCR is engaged by an antigen is well understood, however precisely how TCR engagement triggers intracellular signaling remains unclear. A number of models

have been proposed, several of which are supported by the available evidence (21, 38, 195). It is likely that TCR triggering is initiated by a combination of mechanisms that contribute to signal initiation and amplification.

1.4 Regulation of TCR signaling

T lymphocytes provide protection to the host against specific pathogens during an infection. But they can also be the source of immunopathology in disease associated with misregulation of T cell function (1, 141, 159). In order to prevent excessive and deleterious lymphocyte responses, T cells possess a number of mechanisms to limit the intensity and duration of signals generated by receptor activation.

Modulation of surface TCR

Modulation of surface TCR expression levels is one mechanism for regulating TCR signaling and plays an important role in adjusting T cell responsiveness (159, 199). Following antigen stimulation, the TCR is internalized via endocytosis and degraded in the lysosome (72, 199). Cbl family proteins are involved in negatively regulating T cell activation by promoting ubiquitination and internalization of TCR subunits (160). This process is essential for termination of TCR signaling, as demonstrated by the observation that Cbl knockout T cells, which do not down modulate surface TCR after ligand binding, are hyperresponsive to TCR stimulation (128).

Phosphorylation/dephosphorylation of signaling molecules

TCR stimulation induces the formation of phosphorylation-dependent signaling cascades. The signaling events beginning with TCR engagement and leading to T cell activation involve regulation of a number of protein kinases and phosphatases, and the phosphorylation status of their substrates (102, 127, 198).

Phosphorylation/dephosphorylation of key residues on signaling molecules regulates signal initiation and propagation (127).

One of the earliest detectable events after TCR ligation is the induction of tyrosine phosphorylation by Src family kinase Lck (76, 102, 113). Lck binds to the cytoplasmic domains of the TCR co-receptors CD4 and CD8 and its activity is regulated by the opposing actions of the Csk cytoplasmic kinase and the receptor-like protein phosphatase CD45 (122, 217). Lck is activated by removal of inhibitory C-terminal tyrosine phosphate. The inhibitory tyrosine residue is phosphorylated mainly by the Csk tyrosine kinase and dephosphorylated by members of the CD45 tyrosine phosphatase family (146).

A key initiation event in T cell activation by an antigen is phosphorylation of tyrosine residues within the ITAMs of the cytoplasmic CD3ζ chain by Lck (13, 21, 182). Tyrosine kinase Zap-70 is recruited to phosphorylated ITAMs and activated by phosphorylation. These events result in activation of a number of signaling pathways involving protein kinases, adaptor proteins, and effector enzymes in a highly organized phosphorylation-dependent cascade, and ultimately lead to T cell activation (13, 182).

Ubiquitination

Ubiquitination is an important regulatory mechanism involved in many aspects of TCR signaling, such as targeting signaling molecules for degradation and modulating protein-protein interactions and enzyme activity (83, 119).

Ubiquitination is involved in modulation of surface TCR by targeting the receptor complex for endocytosis. Ubiquitination of subunits of the TCR also serves nonproteolytic regulatory functions, such as reducing its association with Zap-70 (75).

Ubiquitination plays a crucial role in NF-κB activation by targeting inhibitory IκB molecules for proteasomal degradation. The ubiquitin-proteasome pathway is also involved in regulating a number of signaling molecules, including Lck and the p85 subunit of PI3K (44, 163).

Sts proteins

One mechanism for regulating TCR signaling involves two proteins, the Suppressor of TCR Signaling (Sts) proteins. The Sts proteins are two related proteins, Sts-1 and Sts-2, which act as negative regulators of TCR signaling pathways. They are characterized by a unique tripartite structure comprised of an N-terminal ubiquitin-association domain (UBA), a central SH3 domain, and a C-terminal histidine phosphatase (HP) domain (19, 20, 136, 193). The realization that Sts proteins play a role in regulating TCR signaling emerged from analysis of T cells derived from mice which lack the two proteins. *Sts-1/2*$^{-/-}$ T cells are hypersensitive to TCR stimulation, exhibiting a pronounced increase in TCR-induced proliferation relative to wild-type. This phenotype is accompanied by an increase in cytokine production by *Sts-1/2*$^{-/-}$ T cells and significantly increased susceptibility of *Sts-1/2*$^{-/-}$ mice to autoimmunity (19, 20, 125, 193). In addition, *Sts-1/2*$^{-/-}$ T cells display enhanced phosphorylation and activation of Zap-70, a kinase that plays a critical role in relaying activating signals from the TCR (20).

1.5 Zeta-chain-associated protein kinase 70 (Zap-70)

A. Zap-70 structure and function

The TCR has no intrinsic catalytic activity and depends on the recruitment and activation of protein tyrosine kinases for transmission of an activation signal (182). Zap-70 plays a critical role in propagating signals initiated by TCR engagement and is required for activating many signaling pathways downstream of the activated TCR (5, 50, 200).

Zap-70 is a tyrosine kinase of the spleen tyrosine kinase (Syk) family. Its expression is predominantly limited to thymocytes and peripheral T cells (5, 50). Zap-70 contains two N-terminal SH2 domains that are separated by a linker region termed Interdomain A, and a C-terminal kinase domain (Figure 1.3). The region between the second SH2 domain and the kinase domain, Interdomain B, contains three tyrosine residues (Tyr-292, Tyr-315, and Tyr-319) that are critical for Zap-70 regulation (5).

The tandem SH2 domains of Zap-70 bind to doubly phosphorylated ITAMs of the CD3ζ chain during T cell activation. This binding is responsible for the recruitment of Zap-70 to the activated receptor complex, which is critical for its activation (5, 60, 200). The crystal structure of the tandem SH2 domains in complex with an ITAM-derived peptide reveals details of the interaction between the two domains and the phosphorylated ITAMs (51, 60). The phosphotyrosine-binding (PTB) pocket of the N-terminal SH2 domain is formed by residues contributed by both the N- and C-terminal SH2 domains, while the C-terminal PTB pocket is completely composed of residues from the C-terminal

SH2 domain. In the bound state, the two SH2 domains are tightly apposed and make extensive contacts with each other and the phosphorylated ITAMs (Figure 1.4) (51). In addition, interdomain A forms a coiled-coil domain that facilitates contact between the SH2 domains and stabilizes their interaction. The cooperative binding of the tandem SH2 domains confers considerable specificity to ITAM binding and higher affinity for doubly phosphorylated ligand, preventing spurious activation of Zap-70 (5, 60, 144).

Zap-70 kinase domain has a bilobed structure that is conserved among the catalytic domains of protein kinases (5, 101). The smaller N-terminal lobe is composed of a five-stranded antiparallel β-sheet and a single α helix (αC). The larger C-terminal lobe is predominantly helical with short strands of β-sheets. The catalytic site is located at the junction between the N- and C-terminal lobes, and the two lobes are connected by an extended linker region (84). The activation loop in the C-terminal lobe contains two regulatory tyrosine residues (Tyr-492 and Tyr-493). It is thought that phosphorylation of these residues results in a conformational change, leading to displacement of the activation loop from the catalytic site of the kinase (5, 200). Phosphorylation of Tyr-493 is required for activation of Zap-70 *in vivo* (22, 203).

The SH2-kinase linker, interdomain B, contains three tyrosine residues that are phosphorylated following TCR stimulation (Tyr-292, Tyr-315 and Tyr-319) (5, 50). These residues serve important regulatory roles and have been implicated in binding signaling and regulatory molecules including Cbl, Lck, and PLCγ (116, 134, 162, 207). In addition

to acting as binding sites for signaling molecules, Tyr-315 and Tyr-319 are part of an autoinhibitory mechanism which regulates Zap-70 activity (5, 39, 40, 84, 200, 210).

B. Zap-70 in T cell development

Antigen receptor signaling is critical for thymocyte development. Two developmental checkpoints that require TCR signaling control progress to the next developmental stage. The first checkpoint occurs when a $CD4^-CD8^-$ DN cell progresses to the $CD4^+CD8^+$ DP stage (18, 54). Progression beyond this checkpoint requires successful pre-TCR mediated signaling. The second checkpoint occurs when DP thymocytes progress to the more mature $CD4^+$ and $CD8^+$ SP stage (lineage differentiation). Progression past the second checkpoint requires signaling through a mature TCR (18, 54, 91, 189).

At the DP stage, thymocytes undergo a process of selection in which those cells that have TCR complexes which interact productively with peptide–MHC complexes but not so strongly to be autoreactive differentiate into either $CD4^+$ or $CD8^+$ lineage T cells (18, 91). Dynamic regulation of TCR signaling during selection plays a role in discriminating CD4 and CD8 lineage development. Zap-70 is essential in both positive and negative selection of thymocytes and is involved in the discrimination of lineage-specific signaling events (170, 181).

The abundance of Zap-70 is a key determinant of sensitivity of cells to TCR stimulation. Zap-70 expression is upregulated during positive selection of DP thymocytes, functionally increasing the sensitivity of the TCR to stimulation during selection. Signaling

downstream of the TCR induces upregulation of Zap-70 expression, forming a positive feedback circuit. A failure to upregulate Zap-70 expression in DP thymocytes prevents normal generation of $CD8^+$ SP thymocytes (170). Disruption of the positive feedback circuit by replacing Zap-70 with a fixed-expression inducible transgene also compromises the efficiency of thymocyte differentiation to the CD4 lineage. In these cells, suboptimal Zap-70 signaling results in misdirection of MHC Class II-restricted thymocyte development into CD8 lineage precursor DP thymocytes which are unsuccessful in generating long-lived CD8 lineage T cells (181).

Zap-70 and Syk

Zap-70 and its close relative Syk, which is essential for BCR signaling, share a similar domain organization that includes tandem SH2 domains and a C-terminal kinase domain and both kinases bind phosphorylated ITAMs in antigen receptor subunits (85, 208). Zap-70 is exclusively expressed in T cells and NK cells, and is present in all thymocyte subpopulations as well as in mature T cells (5, 50, 196). In contrast, Syk is most abundantly expressed in B cells, but it is also found at low levels in other cell types including T cells. Syk protein levels are higher in thymocytes than in mature T cell populations as its expression decreases after thymocytes reach the DP stage (24, 196).

Zap-70 and Syk have crucial but overlapping functions in pre-TCR signaling and early T cell development. Syk can compensate partially for the lack of Zap-70 in pre-TCR signaling during developmental events to allow transition through the first developmental checkpoint (DN to DP transition) in *Zap-70*$^{-/-}$ mice (85). DN thymocytes in mice lacking

both Syk and Zap-70 undergo β rearrangement but fail to initiate pre-TCR signaling and are incapable of differentiating into DP cells (29, 85). As a result, T cell development is arrested at the DN stage in mice lacking both proteins.

C. Zap-70 regulation

The recruitment and activation of Zap-70 are key steps in initiating the signaling cascade leading to T cell activation. As such, tight regulation and specific activation of Zap-70 are critical for a proper T cell response (5, 200).

Zap-70 activation is a multistep process and is regulated both by binding to phosphorylated ITAMs of CD3ζ chain and by phosphorylation of multiple residues on Zap-70 itself (5, 90, 110, 134, 188, 200).

Binding of Zap-70 to phosphorylated ITAMs of the CD3ζ chain not only serves to localize the kinase to the receptor complex, the binding event also results in a large conformation change in the tandem SH2 domains, which destabilizes its cytosolic autoinhibited conformation (5, 39, 40, 200). In inactive Zap-70, the tandem SH2 domains are docked onto the kinase domain, with interdomain B packed between Interdomain A and the kinase domain. Extensive hydrogen bonding between the two linker regions and the kinase domain reduces the flexibility of the hinge region connecting the two lobes of the kinase domain and stabilizes the inactive conformation. Critical residues stabilizing this conformation include Trp-131 in interdomain A, Tyr-315 and Tyr-319 in interdomain B, and Pro-396 in the kinase domain. Aromatic-aromatic interactions between the side chains of these residues create a stable hydrophobic environment. Upon ITAM binding, the SH2

domains move relative to each other (Figure 1.4), resulting in structural changes in interdomain A leading to disassembly of the inhibitory interface. This exposes regulatory residues Tyr-315 and Tyr-319 in Interdomain B. Phosphorylation of these residues further destabilizes the autoinhibitory interactions since phosphorylated tyrosine residues cannot be accommodated in the hydrophobic environment at the inhibitory interface (39, 40, 51). Phosphorylation of Tyr-493 in the activation loop of the kinase domain results in full activation, leading to the initiation of downstream signaling pathways that are important for T cell proliferation, differentiation, and effector functions (5, 69, 76, 200).

Phosphorylation of specific tyrosine residues has also been implicated in negative regulation of Zap-70 function. Phosphorylation of Tyr-292 promotes association of Zap-70 with negative regulatory molecules, such as Cbl family members (10, 162, 201). Some evidence suggests that phosphorylation of Tyr-492 plays a role in negatively regulating kinase activity, although the underlying mechanism remains unclear (5, 200, 203).

Zap-70 is negatively regulated by the Sts phosphatases, as demonstrated by its hyper-phosphorylation in stimulated *Sts-1/2*$^{-/-}$ T cells relative to stimulated wild-type T cells (19, 20, 125, 193). This increased phosphorylation of Zap-70 includes the native 70 kDa form as well as higher molecular weight forms corresponding to ubiquitinated Zap-70. Recently, it was also demonstrated that Zap-70 is modified by K33-linked polyubiquitination predominantly on Lys-578 following TCR engagement, and that the Sts proteins play a role in the dephosphorylation of polyubiquitinated Zap-70 in

stimulated $CD8^+$ T cells (212). Another study revealed that Otud7b facilitates Zap-70 activation by deubiquitinating Zap-70, thus preventing its association with negative-regulatory phosphatases Sts-1 and Sts-2 (74). Other phosphatases, such as SHP1, have also been implicated in negatively regulating Zap-70 (11, 157).

D. Zap-70 and disease

The entire TCR signaling pathway must be optimally activated to ensure proper T cell development during thymic selection as well as appropriate T cell activation in the periphery during an immune response. Mutations in key enzymes in this pathway that impair signaling are associated with immunodeficiency and autoimmunity (5, 180, 200).

Zap-70 deficiency

Loss of Zap-70 function or expression results in severe developmental and functional defects in the T cell compartment of both humans and mice (4, 23, 45, 66, 200). In humans, lack of Zap-70 or complete loss of its function leads to development of severe combined immunodeficiency (SCID), which is characterized by absence of $CD8^+$ T cells and non-functional $CD4^+$ T cells in the peripheral blood (4, 23, 45, 46, 66). In *Zap-70*$^{-/-}$ mice, thymocyte development is arrested at the $CD4^+$ $CD8^+$ DP stage and there is complete absence of both $CD4^+$ and $CD8^+$ peripheral T cells (46, 85, 131). These observations underscore the importance of Zap-70 signaling in mature T cells and during T cell development.

Hypomorphic Zap-70 mutations

Null mutations that abolish TCR signaling result in rare severe primary immunodeficiencies. However, more common polymorphisms that lead to subtle defects in TCR signaling are paradoxically associated with autoimmunity (180).

TCR signaling pathways must be precisely activated during thymic selection to ensure a diverse and well-balanced peripheral T cell repertoire. The outcome of selection events during T cell development is determined by the strength of the signal delivered by the TCR. Quantitative changes in TCR signal strength affect positive and negative selection thresholds during thymic development and are associated with autoimmunity and immunopathology (180, 200). The signal strength is determined by the affinity of the TCR for self-peptide–MHC complexes and the efficiency of intracellular signal transduction, which is dependent on the availability and function of relevant signaling molecules (129, 180).

In SKG mice, a mouse model for rheumatoid arthritis, a single missense mutation (W163C) in the C-terminal SH2 domain of Zap-70 impairs the ability of Zap-70 to bind to phosphorylated ITAMs of the CD3ζ during T cell activation. This leads to alterations of signaling thresholds in T cells, which result in defects in T cell development and differentiation and lead to autoimmunity (36, 171, 172, 180, 200).

The Zap-70 YYAA mouse strain has knock-in alanine mutations of Tyr-315 and Tyr-319 in interdomain B of Zap-70. YYAA mice show defects similar to SKG mice, including diminished TCR signaling, impaired T cell development, and defective positive

and negative selection (73, 200). Unlike SKG mice, YYAA mice rarely develop autoimmune arthritis (73).

A series of Zap-70 variants with different levels of reduction in TCR signaling impact opposing TCR functions of immunity and tolerance. One variant, *murdock*, moderately decreases TCR signaling and thymic selection without compromising immunological tolerance, whereas a more severe defect, *mrtless*, abolishes positive selection and leads to immunodeficiency (180). Signaling capacities between these two thresholds compromise thymic selection and regulatory T cell development, creating a cellular imbalance between immunogenic and tolerogenic functions that results in paradoxical autoimmune and immunocompromised states (180).

Zap-70 R192W/R360P

A novel human autoimmune syndrome was found to be caused by combined hypomorphic and activating mutations in Zap-70 (25). Mutation R192W in the C-terminal SH2 domain exhibits reduced binding to CD3ζ chain during T cell activation, and mutation R360P in the kinase domain produces a weakly hyperactive protein by disrupting autoinhibitory mechanisms. The two missense mutations in Zap-70 are associated with early onset, severe autoimmune manifestations, including bullous pemphigoid and colitis (25).

Chronic lymphocytic leukemia (CLL)

B cell chronic lymphocytic leukemia (B-CLL) is a B cell malignancy caused by accumulation of B lymphocytes in the blood, bone marrow, spleen, and lymph nodes (30).

Zap-70 expression in B-CLL patients correlates with more aggressive disease and significantly worse prognosis (77, 143, 206). Whether Zap-70 plays a functional role in B-CLL progression remains unclear.

E. Zap-70 as a therapeutic target

Zap-70 plays an essential role in pre-TCR and TCR signaling during T cell development in the thymus and in peripheral T cells (5, 200). Due to its critical role in TCR signaling, Zap-70 is an attractive target for pharmaceutical intervention. Because Zap-70 expression is predominantly limited to thymocytes, peripheral T cells, NK cells, and basophils, effects of specific inhibition would be limited to these cell types. Since NK cells and basophils also express Syk, which may compensate for the lack of Zap-70, inhibition of Zap-70 would specifically target T cell responses, leaving innate immunity intact (131, 200).

Zap-70 catalytic activity and adaptor function are subject to extensive positive and negative regulation, mediated by many regulatory molecules including kinases, phosphatases, and E3 ubiquitin ligases. A better understanding of the specific mechanisms involved in its regulation would provide additional targets for therapeutic intervention in the prevention and treatment of diseases associated with misregulation of T cell function.

1.6 Ubiquitination

Ubiquitin is a 76-amino acid globular protein which is highly conserved across eukaryotes (49, 204). Ubiquitination is an ATP-dependent process involving the covalent attachment of the C-terminal glycine of ubiquitin to the ε-amino group of lysine residues of the target protein (81, 204). Ubiquitination plays a central role in a variety of cellular processes, such as DNA repair, transcription, receptor endocytosis, cell cycle control, apoptosis, and stress responses (103, 204). The functional flexibility of this post-translational modification is due to the large number of ubiquitinating enzymes involved in the formation of distinct ubiquitin chains which encode different signals (103).

Ubiquitination cascade

The covalent attachment of ubiquitin to a substrate is achieved by the sequential action of three types of enzymes (Figure 1.5) (63, 81, 204). First, ubiquitin is activated by a ubiquitin-activating enzyme (E1). The E1 enzyme forms a thiol-ester bond with the C-terminal glycine of ubiquitin in an ATP-dependent reaction. Then, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2) by a trans-thiolation reaction. Finally, a ubiquitin ligase (E3) catalyzes the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the target protein (28, 81, 155, 204).. Substrate specificity is determined by the E3 ligase, alone or in combination with its bound E2 (123, 204).

E3 ubiquitin ligases

The E3 ubiquitin ligases play an important role in the ubiquitination cascade by recognizing specific substrates and facilitating ubiquitin transfer from the E2 to a lysine residue on the substrate (93, 123). E3 ligases can be generally divided into two main groups: the Homologous to the E6-associated protein carboxyl terminus (HECT) domain-containing E3s, and the Really interesting new gene (RING) domain-containing E3 ligases (108, 123). For E3 ligases of the HECT domain family, transfer of ubiquitin from E2 to the target protein involves a thioester intermediate with the catalytic cysteine in the active site of the E3 ligase. RING finger-containing E3 ligases mediate direct transfer of ubiquitin from E2 to the substrate (93, 123). The RING finger family of E3s represents the largest group of E3s and includes the Cbl family proteins. The Cbl family proteins are evolutionarily conserved negative regulators of activated tyrosine kinase-coupled receptor signaling (160, 163). Antigen receptors are prominent targets of negative regulation by Cbl family members. Cbl-mediated ubiquitination of activated receptors and signaling molecules results in attenuation of receptor signals (43, 160, 163).

Types of ubiquitin modifications

A single protein can be modified on one or more lysine residues by a single ubiquitin (monoubiquitination) or a polyubiquitin chain. Such chains are formed by linking a lysine residue on ubiquitin to a new ubiquitin molecule (64, 81). Ubiquitin itself has seven lysine residues (K6, K11, K27, K29, K33, K48, K63), all of which are potential sites of peptide linkage, and the choice of lysine in polyubiquitin chain formation has important functional consequences (Figure 1.5) (81, 103, 156, 204, 209). The diversity of

signaling functions associated with ubiquitination is due to the many different ways in which polyubiquitin chains can be formed, as either uniform (containing ubiquitin molecules linked through the same lysine) or as atypical branched chains with mixed linkages which seem to serve different context-specific functions (94, 124). Modification with polyubiquitin chains linked through lysine 48 (K48) is known to target proteins for proteasomal degradation, with a chain of four or more K48-linked ubiquitin molecules being adequate as a proteasome-targeting signal (81, 93, 94, 204). In contrast, modifications with a single ubiquitin and K63-linked polyubiquitin chain serve to alter the substrate function and interacting partners rather than its stability (64, 81, 103, 204). Other noncanonical polyubiquitin chains (linked via K6, K11, K27, K29, and K33) are less well characterized and have various proteolytic and non-proteolytic functions (28, 94, 156).

Ubiquitin-binding and deubiquitination

Various proteins recognize ubiquitin modifications through Ubiquitin-binding domains (UBD). These domains can directly interact with monoubiquitin or polyubiquitin chains and serve to recognize and interpret ubiquitin modification signals on substrate proteins (65, 79). UBDs are found in proteins that function in many different cellular processes, such as proteins involved in ubiquitination and deubiquitination, proteasomal degradation, and endocytic pathways (65, 94).

Like phosphorylation, ubiquitination is a dynamic and reversible process. Deubiquitinating enzymes (DUBs) are thiol proteases which clave the isopeptide bond between ubiquitin and its substrate protein. DUBs remove ubiquitin from proteins and

disassemble polyubiquitin chains (94, 204). DUBs reverse ubiquitination of target proteins, playing a role analogous to phosphatases in kinase/phosphatase regulatory pathways. Like ubiquitination, deubiquitination is a highly controlled process and has been implicated in the regulation of a variety of cellular functions (94, 164).

1.7 Figures

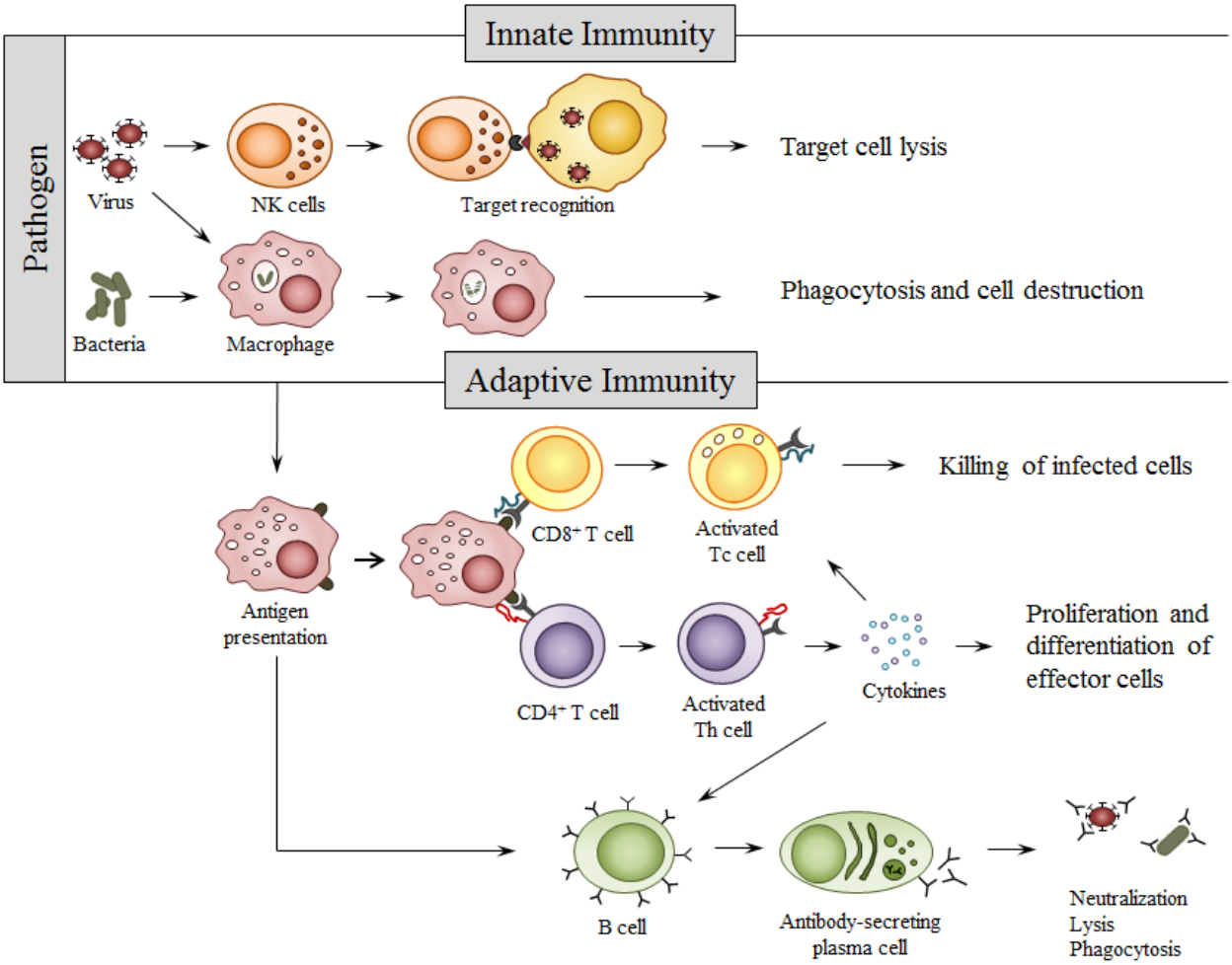

Figure 1.1 The innate and adaptive immune responses. The immune system is divided into two main branches, the innate and adaptive immune systems. Cells of the innate immune system include phagocytes (such as macrophages), dendritic cells and NK cells. NK cells recognize and destroy infected and abnormal cells. Macrophages phagocytose invading microbes and present pathogen-derived peptides to cells of adaptive immune system. The adaptive immune response consists of cell-mediated and humoral immunity. T cells constitute the basis of cell-mediated immunity. They differentiate into $CD4^+$ Th cells, which release cytokines that regulate and assist in the active immune response, and $CD8^+$ Tc cells, which release cytotoxins to destroy infected cells. B cells are involved in humoral immunity and produce and secrete antigen-specific antibodies which aid other components of the immune system.

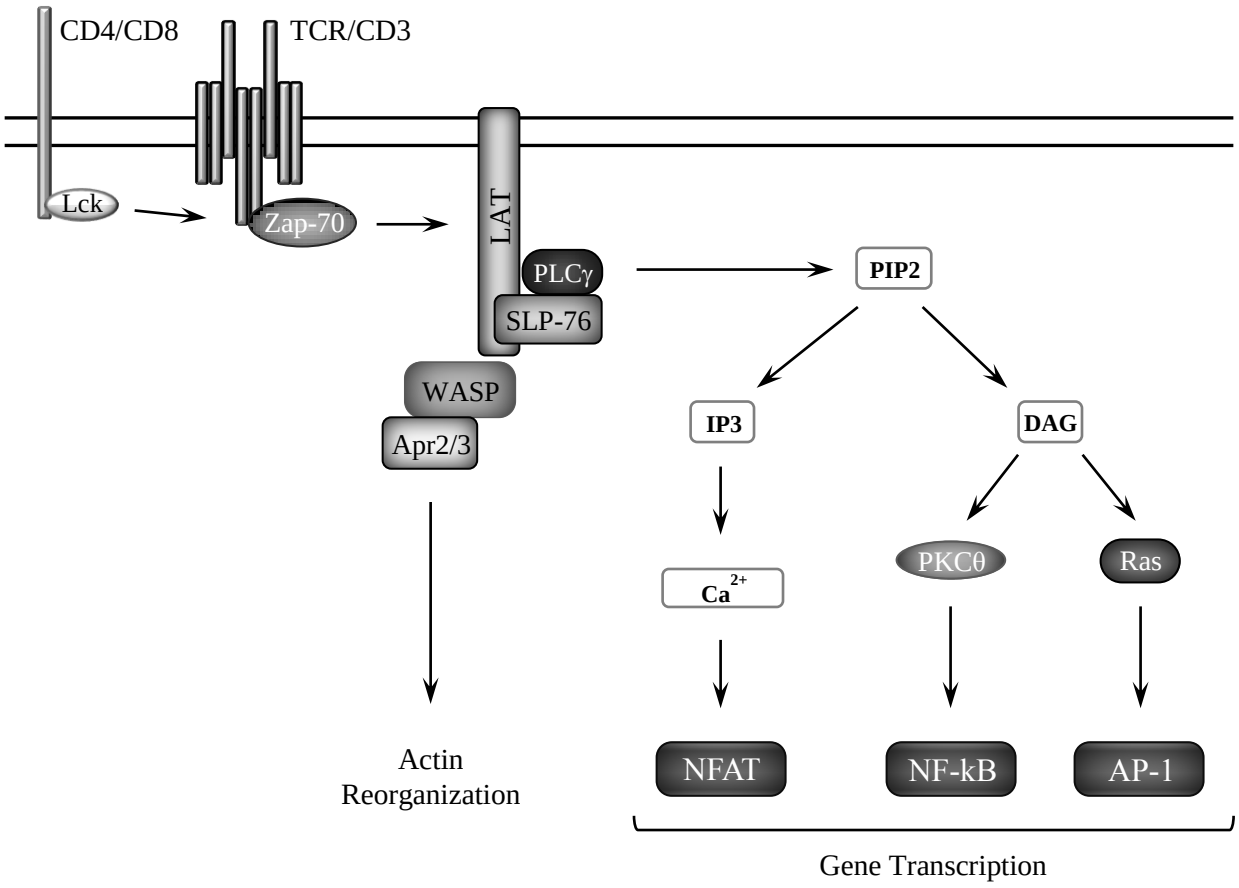

Figure 1.2 Simplified overview of TCR signaling pathways. TCR engagement results in Lck-mediated phosphorylation of tyrosine residues within the ITAMs of the CD3ζ chain. Zap-70 is recruited to phosphorylated ITAMs activated. Zap-70 subsequently phosphorylates signaling adaptor proteins LAT and SLP-76, which serve as scaffolds to recruit other signaling molecules, including PLCγ. PLCγ1 cleaves PIP2 producing second messengers IP3 and DAG. IP3 mediates Ca^{2+} flux and subsequent activation of NFAT. DAG activates a number of proteins including PKC, which is involved in NF-kB activation, and Ras, which leads to formation of transcription factor AP-1. Activation and nuclear localization of these transcription factors leads to the transcription of gene products that allow the T cell to proliferate, differentiate and secrete cytokines. TCR engagement also leads to activation of WASP and recruitment of the actin regulatory complex Arp2/3, resulting in actin polymerization and cytoskeletal reorganization, which ultimately leads to formation of the Immunological synapse.

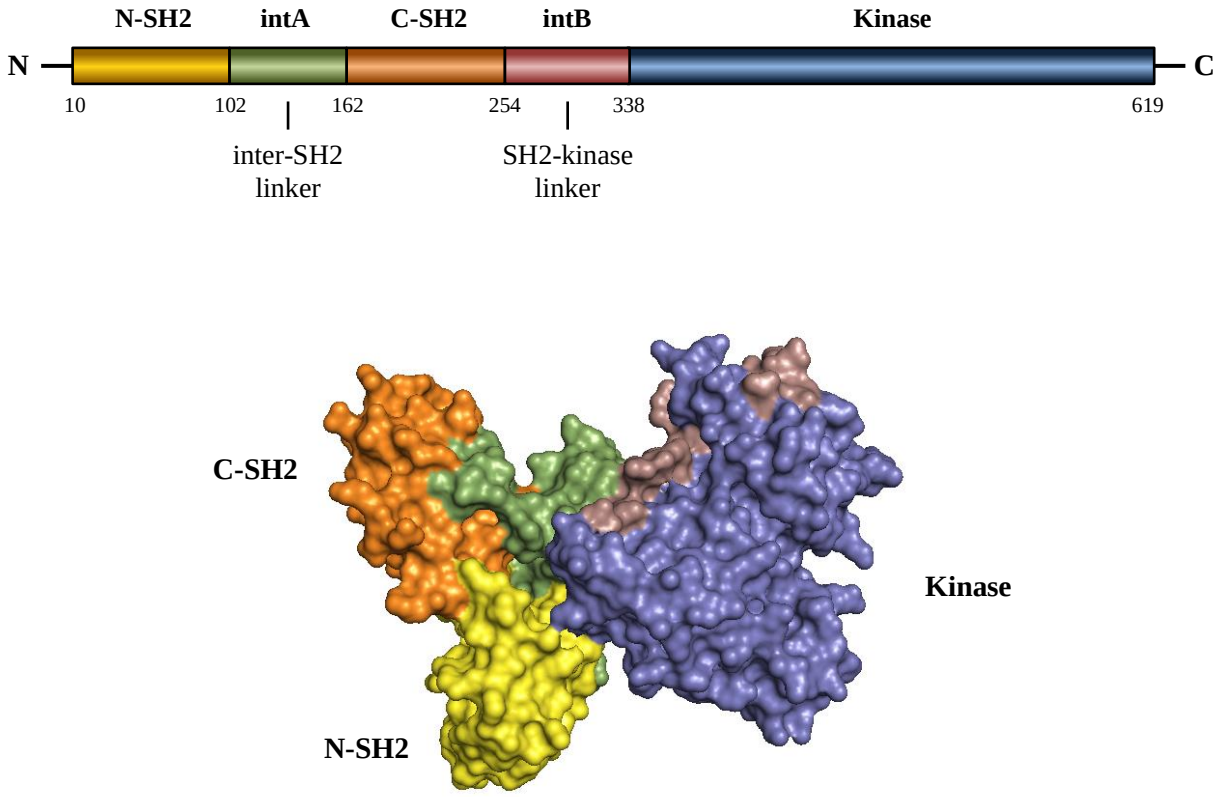

Figure 1.3 Zap-70 structure. Zap-70 domain organization (*top*) and crystal structure of full length inactive Zap-70 (*bottom*) (PDB ID: 2OZO) (39). The N-terminal and C-terminal SH2 domains, interdomain A, interdomain B, and kinase domain are shown in yellow, orange, green, red, and blue, respectively.

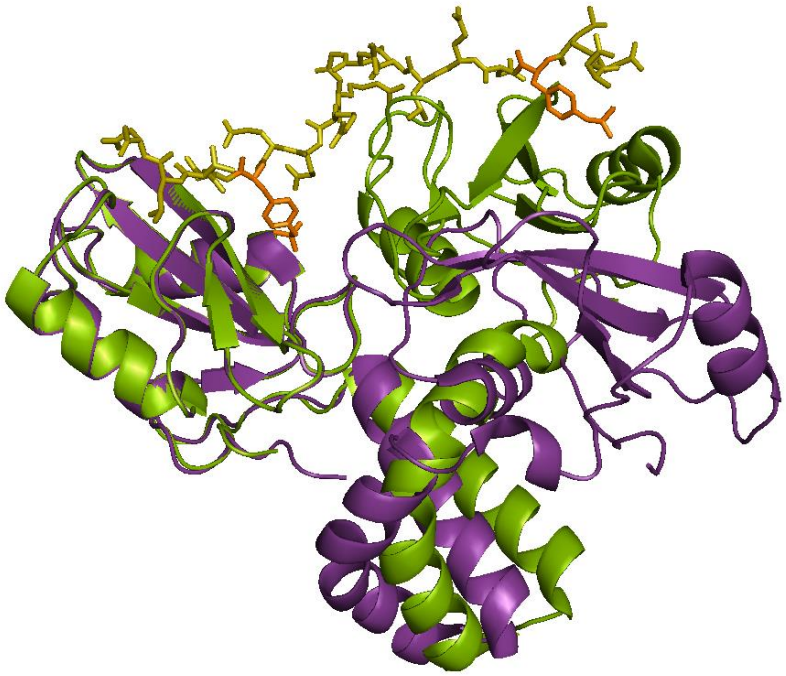

Figure 1.4 Comparison of the structure of unbound and ITAM-bound tandem SH2 domains of Zap-70. Crystal structure of unbound tandem SH2 domains of Zap-70 (PDB ID: 1M61) and crystal structure of Zap-70 SH2 domains in complex with a peptide derived from the first ITAM of the CD3ζ chain (PDB ID: 2OQ1) (52, 60). Unbound SH2 domains are shown in purple, ITAM-bound SH2 domains in green, and ζ1 peptide is shown in yellow with phospho-tyrosines in orange. The structure of the N-terminal SH2 domain (residues 9-105) was used to superimpose the molecules. The figure was generated using PyMOL.

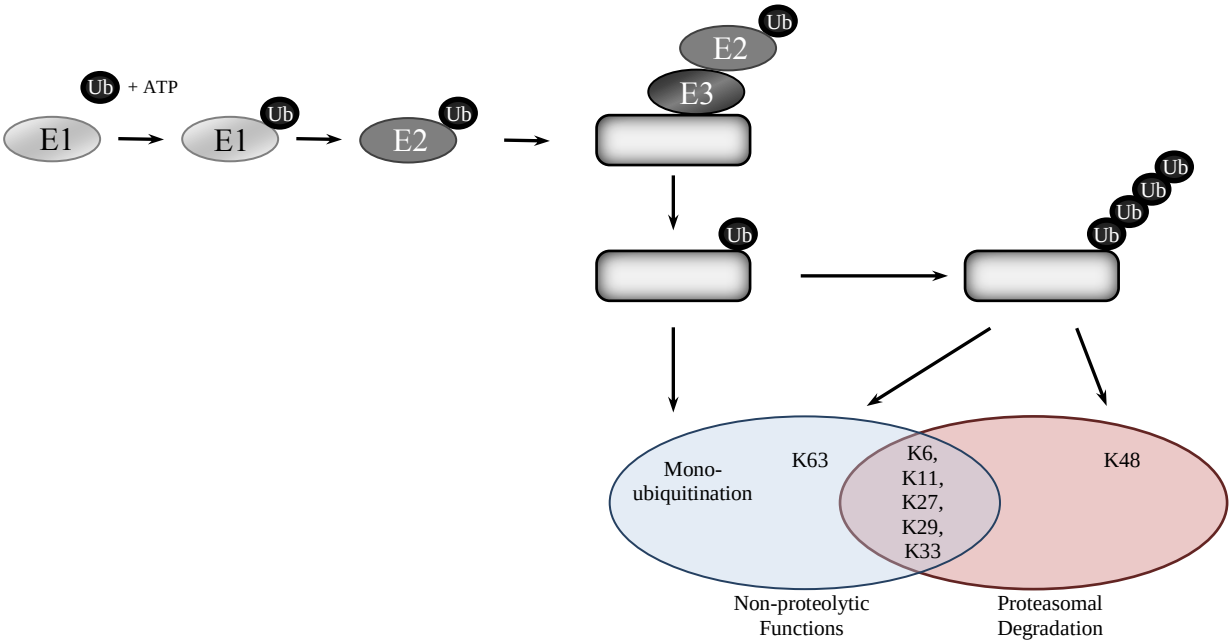

Figure 1.5 The ubiquitination pathway. The covalent attachment of ubiquitin to a substrate is achieved by the sequential action of three types of enzymes: a ubiquitin-activating enzyme (E1) which activates free ubiquitin in an ATP-dependent reaction, a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) which transfers the activated ubiquitin from the E2 enzyme to the substrate. A protein can be modified by a single ubiquitin molecules or different polyubiquitin chains. Monoubiquitination and K63-linked polyubiquitination generally serve non-proteolytic functions, whereas K48-linked polyubiquitin chains target proteins for proteasomal degradation. Polyubiquitin chains of other linkages have various proteolytic and non-proteolytic functions.

Chapter 2: Experimental procedures

2.1 Materials and methods

Mice

The generation of mice containing the Sts mutations, backcrossed 10 generations onto the C57/B6 background, has been described (20). Mice were housed and bred in the Stony Brook University Animal Facility under specific pathogen-free conditions. Mice used for experiments were 8 weeks old. All mice were maintained in accordance with Stony Brook University Division of Laboratory Animal Resources (DLAR) guidelines. All animal experiments were approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC).

Cell culture

Raji B cells (ATCC CCL-86), Jurkat clone E6-1 (ATCC TIV-152), P116 (Zap-70 deficient Jurkat sub-clone; ATCC CRL-2676), and P116 cells expressing wild type or mutant Zap-70 were maintained at 37°C in 5% CO_2 in RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and the appropriate concentration of penicillin and streptomycin. TK-1 cells (ATCC CRL-2396) were cultured in T cell medium (RPMI media containing 10% FBS, 10 mM HEPES (pH 7.0), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 0.1 mM nonessential amino acids, and the appropriate concentration of penicillin/streptomycin). Human Embryonic Kidney 293T

(HEK293T) cells (ATCC CRL-11268) were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. Primary murine T cells were derived from wild-type and *Sts-1/2*$^{-/-}$ mice by crushing spleens in phosphate-buffered saline (PBS) containing 2% FBS, lysing red blood cells in ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA), and removing debris by filtration through a 70 μM filter (BD). T cells were expanded for 2 days in T cell medium supplemented with 0.5 μg/ml anti-CD3ε antibody (145-2C11) and 10 U/ml IL-2. T cells were further expanded for 2 days in fresh T cell media containing IL-2.

Antibodies and reagents

Antibodies against Zap-70 (A-1), SLP-76 (6A462), LAT (FL-233), CD3ζ (6B10.2), and ubiquitin (P4D1) were obtained from Santa Cruz Biotechnology. Anti-pTyr (4G10) antibody was from Upstate Biotechnology, Inc. Polyclonal antibodies against phosphor-Zap-70 Y493, SLP-76, MAPK, phospho-MAPK (Thr 202/Tyr 204), K48-linked polyubiquitin, as well as monoclonal MAPK (3A7) and K63-linked polyubiquitin (D7A11) antibodies were obtained from Cell Signaling Technology. Anti-β-actin (8H10) antibody was from ABM, Inc. Anti-Flag (M2) antibody was purchased from Sigma-Aldrich. For immunoblotting analysis, secondary antibodies were Alexa Fluor® 680-conjugated goat anti-mouse (Molecular Probes) and IRDye800-conjugated goat anti-rabbit (LI-COR). Agarose-conjugated phospho-tyrosine antibody (4G10) used in purification of ubiquitinated Zap-70 was obtained from EMD Millipore. Fluorochrome-conjugated IL-2 antibody (JES6-5H4) used for intracellular cytokine staining was purchased from

BioLegend. Fluorochrome-conjugated Thy1.2 antibody (53-2.1) was purchased from eBioscience. For stimulation of murine T cells, anti-CD3ε (purified 145-2C11 or biotinylated 500A2), biotinylated anti-CD4 (RM4-5), and biotinylated anti-CD8 (53-6.7) were employed. For stimulation of human T cells, biotin-conjugated CD3ε (UCHT1) antibody was used. FITC-streptavidin (BD Pharmingen) was used for cross-linking. Recombinant human IL-2 cytokine was purchased from Peprotech Inc. Partially purified Staphylococcal enterotoxin D (SED) was obtained from Toxin Technology (Sarasota, FL).

T cell isolation and stimulation

Primary murine T cells ($6x10^7$) were labeled on ice for 25 min with 20 μg/ml biotin-conjugated anti-CD3ε (500A2), anti-CD4 (RM4-5) and anti-CD8a (53-6.7) antibodies, washed with PBS with 2% FBS, incubated on ice for 25 minutes with 10 μg/ml FITC-streptavidin in the presence or absence of pervanadate (PV) as indicated, placed in a 37°C water bath for 2 minutes or for the indicated times, and immediately lysed in cold lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing 100 μM PMSF, Complete™ protease inhibitors (Roche), 1 mM sodium orthovanadate and 25 mM N-ethylmaleimide (NEM; Sigma-Aldrich). A stock solution of 50 mM pervanadate was freshly prepared by mixing equal volumes of 100 mM H_2O_2 with 100 mM sodium orthovanadate. For proteasomal inhibition, cells were pre-treated with 100 μM MG-132 (Boston Biochem, Inc.) for 1 hr at 37°C in T cell medium containing IL-2, then stimulated as described with MG-132 present during all the steps of the stimulation.

Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll-Hypaque (Lymphocyte Separation Medium 1.077, Lonza) gradient centrifugation. Peripheral blood T cells were isolated from PBMCs by negative selection using Pan T cell Isolation Kit (Miltenyi Biotec). Primary human T cells ($3.5x10^7$) were stimulated as described above using biotin-conjugated CD3ε (UCHT1) antibody and FITC-streptavidin.

Immunoprecipitation and immunoblotting

Cell extracts were prepared in ice-cold lysis buffer containing 100 μM PMSF, Complete™ protease inhibitors (Roche), 1 mM sodium orthovanadate and 25 mM NEM, clarified by centrifugation and rotated at 4°C with specific antibody for 1 hr and with Protein A-sepharose beads (Sigma) for 2 hrs. After beads were washed 3 times in cold wash buffer (20 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 100 μM PMSF and 1 mM sodium orthovanadate), bound proteins were eluted with Laemmli sample buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane (Whatman) using a semidry transfer apparatus (BioRad). Membranes were blocked with 3% BSA in Tris-buffered saline (TBS, pH 8.0), incubated at 4°C overnight with specific antibody followed by the appropriate secondary antibody, and developed with the ODYSSEY Infrared Imaging System (LI-COR). Densitometry analysis of Western blots was performed using Image Studio Lite software (LI-COR).

Purification of Zap-70 and identification of ubiquitination sites

Activated *Sts-1/2*$^{-/-}$ T cells ($6x10^9$) were stimulated with anti-CD3 antibody for 2 minutes at 37°C and lysed as described above. Cell lysates were clarified by centrifugation and flowed over a column of agarose-conjugated phospho-tyrosine antibody (4G10). Bound proteins were eluted from the column with 3% acetic acid (pH 2.5) containing 0.5% SDS. The elution mixture was then transferred to a tube containing 2M Tris base (pH 11) to neutralize the solution. Eluted fractions were applied to Amicon® Ultra-4 centrifugal filter units (3 kDa MWCO, EMD Millipore) for filtration and buffer exchange into a modified lysis buffer containing 0.0067% RapiGest™ SF Surfactant (Waters). Buffer exchange was followed by Zap-70 immunoprecipitation (IP) with pZap-70 Tyr-493 antibody and protein A magnetic beads (EMD Millipore). Beads were flash-frozen in dry ice-ethanol bath prior to UbiScan® analysis (Cell Signaling Technology, Inc.). For quantitative profiles of ubiquitinated proteins comparing unstimulated and stimulated cells, splenocytes were isolated from spleens of *Sts-1/2*$^{-/-}$ mice and activated in culture. Unstimulated cells and cells stimulated with anti-CD3 antibody for 2 minutes at 37°C ($1.9x10^9$ each) were flash-frozen in a dry-ice ethanol bath prior to UbiScan® analysis.

Peptide preparation, immunoprecipitation, and mass spectrometry (MS) analysis were performed as described (185, 186). Briefly, cell pellets were brought to 10 ml each with urea lysis buffer (9 M sequanal grade urea, 20 mM HEPES, pH 8.0, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 2.5 mM sodium pyrophosphate), sonicated at 15 W output three times for 25 s, and centrifuged 15 min at 20,000 × *g* to remove

insoluble material. The cleared protein extracts were reduced and carboxamidomethylated. Proteins were digested overnight with trypsin. Peptides were separated by solid phase extraction with Sep-Pak C18 cartridges and lyophilized.

Lyophilized peptides were redissolved, and ubiquitinated peptides were isolated using ubiquitin branch antibody (K-ε-GG; Cell Signaling Technology) bound to protein G-agarose beads. Peptides were eluted from antibody resin in 0.15% trifluoroacetic acid (TFA), and eluted peptides were concentrated using Empore C18 tips prior to liquid chromatography–mass spectrometry (LC-MS) analysis.

MS analysis was performed as described (185, 186). Immunoprecipitated peptides were separated in a 75-μm x 10 cm PicoTip emitter packed with Magic C18 AQ (100 Å x 5 μm). Peptides were eluted using a 45-, 72-, or 90-min linear gradient of acetonitrile in 0.125% formic acid delivered at 280 nl/min. Tandem mass spectra were collected with an LTQ-Orbitrap Velos mass spectrometer running XCalibur 2.0.7 SP1 using a top twenty MS/MS method, a dynamic repeat count of one, and a repeat duration of 30 s. MS/MS spectra were evaluated using SEQUEST 3G and the SORCERER 2 platform from Sage-N Research (v4.0, Milpitas CA).

Cloning and site-directed mutagenesis

All cDNA mutants were constructed by polymerase chain reaction (PCR) mutagenesis using full-length murine Zap-70 coding sequence (Genbank Acc. No. U77667.1). Zap-70 coding sequence was amplified by PCR using the indicated primers (see Table 2.1). Restriction sites for endonucleases EcoRI and XhoI were incorporated

into the primer sequence to facilitate cloning into pBluescript II SK(+) (Agilent Technologies). Kozak consensus sequence for expression in mammalian cells was also incorporated into the forward primer sequence (96). Flag epitope tag was added to the C-terminus of Zap-70 by PCR using a reverse primer that contained Flag-tag coding sequence.

Site-directed mutagenesis of Zap-70 was carried out by overlap extension PCR using *Platinum Pfx* DNA Polymerase (Invitrogen) with targeted oligonucleotides (62). Two complementary oligonucleotides containing the desired mutation were synthesized for each mutant (Table 2.1). All constructs generated by PCR were sequenced to confirm the presence of only the desired mutation.

Murine full-length Zap-70 coding sequence and Lys-to-Arg mutants amplified by PCR were cloned into mammalian expression vector pcDNA3 (Invitrogen), lentiviral expression vector pCDH-EF1-MCS-T2A-copGFP (System Biosciences), and retroviral expression vector carrying a bicistronic cassette expressing wild-type or mutant Zap-70 upstream of an IRES-GFP element, which was kindly provided by Dr. Derek A. Persons (St. Jude Children's Research Hospital) (154). The Zap-70 coding sequence was cloned into pcDNA3 using EcoRI and XhoI restriction sites, then cloned into the retroviral expression vector using EcoRI and XhoI restriction sites.

For cloning into lentiviral expression vector pCDH-EF1-MCS-T2A-copGFP, full length Zap-70 coding sequence and Lys-to-Arg mutants were amplified by PCR using a reverse primer designed to remove the stop codon at the 3' end of the target sequence and

place the target sequence in frame with the 2A peptide. To facilitate cloning, a restriction site for endonuclease NotI was also incorporated into the reverse primer (Table 2.1). Zap-70 coding sequence was cloned into pCDH-EF1-MCS-T2A-copGFP vector using EcoRI and NotI restriction sites.

Cell transfection

Transfections were performed using Polyethylenimine (PEI) reagent (Sigma-Aldrich) as described previously (112). Briefly, HEK293T cells were plated 24 hrs prior to transfection to obtain 90% confluence at the time of transfection. The plasmid DNA was diluted in serum-free DMEM (transgene: viral packaging: viral envelope constructs at 2:1:1 DNA ratio). PEI (1 μg/ml) was added to the diluted DNA. The volume of PEI used is based on a 3:1 ratio of PEI (μg): total DNA (μg). The mixture was incubated at room temperate for 30 minutes and then added to the cells. At 8 hrs post-transfection the cell media were replaced with fresh media.

Generation of stable cell lines

HEK293T cells were co-transfected with a pCDH-EF1-MCS-T2A-copGFP lentiviral expression vector containing mutant Zap-70, along with packaging (pPACKH1-Gag and pPACKH1-Rev) and envelope (pVSV-G) plasmids (System Biosciences). The lentiviral expression vector and packaging plasmids were kindly provided by Dr. Nancy Reich-Marshall (Stony Brook University). Supernatant was collected from transfected cells 48 and 72 hrs post-transfection and used to spin-infect P116 cells in the presence of 8 μg/ml Polybrene (EMD Millipore). GFP^+ cells were

isolated using a FACSAria flow cytometer (BD Biosciences) and Zap-70 expression was verified by Western blot.

Luciferase assay

Jurkat cells were transfected by nucleofection with pcDNA3 carrying wild-type or mutant Zap-70, a firefly luciferase expression construct driven by IL-2-binding sequences (Promega) and a Renilla luciferase construct (pRL-CMV; Promega) for normalization. The cells were nucleofected using Nucleofector™ Kit for Jurkat cells (Lonza) and program X-001 of the Nucleofector II device (Amaxa Biosystems). At 24 hrs post-transfection, cells were stimulated by plating on to 96-well plates coated with 4 μg/ml HIT3a antibody (Biolegend) at a density of 2×10^5 cells per well for 8 hrs. After stimulation, cells were lysed and luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) using a Lumat LB 9507 luminometer (Berthold Technologies).

IL-2 assay

P116 cells stably expressing wild-type or mutant Zap-70 were grown in serum-free media for 4 hrs and then stimulated in culture with 25 μg/ml Concanavalin A (ConA; Sigma-Aldrich) or mixed with equal number Raji cells alone or Raji cells and 100 ng/ml Staphylococcal enterotoxin D (SED; Toxin Technology). Cell culture supernatants were collected after 24 hours and IL-2 assayed by enzyme-linked immunosorbent assay (ELISA) with Human IL-2 ELISA Max Deluxe kit (BioLegend) using VersaMax Microplate Reader with SoftMax Pro Software (Molecular Devices).

Retroviral infection and intracellular cytokine analysis

After 24 hours growth in the presence of 0.5 μg/ml anti-CD3 antibody (145-2C11) and 10 U/ml IL-2, wild-type splenocytes were infected with a retrovirus carrying a bicistronic cassette expressing wild-type or mutant Zap-70 upstream of an IRES-GFP element (154). HEK293T cells were co-transfected with the retroviral expression vector, along with pCMV-gag-pol packaging plasmid and pCAG-env envelope plasmid, kindly provided by Dr. Michael J. Hayman (Stony Brook University). Supernatant was collected from transfected cells 48 and 72 hrs post-transfection, incubated with 2 μg/ml Lipofectamine 2000 (Thermo Fisher Scientific) for 30 minutes at room temperature, and used to spin-infect the activated splenocytes. Infected T cells were cultured for 48 hrs in the presence of IL-2, stimulated with anti-CD3 antibody for 6 hrs in the presence of 5 μg/ml Brefeldin A (Cell Signaling Technology, Inc.), and stained for intracellular IL-2 (clone JES6-5H4) using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). GFP^+ cells were analyzed for IL-2 expression using a BD FACScan flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.).

Statistical analysis

Quantitative data for all experiments are expressed as mean ± standard deviation (SD) for each group. Data were analyzed using Prism Software (GraphPad). Unpaired two-tailed Student's t-test was used for all data analysis and P-values <0.05 were considered statistically significant.

2.2 Tables

Primer	Primer Sequence (5'-3')	Description
N-Zap70-KZ-EcoRI	ACTGGAATTC**GCCACCATG**CCCGATCCCGCGGCGCAC	Forward primer with Kozak consensus sequence and EcoRI restriction site (1-21)
N-Zap70-XhoI-R	CAGTCTCGAGGGCCTCTCGCATCATCTCAT	Reverse primer with XhoI restriction site (1158-1149)
C-Zap70-FLAG-XhoI	CAGTCTCGAGCTA**CTTGTCATCGTCATCCTTGTAATC**GCCTCCGCCGCCACATGCAGCCTC	Reverse primer with FLAG tag and XhoI restriction site (1857-1840)
C-Zap70-R	AGCTCTTCCTGAAGCGAGAGAATC	Reverse primer (983-1006)
C-Zap70-NotI	TACTGGCGGCCGCGCCACATGCAGCCTCGGCCA	Reverse primer with NotI restriction site for cloning into lentiviral vector (1834-1854)
Zap70-K193R-F	GAGGCCCCGGAGGGAGCAGGGCAC	Forward mutagenic primer for K193R mutant (567-590)
Zap70-K193R-R	GTGCCCTGCTCCCTCCGGGGCCTC	Reverse mutagenic primer for K193R mutant (567-590)
Zap70-K217R-F	CATCAGCCAGGACAGGGCTGGCAAGTAC	Forward mutagenic primer for K217R mutant (636-663)
Zap70-K217R-F	GTACTTGCCAGCCCTGTCCTGGCTGATG	Reverse mutagenic primer for K217R mutant (636-663)
Zap70-K376R-F	GAAGCAGGGCACAGAGAGGGCCGACAAAGATGAG	Forward mutagenic primer for K376R mutant (1110-1143)
Zap70-K376R-F	CTCATCTTTGTCGGCCCTCTCTGTGCCCTGCTTC	Reverse mutagenic primer for K376R mutant (1110-1143)
C-Zap70-NotI	TACTGGCGGCCGCGCCACATGCAGCCTCGGCCAC	Reverse primer with NotI restriction site (1834-1854)

Table 2.1 Oligonucleotides used for cloning and PCR mutagenesis. The start and end positions within the original template sequence that correspond to the primer sequence are indicated. Kozak consensus sequence is indicated in bold. FLAG tag sequence is bold and underlined. Mismatched bases compared to the original template sequence are underlined in the primer sequence.

Chapter 3: Characterization of Zap-70 ubiquitination

3.1 Introduction

The TCR has no intrinsic catalytic activity and depends on the recruitment and activation of protein tyrosine kinases for transmission of an activation signal (182). Engagement of the TCR by a peptide–MHC complex leads to Lck-dependent phosphorylation of tyrosine residues within the ITAMs of different TCR subunits (113). This allows recruitment and activation of the Syk family kinase Zap-70 to the TCR complex, where it is phosphorylated and activated (102, 182). Zap-70 plays a critical role in propagating signals initiated by TCR engagement and is required for activating many critical signaling pathways downstream of the TCR. Recruitment of Zap-70 and its subsequent activation result in initiation of downstream signaling pathways that are important for T cell proliferation, differentiation, and effector functions (21, 69, 76, 102, 182). The importance of Zap-70 in TCR signaling is underscored by the observation that loss of expression or function of Zap-70 leads to severe developmental and functional defects within the T cell compartment (5, 50, 200).

T cells possess a number of mechanisms to limit the duration and intensity of signals generated by receptor activation in order to prevent excessive and damaging responses (1). Following antigen stimulation, the TCR is rapidly internalized to limit the pool of surface receptor available for stimulation (199). In addition, dephosphorylation of kinase activating residues and phosphorylation of kinase inhibitory residues serve to regulate signal initiation and propagation (147). Ubiquitin ligases are also believed to

target components of the TCR signaling complex for internalization and/or degradation (107). Another mechanism for negatively regulating signals downstream of the TCR involves the Sts proteins. Zap-70 is negatively regulated by the Sts phosphatases, as demonstrated by its hyper-phosphorylation in stimulated *Sts-1/2*$^{-/-}$ T cells relative to wild-type T cells (19, 20, 125, 193). Interestingly, in primary CD4^{+} and CD8^{+} naïve splenic T cells lacking the Sts proteins, increased levels of phosphorylation are visible on both the native 70 kDa form of Zap-70 and on slower migrating forms that are recognized by antibodies to ubiquitin (20). Recently, it was also demonstrated that Zap-70 is modified by K33-linked polyubiquitination predominantly on Lys-578 following TCR engagement, and that the Sts proteins play a role in the dephosphorylation of polyubiquitinated Zap-70 in stimulated CD8^{+} T cells (212). Together, these results demonstrate that ubiquitination of Zap-70 is likely a mechanism involved in the regulation of signaling pathways important for T cell activation.

Here, we demonstrated that ubiquitinated Zap-70 can be observed in human and mouse T cell lines as well as primary human and mouse T cells following TCR stimulation. Zap-70 ubiquitination was revealed to be a transient phenomenon. Levels of ubiquitinated Zap-70 peaked shortly after TCR stimulation and then quickly declined in a manner unaffected by proteasomal inhibition. We demonstrated that, despite the presence of K48-linked polyubiquitin, ubiquitinated Zap-70 is not degraded by the proteasome following TCR stimulation. These results suggest that Zap-70 ubiquitination serves regulatory functions other than signaling for proteasome-mediated degradation.

3.2 Results

A. Ubiquitination of Zap-70 following TCR stimulation

Zap-70 is negatively regulated by the Sts phosphatases, as evidenced by its hyper-phosphorylation in stimulated *Sts-1/2*$^{-/-}$ T cells relative to stimulated wild-type T cells (19, 20). Increased levels of phosphorylation are visible on the native 70 kDa form of Zap-70 as well as on slower migrating forms that are recognized by antibodies to ubiquitin (20). These results suggest that the absence of the Sts proteins in T cells leads to increased levels of Zap-70 that is dually modified by both protein phosphorylation and ubiquitination. Sts deficiency first revealed Zap-70 ubiquitination because ubiquitinated Zap-70 is also phosphorylated in the absence of the Sts proteins. However, anti-ubiquitin Western analysis revealed that there is ubiquitinated Zap-70 in stimulated wild-type T cells as well, but it is not phosphorylated (20). In addition, when wild-type and *Sts-1/2*$^{-/-}$ T cells are treated with phosphatase inhibitor pervanadate (PV), comparable levels of dually modified Zap-70 can be detected (Figure 3.1). It is unclear whether ubiquitinated Zap-70 in wild type T cells remains unphosphorylated or if it is rapidly dephosphorylated following TCR stimulation.

To determine whether Zap-70 ubiquitination can be observed in multiple cell lines, we treated human Jurkat T cells (Figure 3.2A) and murine TK-1 T cells (Figure 3.2B) with TCR stimulatory antibodies for 2 minutes, isolated Zap-70 by immunoprecipitation (IP), and assessed levels of post-translationally modified Zap-70 via immunoblot analysis. Figure 3.2 illustrates that phosphorylated Zap-70 isolated from both human and mouse

T cell lines following TCR stimulation migrates by SDS-PAGE in a characteristic laddering pattern that is suggestive of ubiquitin modification. Treatment of cells with pervanadate, a phosphatase inhibitor, led to significantly increased levels of dually modified Zap-70 relative to non-pervanadate treated cells. We also observed a similar pattern of post-translational modification in primary splenic T cells isolated from wild-type mice (Figure 3.3A) and in primary human T cells isolated from peripheral blood (Figure 3.3B) stimulated with CD3 antibodies. These data suggest that the accumulation of ubiquitinated forms of Zap-70 could play an important regulatory role in TCR signaling.

B. Ubiquitin modification present on Zap-70

Ubiquitination is a highly versatile and dynamic post-translational modification. Attachment of ubiquitin to a substrate can alter the substrate's stability, activity, localization, and interacting partners. The type of ubiquitin modification determines its functional role in targeting the substrate to various cellular pathways (28, 49, 81, 93, 94). Modification with polyubiquitin chains linked through lysine 48 (K48) typically targets proteins for proteasomal degradation, whereas modification with single ubiquitin and K63-linked polyubiquitin chain serves other non-proteolytic functions (28, 81, 94, 103, 204). Because polyubiquitin linkage type is an important determinant of the modification's function, we examined the ubiquitin modification present on Zap-70 following TCR stimulation with polyubiquitin linkage-specific antibodies (137). We detected the presence of K48-linked but not K63-linked polyubiquitin on Zap-70 (Figure 3.4) indicating that one or more lysine residues on Zap-70 are modified with a K48-linked polyubiquitin chain.

Modification with a polyubiquitin chain linked through Lys-48 is known to target substrates to the proteasome for degradation (81, 94, 204). To address the possibility that ubiquitinated Zap-70 is degraded by the proteasome following TCR stimulation, we and assessed the levels of modified Zap-70 following TCR stimulation in cells in which proteasome-mediated degradation is inhibited.

C. Functional characterization of Zap-70 ubiquitination

Splenic T cells were isolated from wild-type mice, activated in culture with CD3 antibody and allowed to expand in the presence of IL-2 for several days. T cells were treated with proteasome inhibitor MG-132 in the presence or absence of pervanadate then stimulated with CD3 antibody for the indicated time, and levels of Zap-70 phosphorylation were examined.

Treatment of T cells with proteasome inhibitor did not alter the levels of accumulation of dually modified Zap-70. As illustrated in Figure 2.5, levels of modified Zap-70 peaked between 2 and 5 minutes following TCR stimulation then gradually declined in a manner that is not affected by MG-132 treatment (Figure 3.5A,C). In contrast, treatment of T cells with pervanadate over a 10 minute time course of stimulation led to significantly increased levels of phosphorylation on both the 70 kDa form of Zap-70, as well as its slower migrating forms (Figure 3.5B). Thus, accumulation of dually modified forms of Zap-70 occurs rapidly following TCR stimulation and inhibition of phosphatase activity increases the relative abundance of the dually modified forms. We observed no difference in the levels of modified Zap-70 in cells treated with either

pervanadate alone or cells treated with both pervanadate and MG-132 (Figure 3.5C). The failure of MG-132 treatment to visibly enhance accumulation of dually modified Zap-70 suggests that, despite the presence of K48-linked polyubiquitination, the majority of ubiquitinated Zap-70 is not degraded in a proteasome-dependent manner following TCR stimulation. Although typically associated with proteasomal degradation, it is possible that K48-linked polyubiquitination of Zap-70 serves non-proteolytic functions. However, it is more likely that there is a fraction of ubiquitinated Zap-70 that is degraded by the proteasome following TCR stimulation but it represents a relatively minor species and we are unable to detect changes in levels of modified Zap-70 resulting from proteasomal inhibition. Whether Zap-70 ubiquitination serves biochemical functions other than signaling for proteasomal degradation is currently unclear.

3.3 Conclusions

Zap-70 ubiquitination can be observed in human and mouse T cell lines following TCR stimulation, as well as in primary mouse and human T cells. Using linkage-specific ubiquitin antibodies we detected the presence of K48-linked polyubiquitin modification on Zap-70. Inhibition of proteasomal degradation failed to enhance accumulation of ubiquitinated Zap-70, indicating that, despite the presence of K48-linked polyubiquitination, Zap-70 is not degraded in a proteasome-dependent manner following TCR stimulation. These results suggest that modification of Zap-70 with ubiquitin serves regulatory functions other than signaling for degradation.

3.4 Figures

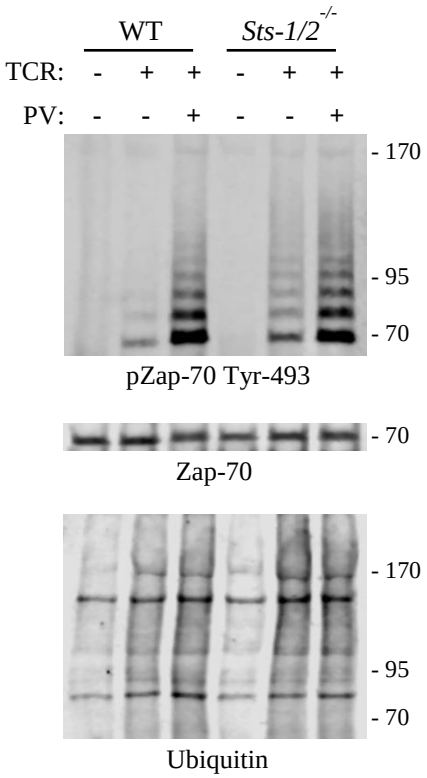

Figure 3.1 Zap-70 is ubiquitinated following TCR stimulation in wild-type and *Sts-1/2*$^{-/-}$ T cells. Pre-activated T cells from wild-type and *Sts-1/2*$^{-/-}$ mice were stimulated, lysed and processed for IP/Western blot analysis. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Tyr-493 (*top*), total Zap-70 (*middle*), and ubiquitin (*bottom*).

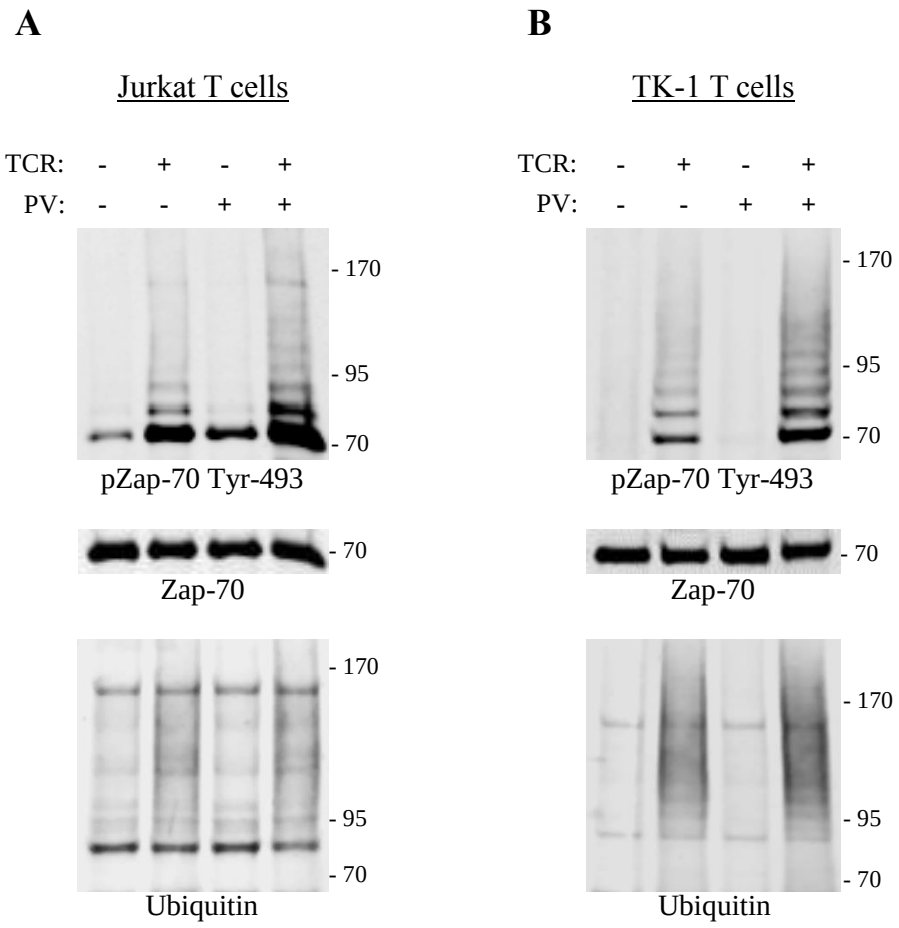

Figure 3.2 Ubiquitination of Zap-70 following TCR stimulation in human and mouse T cell lines. Jurkat T cells (**A**) or TK-1 T cells (**B**), treated with pervanadate as indicated, were stimulated, lysed and processed for IP/Western blot analysis to visualize levels of Zap-70. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Tyr-493 (*top*), total Zap-70 (*middle*), and ubiquitin (*bottom*).

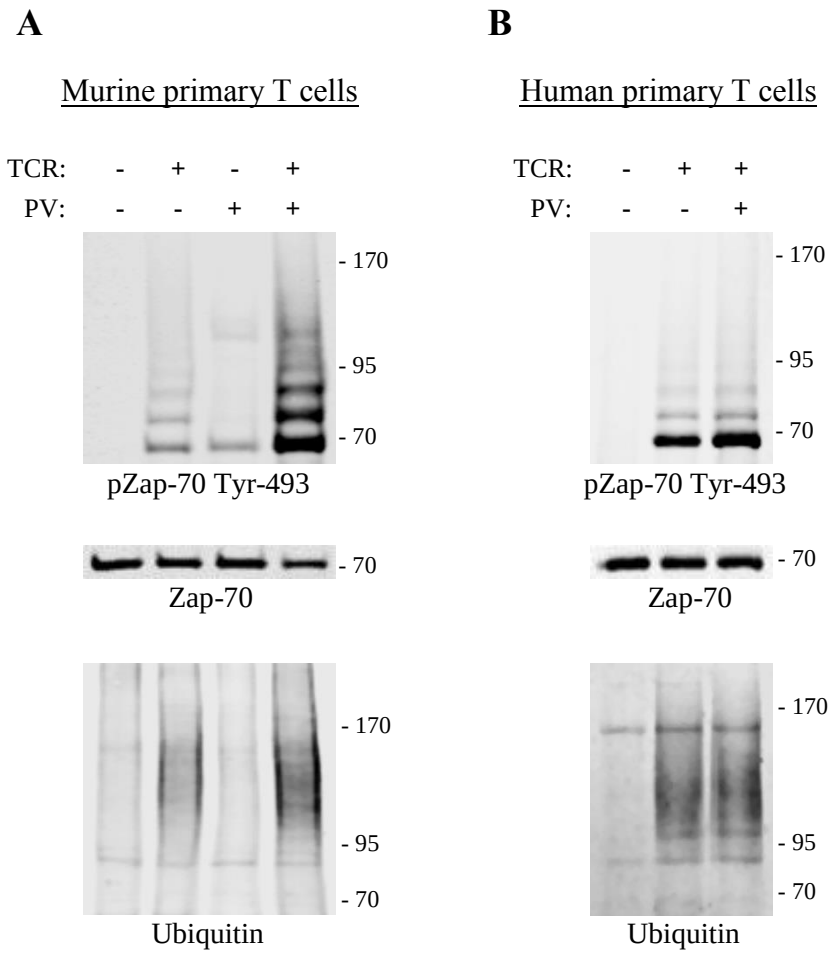

Figure 3.3 Ubiquitination of Zap-70 in stimulated primary T cells. Pre-activated T cells isolated from wild-type mice (**A**) or primary human T cells isolated from peripheral blood (**B**), treated with pervanadate as indicated, were stimulated, lysed and processed for IP/Western blot analysis to visualize levels of Zap-70. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Tyr-493 (*top*), total Zap-70 (*middle*), and ubiquitin (*bottom*).

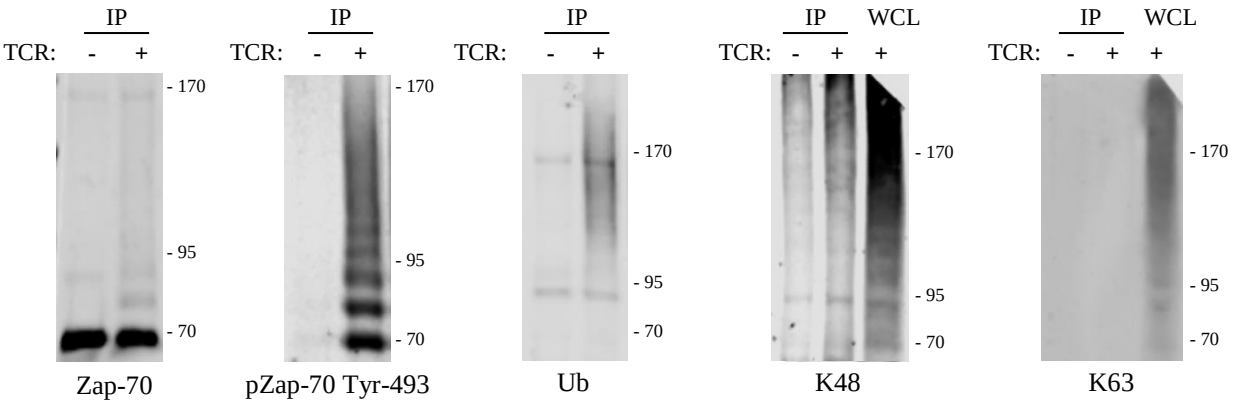

Figure 3.4 Types of polyubiquitin linkages present on Zap-70 following TCR stimulation. Pre-activated T cells isolated from *Sts-1/2*$^{-/-}$ mice were stimulated, lysed and processed for IP/Western blot analysis. Immunoprecipitated Zap-70 (IP) was assessed for levels of total Zap-70, phospho-Zap-70 Tyr-493, total ubiquitin (Ub), K48-linked polyubiquitin (K48), and K63-linked polyubiquitin (K63). Whole cell lysate (WCL) from stimulated *Sts-1/2*$^{-/-}$ T cells was used as positive control for K48 and K63 antibodies.

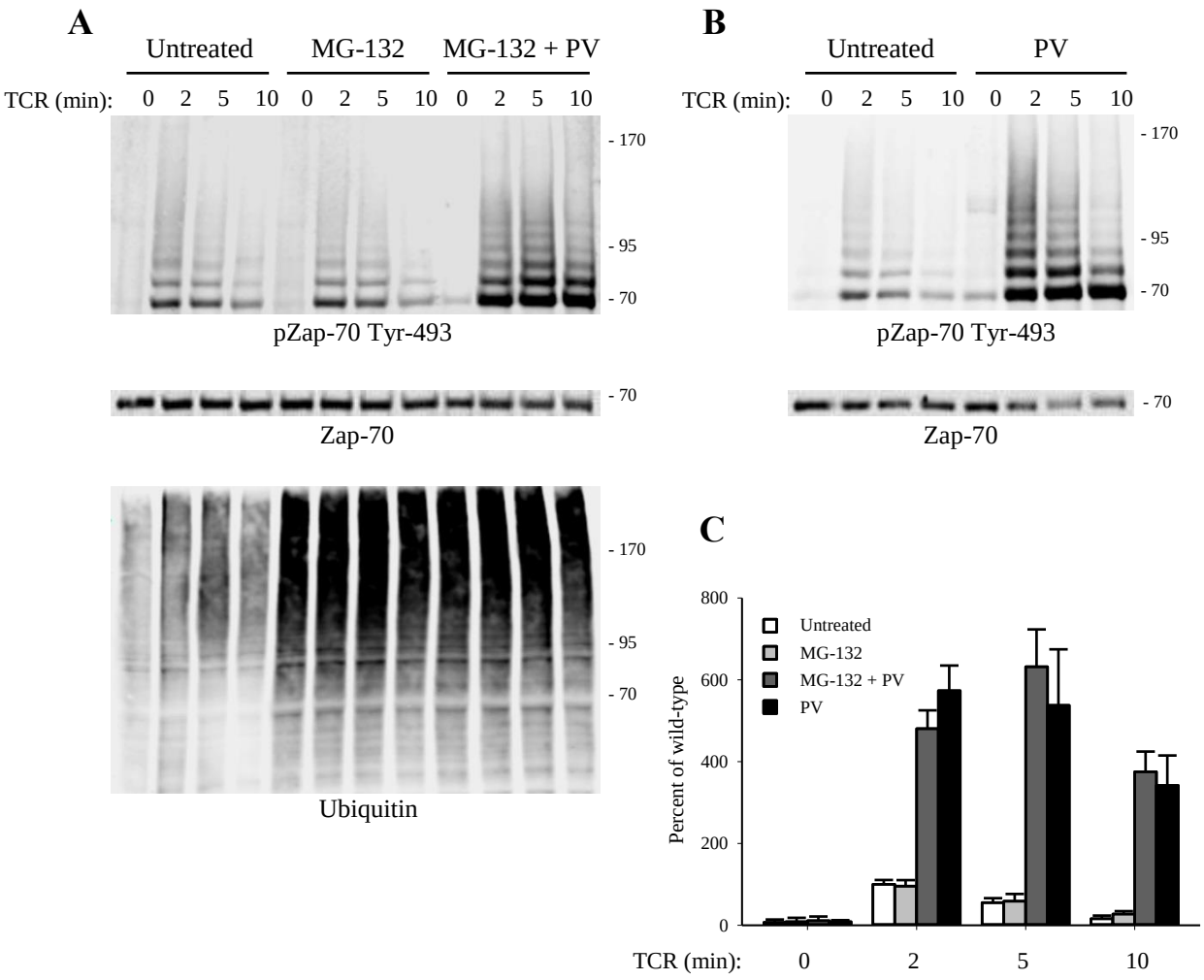

Figure 3.5 Levels of ubiquitinated Zap-70 in stimulated T cells are unaffected by proteasome inhibition. Pre-activated T cells from wild-type mice were left untreated, treated with MG-132, with MG-132 and pervanadate (**A**), or treated with pervanadate alone (**B**). Following treatment they were stimulated, lysed and processed for IP/Western blot analysis. Immunoprecipitated Zap-70 was assessed for levels of phospho-Zap-70 Tyr-493 (*top*) and total Zap-70 (*middle*). Whole cell lysates were separated by SDS-PAGE and subjected to Western blot analysis with anti-ubiquitin antibody (*bottom*). Representative blots are illustrated (**A**,**B**). (**C**). Bar graph shows densitometric analysis of Western blot and represent optical density of the entire lanes between 75 kDa and 170 kDa. Levels of dually modified Zap-70 were quantified and normalized to total Zap-70 in each lane (including 70 kDa form). All sets are shown as percent of levels in untreated cells stimulated for 2 min (*lane 2*). Data represented as mean ± SD for at least three independent experiments. No statistically significant difference was found between untreated and MG-132 treated samples, or between cells treated with MG-132 and pervanadate and those treated with pervanadate alone.

Chapter 4: Purification of Zap-70 and identification of ubiquitination sites

4.1 Introduction

Ubiquitination is a highly diverse post-translational modification. Conjugation of ubiquitin to a substrate can target the substrate to the proteasome for degradation, alter its subcellular localization, modulate catalytic activity, and promote or inhibit interactions with other proteins and regulatory molecules (81, 93, 204). A single protein can be modified on one or multiple lysine residues by a single ubiquitin molecule or a polyubiquitin chain, and the type of modification determines the functional consequences of ubiquitination.

In Chapter 3, we demonstrated that Zap-70 is ubiquitinated following TCR stimulation in human and mouse primary T cells and T cell lines, and that ubiquitination does not target Zap-70 for proteasome-mediated degradation. However, it was unclear whether Zap-70 ubiquitination serves other biochemical functions.

Here, we designed and implemented a strategy for purifying endogenous ubiquitinated Zap-70 by combining affinity-based enrichment of tyrosine phosphorylated proteins with an immunoprecipitation step that isolated Zap-70 from the subsequent pool of phosphorylated proteins. Global ubiquitin remnant analysis and analysis of purified Zap-70 led to identification of ten ubiquitination sites in Zap-70. All identified sites displayed an increase in the level of ubiquitin modification following TCR stimulation, with the largest increases (>20 fold) observed for Lys-193, Lys-217, and Lys-376.

4.2 Results

A. Purification of ubiquitinated Zap-70

In order to evaluate the functional consequences of Zap-70 ubiquitination, we adopted the following strategy to identify the ubiquitin conjugation sites in Zap-70: purification of endogenous ubiquitinated Zap-70 and identification of ubiquitination sites by mass spectrometry. Due to the relatively low abundance of ubiquitinated substrate, purification of ubiquitinated Zap-70 required a multi-step strategy combining affinity-based enrichment of target protein with an immunoprecipitation step (Figure 4.2A).

For the enrichment step, we took advantage of the fact that ubiquitinated Zap-70 is also phosphorylated in the absence of Sts proteins. We pulled down modified Zap-70 from stimulated *Sts-1/2*$^{-/-}$ T cells using agarose-conjugated phosphotyrosine (pTyr) antibody, as can be detected using Zap-70 and phospho-Zap-70 antibodies (Figure 4.1A). Bound proteins were eluted from the pTyr column with a low pH buffer containing SDS (Figure 4.1B).

High SDS concentrations in the elution buffer were found to inhibit antigen-antibody interactions and reduce the efficiency of subsequent immunoaffinity purification steps (Figure 4.1C, *lane 3*). In addition, the presence of SDS or other detergents can interfere with mass spectrometry analysis (214). For this reason, the pTyr column elutions were filtered and concentrated, and the buffer exchanged with a buffer suitable for subsequent steps. We used a buffer containing acid-labile detergent which can solubilize proteins at neutral pH, but can be cleaved at low pH into small molecules that do not interfere with mass spectrometry analysis (139, 166, 214). Filtration did not result in noticeable loss of target protein and improved

immunoprecipitation efficiency significantly (Figure 4.1C). This approach yielded phosphorylated Zap-70 that was immunoreactive to antibodies against pZap-70 Tyr-493 and ubiquitin (Figure 4.2B). Immunoprecipitated Zap-70 bound to protein A-magnetic beads was submitted to Cell Signaling Technology for UbiScan® analysis using the ubiquitin branch motif (K-ε-GG) antibody.

B. Identification of ubiquitination sites in Zap-70

We employed two methods for identification of ubiquitination sites in Zap-70, ubiquitin remnant analysis of purified phosphorylated Zap-70 and a global ubiquitin remnant analysis for ubiquitin profiles comparing unstimulated and stimulated cells.

Because trypsin cuts after the C-terminal arginine in ubiquitin, it removes all but the two terminal glycine residues of ubiquitin from the ubiquitinated peptide. Thus trypsin digestion of ubiquitinated substrates yields peptides showing missed proteolytic cleavage at the modified lysine residue and a 114 Da increase in peptide mass due to the covalent attachment of the two glycine residues to the ubiquitin acceptor lysine (93, 211). This diglycine-modified lysine residue serves as a signature of ubiquitination and identifies sites of ubiquitin conjugation. Modified peptides can be analyzed directly or further enriched using an anti-diglycyl-lysine antibody prior to mass spectrometry analysis (211).

Mass spectrometry analysis of purified Zap-70 revealed two novel ubiquitination sites, Lys-193 and Lys-217 (Table 4.1). Global ubiquitome analysis (see Materials and Methods) confirmed the sites obtained by analysis of purified protein and identified eight additional ubiquitination sites (Table 4.1). There appears to be a basal level of Zap-70 ubiquitination in

unstimulated cells, as ubiquitination at all the identified sites was detected in both unstimulated and stimulated cells. However, all the sites displayed an increase in the level of ubiquitin modification following stimulation, with the largest increases (>20 fold) observed for Lys-193, Lys-217, and Lys-376 (Table 4.1). The remaining identified sites of ubiquitination displayed significantly reduced fold increase in stimulation-dependent modification.

Of the ten Zap-70 ubiquitination sites that were identified in stimulated primary T cells, only two are located in the kinase domain. In particular, Lys-376 is located within the αC helix of the N-lobe of the kinase domain, while Lys-541 is located between the αG helix and αH helix of the kinase domain C-lobe (Figure 4.3A,B). Interestingly, the majority of sites (60%) are located within the tandem SH2 domains, in the region of Zap-70 that interacts with the phosphorylated ITAMs of the TCR/CD3 subunits during T cell activation (Figure 4.3A,C). Lys-25, Lys-75 and Lys-100 are located in the N-terminal SH2 domain, while Lys-193, Lys-206, and Lys-217 are within the C-terminal SH2 domain. Lys-193 is adjacent to three residues (Arg-170, Arg-190, and Arg-192) within the phospho-tyrosine binding pocket that have been demonstrated to make direct hydrogen bonding contact with a phosphate moiety of the activated ITAM (60). Lys-217 is located adjacent to the Zap-70–ITAM binding surface, at the interface of the two SH2 domains. Lys-132 is located in Interdomain A, which links the two SH2 domains, and Lys-328 is within Interdomain B (Figure 4.3A,B), which connects the C-terminal SH2 domain and the kinase domain (6). All ten sites are evolutionarily conserved (Figure 4.4). Based on the large stimulation-dependent fold increase in ubiquitination at the Lys-193, Lys-217, and Lys-376 modification sites, we chose these three sites for further functional analysis, although it

should be noted that the relative abundance of modified Lys-376 was significantly lower than the relative abundance of either Lys-193 or Lys-217 (Table 4.1).

4.3 Conclusions

In this study, we successfully developed and implemented a strategy for purifying endogenous ubiquitinated Zap-70. Ubiquitin remnant analysis of Zap-70 isolated from stimulated *Sts-1/2*$^{-/-}$ T cells identified two ubiquitination sites in activated Zap-70, Lys-193 and Lys-217. Global analysis of lysine ubiquitination in unstimulated and stimulated T cells by ubiquitin remnant immunoaffinity profiling identified eight additional ubiquitination sites in Zap-70 and revealed quantitative differences in ubiquitination at each site following TCR stimulation. All identified sites displayed an increase in levels of ubiquitination in stimulated cells relative to unstimulated cells. The largest fold increase in ubiquitination (>20 fold) was seen at Lys-193, Lys-217, and Lys-376. The remaining identified sites of ubiquitination displayed significantly reduced fold increase in stimulation-dependent modification. Of the ten sites identified, only one has been reported previously (212).

4.4 Tables and figures

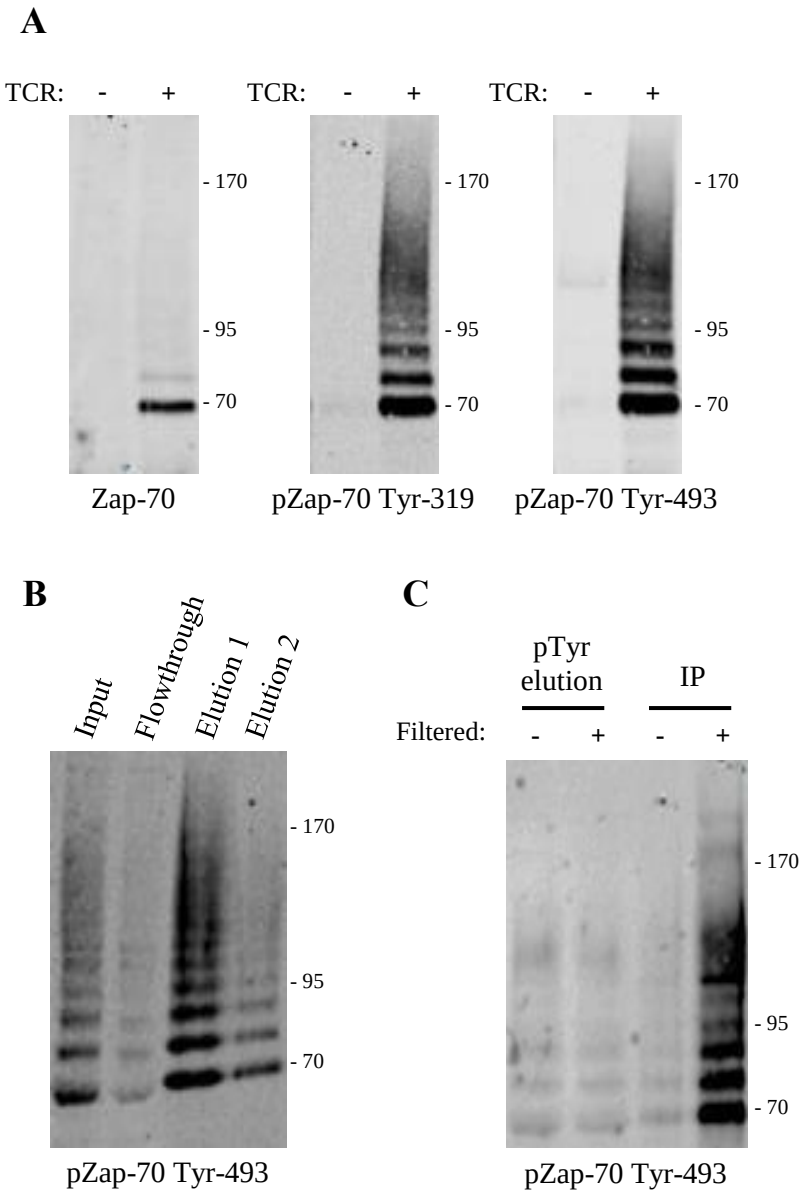

Figure 4.1 Affinity enrichment of modified Zap-70. (**A**) Modified Zap-70 pulled down with phospho-tyrosine antibody can be observed by Western blot using antibodies to Zap-70 (*left*), phospho-Zap-70 Tyr-319 (*middle*), and phospho-Zap-70 Tyr-493 (*right*). (**B**) Modified Zap-70 can be eluted from phospho-tyrosine column with 3% acetic acid containing 0.5% SDS. (**C**) Sample filtration and buffer exchange of pTyr column elutions increases the efficiency of immunoprecipitation.

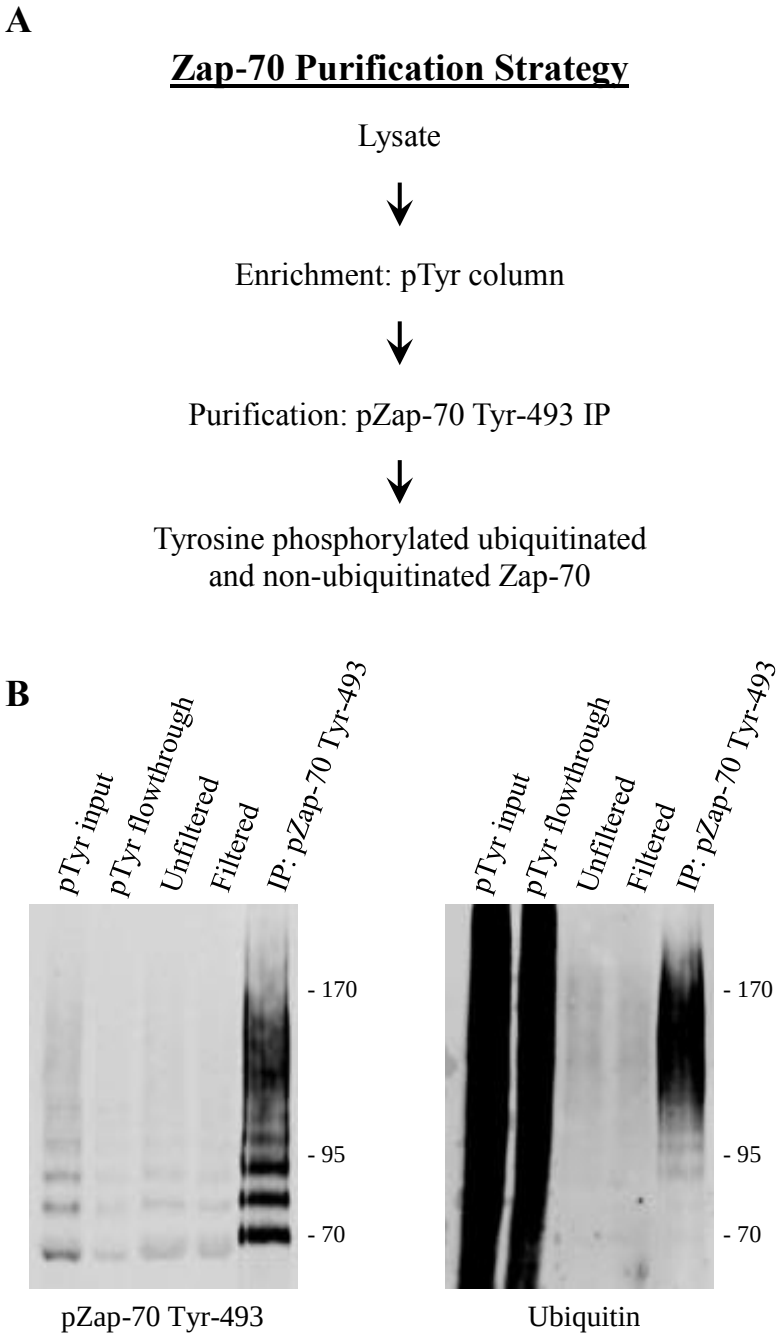

Figure 4.2 Purification of Zap-70 for ubiquitination site identification. (**A**) Strategy for purification of endogenous ubiquitinated Zap-70. (**B**) Enrichment of dually modified Zap-70 can be observed by Western blot using antibodies to phospho-Zap-70 Tyr-493 (*left*) and ubiquitin (*right*).

		Average Peak Intensity		
Site	**Fold change**	**Unstimulated**	**Stimulated**	**Peptide**
25	6.6	5,415,628	27,978,322	AEAEEHLK*LAGMADGLFLLR
75	4	5,431,784	16,922,299	QLNGTYAIAGGK*AHCGPAELCQFYSQDPDGLPCNLR
100	5.9	3,367,094	15,508,399	K*PCNRPPGLEPQPGVFDCLR
132	8.9	12,820,362	89,789,764	QTWK*LEGDALEQAIISQAPQVEK
193	**20.8**	**17,238,167**	**279,258,112**	**K*EQGTYALSLVYGK**
206	2.6	13,720,480	27,763,319	KEQGTYALSLVYGK*TVYHYLISQDK
217	**19.4**	**19,639,150**	**295,996,576**	**TVYHYLISQDK*AGK**
328	4.1	1,086,215	3,477,448	LASSTDKPRPMPMDTSVYESPYSDPEELKDK*K
376	**23.6**	**575,780**	**10,600,356**	**QGTEK*ADKDEMMR**
541	2.9	1,783,042	4,050,919	K*MKGPEVLDFIK

Table 4.1 Ubiquitination sites in Zap-70. Lysine residue position within Zap-70 identified as a site of ubiquitin modification, fold change in ubiquitination, and sequence of the ubiquitinated peptide (modified lysines are marked with *****). Average peak intensity is the parent ion intensity observed at its chromatographic apex and is used to determine the fold increase in ubiquitination following stimulation at each site. Sites chosen for further analysis are shown in bold.

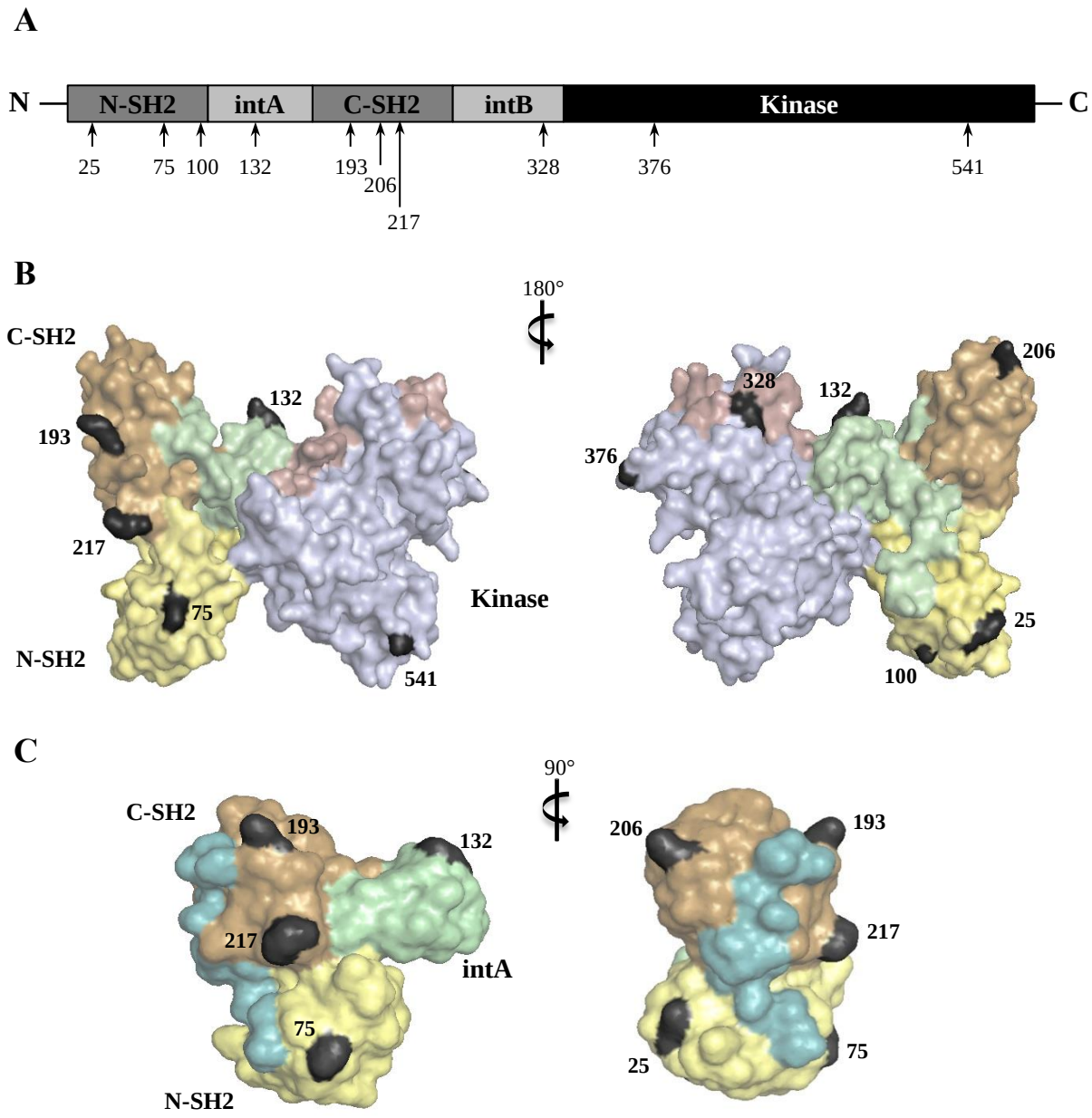

Figure 4.3 Zap-70 ubiquitination sites. (**A**) Schematic representation of full-length Zap-70. Arrows indicate location of ubiquitination sites. (**B**) Crystal structure of full length inactive Zap-70 (PBD ID: 2OZO). The N-terminal and C-terminal SH2 domains, interdomain A, interdomain B, and kinase domain are shown in yellow, orange, green, red, and blue, respectively. Ubiquitination sites identified by UbiScan® analysis are indicated in dark gray. (**C**) Crystal structure of Zap-70 tandem SH2 domains in complex with a peptide derived from the first ITAM of the CD3ζ chain (PDB ID: 2OQ1). CD3ζ peptide is shown in cyan. Ubiquitination sites identified by UbiScan® analysis are shown in dark gray.

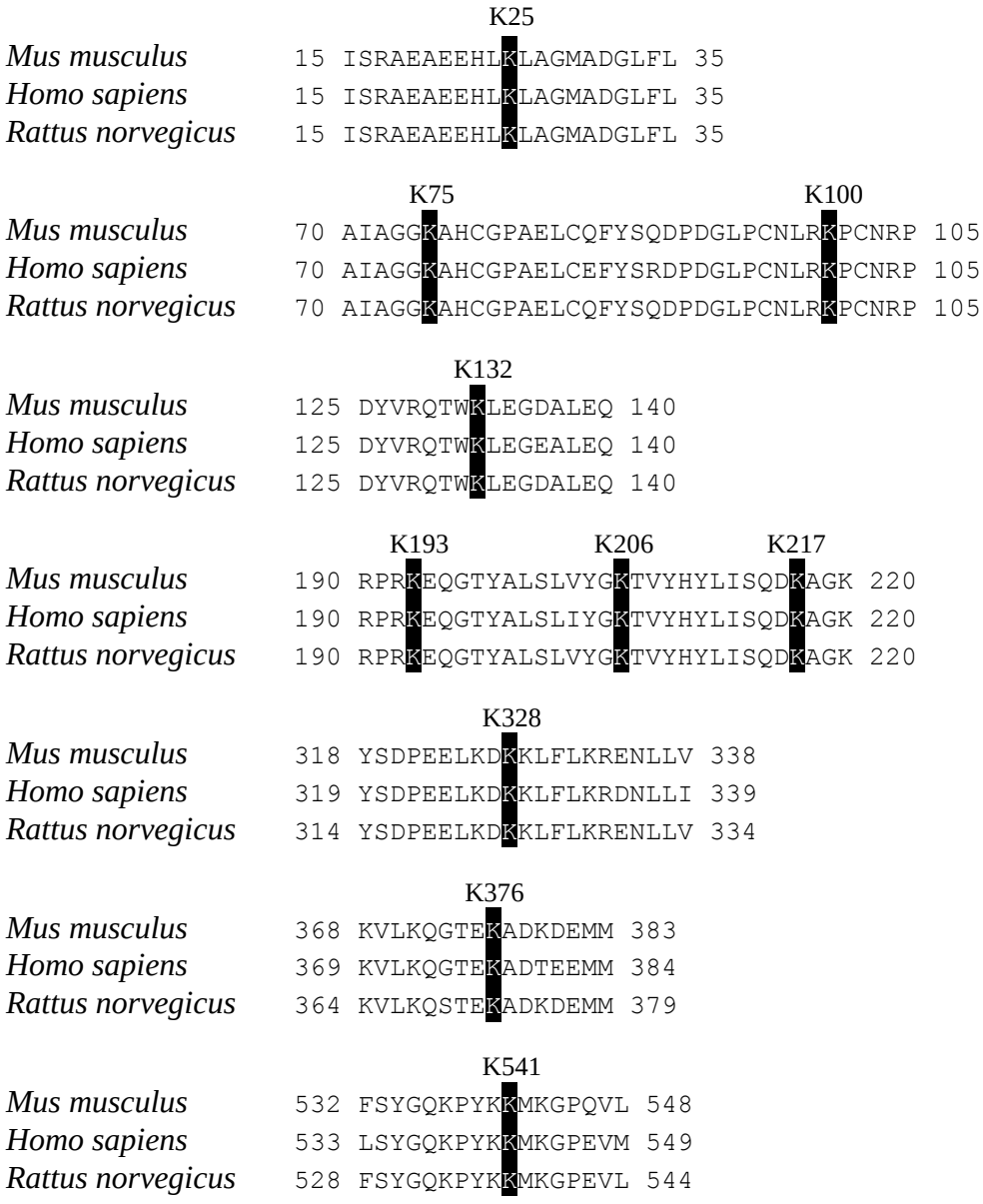

Figure 4.4 Sequence alignment of Zap-70 proteins from indicated species. Lysine residues within Zap-70 identified as sites of ubiquitin modification are evolutionarily conserved.

Chapter 5: Functional characterization of Zap-70 mutants

5.1 Introduction

The TCR has no intrinsic catalytic activity. Propagation of an activating signal following TCR engagement depends on phosphorylation of downstream targets by non-receptor kinase Zap-70 (76, 87, 113). Zap-70 is recruited to the activated receptor complex following TCR engagement, where it is activated by phosphorylation. Once activated, Zap-70 phosphorylates and activates a number of downstream signaling molecules. Two important targets of Zap-70 are signaling adaptor proteins LAT and SLP-76 (1, 76). When phosphorylated, these adaptor proteins serve as scaffolds to recruit other signaling molecules leading to activation of downstream signaling pathways. One pathway that becomes activated downstream of the signaling adaptor proteins is MAPK pathway. Activation of this pathway results in formation of transcription factor AP-1, which is essential for IL-2 expression (13, 76, 117).

In Chapter 4, we demonstrated that Zap-70 is ubiquitinated on multiple lysine residues following TCR stimulation, and identified nine novel ubiquitination sites, including Lys-193, Lys-217, and Lys-376. These sites displayed greater than 20-fold increase in modification levels following TCR stimulation and were chosen for further functional analysis.

Here, we generated Zap-70 mutant in which the ubiquitin acceptor lysine is replaced with arginine, thereby blocking ubiquitination at the specific site. These mutants were transduced into *Zap-70*$^{-/-}$ Jurkat-derived P116 cells via lentiviral vector, generating cell lines stably expressing mutant Zap-70. We utilized these cell lines to assess the effect of abrogating specific ubiquitination sites on Zap-70 activation and the activation of downstream signaling pathways. We found that Zap-70 that lacks the Lys-217

ubiquitination site displays an increase in levels of activation following TCR stimulation. The increase in levels of Zap-70 activation seen in cells expressing Zap-70 K217R mutant was accompanied by increased activation of downstream signaling pathways and enhanced IL-2 secretion relative to wild-type. These results suggest that ubiquitination at Lys-217 plays a negative regulatory role in events involved in T cell activation.

5.2 Results

A. Increased pathway activation in the absence of Zap-70 Lys-217 ubiquitination site

The P116 Jurkat cell line lacks expression of Zap-70, rendering cells unresponsive to TCR stimulation (208). To evaluate the role of Zap-70 ubiquitination in the regulation of signaling pathways downstream of the TCR, we generated P116 Jurkat cell lines stably expressing wild-type Zap-70 or Zap-70 Lys-to-Arg mutants in which ubiquitin-acceptor lysines were replaced by an arginine, thereby blocking ubiquitination at the specific site. The lysine mutants were introduced into Zap-70 deficient Jurkat derived P116 cells using a lentiviral vector which contains GFP as a reporter gene. After infection, GFP^+ cells were collected by fluorescence-activated cell sorting (FACS) to establish stable cell lines (Figure 5.1A). Expression of Zap-70 was comparable in the different P116-derived stable cell lines (Figure 5.1B) and was able to restore TCR signaling pathways (Figure 5.2 and 5.3). Further, the recruitment of wild-type Zap-70 and Zap-70 SH2 domain mutants into the activated TCR complex was comparable, as evinced by the equivalent levels of Zap-70 associated with CD3ζ following stimulation (Figure 5.1C). The expression level of Zap-70 was found to be stable over time (as checked by FACS and western blot; not shown).

To assess levels of Zap-70 activation following TCR stimulation, cells were stimulated and levels of activation loop tyrosine (Tyr-493) phosphorylation were evaluated by Western blot. We consistently noted increased levels of phosphorylated Tyr-493 in cells expressing Zap-70 K217R single mutant and K193/217R double mutant (Figure 5.2A). No difference in Zap-70 activation levels was observed when Lys-193 or Lys-376 were individually altered to prevent

ubiquitin conjugation. These results suggest that ubiquitination of Zap-70 Lys-217 following TCR stimulation plays a negative regulatory role with regard to phosphorylation at Tyr-493 and activation of Zap-70.

A kinetic analysis of kinase activation following TCR stimulation revealed that cells expressing Zap-70 K217R display significantly enhanced levels of activated Zap-70 at early time points (within 2 minutes), but the difference between levels of activated Zap-70 in WT- and K217R-expressing cells becomes less significant by 5 minutes after stimulation (Figure 5.2B). This analysis also revealed that the lack of an effect on cells expressing Zap-70 K193R is not due to a shift in the time of maximal activation of Zap-70, as the levels of activated Zap-70 in cells expressing K193R are similar to wild-type at both earlier (2 min) and later (10 min) time points (Figure 5.2C). These results suggest that ubiquitination of Zap-70 Lys-217 is an acute response that limits Zap-70 activation following TCR stimulation.

We also assessed signaling events downstream of Zap-70. The adaptor proteins LAT and SLP-76 are two important substrates of Zap-70 whose phosphorylation is critical for activation of downstream signaling pathways (95, 118). The stimulation-dependent phosphorylation of both LAT (Figure 5.3A) and SLP-76 (Figure 5.3B) was found to be enhanced in P116 cells expressing Zap-70 K217R and Zap-70 K193R/K217R relative to cells expressing wild-type Zap-70, Zap-70 K193R, or Zap-70 K376R.

Downstream of activated adaptor proteins, levels of phosphorylated MAPK following TCR stimulation were also enhanced in P116 cells expressing Zap-70 K217R and Zap-70 K193R/K217R compared to cells expressing wild-type, K193R, and K376R Zap-70 (Figure 5.3C).

These results suggest that the enhanced tyrosine phosphorylation of Zap-70 observed in the absence of the Lys-217 ubiquitin modification site results in increased activation of downstream signaling pathways.

B. Increased IL-2 production in the absence of Zap-70 Lys-217 ubiquitination site

The functional outcome of early signaling events following TCR engagement is T cell activation leading to T cell proliferation, differentiation and effector cytokine secretion. The induction of IL-2 expression is one of the hallmarks of T cell activation following TCR ligation (183). Because the Zap-70 K217R mutant consistently displayed enhanced activation of proximal elements of the TCR pathway, we sought to determine whether increases in pathway activation observed in the absence of the Lys-217 ubiquitin modification site resulted in downstream functional effects.

We evaluated the IL-2 activity in P116 cells expressing wild-type or mutant Zap-70 utilizing a luciferase reporter assay in which a reporter construct expresses firefly luciferase under the control of the IL-2 promoter. P116 cells were nucleofected with wild-type or mutant Zap-70 and the luciferase reporter construct, and then stimulated. We found increased IL-2 activity in cells expressing K217R and K193/217R mutants, while K193R and K376R single mutants did not alter IL-2 activation levels significantly (Figure 5.4).

In addition, we compared levels of IL-2 secretion in P116 cells stably expressing wild-type Zap-70 and Zap-70 K217R mutant. We detected increased IL-2 secretion in cells expressing Zap-70 K217R relative to cells expressing wild-type Zap-70 following stimulation (Figure 5.5). This increase in IL-2 production is seen in cells stimulated with concanavalin A (Figure 5.5A) as

well as cells co-cultured with Raji cells in the presence of Staphylococcal enterotoxin D (SED) (Figure 5.5B). We also compared levels of IL-2 expression in primary T cells overexpressing wild-type Zap-70 or the K217R mutant. As expected, we detected increased IL-2 expression in cells over-expressing wild-type Zap-70 relative to uninfected cells. Of note, we also observed increased IL-2 expression in cells expressing Zap-70 K217R relative to cells expressing wild-type Zap-70 (Figure 5.6A,B), despite similar levels of kinase expression (Figure 5.6C). These results suggest that the enhanced activation of TCR signaling pathways observed in the absence of the Lys-217 ubiquitin modification site results in increased transcriptional activation, including expression of the cytokine IL-2.

5.3 Conclusions

In this study we showed that Zap-70 is ubiquitinated on multiple lysine residues following TCR stimulation and identified nine novel ubiquitination sites, including Lys-193, Lys-217, and Lys-376. We constructed Zap-70 mutants in which the ubiquitin acceptor lysine is replaced with arginine in order to prevent ubiquitin conjugation at that site. In addition, we generated stable cell lines derived from *Zap-70*$^{-/-}$ Jurkat P116 cells which express these Zap-70 lysine mutants and utilized them to evaluate the effect of different ubiquitination sites on TCR signaling pathways. Zap-70 that lacks the Lys-217 ubiquitination site displayed a marked increase in levels of activation following TCR stimulation relative to wild-type Zap-70. The increase in levels of activated Zap-70 led to increased phosphorylation of adaptor proteins LAT and SLP-76, which are direct targets of Zap-70, as well as increased activation of downstream signaling MAP kinases Erk1/2. Enhanced signaling pathway activation was accompanied by an increase in cytokine production by cells expressing Zap-70 K217R mutant. These results suggest that ubiquitination at Lys-217 has a non-redundant role in negatively regulating Zap-70 signaling function.

5.4 Figures

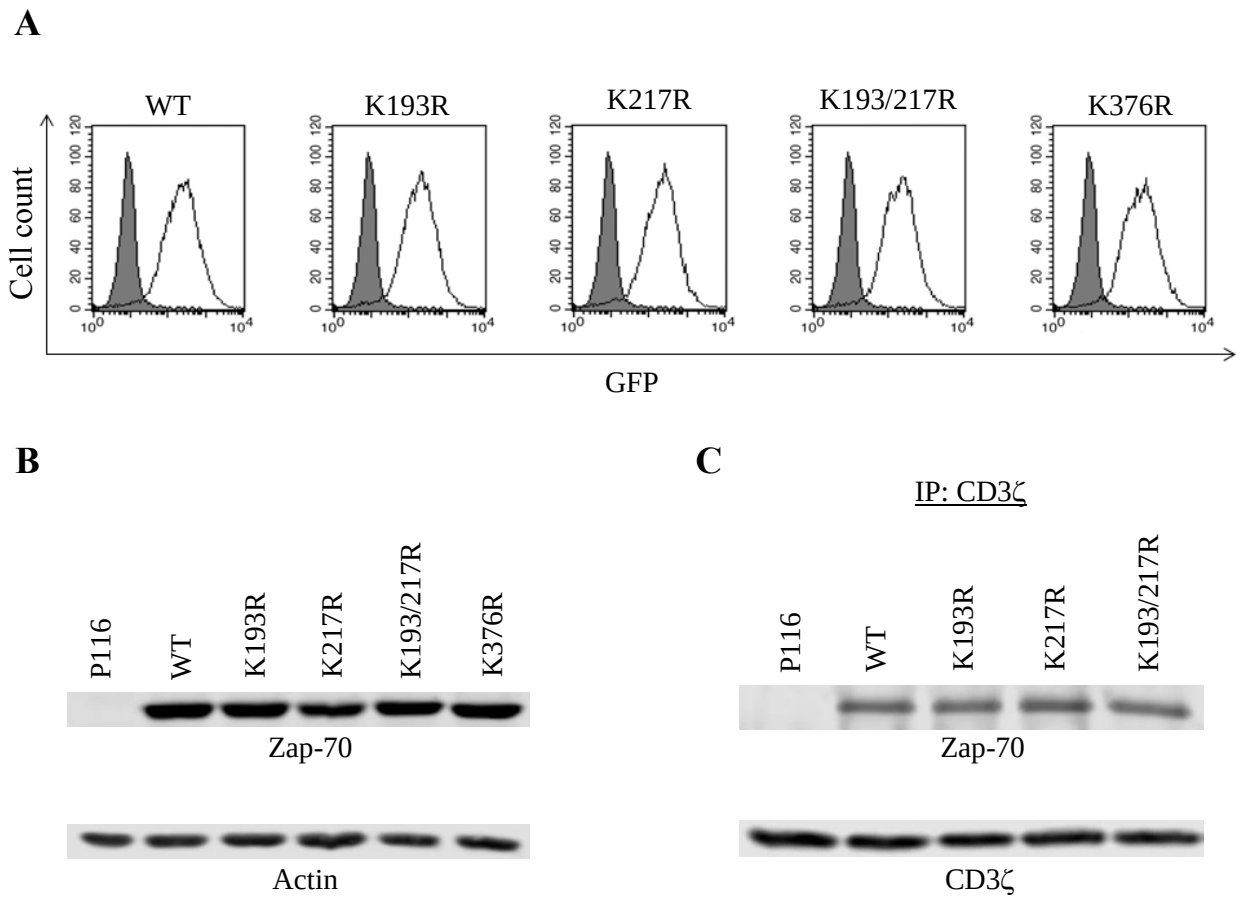

Figure 5.1 Stable cell lines expressing mutant Zap-70. (**A**) Zap-70 expression of stable cell lines expressing mutant Zap-70 was analyzed by flow cytometry. Representative fluorescent histogram plots show the GFP fluorescence intensities. Gray filled histograms show the fluorescent signal of parental *Zap-70*$^{-/-}$ P116 cells, whereas unfilled histograms represent the fluorescent intensities of the Zap-70-expressing cells (GFP^{+}). (**B**) P116 cells stably expressing Zap-70 mutants were lysed and levels of Zap-70 were assessed by Western blot using anti-Zap-70 antibody. The same blot was re-probed with an anti-actin antibody as loading control. (**C**) P116 cells stably expressing Zap-70 mutants were stimulated with CD3 antibody and lysed. CD3ζ was immunoprecipitated and levels of Zap-70 associated with CD3ζ were assessed by Western blot with Zap-70 and CD3ζ antibodies.

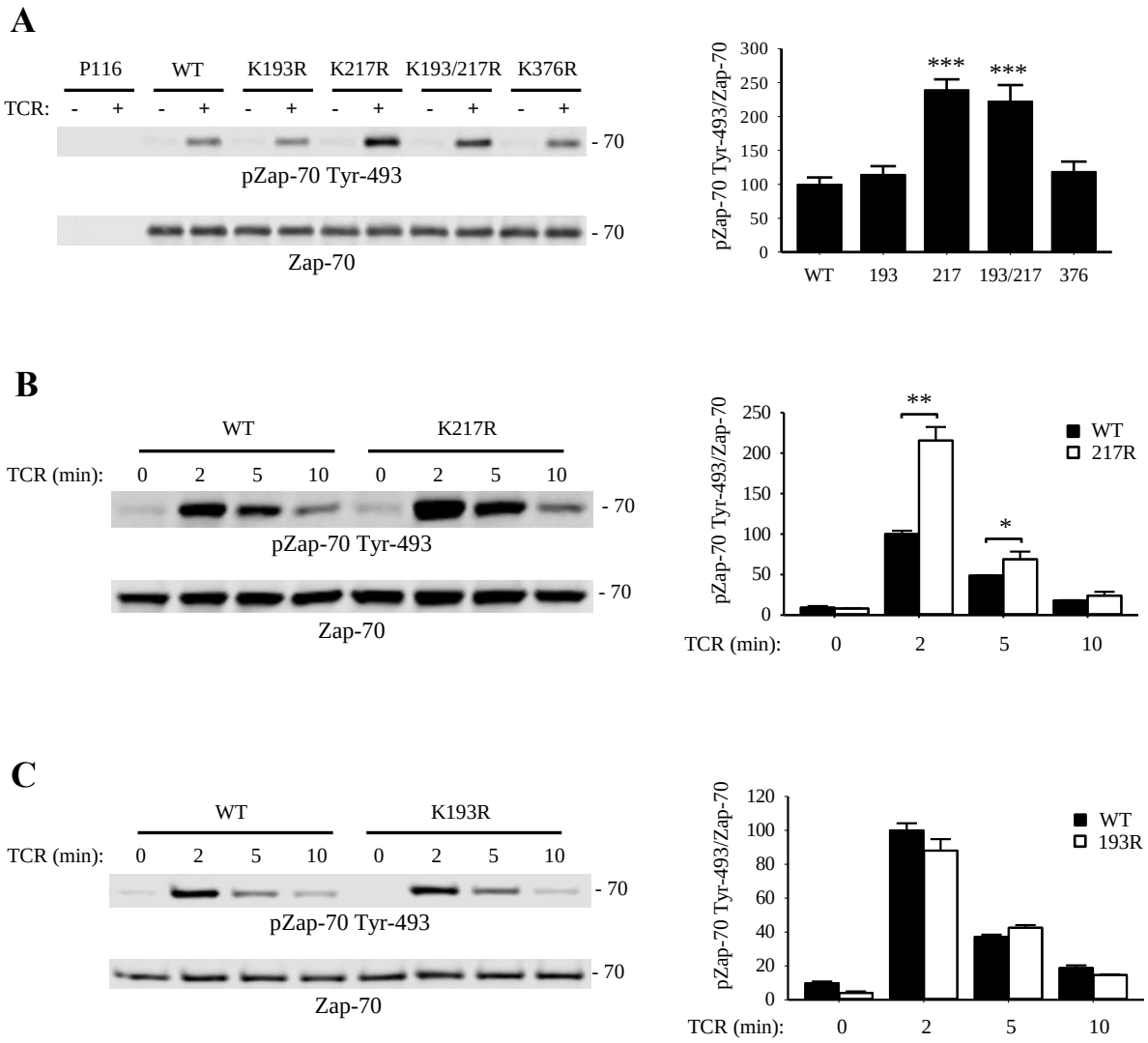

Figure 5.2 Increased levels of activated Zap-70 in the absence of Zap-70 Lys-217 ubiquitination site. (**A**) P116 cells stably expressing the indicated Zap-70 Lys-to-Arg mutants were stimulated with CD3 antibody for 2 minutes, lysed and processed for IP/Western analysis. Additionally, P116 cells stably expressing WT Zap-70 and K217R (**B**) or K193R (**C**) mutants were stimulated with CD3 antibody for the indicated time, lysed and processed for IP/Western blot analysis. Levels of phosphorylated Zap-70 were assessed. Representative results for are displayed (*left panel*). Bar graphs (*right panel*) show densitometric analysis of Western blots. Relative phosphorylation of Zap-70 in stimulated cells was normalized to total Zap-70 for each sample and shown as percent of wild-type levels. Data represented as mean ± SD for at least three independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. WT).

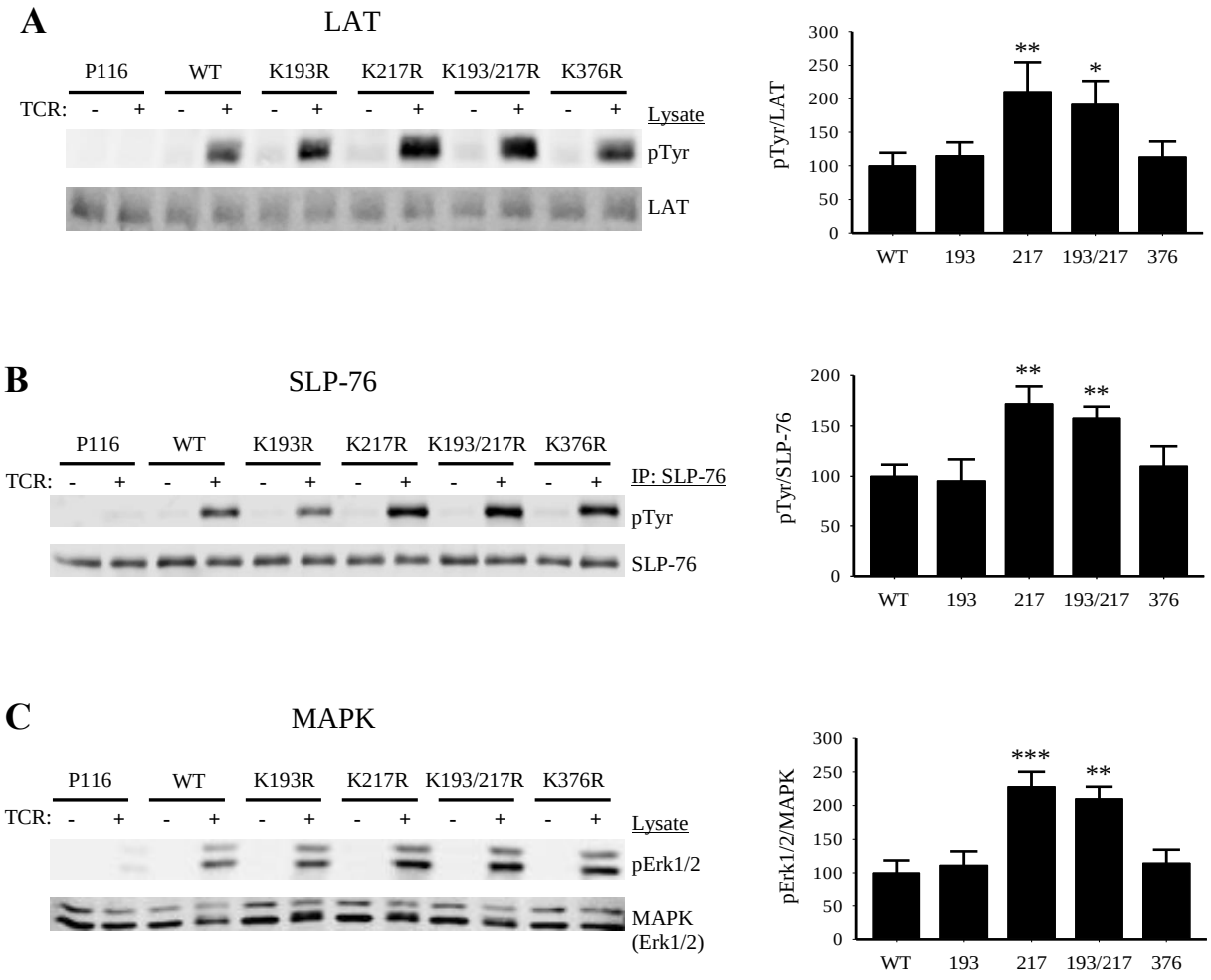

Figure 5.3 Enhanced signaling in the absence of Zap-70 Lys-217 ubiquitination site. P116 cells stably expressing the indicated Zap-70 Lys-to-Arg mutants were stimulated with CD3 antibody for 2 minutes, lysed and processed for IP/Western analysis. Levels of phosphorylated LAT (**A**), SLP-76 (**B**), and activated MAPK (**C**) were assessed as indicated. Representative results for each signaling molecule are displayed (*left panel*). Bar graphs (*right panel*) show densitometric analysis of Western blots. Relative phosphorylation of the indicated proteins in stimulated cells was normalized to total protein for each sample and shown as percent of wild-type levels. Data represented as mean ± SD for at least three independent experiments (*p<0.05, **p<0.01, ***p<0.001 vs. WT).

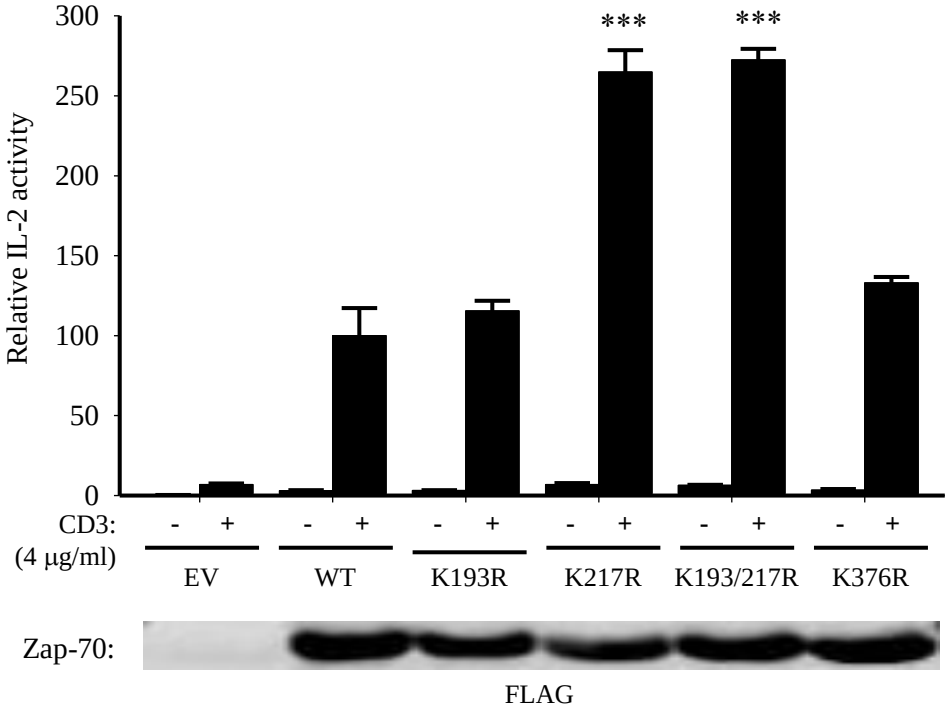

Figure 5.4 Increased activation of transcription from the IL-2 promoter in the absence of Zap-70 Lys-217 ubiquitination site. P116 cells were nucleofected with a firefly luciferase reporter construct under the control of IL-2 promoter, a Renilla luciferase control plasmid and plasmids encoding wild-type or mutant Zap-70. The cells were stimulated with plate-bound CD3 antibody for 8 hrs and relative luciferase activity determined. The histogram represents luciferase activity (shown as percent of wild-type levels). Data represented as mean ± SD for at least three independent experiments (***$p<0.001$ vs. WT). Expression levels of wild-type and mutant Zap-70 (FLAG) are shown below the bar graph.

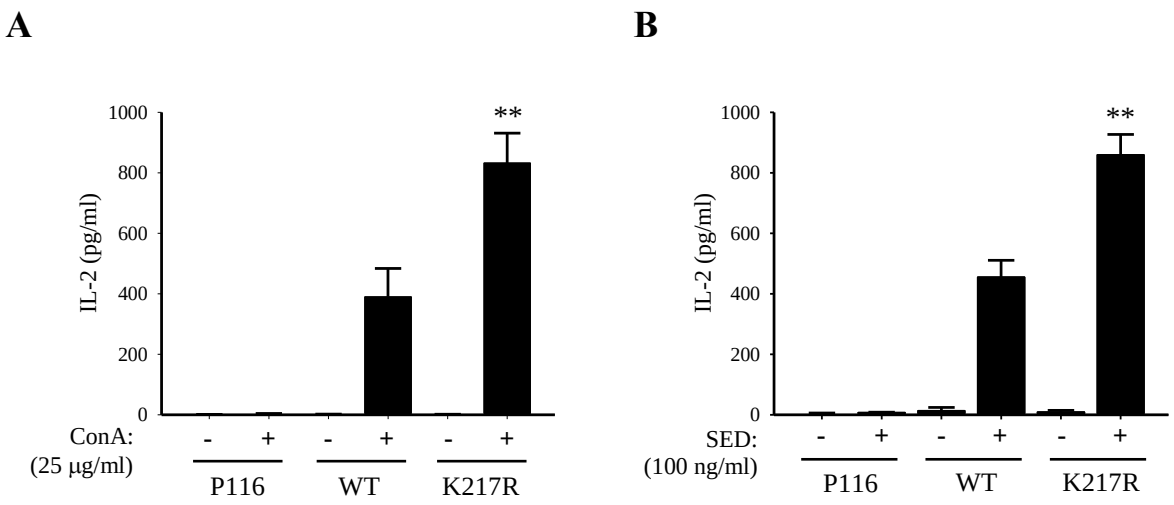

Figure 5.5 Secretion of IL-2 is enhanced in P116 cells stably expressing Zap-70 lacking the Lys-217 ubiquitination site. P116 cells stably expressing wild-type Zap-70 or Zap-70 K217R mutant were stimulated with Concanavalin A (**A**) or Raji/SED (**B**). After 24 hours in culture, supernatants were collected and assayed for IL-2 content by ELISA. Results are representative of three independent experiments for each condition (mean ± SD; **$p<0.01$ vs. WT).

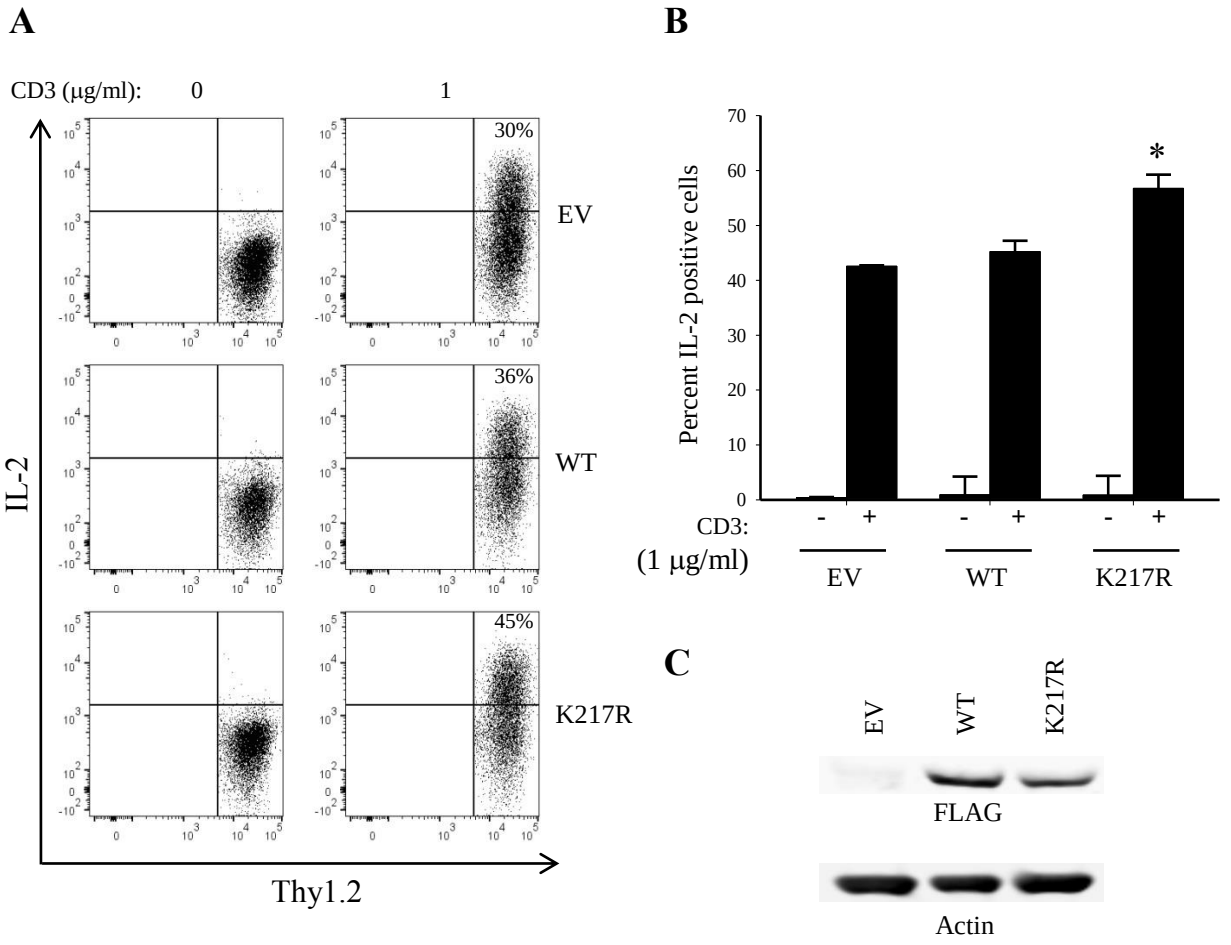

Figure 5.6 Increased IL-2 production in primary T cells over-expressing Zap-70 lacking the Lys-217 ubiquitination site. Primary wild-type T cells were infected with empty vector, or retrovirus expressing wild-type Zap-70 (WT) or Zap-70 K217R mutant. Infected cells were stimulated with CD3 antibody (1 μg/ml) for 6 hours. Following stimulation, cells were stained with fluorochrome-labeled antibodies to Thy1.2 (T cell marker) and IL-2, and analyzed by flow cytometry to assess levels of IL-2 expression. Cytokine-expressing T cells are visible in the upper right-hand quadrant with the percentage of cells indicated. Representative plots (**A**) and the summary data of three independent experiments (**B**) are illustrated (mean ± SD; *$p \leq 0.01$ vs. WT). (**C**) GFP^+ reconstituted T cells were sorted by flow cytometry, lysed and the levels of exogenous Zap-70 protein was assessed by Western blot analysis using an anti-FLAG antibody. The same blot was re-probed with an anti-actin antibody as loading control.

Chapter 6: Discussion

6.1 Discussion

This study identifies Zap-70 ubiquitination as a novel mechanism for regulating Zap-70 function downstream of the TCR. Protein ubiquitination is a highly versatile, dynamic, and functionally important post-translational modification. Indeed, it is well established that post-translational modification of a protein substrate by either a single ubiquitin polypeptide or long chains of polymeric ubiquitin can alter a protein's stability, activity, localization, and interacting partners (94, 103, 204). While the role of ubiquitination in targeting protein substrates for proteasome-mediated degradation has been extensively characterized, additional functional consequences of ubiquitin modification have emerged more recently (94). The ubiquitination of Zap-70 was first observed in mutant T cells lacking the Sts regulatory proteins, due to the hyper-phosphorylation of both native Zap-70 and its ubiquitin-modified forms in the absence of the Sts phosphatases (20). An important question that arose from this observation was whether Zap-70 ubiquitination was limited to *Sts-1/2*$^{-/-}$ T cells, in which the normal biochemical pathways that control TCR signaling are deregulated. Our current study demonstrates that ubiquitination of Zap-70 following T cell stimulation is a phenomenon that can be observed in human and mouse T cells lines, and in wild-type primary murine T cells. Importantly, we have also demonstrated the appearance of Zap-70 ubiquitination in primary human T cells. Taken together, these observations demonstrate the likelihood that Zap-70 ubiquitination plays a widespread and critical role in regulating

Zap-70 signaling function as part of a mechanism that controls the extent of T cell activation following antigen stimulation.

Using linkage-specific ubiquitin antibodies we detected the presence of K48-linked polyubiquitin on Zap-70 following TCR stimulation. Although this type of modification is generally known for targeting proteins for proteasomal degradation, that does not appear to be the primary function of Zap-70 ubiquitination. Inhibition of proteasome function failed to enhance accumulation of ubiquitinated Zap-70, suggesting that the majority of ubiquitinated Zap-70 is not degraded in a proteasome-dependent manner following TCR stimulation.

Zap-70 and its close relative Syk are involved in antigen receptor signaling. While Zap-70 is essential in signaling pathways downstream of the TCR, Syk plays a critical role in B cell activation (71, 85, 196, 208). It is known that Cbl family E3 ligases bind to phosphorylated Syk and negatively regulate BCR signaling through ubiquitination and degradation of Syk after BCR stimulation (161, 184). Although Zap-70 similarly binds Cbl and promotes ubiquitination of TCR subunits, it is thought to primarily act as an adaptor rather than a direct target for Cbl-mediated ubiquitination (200). Moreover, recent studies, including this one, suggest that ubiquitination of Zap-70 negatively regulates TCR signaling in non-proteolytic fashion (74, 212).

In order to better understand the functional role of Zap-70 ubiquitination, we developed and implemented a strategy for purifying endogenous ubiquitinated Zap-70. Ubiquitin remnant affinity purification followed by mass spectrometry analysis was

carried out on the purified sample. In addition, global ubiquitin remnant analysis was performed on unstimulated and stimulated T cells for quantitative profiles of ubiquitinated proteins. Our analysis identified ten lysine residues within Zap-70 that are sites of ubiquitin conjugation, three of which displayed greater than 20-fold increase in levels of modification following TCR stimulation.

Zap-70 function in T cell activation is regulated in part by multiple phosphorylation events. Ubiquitination has been shown previously to mediate dephosphorylation of Zap-70 by the Sts phosphatases (74, 212). In a recent study, ubiquitination at Lys-578 was shown to promote dephosphorylation of Zap-70 by the Sts proteins (212). The role of ubiquitination as a mechanism that targets Zap-70 for dephosphorylation by the Sts phosphatases is supported by another study, which demonstrated that DUB Otud7b facilitates Zap-70 activation and promotes TCR signaling by deubiquitinating Zap-70, with Lys-544 as the main regulatory ubiquitination site. Deubiquitination of Zap-70 was shown to reduce its association with Sts-1 and Sts-2, thus preventing its subsequent dephosphorylation (74). It is possible that other ubiquitination sites similarly act as binding sites for phosphatases.

We observed that Zap-70 mutant that lacks the Lys-217 ubiquitination site displays a marked increase in levels of activation following TCR stimulation relative to wild-type Zap-70. Increased phosphorylation of downstream molecules LAT, SLP-76, and MAPK was also evident in cells reconstituted with Zap-70 K217R. The increase in activation of signaling pathways was accompanied by enhanced IL-2 production downstream of the

TCR. Together, these results suggest that Zap-70 ubiquitination at Lys-217 acts in a non-redundant fashion to negatively regulate Zap-70 signaling function. In particular, these results are consistent with a role for ubiquitination in regulating the phosphorylation and activation of Zap-70 in a proteolysis-independent manner. One possibility is that ubiquitination at Lys-217 creates a binding site for regulatory molecules that directly or indirectly target Zap-70 phosphorylation sites. An intriguing question yet to be resolved is whether simultaneous modification of Zap-70 by tyrosine phosphorylation and ubiquitination has specific functional consequences. Ubiquitination of residues adjacent to phosphorylation sites could alter conformational changes induced by phosphorylation, or restrict the formation of multi-protein signaling complexes by interfering with phosphorylation-induced recruitment of signaling molecules.

It is also important to note that it is not known what E3 ubiquitin ligase is involved in ubiquitinating the sites in Zap-70 identified in this study. While the well-studied Cbl family of E3 ligases have been demonstrated to interact with Zap-70, the latter is thought to act primarily as an adaptor protein that recruits Cbl to the activated receptor complex (162, 201). Indeed, the targets of the Cbl ligases are considered to be the TCR, Lck, and the p85 subunit of PI3K, and no study to date has linked their activity with direct Zap-70 ubiquitination (44, 47, 160, 201). Recently, E3 ligase Nrdp1 was identified as a negative regulator of TCR signaling. Nrdp1-mediated polyubiquitination of Zap-70 was shown to induce Zap-70 dephosphorylation by Sts-1 and Sts-2 (212). Yang et al. also identified six ubiquitination sites in Zap70, with Lys-578 as the main ubiquitin acceptor site (Figure 6.1). In contrast, Hu et al. identified three other sites, with Lys-544 as the main regulatory

ubiquitination site (74, 212). Of the sites identified, only one site (Lys-328), reported by Yang et al., overlaps with the ubiquitin acceptor sites identified in our study. This discrepancy might be due to the differences in cell types or methods used by our study. For example, Yang et al. identified modified lysine residues following co-expression of Zap-70 and Nrdp1 in HEK293T cells, whereas Hu et al. identified ubiquitination sites in Zap-70 isolated from stimulated EL4 cells (74, 212). In contrast, we determined TCR stimulation-induced ubiquitination sites on Zap-70 in stimulated primary T cells isolated from *Sts-1/2*$^{-/-}$ mice. Given the large number of Zap-70 ubiquitination sites and their distinct locations on the surface of the molecule, an interesting possibility is that multiple E3 ligases target distinct sites within Zap-70 for ubiquitination in a spatially and temporally distinct manner.

Our analysis identified ten lysine residues within Zap-70 that are sites of ubiquitin conjugation. Six of the identified ubiquitination sites are located within the Zap-70 N-terminal SH2 domains (Figure 4.3). The N-terminal region mediates interaction of the kinase with phosphorylated ITAMs of the activated TCR complex, and we surmise that steric constraints could make ubiquitin post-translational modification of Zap-70 incompatible with its localization in the TCR complex. Thus, it is not clear whether Zap-70 is ubiquitinated while it is bound to TCR subunits. In our analysis of P116 cells stably expressing Zap-70 Lys-to-Arg mutants, we did not observe increased association of SH2 domain mutants with the CD3ζ chain. However, this observation does not preclude a role of Zap-70 ubiquitination in modulation of Zap-70–CD3ζ interactions. Fluorescent studies of T cell activation and immunological synapse formation show that signaling is initiated in

small TCR-containing clusters within seconds of T cell–APC contact and Zap-70 is rapidly recruited to these clusters. Zap-70 remains associated with CD3ζ microclusters until they migrate towards the center of the contact and form the cSMAC (3 min after contact) (15, 213). Additionally, fluorescence recovery after photobleaching (FRAP) experiments show that individual Zap-70 molecules are not stably bound to CD3ζ microclusters (15). It is possible that ubiquitination of certain sites in Zap-70 (e.g. Lys-193) does not affect the rate of Zap-70 dissociation from the TCR complex, but instead prevents re-binding to CD3ζ.

TCR ligation triggers intracellular signaling pathways which may result in the initiation of markedly different cellular responses, including proliferation and differentiation, or unresponsiveness and apoptosis (7, 57, 68, 88, 121). The magnitude and duration of activating signals is a critical determinant of the functional outcome of TCR ligation (121). Previous studies have shown that T cell stimulation with antibodies crosslinked in solution elicits a strong and transient activation of signaling pathways, and is associated with unresponsiveness and apoptosis (3, 158). In contrast, stimulation with antibodies immobilized on microbeads leads to weak but sustained signaling which promotes proliferation (3, 158). Analysis of signaling pathways revealed the presence of ubiquitinated Zap-70 following TCR stimulation under conditions that promote unresponsiveness which is absent from cells stimulated under proliferation-inducing conditions (158, 202). These observations suggest that Zap-70 ubiquitination could be part of an inhibitory feedback mechanism responsible for terminating TCR-mediated signaling and promoting unresponsiveness and apoptosis.

We do not currently know the stepwise series of biochemical events that induce or promote Zap-70 ubiquitination at the varied lysine residues, or whether the recruitment of Zap-70 to the TCR and its subsequent activation by phosphorylation are necessary intermediate signaling steps to the activation of an E3 ligase that targets Zap-70. We favor a stepwise model in which Zap-70 recruitment to the activated receptor complex, its subsequent phosphorylation by Lck, and its activation of downstream signaling cascades precede the activation of a negative feedback loop that results in Zap-70 ubiquitination and functional inhibition. In our model, ubiquitination at certain residues could directly promote Zap-70 dephosphorylation and inactivation (Figure 6.2), while ubiquitination at other lysines may inhibit Zap-70–CD3ζ association so that ubiquitinated Zap-70 that is inactivated by dephosphorylation after dissociating from the TCR would be unable to re-bind the CD3ζ chain and would remain inactive (Figure 6.3). In this way, ubiquitination could serve a negative regulatory role by directly promoting Zap-70 inactivation and limiting the pool of available Zap-70. This model is consistent with a role of Zap-70 ubiquitination in termination of TCR signaling during abortive T cell responses which result in unresponsiveness and apoptosis.

6.2 Future Directions

With these studies we have established Zap-70 ubiquitination as a novel regulatory mechanism in TCR signaling. Many open questions remain regarding specific mechanisms involved in regulation of Zap-70 by ubiquitination at different sites as well as regulatory molecules involved in these processes.

A. Ubiquitin ligase(s) involved in Zap-70 ubiquitination

One important question that remains to be answered is the identity of the E3 ubiquitin ligase responsible for Zap-70 ubiquitination. A recent study demonstrated that E3 ligase Nrdp1 negatively regulates Zap-70 through ubiquitination at Lys-578. However, it is not clear whether Nrdp1 is involved in ubiquitinating any of the sites identified in our study, especially Lys-217. Given the large number of ubiquitination sites, it is likely that multiple ligases target distinct sites within Zap-70. Determining the identity of the ligase(s) involved in ubiquitinating Zap-70 could provide important information regarding potential functions of this modification during different stages of T cell development and activation. There are a number of ubiquitin ligases involved in various aspects of the immune system, including development, differentiation and activation of lymphocytes, antigen presentation, and induction of T cell tolerance (108, 109, 140, 149, 151). Several E3 ligases have been identified whose absence and impaired function are implicated in development of autoimmunity and immunopathologies. Members of the Cbl family have been shown to act as negative regulators of T cell activation and their absence predisposes to autoimmunity (141). Deficiency of Itch E3 ligase has been shown to cause severe

multisystem autoimmune disease in mice (111). GRAIL E3 ligase is associated with induction of T cell tolerance and GRAIL deficiency results in increased susceptibility to autoimmunity (140). Identifying the E3 ubiquitin ligase(s) responsible for Zap-70 ubiquitination could contribute to understanding the mechanisms involved in autoimmune disease development associated with similar deficiencies.

B. Role of Sts proteins

In this study we identified ten residues in Zap-70 which are modified with ubiquitin following TCR stimulation. We were able to show that ubiquitination at Lys-217 serves a negative regulatory function and TCR signaling is enhanced in the absence of the modification. Yet the precise mechanism by which ubiquitination modulates Zap-70 function remains unclear. One likely explanation is that ubiquitination at that site serves to create a binding site for negative regulatory molecules, such as phosphatases. This is supported by the increase in activated Zap-70 observed in the absence of the Lys-217 ubiquitination site. A recent study demonstrated that ubiquitination of Zap-70 Lys-578 by Nrdp1 promotes Zap-70 dephosphorylation by Sts-1 and Sts-2 (212). It is possible that other ubiquitination sites regulate Zap-70 phosphorylation in a similar fashion by facilitating its recognition by Sts-1/2 or other phosphatases.

Sts-1 and Sts-2 have been shown to have a role in regulating the phosphorylation status of Zap-70 (19, 20, 175, 212). It is not clear whether this is due to direct dephosphorylation of Zap-70 by one or both proteins. Both Sts proteins contain UBA domains as well as phosphatase domains, and Sts-1 has been shown to be capable of

binding to and dephosphorylating Zap-70 in vitro (175). It would be of interest to examine the role of the Sts proteins in regulation of Zap-70 via ubiquitination at Lys-217.

C. TCR microclusters and signaling complexes

Immediately after TCR stimulation, small clusters containing TCR, kinases and signaling molecules form at the site of contact of the APC and T cell. During the first 1-2 minutes after T cell–APC contact, the T cell expands the contact and CD3 microclusters are generated. After reaching maximum cell spreading, the CD3 microclusters move towards the center of the contact and form the central supramolecular activation cluster (cSMAC) of the immunological synapse (16, 213). Fluorescent microscopy studies show that Zap-70 co-localizes with CD3 in the same microclusters during the expansion phase, but is lost from CD3-containing microclusters at later time points and does not translocate to the cSMAC (213). It would be interesting to evaluate the potential role of ubiquitination in Zap-70 association with other signaling molecules and the formation of functional signaling complexes during T cell activation.

D. Phosphorylation and ubiquitination

Zap-70 is modified by both phosphorylation and ubiquitination following TCR stimulation. However, it remains unclear whether Zap-70 phosphorylation and activation are necessary for subsequent ubiquitination or if recruitment of Zap-70 to the receptor complex is sufficient for its ubiquitination. Zap-70 mutants containing mutations in one or more of the tyrosine residues that are phosphorylated following TCR stimulation (Tyr-292, Tyr-315, Tyr-319, and Tyr-493) can be constructed. Utilizing these mutants

could help determine whether ubiquitination can occur in the absence of phosphorylation or whether phosphorylation at certain sites in Zap-70 is required for ubiquitin conjugation.

E. Zap-70 K217R knock-in mouse

Absence of Lys-217 ubiquitination site in Zap-70 results in increased activation of signaling pathways following TCR stimulation, which correlates with increased cytokine production. All the studies of Zap-70 Lys-217 mutant conducted so far have been in vitro studies. However, many questions remain unresolved regarding the role of this modification in T cell development and function, as well the physiological consequences of the altered TCR signaling that results from its absence. TCR signal strength determines the outcome of positive and negative selection and is a key determinant of lineage choice during T cell development (18, 54). An activating mutation in Zap-70 could lead to increased activation of TCR signaling pathways and, consequently, increased sensitivity of developing thymocytes to TCR stimulation. This could result in alterations of signaling thresholds with potential effects on the selection of the T cell repertoire which could compromise immune function. Generating a Zap-70 K217R knock-in mouse would be an invaluable tool in addressing the functional consequences of Zap-70 ubiquitination in T cell development, immune system regulation and function, and potential involvement in immunopathology.

F. Global ubiquitin remnant immunoaffinity profiling

The global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling used to identify ubiquitination sites in Zap-70 also identified over 400 unique

proteins which are ubiquitinated following TCR stimulation (Table 6.1). This information can be used to identify major signaling components which are modified with ubiquitin during T cell activation in an effort to better understand TCR-dependent ubiquitination networks. This could significantly add to our understanding of the role of ubiquitination in regulation of T cell activation.

6.3 Impact of studies

T cell development, differentiation and function are all a result of selections events controlled by signals though the TCR. Zap-70 plays a critical role in events involved in T cell activation during an immune response. It is a key molecule which couples engagement of the TCR by an antigen to initiation of signaling pathways that lead to T cell activation. This study provides valuable insights into the regulation of Zap-70 function in T cell activation and adds a new dimension to our understanding of the role of Zap-70 in fine-tuning TCR signaling. Addressing some of the questions raised by this study could have significant implications for prevention and treatment of diseases associated with misregulation of T cell function and could aid in identifying new therapeutic targets.

6.4 Tables and figures

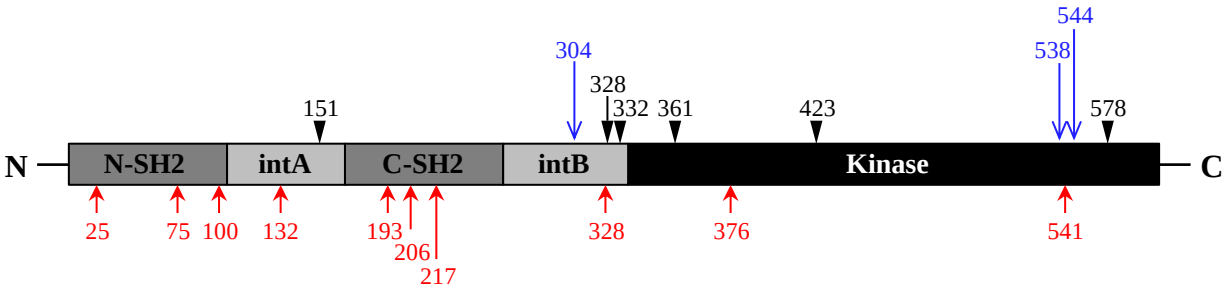

Figure 6.1 Ubiquitination sites identified in Zap-70. Schematic representation of full-length Zap-70. Red arrows (↑) indicate location of ubiquitination sites identified in this study. Ubiquitination sites identified by Yang et. al. 2015 are marked with (▼) (212). Ubiquitination sites identified by Hu et. al. 2016 are marked with blue arrows (↓) (74).

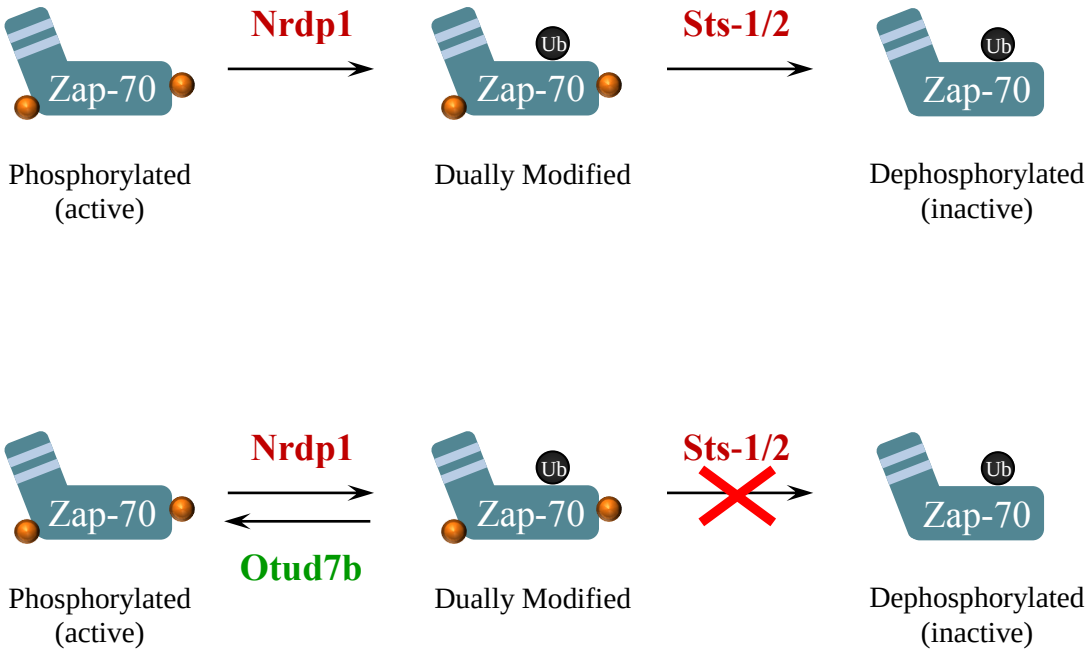

Figure 6.2 Model for regulation of Zap-70 phosphorylation by ubiquitination. Ubiquitination of Zap-70 by E3 ligases (e.g. Nrdp1) results in its dephosphorylation and inactivation by Sts-1/2 phosphatases (*top*). Deubiquitination of Zap-70 by DUBs (e.g. Otud7b) promotes signaling by inhibiting Zap-70 association with Sts proteins and its subsequent dephosphorylation (*bottom*).

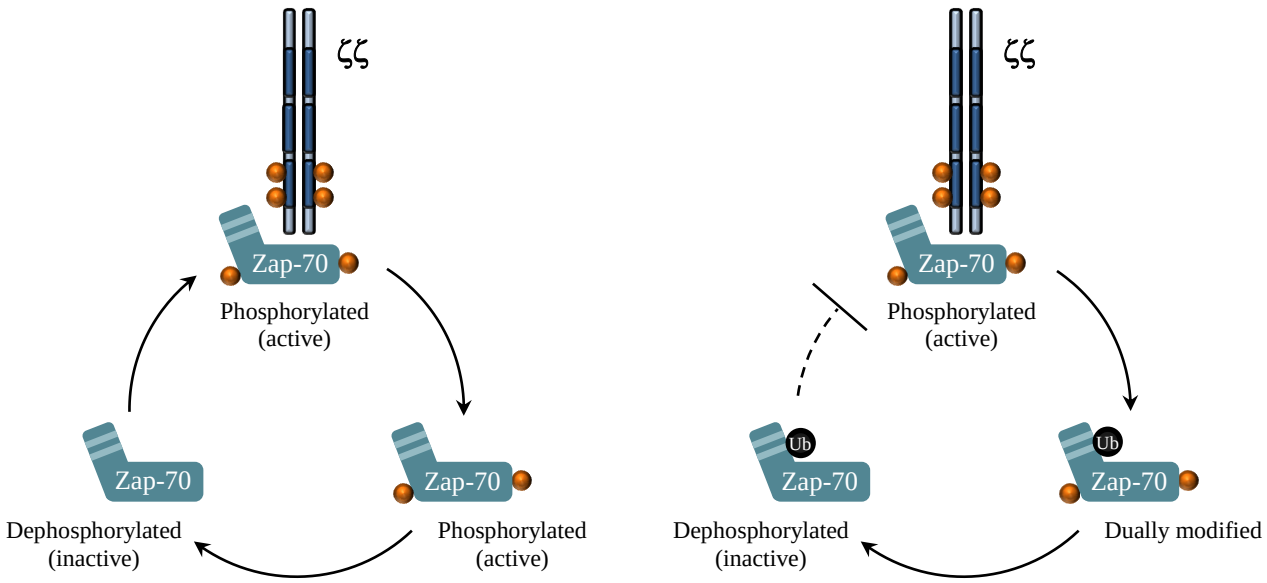

Figure 6.3 Model for regulation of Zap-70–CD3ζ binding by ubiquitination. Zap-70 binds to phosphorylated ITAMs of CD3ζ and is activated by phosphorylation. After activation, Zap-70 dissociates from the receptor and is subsequently dephosphorylated and inactivated. Inactive Zap-70 not modified with ubiquitin can then rebind the CD3ζ chain and get reactivated (*left*). In contrast, ubiquitinated Zap-70 is unable to rebind and remains inactive (*right*).

Fold Change	Protein Name	Site	Description
Adaptor/scaffold			
4.8	Cbl	201	E3 ubiquitin-protein ligase CBL
8.8	Cbl-b	68	E3 ubiquitin-protein ligase CBL-B
-8.4	GGA1	23	ADP-ribosylation factor-binding protein GGA1
9.1	LAT	53	linker for activation of T-cells family member 1
-7.5	UBE2M	45	NEDD8-conjugating enzyme Ubc12 isoform 2
Adhesion or extracellular matrix protein			
3.8	ITGβ2	363	integrin beta-2
-7.4	LSP1	92	lymphocyte-specific protein 1 isoform 1
9.2	RhoQ	169	rho-related GTP-binding protein RhoQ precursor
-4.7	TOP2β	425	DNA topoisomerase 2-beta
Cell cycle regulation			
-6.5	Cdc20	259	cell division cycle protein 20 homolog
Chromatin, DNA-binding, DNA repair or DNA replication protein			
9.7	H4; H4H4	21	histone H4
5.1	HIST2H2AC	125	histone H2A type 2-C
-8.5	SMARCA3	95	helicase-like transcription factor isoform 1
19.2	LIG1	312	DNA ligase 1
-10.9	RFC1; RFC1 iso4	260	replication factor C subunit 1
Phosphatase			
4.1	PTPN22	344	tyrosine-protein phosphatase non-receptor type 22
2.6	CD45	790	receptor-type tyrosine-protein phosphatase C isoform 1
Protein kinase, Ser/Thr (non-receptor)			
7.3	HPK1	290	mitogen-activated protein kinase kinase kinase kinase 1
-13.8	RIPK1	376	receptor-interacting serine/threonine-protein kinase 1
Protein kinase, Tyr (non-receptor)			
23.9	Lck	246	proto-oncogene tyrosine-protein kinase LCK isoform a
23.6	ZAP70	376	tyrosine-protein kinase ZAP-70
Receptor, channel, transporter or cell surface protein			
-9.2	AGTRAP	153	type-1 angiotensin II receptor-associated protein
23.8	CD3ζ	118	CD247 antigen isoform zeta precursor
16.0	CD3ε	159	T-cell surface glycoprotein CD3 epsilon chain precursor
18.2	CD4	442	T-cell surface glycoprotein CD4 precursor
27.0	CD8α	245	T-cell surface glycoprotein CD8 alpha chain isoform 1
13.3	FcεR1γ	80	high affinity immunoglobulin epsilon receptor subunit gamma
-4.3	NUP153	264	nucleoporin 153
-7.3	VAPA	161	vesicle-associated membrane protein-associated protein A
Transcriptional regulator			
-20.6	ataxin-3	291	ataxin-3 isoform 2
12.4	C/EBP-zeta	719	CCAAT/enhancer-binding protein zeta
-6.0	FAF1	443	FAS-associated factor 1
11.6	MXD3	126	max dimerization protein 3
-19.3	SIRT1	491	NAD-dependent deacetylase sirtuin-1 isoform 2
45.3	STAT5B	586	signal transducer and activator of transcription 5B
-9.7	RAP80	31	BRCA1-A complex subunit RAP80
Ubiquitin conjugating system			
-4.0	ITCH	439	E3 ubiquitin-protein ligase Itch
3.1	TRAF2	148	TNF receptor-associated factor 2

Table 6.1 Global ubiquitin remnant immunoaffinity profiling. Global ubiquitin remnant analysis of unstimulated and stimulated T cells identified a large number of proteins modified with ubiquitin. Some of the identified proteins are shown, along with sites of ubiquitin modification and fold change in ubiquitination at those sites. An increase in ubiquitination levels at a specific site following TCR stimulation is shown in green and a decrease is shown in red.

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