Redox-mediated regulation of the tyrosine kinase Zap70

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von M.Sc. Christoph Thurm

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Gutachter: apl. Prof. Dr. Luca Simeoni

PD Dr. rer. nat. Marcus Lettau

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I. Eigenständigkeitserklärung

Christoph Thurm
Halberstädter Straße 29
39112 Magdeburg

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema

Redox-mediated regulation of the tyrosine kinase Zap70

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades *doctor rerum naturalium* (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 02.02.2018

M.Sc. Christoph Thurm

II. Acknowledgements

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V. Abbreviations

A	- alanine
ADAP	- adhesion and degranulation promoting adapter protein
AP1	- Activator protein 1
APC	- Antigen-presenting cell
APS	- ammonium persulfate
B-CLL	- B-cell chronic lymphocytic leukemia
BCR	- B-cell receptor
BSA	- bovine serum albumin
BST	- biotin-switch technique
C	- cysteine
C lobe	- C-terminal lobe
c-Cbl	- Casitas B-lineage Lymphoma
CD	- Cluster of differentiation
Cdc37	- cell division cycle 37
CHX	- cycloheximide
Ci	- Curie
CLP	- common lymphoid progenitors
CrkII	- CT10 regulator of kinase II
Csk	- C-terminal Src kinase
D	- aspartic acid
DAG	- diacylglycerol
DMEM	- Dulbecco's modified Eagle's medium
DMF	- dimethylformamide
DMSO	- dimethylsulfoxide
DN	- double negative
DNA	- deoxyribonucleic acid
DP	- double positive
E	- glutamic acid
ECL	- electrochemiluminescence
EDTA	- ethylenediaminetetraacetic acid
EGF	- epidermal growth factor
EGFR	- epidermal growth factor receptor
ER	- endoplasmic reticulum
F	- phenylalanine
FBS	- fetal bovine serum
FSC	- forward-scattered light
GADS	- GRB2-related adaptor protein
GEF	- guanine exchange factor
Grb2	- growth factor receptor-bound protein 2
GSH	- glutathione
GTPase	- guanosine triphosphatase
H	- histidine
HRP	- horseradish peroxidase
HSP	- heat shock protein
HSC70	- heat shock cognate 70kDa protein
Hsp40	- heat shock protein 40
Hsp90	- heat shock protein 90
iAbs	- immobilized antibodies
InsP3	- inositol(1,4,5)trisphosphate
IRK	- insulin receptor kinase
ITAM	- immunoreceptor tyrosine-based activation motif

ITK	- interleukin-2-inducible T cell kinase
K	- lysine
L	- leucine
Lck	- lymphocyte-specific protein tyrosine kinase
M	- mol/L
M	- methionine
MAPK	- mitogen-activated protein kinase
MHC	- major histocompatibility
MMTS	- methyl methanethiosulfonate
N lobe	- N-terminal lobe
NAC	- N-Acetyl-L-cysteine
NADPH	- nicotinamide adenine dinucleotide phosphate
NFAT	- nuclear factor of activated T cells
NFkB	- nuclear factor-κB
NO	- nitric oxide
NOS	- nitric oxide synthases
NOX	- NADPH oxidase
nRTK	- non-receptor tyrosine kinases
PBS	- phosphate buffered saline
PDGFR	- Platelet-derived growth factor receptor
PH	- pleckstrin homology
PKC	- protein kinase C
PLCγ1	- phospholipase Cγ1
PMA	- phorbol-12-myristat-13-acetat,
PMSF	- phenylmethylsulfonylfluorid
PTB	- phosphotyrosine-binding pocket
PtdIIns(4,5)P2	- phosphatidylinositol-4,5-bisphosphate
PTK	- protein tyrosine kinase
PTP	- protein tyrosine phosphatase
Q	- glutamine
R	- arginine
RASGRP1	- RAS guanyl-releasing protein 1
RNS	- reactive nitrogen species
ROS	- reactive oxygen species
RPMI	- Roswell Park Memorial Institute
RTK	- receptor tyrosine kinases
S	- serine
sAbs	- soluble antibodies
SDS	- sodium dodecyl sulfate
SFK	- Src-family kinases
SH2	- Src-homolgy 2
SH3	- Src-homolgy 3
SLP76	- SH2 domain-containing leukocyte protein of 76 kDa
SNP	- sodium nitroprusside
SOD	- superoxide dismutase
SP	- single positive
SSC	- side-scattered light
T	- tyrosine
TCR	- T-cell receptor
TEMED	- tetramethylethylendiamine
TRX	- thioredoxin
U	- Unit
UPS	- Ubiquitin-Proteasome System
W	- tryptophan
wt	- wildtype

Y	- tyrosine
Zap70	- ζ-chain associated protein of 70 kDa

VI. Abstract

Protein tyrosine kinases (PTKs) are central players in many signal transduction processes. Therefore, the regulation of the activation of these proteins requires sophisticated mechanisms in order to prevent uncontrolled activity. In the past decades, it became evident that also oxidation of cysteine residues by reactive oxygen (ROS) and nitrogen species (RNS) can take part in the regulation of the activity of PTKs. However, the molecular mechanisms of how ROS and RNS modulate the activity of PTKs remains largely unknown. Recently, this research field has gained much attention because it has the potential to open a new avenue for the development of more specific PTK inhibitors.

In my thesis, I analyzed the role of oxidation in the regulation of Zap70, a tyrosine kinase which is essential for the initiation of T-cell receptor (TCR) signaling and therefore indispensable for the execution of an adaptive immune response. In the first part of my work, I conducted a functional screening of Zap70 mutants harboring Cys-to-Ala substitutions. The selected cysteine residues were either highly conserved among closely related tyrosine kinases or were proposed to undergo oxidation. The screening revealed that three cysteine mutants displayed altered function. The Zap70C564A mutant showed increased activity, whereas the activity of the Zap70C39A and Zap70C575A mutants was reduced.

In the second part of my work, I conducted an in-depth analysis of Zap70C575A. I have shown that C575 is a major regulatory site of Zap70 stability. Mutation of C575 to alanine results in protein instability and degradation at least in part via the proteasome. Also, the co-chaperone Cdc37 appears to be involved in the degradation of Zap70C575A. Indeed, Cdc37 overexpression partially rescued the expression of Zap70C575A. Additionally, Cdc37 fully rescued Zap70C575A activity. Finally, I have shown that C575 in Zap70 is embedded in a highly conserved $Mx_{(2)}CWx_{(6)}R$ motif which is required for the oxidation of C575. Interestingly, this motif is present in all human tyrosine kinases and appears to represent a universal consensus sequence regulating the stability and/or activity of PTKs.

Collectively, I showed that C39, C564, and C575 are functionally important for the regulation of Zap70. In the future, it will be important to further assess how oxidation contributes to the regulation of Zap70. It will be of particular interest to test whether modifications of the cysteines studied in my work using compounds reactive with cysteines will affect Zap70 functions.

VII. Zusammenfassung

Tyrosinkinasen sind von zentraler Bedeutung für die Initiation von Signalkaskaden sowie die Transduktion dieser Signale innerhalb der Zellen. Oftmals führen diese Prozesse zur Aktvierung und Proliferation der Zielzelle. Um eine unkontrollierte Aktivierung von Tyrosinkinasen und den damit assoziierten Kaskaden zu verhindern, sind diese Enzyme komplexen Regulationsmechanismen unterworfen. Ein klassisches Prinzip stellt dabei die Phosphorylierung und Dephosphorylierung an essentiellen Tyrosin-, Serin- und Threoninresten dar. Diese posttranslationalen Modifikationen führen zu einer direkten Modulation der Aktivität und/oder zu einer veränderten Assoziation mit Interaktionspartnern. Weiterhin können Tyrosinkinasen über alternative Modifikationen, wie Ubiquitinylierung oder Sumoylierung, reguliert werden die dann zur Degradation des Zielproteins führen können.

Die vergangenen Dekaden haben gezeigt, dass neben diesen klassischen Prinzipien alternative Mechanismen zur direkten Regulation von Tyrosinkinasen existieren. Eine wichtige Rolle nimmt dabei die Oxidation von Cysteinresten mittels reaktiver Sauerstoff- (ROS, von engl. *reactive oxygen species*) und Stickstoffspezies (RNS, von engl. *reactive nitrogen species*) ein. Über viele Jahre herrschte das Dogma, dass ROS und RNS lediglich Beiprodukte der Atmungskette darstellen und keine Rolle in der Signaltransduktion spielen. Erst die Entdeckung, dass Zellen nach bestimmten Stimuli spezifisch ROS und RNS produzieren stellte dieses Dogma in Frage. Dass diese sekundären Botenstoffe Einfluss auf viele Signaltransduktionsprozesse nehmen und auch zur direkten Regulation von Tyrosinkinasen beitragen, ist heutzutage ein akzeptierter Fakt. Allerdings fehlen bisher für viele Tyrosinkinasen detaillierte Untersuchungen zur Rolle von Cysteinoxidation in deren Regulation.

In der vorliegenden Arbeit wurde untersucht, ob die Tyrosinkinase Zap70 mittels Oxidation von Cysteinresten reguliert werden kann. Zap70 ist von essentieller Bedeutung für die Initiation der T-Zell-Rezeptor-Kaskade und damit für die Aktvierung von T-Zellen des Immunsystems. Im ersten Teil dieser Arbeit sollten Cysteinreste identifiziert werden, die von funktioneller Bedeutung für Zap70 sind. Dafür wurden Cysteine ausgewählt, die entweder in verwandten Tyrosinkinasen konserviert sind oder bereits als potentielles Ziel für Oxidation beschrieben wurden. Diese Cysteine wurden anschließend mit Alanin substituiert und die Fähigkeit der mutierten Kinasen das T-Zell-Rezeptor-Signal zu rekonstituierten untersucht. Hierfür wurden verschiedene Methoden der Stimulation analysiert, die Rezeptorsignale unterschiedlicher Stärke induzieren. Diese Untersuchungen zeigten, dass ein Großteil der untersuchten Cysteinreste keine funktionelle Bedeutung für Zap70 haben. Lediglich Mutation

der Cysteine an den Positionen 39, 564 und 575 führte zu einer veränderten Rekonstitution der T-Zell-Rezeptor-Kaskade. Dabei zeigte sich, dass Substitution von Cystein 564 mit Alanin, im Vergleich zum wildtypischen Zap70, zu einer verstärkten Rekonstitution des Rezeptorsignals führt. Im Gegensatz dazu führte die Mutation der Cysteine 39 und 575 zu einer Abschwächung des Signals im Vergleich zur wildtypischen Kinase.

Der zweite Teil dieser Arbeit umfasst eine detaillierte Analyse der Zap70C575A Mutante. Dabei konnte ich zeigen, dass C575 nicht nur essentiell für die Regulation der Aktivität, sondern auch der Stabilität von Zap70 ist. Mutation dieses Restes führt zu einer stark reduzieren Expression der Kinase. Mittels *pulse-chase* Untersuchungen sowie der Inhibition der Proteinsynthese konnte ich nachweisen, dass eine Reduktion der Stabilität des mutieren Proteins für die verringerte Expression verantwortlich ist. Dies führt zum Teil zu einer Degradation des Proteins über das Proteasom. Weiterhin konnte gezeigt werden, dass das Co-Chaperon Cdc37 an der Reduktion der Stabilität beteiligt ist. Eine der Hauptaufgaben dieses Chaperons ist die Bindung und Stabilisierung der Kinasedomäne von Tyrosinkinasen. Überexpression von Cdc37 führte zu einer teilweisen Rekonstitution der Proteinexpression, wohingegen der funktionelle Defekt komplett behoben werden konnte.

Die zentrale Rolle von C575 für die Regulation der Stabilität und Aktivität von Zap70 ließ eine Oxidation dieses Cysteins vermuten. Daher wurde unter Zuhilfenahme zweier biochemischer Methoden untersucht, ob C575 sulfenyliert oder nitrosyliert ist. Es konnte gezeigt werden, dass dieses Cystein konstitutiv sulfenyliert ist, wohingegen keine Nitrosylierung nachgewiesen werden konnte. Zusätzlich konnte dieses Cystein mittels Sequenzanalyse als Bestandteil eines $Mx_{(2)}CWx_{(6)}R$ Motifs identifiziert werden, welches in allen humanen Tyrosinkinasen hoch konserviert ist. Eine detaillierte Untersuchung zeigte, dass dieses Motif essentiell für die Oxidation von C575 ist und dass die Mutation aller Aminosäuren des Motifs die Expression von Zap70 herabsetzten.

Zusammenfassend konnte ich zeigen, dass Zap70 funktionell relevante Cysteinreste besitzt, welche die Aktivität der Kinase entweder positiv oder negativ regulieren. Eine wichtige Fragestellung für die Zukunft wird sein, ob diese Ergebnisse zur die Entwicklung neuer spezifischer Zap70 Inhibitoren beitragen, welche sich die Modifikation von Cysteinresten zu Nutze machen.

1. Introduction

1.1 Signaling in eukaryotic cells

Living in the 21st century means living in a society which during the last centuries has undergone major changes and reorganizations on different levels, like economy, politics, sociology, and science. Especially, advances in science have altered the view on ourselves as human beings and our environment. This means that knowledge which was acquired over centuries is taken for granted nowadays. For instance, before the 16th century it was believed that piles of straw could spontaneously turn into mice. That there is no such thing as spontaneous conversion of a non-living thing into a living organism, today parents already teach to their children. Despite the fact that this is considered basic knowledge today, also this finding required research which eventually inspired the framing of the so-called "characteristics of living things". These describe the requirements which must be met to be considered living. One of these conditions is the response to the environment. Even the smallest unit of a eukaryotic organism, the cell, can implement environmental insults and translate them into a cellular reaction by a complex network of signaling cascades. Of central importance for these processes are molecules which are able to transmit these extracellular signals into the cell and to initiate the appropriate responses. Many of these signaling cascades rely on the action of protein tyrosine kinases (PTKs) as these molecules can propagate an extracellular signal by phosphorylation of target molecules and thereby, for instance changing their activation status, localization, or molecular properties.

1.2 Protein Tyrosine Kinases

PTKs are integral parts of various signaling cascades giving rise to cellular responses like proliferation, differentiation, migration, or metabolic changes. A prominent example for the function of PTKs is the initiation of signaling in naïve T cells upon activation by antigens. Antigen recognition by the receptor expressed on T cells (T-cell receptor, TCR) triggers a signaling cascade which is initially coordinated by the PTKs lymphocyte-specific protein tyrosine kinase (Lck) and ζ-chain associated protein of 70 kDa (Zap70). Signaling initiated by Lck and Zap70 is further propagated to the nucleus which ultimately leads to cell activation, differentiation, and proliferation. In general, PTKs are classified into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). The main function of both groups is the transmission of a signal inside the cell by phosphorylation of tyrosine residues on their respective target proteins. To do so, PTKs require ATP which is hydrolyzed and the γ-phosphate is transferred to the hydroxyl-moiety of the tyrosine. RTKs and nRTKs share a common architecture of their catalytic domains. The kinase domain of PTKs exhibits a bi-lobed structure. The N-terminal lobe (N lobe) is characterized by a five-stranded antiparallel β-sheet (β1-β5) which is complemented by an α-helix (αC). In contrast, the larger C-terminal lobe (C lobe) mainly consists of seven α-helices (αD-αI) and additional four short β-strands (β6-β9) (**Figure 1.1**). In some kinases, this common structure is extended by additional helices. A polypeptide chain between β5 and αD connects the N and C lobes. ATP binding is achieved by the cleft between the two lobes and is coordinated mainly by residues of the N lobe. In particular, the loop between β1 and β2 is essential as this is known to be the nucleotide binding loop (also known as glycine-rich loop or p-loop). Mandatory for ATP-binding is also a salt bridge between a lysine residue in β3 and a glutamic acid in αC. The glutamic acid keeps the lysine in the correct orientation for binding to the α- and β-phosphate groups of ATP. Mutation of the lysine renders the kinase inactive and therefore K-to-R substitutions are widely used as kinase dead mutants. The transfer of the phosphate is catalyzed by residues in the catalytic loop which are placed in the C lobe comprising a conserved aspartic acid responsible for phosphate transfer and an asparagine which stabilizes the ATP-associated Mg^{2+}. The catalytic loop is complemented by the activation loop which provides an additional aspartic acid for the stabilization of Mg^{2+}. In most PTKs, the activation loop also regulates the accessibility of the substrate into the catalytic center and hence acts as a gatekeeper of the kinase activity. The function of the activation loop is regulated by phosphorylation of tyrosine residues within the loop which results in the activation of the kinase (Dennis, Bradshaw 2010).

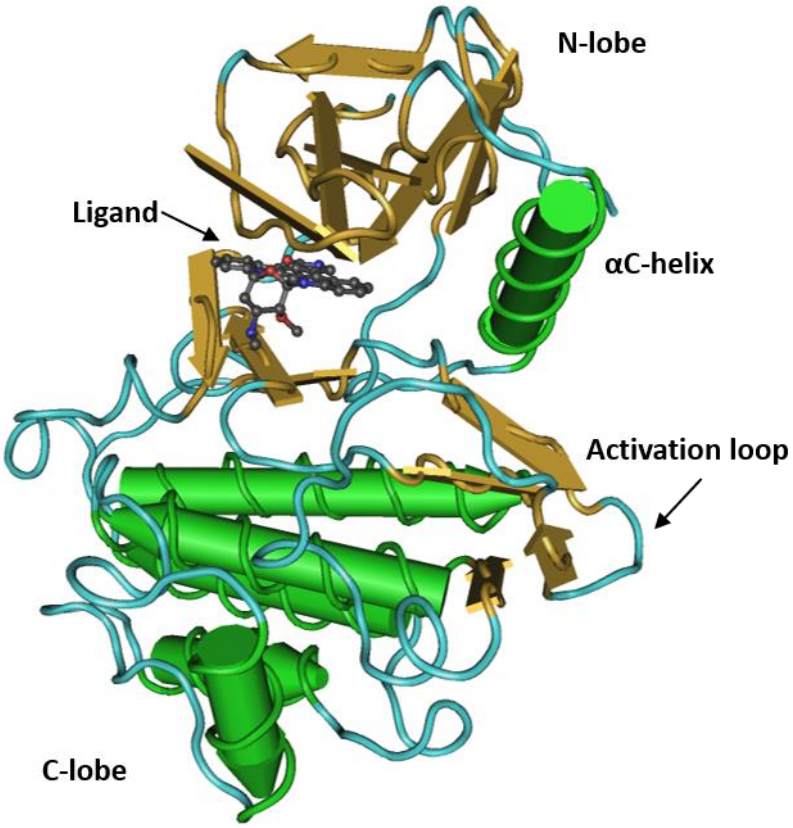


Figure 1.1: **Structure of the kinase domain of PTKs**. The most important structural features are indicated. Unstructured regions are depicted in cyan, α-helices in green, and β-sheets in yellow. The structure of the kinase domain of Zap70 is shown. PDB: 1U59

1.2.1 Receptor Tyrosine Kinases

As the name already suggests, RTKs are transmembrane receptors which are characterized by an extracellular part required for ligand binding, a transmembrane domain, and an intracellular part containing the catalytic domain. The human kinome comprises 58 genes encoding for RTKs which are divided into 20 subfamilies according to similarities in the structure of their extracellular and intracellular domains. The extracellular domains of RTKs are comprised of globular domains like immunoglobulin (Ig)-like domains, fibronectin type III–like domains, cysteine-rich domains, or EGF-like domains. The different RTK families share a general domain organization of their extracellular parts (**Figure 1.2**). Additionally, the RTK subfamilies share similarities in the amino acid sequences of their kinase domains.

RTKs regulate fundamental processes in the human organism. For instance, the members of the epidermal growth factor receptors (EGFRs) are crucial for the development of epithelial tissues. The receptors of the vascular endothelial cell growth factor (VEGFR) family are mainly expressed in endothelial cells and regulate angio-, vasculo-, and lymphangiogenesis. Misregulation in the expression or activation of these molecules leads to the development of many severe human malignancies (Li, Hristova 2006).

Activation of RTKs strongly depends on the binding of the respective ligand, which induces dimerization of the receptors leading to autophosphorylation in *trans* (hereafter referred as transphosphorylation). Targets for transphosphorylation are tyrosine residues inside and outside of the kinase domain. RTKs possess different modes for receptor dimerization. The ligand for the platelet-derived growth factor receptor (PDGFR) is *per se* a dimeric molecule which is connected by disulfide bonds. Therefore, binding of the ligand leads to the formation of a receptor complex consisting of two receptors and one ligand. In contrast, triggering of the ephrin receptor requires two ephrin molecules as these are monomers. For productive triggering, every receptor needs to bind two ligands and every ligand two receptors. Therefore, in the ligand-bound state, a receptor complex of two receptors and two ligands is formed. In both cases, transphosphorylation leads to full activation of the kinase which is required for the propagation of the signal to its downstream target molecules (Dennis, Bradshaw 2010).

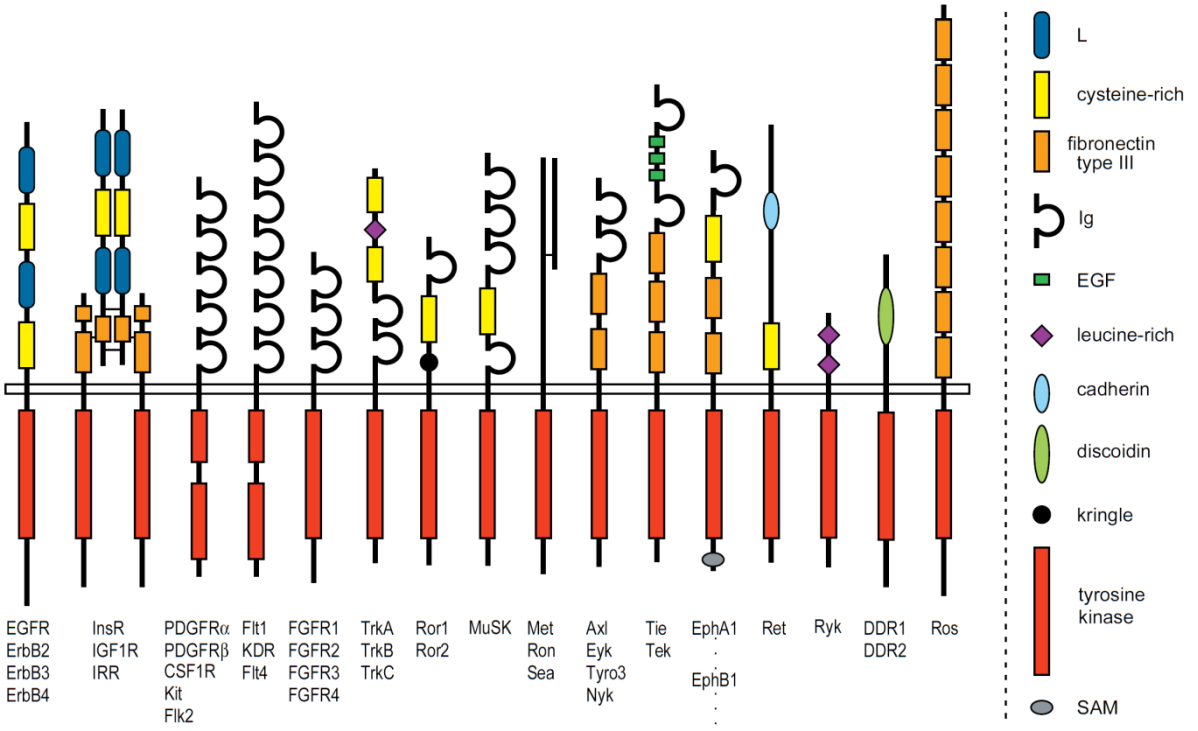


Figure 1.2: **Structure of a subset of RTK families.** The extracellular part (top) and the cytosolic domain (bottom) are represented. L: Leucine rich repeat; Ig: immunoglobulin-like domain; SAM: sterile alpha motif; EGF: EGF-like domain (Modified from Hubbard, Till 2000).

1.2.2 Non-receptor tyrosine kinases

Due to similarities in their domain organization also nRTKs are grouped into subfamilies. The 22 nRTK families share a number of modular domains such as SH3- (Src-homology 3) and SH2 (Src-homology 2)-domains, PH (pleckstrin homology)-domains or DNA-binding domains which are important for subcellular localization, interaction with other molecules, or may even have a function in the regulation of the enzymatic activity (**Figure 1.3**).

Similar to RTKs, also nRTKs are essential mediators of various signaling processes. Unlike RTKs their action is not limited to the cell membrane, as most of them are cytosolic molecules. Therefore, nRTKs are also involved in signal transmission in different intracellular compartments like the nucleus or the endoplasmic reticulum (Krauss 2014).

Due to a great structural variability, nRTKs do not share a mechanism of activation common for all members, like dimerization for RTKs. However, individual nRTK families exhibit conserved mechanisms for the regulation of their activity. Due to the great complexity of these mechanisms, only the regulation of Src-kinases, one of the most important families, will be discussed in detail hereafter. The members of the Src-family kinases (SFKs) are characterized by a similar domain organization consisting of a N-terminal myristylation and palmitoylation sequence, a unique region with low homology, an SH3-domain, an SH2-domain, a kinase domain, and a negative regulatory tail at the C-terminus. The members of the SFKs are mainly regulated by the phosphorylation of two conserved tyrosine resides. In the prototypic member of the SFKs, c-Src, these are Y527 and Y416. Phosphorylation of Y527 in the negative regulatory tail represses the kinase activity due to an intramolecular pTyrosine-SH2 interaction giving rise to a closed conformation. In the contrary, autophosphorylation of Y416 in the activation loop of the kinase domain renders the kinase active and prevents the closed conformation which ultimately leads to the phosphorylation of downstream targets and the propagation of signaling (Dennis, Bradshaw 2010; Hubbard, Till 2000).

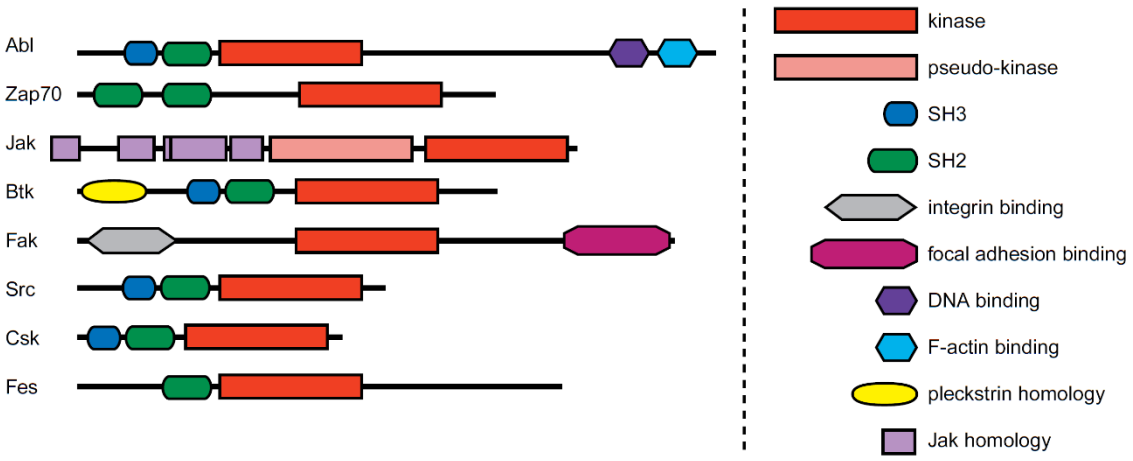


Figure 1.3: **Structure of the main families of the nRTKs.** Linear representation of the structural organization of the main families of the nRTKs from the N-terminus (left) to the C-terminus (right). Shown are Abl, Zap70 (Syk family kinases), Jak (Jak family kinases), Btk (Tec family kinases), Fak (Fak family kinases), Src (Src family kinases), Csk, and Fes (Fes family kinases) (Modified from Hubbard, Till 2000).

1.3 The tyrosine kinase Zap70

In 1992, the group of Art Weiss described a protein with tyrosine kinase activity which was associated with the ζ-chains of the TCR complex which was subsequently named ζ-chain associated protein of 70 kDa – Zap70 (Chan et al. 1992). This represents the first of many following studies which collectively have demonstrated an essential role of Zap70 in the initiation of TCR signaling. Shortly after its discovery, a Zap70-deficient Jurkat T-cell line has been established (P116) which serves as a cellular model for studying the function of Zap70 until today. Additionally, these cells are an impressive example for the importance of Zap70 in the initiation of TCR signaling, as these cells are not able to transmit a signal upon triggering of the TCR (Williams et al. 1998).

Zap70 is a cytosolic kinase which is mainly expressed in T cells and NK cells. Together with Syk, Zap70 constitutes the Syk family of tyrosine kinases. These kinases share a similar structural organization including two tandem SH2 domains at the N-terminus and the catalytic domain at the C-terminus (**Figure 1.4**). The two SH2 domains are separated by a short linker, the so-called InterdomainA. The tandem SH2-domain of Zap70 mediates the binding to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 and ζ-chains of the TCR. The more C-terminal SH2 domain is connected to the kinase domain by the InterdomainB, which harbors three major regulatory tyrosines (Y292, Y315, and Y319 in Zap70). In the C-terminal kinase domain two additional tyrosine residues (Y492 and Y493 in Zap70) are located which are essential for the regulation of the enzymatic activity (**Figure 1.4**).

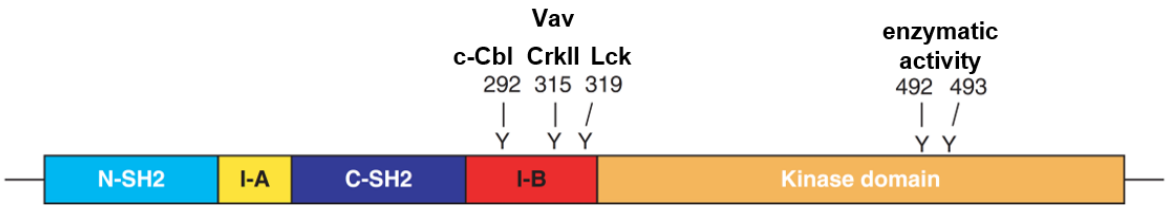


Figure 1.4: **Structural organization of Zap70.** The different domains are indicated by different colors. The position of the major regulatory tyrosines and the respective binding partners are indicated. N-SH2: N-terminal SH2-domain; I-A: InterdomainA; C-SH2: C-terminal SH2-domain; I-B: InterdomainB; c-Cbl: Casitas B-lineage Lymphoma; CrkII: CT10 regulator of kinase II; Lck: lymphocyte-specific protein tyrosine kinase (Modified from Wang et al. 2010).

1.3.1 Function of Zap70

1.3.1.1 T cells and TCR signaling

Every day the human organism is attacked by a plethora of different, potentially lethal, pathogens. T cells orchestrate the response to eliminate these pathogens and to protect the body (immune response). In order to accomplish this task, T cells require specific activation mechanisms to assure an appropriate response to infection. T cells express the TCR, a multimeric receptor complex which is specifically triggered by pathogens. Stimulation of the TCR is achieved by the presentation of antigens on the major histocompatibility (MHC) molecules expressed on antigen presenting cells, like dendritic cells or B cells. As for many other receptors, a consequence of TCR engagement is the translation of a mechanical signal (receptor/ligand interaction) into a biochemical (e.g. phosphorylation of downstream signaling molecules). However, in contrast to most other receptors, the TCR does not possess intrinsic kinase activity and is therefore not able to translate the signal. Hence, the initiation of the signal requires the participation of non-receptor tyrosine kinases. One of these is represented by the Lck, a member of the SFKs. Lck is associated with the co-receptors CD4 and CD8, which also bind to MHC molecules, and thereby recruit Lck to the TCR upon triggering. In turn, Lck phosphorylates the ITAMs at key tyrosines located within the $Yxx(L/I)x_{(6–8)}Yxx(L/I)$ consensus sequence (**Figure 1.5**). As a direct consequence of ITAM phosphorylation, Zap70 is recruited to the TCR. In resting T cells, Zap70 is located in the cytosol in an auto-inhibited conformation. Once the ITAMs of the TCR-ζ-chains are phosphorylated, Zap70 binds to them via the tandem SH2-domain. This binding process represents one of Zap70´s unique modes of regulation as this must meet certain requirements (see below). Binding to the TCR-ζ-chains releases Zap70 from its auto-inhibited conformation which renders the regulatory tyrosine residues in the InterdomainB accessible for phosphorylation by Lck. In turn, Lck phosphorylates the tyrosine residues, an event which further stabilizes the open conformation of Zap70. Upon binding of Lck to Zap70 (via Y319) and the phosphorylation of a tyrosine in the kinase domain of Zap70 (Y493), Zap70 acquires full kinase activity. Subsequently, Zap70 promotes the formation of a signaling hub essential for further signal diversification – the Lat (linker for activation of T cell) signalosome. Zap70 phosphorylates four key tyrosine residues of the transmembrane adaptor protein Lat. This leads to the assembly of a multi-protein complex involving, amongst others, SH2 domain-containing leukocyte protein of 76 kDa (Slp76), Vav-1, phospholipase Cγ1 (Plcγ1), and interleukin-2-inducible T cell kinase (Itk). Once SLP76 is bound to Lat, it is also phosphorylated by Zap70. Finally, these events lead to the branching of the TCR signal (I) by inducing the mobilization of Ca^{2+}-flux from intracellular stores and influx of extracellular

Ca^{2+} leading to nuclear factor of activated T cells (NFAT) activation, (II) by activation of the mitogen-activated protein kinase (MAPK) pathway leading to AP1 activation, and (III) by activation of protein kinase C (PKC)-signaling and nuclear factor-κB (NF-κB). Together, these pathways induce gene transcription giving rise to activation and differentiation of the T cell (**Figure 1.5**) (Brownlie, Zamoyska 2013).

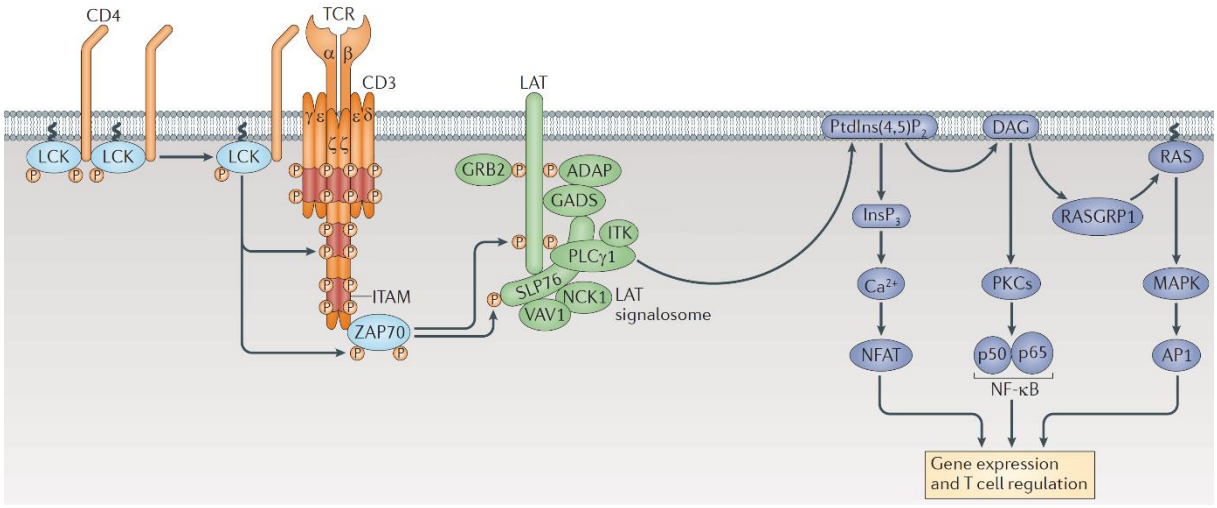


Figure 1.5: **Illustration of the TCR signaling cascade.** Upon triggering of the TCR by antigen/MHC-complexes, Lck is recruited to the TCR complex via its association with the co-receptor CD4. This leads to the activation of Lck and the phosphorylation of the ITAMs of the CD3- and the TCR-ζ-chains. In turn, Zap70 is recruited to the phosphorylated ITAMs via binding of its tandem SH2-domains. Subsequently, Zap70 is phosphorylated by Lck and becomes fully active. In the next step, Zap70 phosphorylates tyrosine residues on Lat. This is a mandatory event for the assembly to the Lat signalosome. During this process, SLP76, a second substrate of Zap70, is recruited to the membrane and becomes phosphorylated by Zap70. These events lead to the activation of Plcγ1 which hydrolyzes phosphatidylinositol-4,5-bisphosphate ($PtdIIns(4,5)P_2$) to diacylglycerole (DAG) and inositol(1,4,5)trisphosphate ($InsP_3$). $InsP_3$ leads to the mobilization of Ca^{2+}-flux and to the activation of the transcription factor NFAT. DAG activates two additional pathways: the PKC pathway activating NF-κB and the MAPK pathway leading to AP1 activation. Together, these pathways regulate gene expression in the activated T cell. GRB2: growth factor receptor-bound protein 2; GADS: GRB2-related adaptor protein; ADAP: adhesion- and degranulation-promoting adaptor protein; ITK: interleukin-2-inducible T cell kinase; RASGRP1: RAS guanyl-releasing protein 1; MAPK: mitogen-activated protein kinase; PKC: protein kinase C (modified from Brownlie, Zamoyska 2013)

1.3.1.2 T-cell development

Thymic development is a tightly regulated sequential process ensuring the generation of T cells expressing a functional TCR recognizing foreign antigens but not reacting with self-peptides. Early thymic progenitor cells are generated in the bone marrow and are known as common lymphoid progenitors (CLP). Once they enter the thymus, CLP undergo four different developmental stages characterized by the expression of CD4 and CD8 (**Figure 1.6**). Double negative (DN) thymocytes are characterized by the absence of CD4 and CD8 on their surface and represent the most immature stage. DN cells are further subdivided into DN1, DN2, DN3, and DN4. DN4 further mature in double positive (DP) thymocytes in which the co-receptors CD4 and CD8 are upregulated. Eventually, they become single positive (SP) for either CD4 or CD8 and are ready for leaving the thymus. The successful progression of an individual T cell through the different developmental stages is strongly dependent on the activity of the Syk-family kinases Zap70 and Syk. During these stages Zap70 and Syk are inversely expressed. Whereas Syk is highly expressed during DN1-3, Zap70 expression is upregulated at the DN4 stage. The expression of both kinases overlaps at the DN3 and DN4 stages (**Figure 1.6**) (Palacios, Weiss 2007). In the course of thymic development, immature T cells undergo two essential checkpoints. The so-called β-selection takes place at the DN3 stage. Here, the cells express the TCRβ-chain together with a surrogate α-chain, the pre-TCRα-chain, which allows the assembly of a complete pre-TCR. At this stage, the pre-TCR, when correctly assembled, induces a signal in a ligand-independent manner which drives maturation to the next developmental stage. Deficiency of either Zap70 or Syk alone does not affect the progression at this checkpoint due to redundant function of the kinases. However, a double-deficiency of both Zap70 and Syk causes a developmental block at the DN3 stage. At the DP stage, thymocytes express a fully assembled TCR and undergo a second checkpoint. Here, the ability of the newly generated TCRs to interact with MHC molecules loaded with self-peptides will be tested. DP cells expressing a TCR unable to interact with MHC molecules will be deleted (death by neglect). Similarly, also DP thymocytes expressing a TCR with high affinity for MHC/self-peptide complexes will be eliminated (negative selection), as they represent autoreactive cells. Finally, only those DP cells expressing TCR with moderate affinity for MHC/self-peptide complexes will mature, thus becoming either $CD4^+$ or $CD8^+$ T cells (**Figure 1.6**). Zap70-deficiency causes an arrest in T-cell development at the DP stage. In fact, mice lacking Zap70 expression do not possess $CD4^+$ or $CD8^+$ T cells, neither as SP cells in the thymus nor as peripheral T cells. Zap70 deficiency in humans leads to the complete absence of $CD8^+$ T cells and the production of signaling incompetent $CD4^+$ T cells (discussed below). In contrast, Syk-deficiency does not affect T-cell development at the DP stage which reflects the decreasing importance of Syk-

activity during the later stages of thymocyte development as at this stage Zap70 is the dominating kinase. These findings merge in the current view on the role of Syk-kinases in T-cell development in which both Syk and Zap70 are of major importance for pre-TCR signaling, whereas Zap70 is crucial for later developmental stages. A still unsolved mystery in T-cell development is why evolution has required Zap70 for the regulation of the maturation of DP thymocytes. So far, it has only been hypothesized that the switch from Syk to Zap70 at the DN4 to DP transition is due to the unique features of Zap70. For example, conversely to Syk, Zap70 can directly phosphorylate p38. Indeed, a study addressing the role of p38 signaling during the transition of the DN4 to the DP stage has shown that pharmacologic blockade of p38 resulted in the inhibition of DP thymocyte development. Additionally, it is well known that, compared to Syk, Zap70 exhibits a stronger dependency on upstream Src-kinases since it is unable to auto-phosphorylate (Shah et al. 2016). Furthermore, due to the special architecture of Zap70´s tandem-SH2 domain the avidity to the ITAMs is reduced in comparison to Syk. Both facts suggest a tighter control of the initiation of signaling by events upstream of Zap70 (due to weaker ITAM binding which requires more robust signaling for productive transmission of the signal and the necessity of Lck to activate Zap70) which reduces the likelihood of uncontrolled T cell activation (Wang et al. 2010; Au-Yeung et al. 2009).

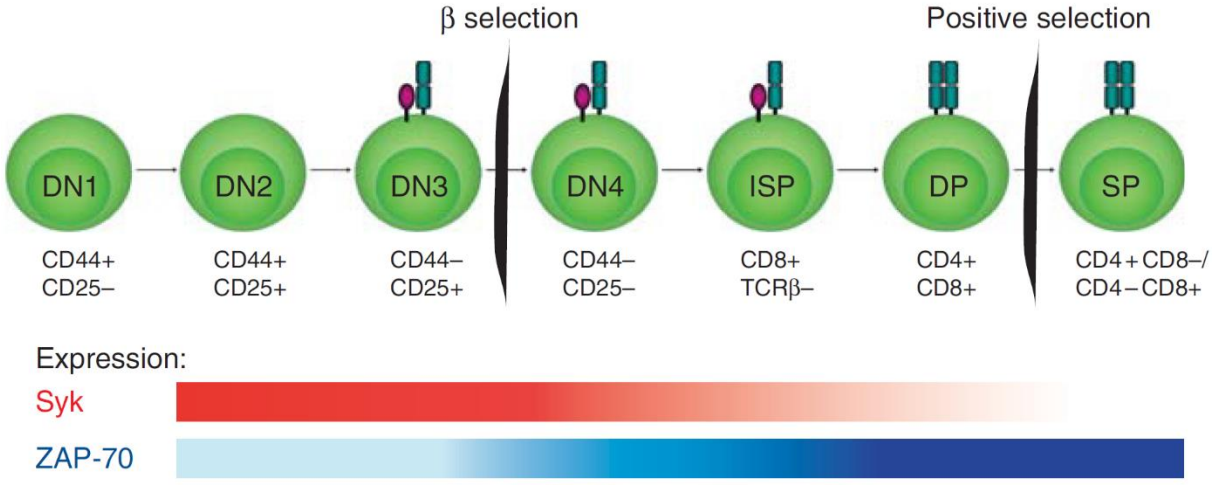


Figure 1.6: **The role of Syk-kinases during T-cell development.** T cells undergo different developmental stages characterized by the presence or absence of CD4 and CD8 (upper panel). DN (double-negative) cells do not express CD4 and CD8. According to the differential expression of the surface markers CD44 and CD25, DN thymocytes can be further subdivided in DN1, DN2, DN3, and DN4. At the DN3 stage, the pre-TCR is assembled and expressed. β-selection will ensure that only DN3 cells expressing a fully rearranged β-chain will further mature to the DN4 stage. DN4 cells develop into double positive (DP) thymocytes which will undergo additional selection processes (i.e. death-by-neglect, positive and negative selection). Finally, positively selected DP cells will mature into single positive (SP) thymocytes. Syk expression declines during thymic development, whereas Zap70 expression increases (lower panel) (modified from Wang et al. 2010).

1.3.2 Zap70 in diseases

In addition to its physiological role in T-cell development and activation, alterations in Zap70 expression and function are also at the basis of a number of pathological conditions ranging from immunodeficiency, to cancer, and to autoimmunity, which are usually fatal. Nevertheless, Zap70-related immune-pathologies are rare, probably because mutations affecting Zap70 are recessive.

1.3.2.1 Immunodeficiency

Loss of Zap70 expression or activity is the cause of one of the most severe forms of immunodeficiency in humans (severe combined immunodeficiency, SCID). To date, the only available treatment for this disease is an allogenic stem cell transplantation to reconstitute the immune system of the patient (Fagioli et al. 2003). The first description of a Zap70-related immunodeficiency dates to 1994, when a Zap70 mutation in a Mennonite kindred was described. To date, a number of Zap70 mutations leading to immunodeficiency have been described (**Figure 1.7** and **Table 1.1**) (Shirkani et al. 2017). Zap70-deficiency is listed as a rare disease affecting 1 in 2000 people according to the Orphanet consortium (www.orpha.net). The onset of this disease is usually within the first months of life. These children suffer from recurrent viral, bacterial, and opportunistic infections, diarrhea, and failure to thrive. Loss of Zap70 function largely affects the development and functionality of T cells in these patients. They possess normal numbers of peripheral blood $CD3^+/CD4^+$ T cells but lack $CD8^+$ T cells (Hauck et al. 2015; Arpaia et al. 1994; Noraz et al. 2000). Despite the fact that $CD4^+$ T cells are present in normal numbers, they are functionally defective. In fact, they do not proliferate in response to stimulation with CD3-antibodies or mitogens (Roifman et al. 2010; Roifman et al. 2012). Conversely, stimulation with PMA/Ionomycin results in proliferation, thus indicating that Zap70-deficient T cells display a defect at the TCR proximal level (Elder et al. 1994; Elder 1997).

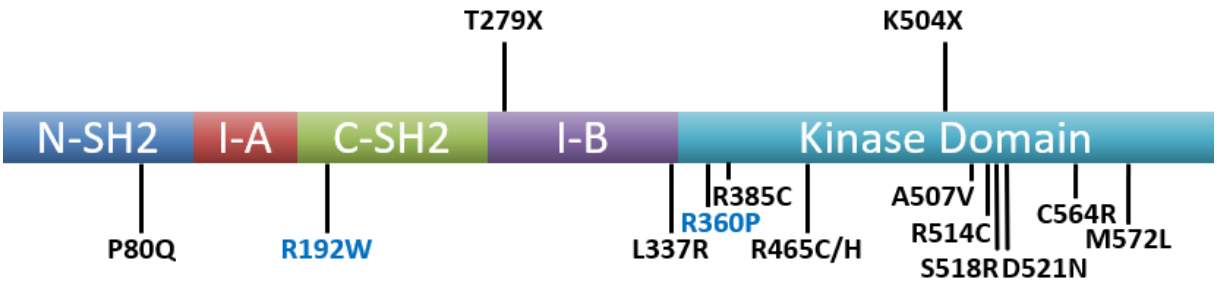


Figure 1.7: **Schematic representation of the location of mutations in Zap70 resulting in severe combined immunodeficiency or autoimmunity.** Structure of Zap70 and position of known mutations causing immunodeficiency (black) or autoimmunity (blue). The X indicates mutations leading to a premature stop codon. N-SH2: N-terminal SH2-domain; I-A: InterdomainA; C-SH2: C-terminal SH2-domain; I-B: InterdomainB (modified from Shirkani et al. 2017; Gavino et al. 2017)

Table 1.1: **Zap70 mutations and their effect on Zap70 function.** (Shirkani et al. 2017; Gavino et al. 2017)

Mutation	Effect on Zap70	Disease
P80Q/M572L	Temperature sensitivity	immunodeficiency
R192W/R360P	Reduced ITAM binding/hyperactivation	autoimmunity
T279X	Introduction of a premature stop codon	immunodeficiency
L337R	Absence of protein expression	immunodeficiency
R385C	Absence of protein expression	immunodeficiency
R465C/H	Impaired kinase activity	immunodeficiency
K504X	Introduction of a premature stop codon	immunodeficiency
A507V	Absence of protein expression	immunodeficiency
R514C	Conformational change in the kinase domain	immunodeficiency
S518R/K541-K542insLEQ	Absence of protein expression	immunodeficiency
D521N	Predicted impaired protein function	immunodeficiency
C564R	Absence of protein expression	immunodeficiency

1.3.2.2 Autoimmunity

As described above**,** Zap70 plays an important role in thymic development and alterations in Zap70 expression or function results in immunodeficiency. However, mutations in Zap70 can also be associated with the development of autoimmunity. In 2003 Sakaguchi and colleagues described a mouse model for rheumatoid arthritis, the SKG mice (Hata et al. 2004). T cells from these mice cause spontaneous inflammatory arthritis even when they are adoptively transferred into healthy donor mice. This disease is characterized by T-cell infiltration in the synovia, hypergammaglobulinemia, anti-self antibodies, and high titers of rheumatoid factor (Sakaguchi et al. 2006). Molecular analyses aimed at the identification of genetic defects responsible for the induction of the disease revealed that Zap70 is mutated in SGK mice. Mutated Zap70 carries a W163C substitution in the C-terminal SH2-domain, which precludes the binding of the kinase to the ζ-chains of the TCR/CD3-complex. As a direct consequence of this defect, TCR signaling is impaired in these mice leading to reduced mobilization of Ca^{2+} and phosphorylation of Lat upon TCR stimulation (Sakaguchi et al. 2003). Therefore, thymic selection in these mice is altered because of reduced TCR signaling. Consequently, negative selection is likely converted to positive selection, thus leading to the maturation of auto-reactive T cells giving rise to autoimmunity.

Zap70-associated autoimmunity in humans is extremely rare. So far, only one case has been reported in which Zap70 caused inappropriate T-cell activation giving rise to early onset of bullous pemphigoid in two siblings. These children carried two independent mutations in Zap70 each affecting its function. The first mutation (R192W) decreased the binding of Zap70 to the ζ-chain, similarly to SKG mice (**Figure 1.7** and **Table 1.1**). The second mutation (R360P) is located in the N-terminal end of the kinase domain, a region which is known to be critical to stabilize Zap70 in its inactive conformation. Likely, this mutation releases Zap70 from its autoinhibitory conformation which renders the kinase more active (Chan et al. 2016). Finally, it is believed that both mutations in conjunction caused a defect in negative selection leading to the maturation of auto-reactive T cells. However, a detailed analysis of the impact of the described mutations on thymic selection is not available so far. Therefore, the exact nature of the effect of this mutation on thymocyte development is unknown.

1.3.2.3 Leukemia

In healthy humans, the expression of Zap70 is restricted to mature T cells, thymocytes, and NK cells. However, Zap70 expression is induced in B cells from some patients suffering from B-cell chronic lymphocytic leukemia (B-CLL) (Rosenwald et al. 2001). B-CLL itself is characterized by its clinical heterogeneity with patients exhibiting a stable and non-progressing disease, whereas in others the disease progresses rapidly with a fatal outcome (Del Principe et al. 2006). Expression of Zap70 is associated with a poor prognosis and is therefore found in the latter group. Thus, during the past years, Zap70 expression has become an accurate marker to predict disease outcome in B-CLL patients (Orchard et al. 2004). The molecular mechanism leading to increased malignancy of B cells in B-CLL patients with Zap70 expression is still under debate. Currently, two major hypotheses have been proposed. It has been shown that Zap70 expression enhances B-cell receptor (BCR) signaling in the leukemic B-cells which is believed to be due to the enzymatic activity of Zap70 (Chen et al. 2002). However, this idea is contradicted by the fact that enhanced BCR signaling in B-CLL is independent of Zap70´s catalytic activity. Indeed, a kinase dead mutant of Zap70 also induces elevated Ca^{2+}-flux upon BCR stimulation. Therefore, it is more likely that Zap70 acts as an adaptor protein or associates with regulators of the closely related kinase Syk. Hence, Zap70 would induce more efficient BCR-signaling by reducing the activation threshold causing the onset of the disease (Fischer et al. 2010).

1.3.3 Regulation of Zap70

The transition from the inactive to the active form of Zap70 is tightly regulated in a sequential manner. Since its discovery in 1992, the regulation of Zap70 has been intensively studied and many mechanistic details of its regulation have been discovered. For example, Zap70 is a cytosolic kinase and in order to phosphorylate its substrates (e.g. the transmembrane adaptor protein Lat) it has to be recruited to the plasma membrane. Additionally, we have learned that, in order to be fully activated, key tyrosine residues need to be phosphorylated. Finally, also termination of Zap70´s activity has been dissected in detail and it is now clear that this is associated with other post-translational modifications like ubiquitination. In the following paragraph, I will present the molecular mechanisms involved in the regulation of the activity of Zap70.

1.3.3.1 Membrane recruitment

The first important event at the basis of Zap70 activation is the recruitment to the plasma membrane. The ITAMs of the TCR-ζ-chains of the TCR/CD3 complex are key players in the recruitment of Zap70. ITAMs are conserved motifs in the cytoplasmic tails of the TCR-ζ-chains which share the Yxx(L/I)$x_{(6–8)}$Yxx(L/I) consensus sequence. Upon phosphorylation, the key tyrosines within the ITAMs act as docking sites for the tandem SH2-domain of Zap70. Pioneering work in the understanding how this docking is achieved was done by Hatada and colleagues in 1995, when they solved the structure of the ITAM-bound tandem SH2-domain of Zap70 (Hatada et al. 1995). This work has shown that, once bound to the phosphorylated ITAMs, the SH2-domains together with the InterdomainA build up a Y-shaped structure (**Figure 1.8A**). The InterdomainA is arranged in a coiled-coil creating a more rigid structure, which keeps the SH2-domains aligned and in close contact. The close proximity of the SH2-domains is necessary because the phospho-tyrosine binding pocket of the N-terminal SH2-domain is incomplete and is completed by the C-terminal SH2 domain (**Figure 1.8B**). This sophisticated mode of regulation is likely needed to reduce the probability of random ITAM-Zap70 interactions which could lead to unwanted signaling. Binding to the ITAMs induces a second major structural rearrangement facilitating Zap70 activation. This promotes the disassembly of the autoinhibited conformation, thus leading to the exposure of tyrosine residues within the InterdomainB which are now accessible for phosphorylation (**Figure 1.8B**) (Wang et al. 2010).

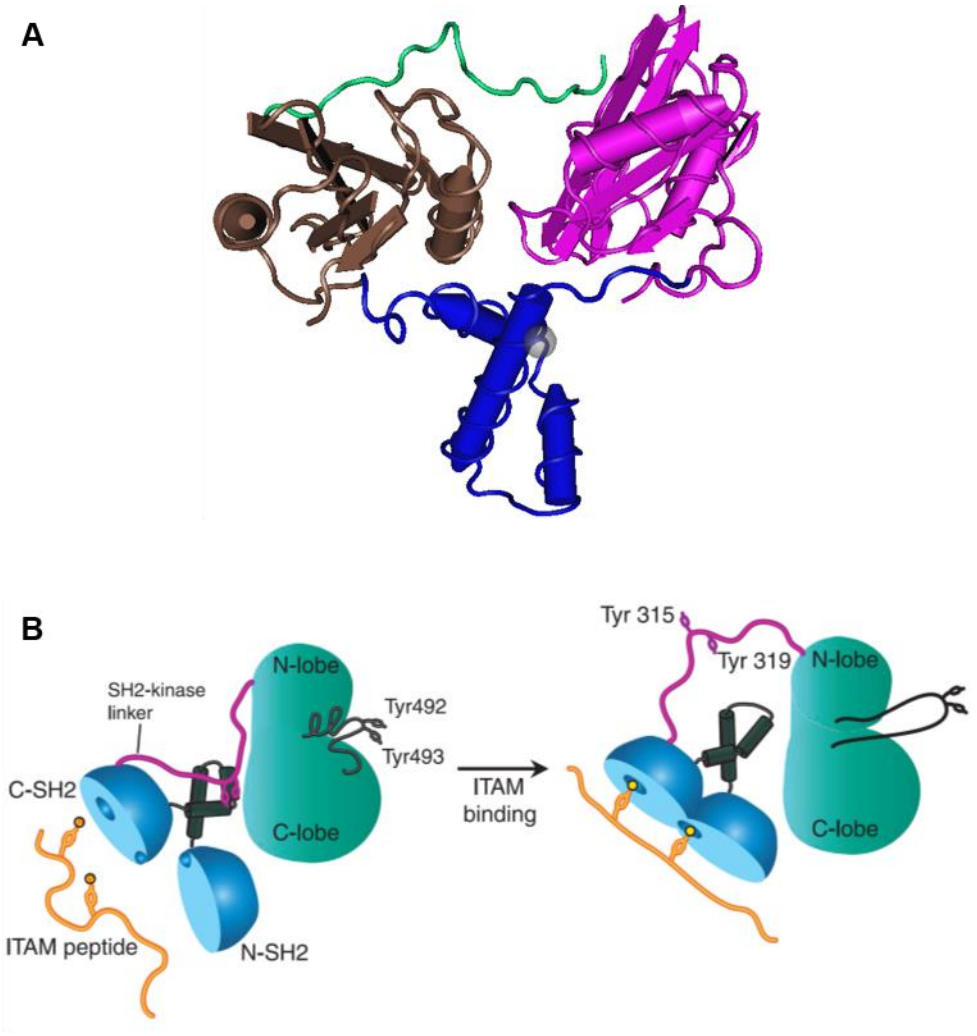


Figure 1.8: **ITAM-binding of Zap70.**

(A) Ribbon diagram of the Zap70 tandem SH2-domain bound to the ITAM. Brown: N-terminal SH2-domain, Magenta: C-terminal SH2-domain, Blue: InterdomainA, Green: ITAM peptide PDB ID 2OQ1

(B) Schematic representation of the binding of the Zap70 SH2-domains to the phosphorylated ITAM peptide (modified from Au-Yeung et al. 2009).

1.3.3.2 Tyrosine phosphorylation

Once Zap70 is stably bound to the TCR, it is phosphorylated on 5 key residues within the InterdomainB and the kinase domain (**Figure 1.4**). Three of these residues (Y315, Y319, and Y493) act as positive regulators, whereas two (Y292 and Y492) inhibit Zap70 function.

Y292 is located within the InterdomainB and is phosphorylated by Lck upon TCR stimulation. Early studies using the non-phosphorylatable Y292F mutant identified this residue as a negative regulator of Zap70 function. Reconstitution of a Syk-deficient B-cell line with Zap70Y292F resulted in enhanced activation of the transcription factor NFAT upon BCR stimulation compared to Zap70wt (Kong et al. 1996; Zhao, Weiss 1996). This was further supported by the analysis of a knock-in mouse carrying the Y292F mutation. These mice exhibit hyperphosphorylation of the Zap70 substrates Lat and SLP76 (Magnan et al. 2001). It is believed that the phosphorylated Y292 represents a binding site for the E3 ubiquitin ligase c-Cbl, which in turn ubiquitinates Zap70 itself and the TCR to dampen signaling (Lupher et al. 1997; Wang et al. 2001). In line with these data are studies showing impaired internalization of the TCR in T cells expressing Zap70Y292F (Magnan et al. 2001). However, the idea that the major function of Y292 in Zap70 is the binding of c-Cbl is contradicted by the finding that the effect of c-Cbl on TCR signaling is independent of its tyrosine-kinase binding domain as expression of a binding-deficient c-Cbl mutant (which is unable to bind Y292 in Zap70) does not resemble the effect of the Y292F mutation (Au-Yeung et al. 2009).

Y315 is also located in the InterdomainB and is also phosphorylated by Lck upon TCR stimulation. This residue has two major roles in the regulation of Zap70. In the autoinhibited conformation, Y315 faces a hydrophobic region of the InterdomainA which stabilizes this conformation (**Figure 1.9**). Phosphorylation of Y315 generates a negative charge and therefore disturbs the hydrophobic interactions preventing the autoinhibited conformation and stabilizing the open conformation (Yan et al. 2013). Upon phosphorylation, Y315 additionally serves as a binding site for several interaction partners. One of the main binding partners of Y315 is Vav, a GEF (guanine exchange factor) for Rho guanosine triphosphatases (GTPases). It has been shown that TCR stimulation triggers the formation of a complex involving Zap70, Vav, Grb2 (growth factor receptor-bound protein 2), and Sos (son of sevenless) which is required for the efficient phosphorylation and activation of Vav. These processes are essential for the activation of the GTPases Ras and Rac (Salojin et al. 2000). Interestingly, in murine T cells, Zap70Y315F has been shown to have a decreased binding affinity for the ζ-chains, which results in an impaired TCR signaling (Di Bartolo et al. 1999). In addition to Vav, also the adaptor molecule CrkII is thought to be a binding partner of pY315 (Gelkop et al. 2005). Upon TCR stimulation, CrkII binds to Zap70, thus promoting the

association of Zap70 with the CrkL-WIP-WASP complex. Therefore, CrkII is required to localize the actin polymerization complex at immunological synapse (Wang et al. 2010).

Y319, the last regulatory tyrosine residue within the InterdomainB, has also a bifunctional role in the regulation of Zap70. Similarly to Y315, upon phosphorylation by Lck, Y319 stabilizes the open conformation of Zap70. However, unlike Y315, in the autoinhibitory conformation this residue interacts with residues of the kinase domain. Y319 participates in a complex hydrogen-bond network keeping the kinase domain in an inactive conformation (**Figure 1.9**). Introduction of a negative charge by phosphorylation releases the kinase domain from the InterdomainB further stabilizing the active conformation of Zap70 (Yan et al. 2013). Additionally, several studies showed that Y319 serves as a binding partner for Lck and PLCγ1. Expression of the Zap70Y319F mutant in TCR-stimulated Jurkat T cells revealed a dominant-negative effect on the activation of NFAT and IL2 production. This was attributed to a defective induction of the kinase activity of the Zap70 mutant which was associated with a reduced phosphorylation of the Zap70 substrates Lat and SLP76 (Di Bartolo et al. 1999). These defects were thought to be caused by the loss of association between Zap70Y319F mutant and Lck (Williams et al. 1999). In line with these observations, a Y319F mutant mice displayed defective mobilization of Ca^{2+} upon TCR stimulation and defective thymocyte development (Gong et al. 2001).

The kinase domain bears two additional tyrosine residues which are critical for Zap70 regulation. Y492 and Y493 are located within the activation loop of the kinase domain, a region which is crucial for the function of tyrosine kinases (Chan et al. 1995). It is believed that these residues become phosphorylated upon TCR stimulation by Lck and/or Zap70 itself (Kong et al. 1996; Watts et al. 1994; Chan et al. 1994). However, a recent study proposed phosphorylation exclusively by Lck as the architecture of the kinase domain of Zap70 does not allow autophosphorylation due to electrostatic repulsion (Shah et al. 2016). Both residues have opposing roles in the regulation of Zap70. Y493 is a positive regulator and phosphorylation is required for full kinase activity, whereas Y492 negatively influences the activation of Zap70. This is demonstrated by the respective Y-to-F substitutions. Zap70Y493F exhibits a decreased and Zap70Y492F and increased kinase activity in comparison to the wt molecule (Chan et al. 1995). Phosphorylation of Y492 seems not to be required for protein function as expression of Zap70Y492F in Syk-deficient DT40 cells restored signaling via the BCR. However, an exact molecular mechanism how these residues regulate Zap70 function is still missing (Au-Yeung et al. 2009).

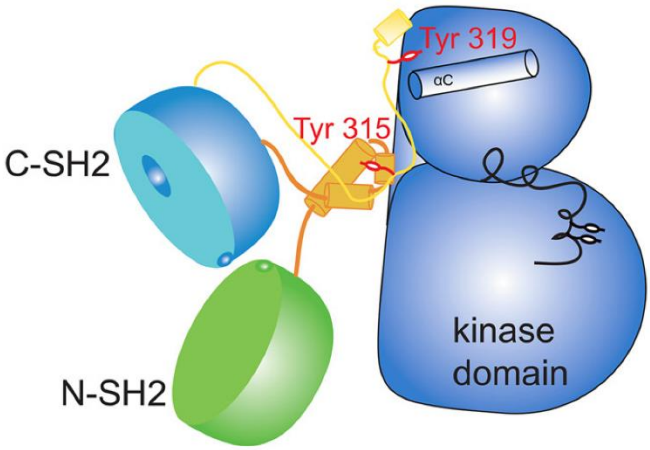


Figure 1.9: **Autoinhibited conformation of Zap70.** In the autoinhibited conformation, the InterdomainA (orange) interacts with the InterdomainB (yellow) via hydrophobic interactions involving Y315. Furthermore, the autoinhibited conformation is stabilized by hydrogen-bonds between Y319 in the InterdomainB and the αC-helix of the kinase domain (blue) (Yan et al. 2013).

1.3.3.3 Ubiquitination

In addition to tyrosine phosphorylation, Zap70 activity can also be regulated by other post-translational modifications such as ubiquitination (Wang et al. 2008; Poltorak et al. 2013). In the last years, several studies have identified a number of enzymes involved in the ubiquitination of Zap70. These studies revealed the existence of a network of ubiquitinating and de-ubiquitinating enzymes which orchestrate the regulation of Zap70 expression and activity. As mentioned above, c-Cbl is a major E3 ubiquitin ligase involved in the regulation of Zap70 degradation. In addition, also other E3 ligases have been identified to regulate Zap70 activity. For instance, it has recently been described that Nrdp1 mediates the K33-linked polyubiquitination of K578 in Zap70. In turn, this favors the interaction of Zap70 with the tyrosine phosphatases Sts1/2 which have been shown to dephosphorylate Zap70 in a ubiquitination-dependent manner. However, this seems to hold true only for $CD8^+$ T cells as $CD4^+$ T cells only express low levels of Nrdp1 (Yang et al. 2015).

The action of E3 ubiquitin ligases is counteracted by de-ubiquitinating enzymes which are also key molecules in the regulation of Zap70. A defective function of these enzymes can lead to Zap70 impairment as shown for Usp9X and Otud7b. Knock-out of the de-ubiquitinating enzymes attenuated TCR signaling in primary T cells. These effects were shown to be due to excessive Zap70 ubiquitination and therefore degradation or inactivation (Naik, Dixit 2016; Hu et al. 2016). The Otud7b-ko revealed that ubiquitination does not only regulate Zap70 degradation as de-ubiquitination by Otud7b prevents association of Sts1/2 to Zap70 which prevents Zap70 dephosphorylation hence keeping the kinase active.

The role of ubiquitination for the regulation of Zap70 has also been addressed in a theoretical manner. Molecular dynamics simulations suggested two key lysine residues in the kinase domain of Zap70 which could contribute to the modulation of Zap70 activity. The residues are in close proximity to the αC-helix and when ubiquitinated could disrupt (K377) or stabilize (K476) the active conformation (Ball et al. 2016). However, the role of these modifications *in vivo* needs to be proven.

1.3.3.4 Oxidation

In the past years, our view on reactive oxygen species (ROS) as simple by-products of cellular metabolism has changed. In fact, it has been shown that ROS can act as signaling mediators by modulating the activity of different enzymes. As regarding Zap70, a work published in 1996 by Stefanová and colleagues suggested that oxidation may regulate Zap70 structure (Stefanová et al. 1996). In this work, the authors showed that Zap70 expression was not detectable by specific antibodies in western blot experiments using lysates from T cells of symptomatic HIV-patients. However, treatment with DTT restored the binding of the antibodies, thus indicating structural changes in Zap70 which are mediated by oxidation. Shortly after this work, another publication described that Zap70 reacts with S-reactive isothiazolone compounds. This observation suggests the presence of reactive cysteine residues in Zap70 which are very likely susceptible to oxidation (Trevillyan et al. 1999). After this report, 15 years passed by without major progression in the field of Zap70 oxidation. In 2013, Zap70 oxidation became of interest again when the existence of particular cysteine residues in the SH2-domain of Zap70 and Syk were proposed to modulate the binding to the ITAMs. Furthermore, it was shown for the first time that binding of Zap70 to the ITAMs is dependent on the redox-milieu and that excessive ROS production could reduce the binding affinity of Zap70 to the ITAMs (Visperas et al. 2015).

1.4 Regulation of tyrosine kinases by oxidation

The complex signaling processes that occur in eukaryotic cells require highly sophisticated ways of regulation. The past 25 years of research have revealed the existence of novel unexpected mechanisms that, similarly to the “classical” post-translational modifications like phosphorylation, are involved in the regulation of signaling processes. The role of oxidation and its mediators ROS and RNS (reactive nitrogen species) in cellular signaling processes has been neglected for years as these have been thought to be mere by-products of metabolism. However, a growing body of evidence suggests that oxidation can modulate the activity of signaling molecules in a local and highly specific way. In particular, the redox-mediated regulation of kinases represents one of the faster growing research areas, since the oxidation of cysteine residues in these proteins has emerged as a specific mechanism to regulate their activity and therefore cysteine oxidation has the potential to become a new target for the development of kinase specific therapeutics (see below). Indeed, to date a number of disease-relevant kinases, including c-Src, EGFR, or c-Abl have been identified to be regulated via oxidation (for a review see Corcoran, Cotter 2013).

Regulation of tyrosine kinases by oxidation is mainly based on reversible modifications of cysteine residues. The thiol groups of these amino acids can undergo reactions with ROS or RNS. The exact nature of these modifications depends on the nature of the oxidant (ROS or RNS) and on the modified protein itself (see below). In the following paragraph, I will shortly introduce the different signaling relevant modifications induced by ROS or RNS.

The most abundant and stable form of ROS in the cell is hydrogen peroxide (H_2O_2). Among other forms of ROS, H_2O_2 is especially suited for signal transduction as its uncharged nature allows it to freely diffuse across membranes. Furthermore, studies suggest that the transport of H_2O_2 across the plasma membrane is additionally facilitated by the help of aquaporins (Miller et al. 2010). Reaction of H_2O_2 with cysteines initially leads to the formation of sulfenic acids (SOH) (**Figure 1.10**). This oxidation state is especially interesting for signaling purposes as it represents a modification which can easily be reverted to the thiol or form disulfide bonds. Alternatively, dependent on the protein environment sulfenylated cysteines can be stabilized or undergo other modifications like formation of a sulfonamide or S-glutathionylation. However, under conditions of excessive ROS production sulfenic acids can further be oxidized to sulfinic and sulfonic acids which are irreversible and usually a sign of oxidative stress and cell damage (**Figure 1.10**) (Truong, Carroll 2013).

Reaction of cysteines with RNS, like NO, leads to the formation of nitrosothiols. Additionally, nitrosylation of cysteine residues can be achieved in a process called trans-nitrosylation in which the modification is typically transferred from a low molecular weight nitrosothiol (e.g.

Nitrosoglutathione) to a cysteine residue in the target protein (Monteiro et al. 2015). Similar to sulfenylation, S-nitrosylation is a reversible process and hence it has the potential to be a mechanism for the regulation of signaling processes. These modifications are well known to e.g. facilitate protein-protein interactions or to modulate the activity of certain target proteins and are therefore of major importance for cellular signaling (Hess et al. 2005). Therefore, both S-nitrosylation and sulfenylation can be considered as the most relevant oxidative post-translational modifications in the context of the regulation of kinases.

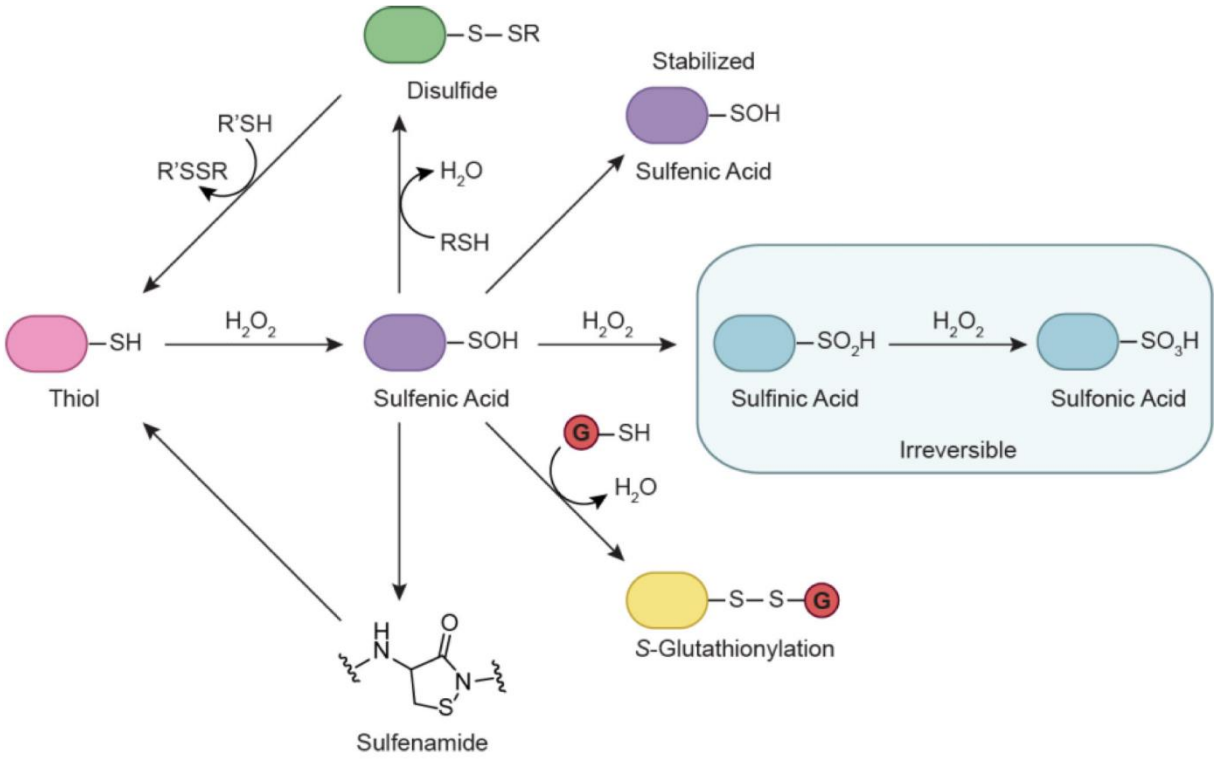


Figure 1.10: **Posttranslational modifications of cysteine residues upon reaction with H_2O_2.** Reaction of a cysteine with H_2O_2 leads to the formation of a sulfenic acid (SOH). The SOH can, depending on the protein-microenvironment, undergo different reactions: reduction to the thiol state; Disulfide bond formation with a free thiol, followed by reduction to the thiol; S-Glutathionylation; sulfenamide formation; stabilization of the SOH; irreversible oxidation by H_2O_2 to sulfinic or sulfonic acids. (modified from Truong, Carroll 2013)

1.4.1 Cellular sources of ROS and RNS

For signaling purposes, the production of ROS and RNS needs to be organized in a tempo-spatial manner to assure specific targeting of the respective substrates. Upon stimulation, cells can produce ROS and RNS by different sources, like organelles or enzymes (**Figure 1.11**).

As already stated before, H_2O_2 is the predominant form of ROS in the cellular context. H_2O_2 production is achieved from various sources. Amongst them, the NOX (NADPH oxidase)-enzymes are one of the most important. These enzyme complexes can produce H_2O_2 via unstable intermediates. NOX are multimeric flavin and haem-containing protein complexes which transfer electrons from cytosolic nicotinamide adenine dinucleotide phosphate (NADPH) across the cell membrane to reduce molecular oxygen. Product of this reaction is the highly reactive superoxide anion ($O_2^{\cdot-}$) which dismutates to produce H_2O_2 (**Figure 1.11**). The prototypical member of the NOX-enzymes (NOX2) was initially described in phagocytes, like neutrophils, where it is, together with other proteins, responsible for the respiratory burst to kill invading pathogens (Hampton et al. 1998). For a long time, expression of these enzymes was thought to be limited to phagocytes but the discovery of other members of the family (NOX1–5 and Duox1–2) in non-phagocytic cells suggested that they may have additional functions. Nowadays, it is known that in other cells and tissues NOX-dependent ROS production is increased upon receptor stimulation induced by different ligands, including TNF, PDGF, EGF, and angiotensin II (Holmström, Finkel 2014).

The second major source of signaling-competent ROS are the mitochondria (**Figure 1.11**). The ROS generated in these organelles is a by-product of the electron transport chain which is used to catalyze the reduction of molecular oxygen to water. ROS is formed when electrons accidentally leak and incompletely reduce molecular oxygen to $O_2^{\cdot-}$ (St-Pierre et al. 2002). This finding initially created the dogma that ROS production by mitochondria is an unavoidable consequence of the aerobic lifestyle. However, the identification of factors initiating controlled mitochondrial ROS production (e.g. $p66^{Shc}$) contradicted this theory (Giorgio et al. 2005). The high reactivity and instability of the superoxide generated by mitochondria makes it dangerous for the cell and limits its function as redox second messenger. Therefore, it is enzymatically converted to H_2O_2 by superoxide dismutases (SODs) directly in the mitochondria or the cytosol, thus contributing to the cellular pool of H_2O_2 (Simeoni et al. 2016) (**Figure 1.11**).

Besides NOX enzymes and mitochondria, also other enzymes, like xanthine oxidases, cyclooxygenases, cytochrome P-450 enzymes, and lipoxygenases or organelles, like peroxisomes or the endoplasmic reticulum (ER) are well known to generate ROS.

Traditionally, ROS derived from these sources is thought to produce highly reactive oxidants by fenton chemistry, like $OH^{\cdot}$, which are not suitable for signaling due to their high reactivity. However, this view changes nowadays as it is evident that also these sources can take part in signaling, like the regulation of corticosteroid production within the adrenal cortex by H_2O_2 produced from adrenal cytochrome P-450 enzymes (Holmström, Finkel 2014) (**Figure 1.11**).

To prevent excessive ROS production, cells possess antioxidant systems and enzymes which are able to specifically detoxify ROS or to reverse oxidation on target proteins. H_2O_2 is especially targeted by the thioredoxin (TRX) and the glutathione (GSH) systems. These cellular redox-buffer systems utilize NADPH to directly reduce H_2O_2 or its oxidation products on cysteines. Additionally, cells express catalase, an enzyme which is able to reduce H_2O_2 to O_2 and H_2O (Simeoni et al. 2016) (**Figure 1.11**).

Sources of RNS are mainly the so-called nitric oxide synthases (NOS). These enzymes catalyze the production of NO from L-arginine. In mammals, three isoforms of the NOS enzymes are expressed – nNOS, iNOS, and eNOS. nNOS (neuronal NOS) is an isoform which is mainly expressed in nervous tissue where it is used for cell-cell communication. The immune system expresses iNOS (inducible NOS) upon stimulation with pro-inflammatory cytokines which takes part in the defense against pathogens. eNOS (endothelial NOS) is found in the endothelium where it is important for vasodilatation (Stuehr 1999). NO produced by these enzymes seems to be an important player in cellular signaling as to date more than 3000 s-nitrosoproteins have been identified. Some researchers even suggest that the regulatory effect of nitrosylation on cellular signaling is as important as that mediated by phosphorylation (Hess, Stamler 2012).

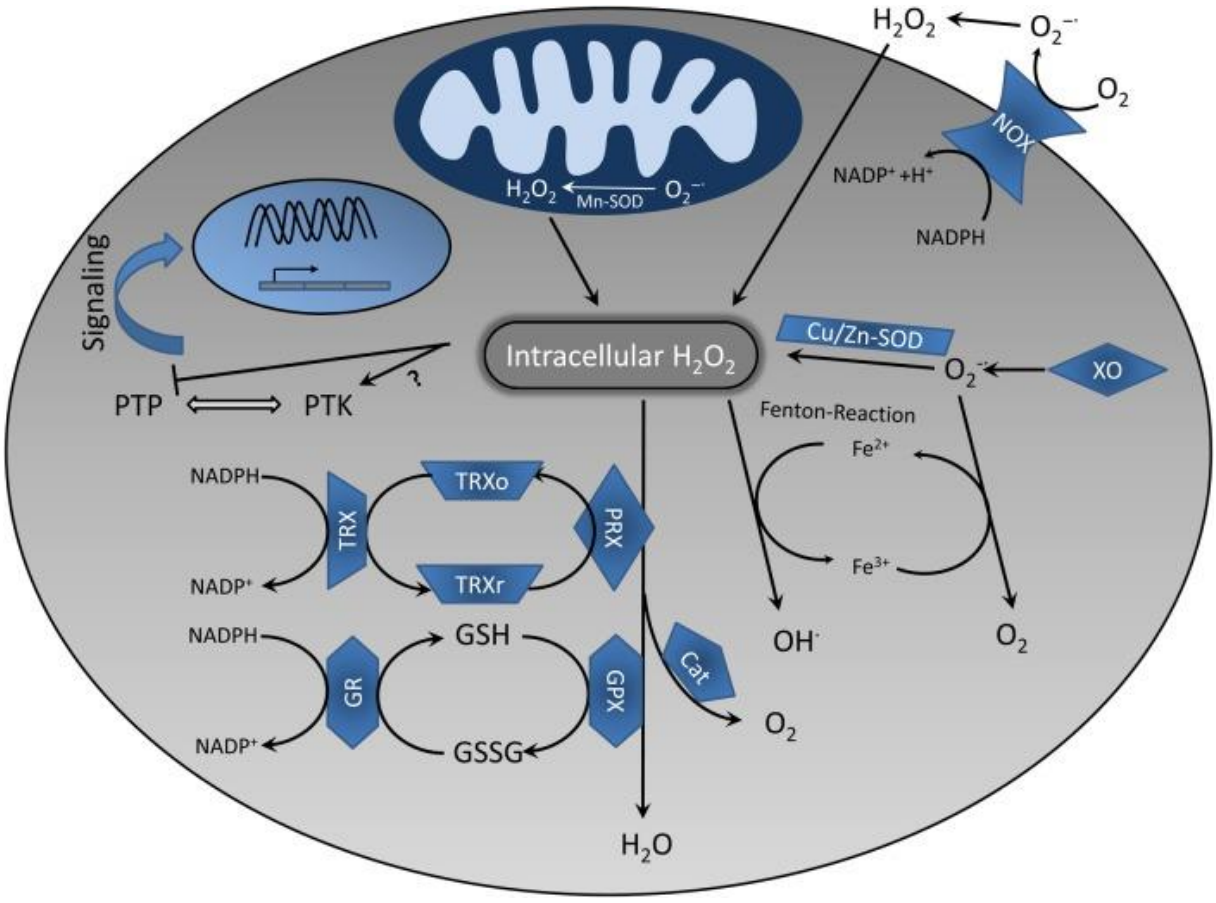


Figure 1.11: **Intracellular sources of reactive oxygen species.** Eukaryotic cells possess various enzymatic and non-enzymatic systems to produce ROS. NOX, NADPH oxidase; XO, xanthine oxidase; $O2^{\cdot -}$, superoxide anion; SOD, superoxide dismutase; Cat, catalase; GSH, glutathione; GSSG, glutathione disulfide; TRX, thioredoxin; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; TRXr, reduced thioredoxin; TRXo, oxidized thioredoxin; PRX, peroxiredoxin, GR, glutathione reductase; GPX, glutathione peroxidase. (Simeoni et al. 2016)

1.4.2 Modulation of the activity of tyrosine kinases by oxidation

1.4.2.1 Direct regulation of kinases via ROS

The impact of oxidation on the regulation of kinases has been shown for a number of different RTKs and nRTKs. Probably the best studied nRTK in this regard is the prototypic kinase of the SFKs, c-Src. Due to its ubiquitous expression, oxidation of c-Src has been reported in various cell types, like epithelial cells (Giannoni et al. 2008), vascular smooth muscle cells (Montezano et al. 2008), or mesangial cells (Block et al. 2008). The interest in the redox-regulation of c-Src lasts already almost twenty years. One of the first studies described that the enzymatic activity of c-Src increased upon treatment with RNS *in vitro* (Akhand et al. 1999). Further studies showed that oxidation of c-Src can give rise to diametrically opposite effects. In one model for the redox-mediated regulation of c-Src, two critical cysteines, C245 and C487, form an intramolecular disulfide bond which leads to an increase in kinase activity (Giannoni et al. 2005). This finding led to a conceptual revision of the regulation of c-Src activation. This new view suggests that initial activation of the kinase is regulated by phosphorylation and de-phosphorylation of two critical tyrosine residues (Y416 and Y527) which is followed by the transition to a hyperactive state upon oxidation of the critical cysteines (Corcoran, Cotter 2013). Conversely to this model, other data suggest that oxidation of C277 results in the formation of an intermolecular disulfide bridge leading to c-Src homodimerization and inactivation (Kemble, Sun 2009). In addition to c-Src, also other tyrosine kinases including c-Abl (Leonberg, Chai 2007), Lck (Lander et al. 1993), or Lyn (Yoo et al. 2011) have been shown to be oxidized.

With respect to the regulation of RTKs, an interesting work has shown that stimulation of EGFR with its ligand EGF (epidermal growth factor) leads to the sulfenylation of C797 in the kinase domain which appears to increase the kinase activity (Paulsen et al. 2011). C797 is located within the ATP-binding site of the kinase and is conserved within nine other kinases. Although a detailed mechanism for the role of C797 has not been identified so far, it can be speculated that a conserved redox-mediated mechanism may regulate the activity of a group of RTKs. This direct effect of oxidation on EGFR is complemented by the colocalization with NOX2 and the redox-sensitive phosphatase SHP2 upon stimulation which leads to inactivation of SHP2 and promotes EGFR-signaling (for the redox-mediated regulation of phosphatases see below).

The proto-oncogene c-Ret is another example of redox-mediated regulation of RTKs (Kato et al. 2000b). Upon oxidation of C376, c-Ret dimers are formed via disulfide bonds leading to

enhanced kinase activity. Furthermore, this cysteine is known to be crucial for basal kinase activity and the increase in c-Ret activity in response to UV-irradiation.

1.4.2.2 Regulation of phosphatases by ROS

Besides the regulation of PTKs by ROS also other targets of redox-regulation have been identified. One of the best studied examples are protein tyrosine phosphatases (PTPs). This is due to the fact that PTPs share a cysteine residue within their active site which is known to undergo sulfenylation and thereby inactivates the enzyme. However, despite this similarity, the conditions under which the active site cysteine is oxidized are strongly dependent on the PTP itself, the form of ROS, and the cellular system which makes regulation of PTPs by ROS a highly complex process. For instance, PTP1B is an excellent example which proves that regulation of PTPs is strongly dependent on the form of ROS. As for all other PTPs, the active site cysteine of PTP1B exhibits a high chemical reactivity. However, inactivation of PTP1B is only induced by H_2O_2 whereas $O2^{\cdot -}$ is not able to inactive it despite its generally higher reactivity (Juarez et al. 2008).

The influence of the cell system and the stimulus on the oxidation of certain PTPs can nicely be shown for the PTPs SHP1 and SHP2. SHP1 is oxidized in hematopoietic progenitors, due to ROS production upon stimulation with the macrophage stimulating colony factor (Corcoran, Cotter 2013). In the same system, SHP2, which has been shown to be oxidized in other cell types like fibroblasts (Chen et al. 2006) or vascular smooth muscle cells (Tabet et al. 2008), is not oxidized despite the high sequence homology of SHP1 and SHP2. Interestingly, SHP1 inactivation seems only to affect the PI3K/AKT pathway whereas the MAPK pathway is unchanged even though both pathways are regulated by SHP1. This shows that even in the same cellular system with the same stimulus certain pathways are differentially regulated by the same PTP.

1.4.2.3 Perspectives in therapy

PTKs are key players in many cellular signaling processes. Therefore, in many diseases de-regulated kinases are drivers of disease progression due to constitutive activation of signaling cascades. This triggers a constant need of novel drugs able to modulate the activity of kinases. To date, the majority of the approved kinase inhibitors are directed against the ATP-binding site. This causes, unfortunately, a lack of specificity for many inhibitors, as the ATP-binding site of kinases exhibits high conservation among different families (Fabbro 2015). Therefore, it is of major importance to identify alternative druggable targets which are highly specific for certain kinases.

A major challenge in the development of therapeutic strategies to modulate the activity of kinases is the fact that this requires deep knowledge of the molecular mechanisms underlying their regulation. Unfortunately, in many cases the regulation of kinases by ROS and RNS is only reported in a descriptive manner without a complete understanding of the molecular details beyond this process. This, however, is absolutely mandatory for the development of drugs targeting specific redox-regulation pathways. Nevertheless, in some cases detailed characterization of the molecular mechanisms of cysteine oxidation led to approaches of specific treatments.

A promising example is the development of inhibitors of EGFR. This RTK is overexpressed in several forms of carcinomas affecting the breast or lungs. The identification of C797 as a crucial regulator of EGFR activation (see above) has allowed the development of agents which covalently modify this residue (Engelman et al. 2007; Smaill et al. 2000; Li et al. 2008; Yoshimura et al. 2006). Indeed, these agents exhibit a high specificity for EGFR and the closely related RTK Her2 whereas other kinases seem not to be affected. Most interestingly, some of these agents have been shown to be highly active against lung cancer, in both, preclinical models, and patients. However, due to the specificity of these agents for the thiol-form of the cysteine new pitfalls could merge. Some cancers are characterized by increased generation of ROS which could shift the redox-equilibrium of this residue towards sulfenylation and disulfide formation. This would render the inhibitor inactive as the thiol-form is not available anymore. Hence, efficient treatment strategies require also inhibitors specific for the oxidized states (Truong, Carroll 2013).

The EGFR is not the only kinase which has successfully targeted by covalent inhibitors of cysteine residues. The TEC family kinases, which comprise TEC, ITK, TXK, BMX, and BTK, possess a cysteine residue in an identical location to C797 of EGFR. Based on this, researchers developed an agent which inhibits BTK with high specificity by covalent modification of the respective C481 (Honigberg et al. 2010). Additionally, this compound shows also inhibitory potential against other kinases like BLK, GMX, EGFR, Her2, ITK, JAK3, and TEC all containing an analogous cysteine. Kinases lacking this residue are not targeted by this inhibitor.

These examples show nicely that research on the redox-regulation of kinases has the potential to be transferred to drug development and some promising candidates have already reached clinical trials (Liu et al. 2013). However, for many pathways the exact molecular details have not been examined. Therefore, it is essential that effort is spent to unravel new and specific ways for the redox-regulation of kinases and to present new targets for drug development.

1.5 Aims of the study

So far, the redox-mediated regulation of the tyrosine kinase Zap70 has not been intensively studied. Due to its essential role in the initiation of TCR signaling and in the development of diseases like immunodeficiency or leukemia, understanding of Zap70´s regulation is of central importance. Zap70 possesses 8 conserved cysteine residues with so far unknown function. Additionally, one cysteine residue within the C-terminal SH2-domain has been identified to be susceptible to oxidation by H_2O_2 (Visperas et al. 2015). However, a functional analysis of this mutant is missing. Therefore, in my study I wanted to shed light into the field of redox-regulation of Zap70. To do so I addressed two main questions.

Which of the selected cysteine residues contributes to the regulation of Zap70?

To study the functional role of these residues, I generated Cys-to-Ala mutants at the respective sites as this is a well-established method to characterize the function of cysteines. To functionally analyze these mutants, I expressed the proteins in P116 cells, a Zap70-deficient Jurkat T-cell line widely used to study TCR signaling. To examine possible functional alterations of these mutants, I applied different stimulations and used various readouts to verify the reconstitution of TCR signaling.

What is the functional role of cysteine 575?

The screening of the Cys-to-Ala-mutants revealed C575 to be crucial for Zap70 stability and function as the C575A mutant is strongly impaired in both. Therefore. I analyzed protein stability in detail and addressed the role of chaperones in the stabilization of Zap70. Furthermore, I studied whether C575 is a redox-active cysteine and which modifications at this site occur. Lastly, the role of a highly conserved $Mx_{(2)}CWx_{(6)}R$ motif in which C575 is located is dissected with a special focus on the protein stability and oxidation.

2. Materials and methods

2.1 Chemicals, Antibodies, and other reagents

2.1.1 Chemicals

Table 2.1: Chemicals and reagents and their suppliers.

Chemical	Supplier
[S35]Methionine/Cysteine	Hartmann Analytic GmbH
Acetone	VWR
Ascorbate	Sigma Aldrich
BafilomycinA1	Sigma Aldrich
Cycloheximide	Carl Roth
DCP-Bio1	MerckMillipore
DMF	Sigma Aldrich
DMSO	Sigma Aldrich
E64	Sigma Aldrich
Geldanamycin	Sigma Aldrich
HDPD-Biotin	ThermoFischer Scientific
High Capacity Streptavidin Agarose	Pierce
HRP-conjugated Streptavidin	ThermoFischer Scientific
Indo-1 AM	Life Technologies GmbH
Ionomycin	Sigma Aldrich
IRDye 800CW Streptavidin	LiCor
Leupeptin	Sigma Aldrich
Methanol	VWR
MG132	Sigma Aldrich
MMTS	Sigma Aldrich
N-Acetyl-Cysteine	Sigma Aldrich
PBS	MerckMillipore
Pepstatin	Sigma Aldrich
Protein G PLUS-Agarose	SantaCruz Biotechnology
Sodium nitroprusside	Sigma Aldrich
Streptavidin Coated Microspheres 9.94µm	Polysciences Europe GmbH

2.1.2 Antibodies for western blot and immunoprecipitation

Table 2.2: Antibodies for western blot and immunoprecipitation. WB: western blot; IP: immunoprecipitation

Antigen	Host	Clone	Supplier	Application
Cdc37	mouse	E-4	SantaCruz Biotechnology	WB
c-Myc	mouse	9E10	SantaCruz Biotechnology	WB/IP
c-Myc	mouse	08	SinoBiological	WB
FLAG	rabbit	-	Sigma Aldrich	WB/IP
HSP90 alpha	mouse	Hyb-K41009	Acris	WB
IRDye® 800CW/680LT anti-Mouse	goat	-	LiCor	WB
IRDye® 800CW/680LT anti-Rabbit	goat	-	LiCor	WB
OctA probe (FLAG)	mouse	H-5	SantaCruz Biotechnology	WB
Zap70	mouse	1E7.2	SantaCruz Biotechnology	WB/IP
Zap70	rabbit	99F2	CellSignaling Technology	WB
β-Actin	mouse	AC-15	Sigma Aldrich	WB

2.1.3 Antibodies for intracellular FACS analysis

Table 2.3: Antibodies for intracellular FACS analysis.

Antigen	Host	Clone	Supplier
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	rabbit	D13.14.4E	CellSignaling Technology
F(ab´)2 anti-rabbit-APC	goat	pAk	Dianova

2.1.4 Antibodies for stimulation

Table 2.4: Antibodies for stimulation.

Antigen	Host	Clone	Supplier
human CD3 Antibody - biotin	mouse	UCHT1	Biolegend
Jurkat T-Cell Receptor	mouse	C305	hybridoma

2.2 Plasmids

Table 2.5: Plasmids and their suppliers

Plasmid	Supplier
pEYFP-N1 Zap70	kindly provided by Prof. L. Samuelson
pCMV6-Entry Zap70-Myc-DDK	Origene
pVB-CMV N-Lck-Zap70-Flag	Vector Builder, customized
pVB-CMV Zap70	Vector Builder, customized
pCMV6-Entry Cdc37-Myc-DDK	Origene
pVB-CMV HSP90aa1-twin strep	Origene
pCMV3-C-FLAG ABL1	Sino Biological
pCMV2-SYK-Myc	Sino Biological
pCMV3-Met-Myc	Sino Biological
pCMV3-Flag-SRC	Sino Biological
pCMV3-Myc-JAK2	Sino Biological
pCMV3-BTK-Myc	Sino Biological

2.3 Buffers and solutions

2xHBS

50mM HEPES (pH 7.05; Gibco)

280mM NaCl (Carl Roth GmbH)

10mM KCl (Carl Roth GmbH)

1.5mM $Na_2HPO_4 \times 2\ H_2O$ (Carl Roth GmbH)

12mM Glucose

Lysis buffer

50mM Tris (pH 7.4; Carl Roth GmbH)

1% NP-40 (Sigma Aldrich)

1% Lauryl maltoside (Calbiochem)

165mM NaCl (Carl Roth GmbH)

10mM EDTA (pH 7.5; Carl Roth GmbH)

10mM NaF (Sigma Aldrich)

1mM Na_3VO_4 (Sigma Aldrich)

1mM PMSF (Sigma Aldrich)

5x sample buffer

100mM Tris (pH 6.8; Carl Roth GmbH)

50% Glycerol (Sigma Aldrich)

0.25% Bromphenol blue (Carl Roth GmbH)

5% SDS (Calbiochem)

For reducing conditions 10% β-mercaptoethanol (Sigma Aldrich)

IP wash buffer

50mM Tris (pH 7.4; Carl Roth GmbH)

0.1% NP-40 (Sigma Aldrich)

0.1% Lauryl maltoside (Calbiochem)

165mM NaCl (Carl Roth GmbH)

10mM NaF (Sigma Aldrich)

1mM PMSF (Sigma Aldrich)

BST lysis buffer

25mM HEPES (pH 7.4; Gibco)

50mM NaCl (Carl Roth GmbH)

0.1mM EDTA (pH 7.5, Carl Roth GmbH)

1% NP-40 (Sigma Aldrich)

0.5 mM PMSF (Sigma Aldrich)

HEN buffer

100mM HEPES (pH 7.5; Gibco)

1mM EDTA (pH 7.5; Carl Roth GmbH)

0.1mM neocuproine (pH 8.0; SigmaAldrich)

HENS buffer

HEN buffer with 1% SDS (w/v)

HEN/10 buffer

HEN buffer diluted 10-fold with ddH_2O

HENS/10 buffer

HEN/10 buffer with 1% SDS (w/v)

Neutralization buffer

25mM HEPES (pH 7.5; Gibco)
100mM NaCl (Carl Roth GmbH)
1mM EDTA (pH 7.5; Carl Roth GmbH)
0.5% Triton X-100 (Sigma Aldrich)

BST wash buffer

neutralization buffer containing 600mM NaCl

RIPA buffer

20mM Tris (pH 7.5; Carl Roth GmbH)
1% Triton X-100 (Sigma Aldrich)
1mMEDTA (pH 7.5; Carl Roth GmbH)
100mM NaCl (Carl Roth GmbH)
0.5% sodium deoxycholate (Sigma Aldrich)
0.1% SDS (Calbiochem)
protease inhibitor cocktail (Roche)

SDS-PAGE resolving gel (10%)

400mM Tris (pH 8.8; Carl Roth GmbH)
10% acrylamide (BioRad)
0.1% SDS (Calbiochem)
0.1% APS (Carl Roth GmbH)
1% TEMED (Carl Roth GmbH)

SDS-PAGE stacking gel (5%)

120mM Tris (pH 6.8; Carl Roth GmbH)
5% acrylamide (BioRad)
0.1% SDS (Calbiochem)
0.1% APS (Carl Roth GmbH)
1% TEMED (Carl Roth GmbH)

Transfer buffer

50mM Tris (pH 6.8; Carl Roth GmbH)

40mM Glycine (Carl Roth GmbH)

0.000375% SDS (Calbiochem)

20% Methanol (Carl Roth GmbH)

TBS

25mM Tris (pH 8; Carl Roth GmbH)

135mM NaCl (Carl Roth GmbH)

2.5mM KCl (Sigma Aldrich)

Membrane washing buffer

TBS

0.1% Tween 20

Blocking buffer/Antibody buffer

TBS

5% BSA/milk powder

2.4 Cell culture

2.4.1 Jurkat cell culture

JE6 and P116 cells were maintained at 1-4x10^5cells/mL, 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) FBS (PAN Biotech), 100U/mL penicillin (Biochrom AG) and 100µg/mL streptomycin (Biochrom AG).

2.4.2 HEK293T cell culture

HEK293T cells (human embryonic kidney 293T cells) were cultured with 10-80% confluency, at 37°C and 5% CO_2 in DMEM supplemented with 10% (v/v) FBS, 100U/mL penicillin and 100µg/mL streptomycin.

2.4.3 Treatments with inhibitors and antioxidants

Treatment of Jurkat T cells with inhibitors and antioxidants was carried out in RPMI 1640 supplemented with 10% (v/v) FBS, 100U/mL penicillin and 100µg/mL streptomycin, at 37°C and 5% CO_2. Duration of treatments and concentrations of the substances are indicated in the respective experiments.

2.5 Site directed mutagenesis

For site directed mutagenesis of the plasmids the Agilent Quick Change II XL (Agilent) system was used according to the manufacturer's instructions. The primers required for the mutagenesis reactions were purchased Biomers (Biomers.net GmbH) and the success was confirmed by sequencing (GATC Biotech GmbH). The primers used for mutagenesis are listed in **Table 2.6**:

Table 2.6: List of the primers used for site directed mutagenesis.

mutation	primer fwd	primer rev
Zap70-C39A	cctgctgcgccaggccctgcgctcgctg	cagcgagcgcagggcctggcgcagcagg
Zap70-C84A	gaccggcagagctcgccgagttctactcgc	gcgagtagaactcggcgagctctgccggtc
Zap70-C96A	cgacgggctgcccgccaacctgcgcaag	cttgcgcaggttggcgggcagcccgtcg
Zap70-C102A	cctgcgcaagccggccaaccggccgtcg	cgacggccggttggccggcttgcgcagg
Zap70-C405A	gctcattggcgtcgcccaggccgaggcc	ggcctcggcctgggcgacgccaatgagc
Zap70-C510A	gtggtacgcacccgaagccatcaacttccgcaag	cttgcggaagttgatggcttcgggtgcgtaccac
Zap70-C560A	gcaagcggatggaggccccaccagagtgtc	gacactctggtggggcctccatccgcttgc
Zap70-C564A	gagtgcccaccagaggctccacccgaactgta	tacagttcgggtggagcctctggtgggcactc
Zap70-C575A	acgcactcatgagtgacgcctggatctacaagtggg	cccacttgtagatccaggcgtcactcatgagtgcgt
Zap70-C575A/K579R	cgcctggatctacaggtgggaggatcgcc	ggcgatcctcccacctgtagatccaggcg
Zap70-C575K	gtacgcactcatgagtgacaagtggatctacaagtgggagg	cctcccacttgtagatccacttgtcactcatgagtgcgtac
Zap70-Y292F	caccctcaactcagatggattcacccctgagc	gctcaggggtgaatccatctgagttgagggtg
Zap70-M572L	cgaactgtacgcactcttgagtgactgcttggat	atccagcagtcactcaagagtgcgtacagttcg
Zap70-M572A	ccgaactgtacgcactcgcgagtgact	agatccagcagtcactcgcgagtgcgtaca

	gctggatct	gttcgg
Zap70-M572E	ccgaactgtacgcactcgagagtgact gctggatct	agatccagcagtcactctcgagtgcgtacag ttcgg
Zap70-M572K	gaactgtacgcactcaagagtgactg ctggatc	gatccagcagtcactcttgagtgcgtacagtt c
Zap70-M572C	cccgaactgtacgcactctgcagtgac tgctggatctac	gtagatccagcagtcactgcagagtgcgtac agttcggg
Zap70-M572T	gaactgtacgcactcacgagtgactgc tggatc	gatccagcagtcactcgtgagtgcgtacagtt c
Zap70-D574A	gtacgcactcatgagtgcctgctggatc tacaagt	acttgtagatccagcaggcactcatgagtgc gtac
Zap70-C575W	cactcatgagtgactggtggatctaca agtggg	cccacttgtagatccaccagtcactcatgagt g
Zap70-C575T	acgcactcatgagtgacacctggatct acaagtggg	cccacttgtagatccaggtgtcactcatgagt gcgt
Zap70-C575M	gtacgcactcatgagtgacatgtggat ctacaagtgggagg	cctcccacttgtagatccacatgtcactcatg agtgcgtac
Zap70-C575E	gtacgcactcatgagtgacgagtggat ctacaagtgggagg	cctcccacttgtagatccactcgtcactcatga gtgcgtac
Zap70-W576L	actcatgagtgactgcttgatctacaag tgggagg	cctcccacttgtagatcaagcagtcactcatg agt
Zap70-W576Y	gcactcatgagtgactgctatatctaca agtgggaggatc	gatcctcccacttgtagatatagcagtcactc atgagtgc
Zap70-W576F	gcactcatgagtgactgcttcatctaca agtgggaggatc	gatcctcccacttgtagatgaagcagtcactc atgagtgc
Zap70-W576H	acgcactcatgagtgactgccatatcta caagtgggaggatcg	cgatcctcccacttgtagatatggcagtcact catgagtgcgt
Zap70-R583A	tggatctacaagtgggaggatgcccc cgacttcc	ggaagtcgggggcatcctcccacttgtagat cca
Zap70-R583K	gctggatctacaagtgggaggataag cccgacttcctg	caggaagtcgggcttatcctcccacttgtaga tccagc
Zap70-R583E	gctggatctacaagtgggaggatgag cccgacttcctg	caggaagtcgggctcatcctcccacttgtag atccagc
Zap70-R583Q	acaagtgggaggatcagcccgacttc ctgacc	ggtcaggaagtcgggctgatcctcccacttgt
c-Abl1b-C494A	tatgaactcatgcgagcagcttggcag	gagggattccactgccaagctgctcgcatga

	tggaatccctc	gttcata
BTK-C633A	ggtatataccatcatgtacagtgcttgg catgagaaagcagatgag	ctcatctgctttctcatgccaagcactgtacat gatggtatatacc
c-Met-C1319A	agacccctatatgaagtaatgctaaa agcctggcaccctaaagc	gctttagggtgccaggcttttagcattacttca tataaggggtct
c-Src-C501A	acctcatgtgccaggcctggcggaag gagc	gctccttccgccaggcctggcacatgaggt
Jak2-C1105A	gcccagatgagatctatatgatcatga cagaagcctggaacaataatgtaa	ttacattattgttccaggcttctgtcatgatcata tagatctcatctgggc
Syk-C608A	ttccacatcgtatgtccaggccagattc atgagatcgtac	gtacgatctcatgaatctggcctggacatacg atgtggaa

2.6 Transfection

2.6.1 Transfection by calcium phosphate

For the transfection of HEK293T cells, transfection with calcium phosphate was used. Briefly, one day prior the experiment cells were seeded onto culture plates to reach a confluency of approx. 30-40% on the day of the experiment. For transfection, a mixture of plasmid DNA in 250mM CaCl was prepared and added dropwise under constant agitation to an equal amount of 2xHBS (**Table 2.7**). After incubation for 25min at room temperature, growth medium (DMEM, 10%FBS, 100U/mL penicillin and 100µg/mL streptomycin) was added and the mixture was slowly pipetted into the culture plates. The cells were incubated for 24h at 37°C and 5% CO_2.

Table 2.7: Reagents for the transfection of HEK293T cells in different culture plates.

	24-well	**6-well**	**10 cm plate**
Cells plated	$200x10^3$	10^6	$6x10^6$
DNA	1µg	10µg	20µg
2 M CaCl (final 250mM)	4µL	37µL	62µL
Fill to with ddH_2O	30µL	300µL	500µL
2xHBS	30µL	300µL	500µl
Growth medium	300µL	3mL	6mL

2.6.2 Transfection by electroporation

Jurkat T cells were transfected by electroporation using the Gene Pulser II System (BIORAD, Hercules, CA, USA). Briefly, $20x10^6$ cells were resuspended in 350µL RPMI 1640/10% FBS and transferred into a 4mm electroporation cuvette (VWR) together with 5-30µg DNA. Electroporations were carried out with 230V and 950µF. Next, cells were immediately transferred into 50mL pre-warmed growth medium without antibiotics (50% fresh/50% conditioned) and cultured overnight.

2.7 Cell stimulation

2.7.1 Stimulation with soluble Antibodies (sAbs)

For the stimulation of Jurkat T cells with soluble antibodies, 10^6 cells were resuspended in 100µL RPMI 1640. Subsequently, 100µL of anti-CD3 antibody (C305) was added, the samples were mixed and incubated at 37°C for the indicated time points. Stimulation was stopped by addition of 1mL ice-cold PBS and samples were forwarded to downstream analysis.

2.7.2 Ca^{2+}-Flux measurements

P116 cells transfected with Zap70wt-eYFP or mutant protein were washed in RPMI 1640 (w/o phenol red) supplemented with 10% FBS and resuspended to achieve a final concentration of $0,5x10^6$cells/mL. 1mL of the cell suspension was incubated in the presence of Indo1-AM for 45min at 37°C followed by addition of 50mL RPMI 1640 (w/o phenol red) supplemented with 10% FBS and incubated for another 45min at 37°C. Cells were washed and resuspended at a concentration of $0,5x10^6$ cells/mL in RPMI 1640 (w/o phenol red) supplemented with 10% FBS. Loaded cells were kept on ice until Ca^{2+}-Flux measurement. Ca^{2+}-Flux was measured using a FACS LSR. For each sample, baseline Ca^{2+}-Flux was measured for 30s followed by addition of 200µL pre-warmed anti-CD3 antibody (C305). At the end of every measurement equal Indo1-AM loading was confirmed by addition of Ionomycin. Data were analyzed using FlowJo 7.6.5 (Tree Star).

2.7.3 Stimulation with antibodies immobilized on microspheres (iAbs)

To initiate a sustained T-cell receptor signal in Jurkat T cells, cells were stimulated with anti-CD3 antibodies immobilized on microspheres as described before (Arndt et al. 2013). Briefly, SuperAvidin™-coated polystyrene microspheres (Ø~10µm) were coated with 5µg/mL biotinylated anti-CD3 antibody (UCHT1) for 30min at room temperature in RPMI 1640. Subsequently, microspheres were washed in PBS and the stimulation was initiated by addition of $5x10^5$ microspheres to 10^6 Jurkat T cells. Cells and microspheres were pelleted

by centrifugation (100g, 10s) and incubated at 37°C for the indicated time points. Stimulation was stopped by addition of 1mL ice-cold PBS and samples were forwarded to downstream analysis.

2.8 Western blotting

2.8.1 General procedure

Jurkat T cells were pelleted by centrifugation (16,000g, 10s, 4°C) and the supernatant was aspirated. Subsequently, 10^6 cells were lysed in 30µL lysis buffer and incubated on ice for 20min. Next, lysates were clarified by centrifugation for 10min at 16,000g and 4°C.

HEK293T cells were washed with PBS and lysed in the cell culture plate (200µL for 24-well, 1mL for 6-well). The lysate was transferred into a clean microcentrifuge tube and incubated on ice for 20min followed by centrifugation for 10min at 16,000g and 4°C.

Supernatants were transferred into new microcentrifuge tubes containing 5x reducing sample buffer and were incubated for 5min at 99°C. Subsequently, lysates were separated by a SDS-PAGE system (Bio-Rad) and transferred onto nitrocellulose membranes (Amersham Biosciences) using a semi-dry Western blotting system (Bio-rad).

2.8.2 Detection by chemiluminescence

For detection with electrochemiluminescence (ECL), the membranes were incubated with specific primary antibodies and the respective secondary antibodies labeled with horseradish peroxidase (Dianova). Signals were detected using the enhanced chemiluminescence system and X-ray film (Amersham GE Healthcare). For data analysis, films were scanned using the Epson V700 scanner (16-bit greyscale) and band intensities were quantified using the Kodak 1D ImageQuant software. Intensities were normalized against the loading control (β-Actin). Additional normalizations are indicated at the respective experiment.

2.8.3 Detection by fluorescence

The detection with fluorophore-labelled secondary antibodies was carried out using an Odyssey Classic infrared imager system (LI-COR Biosciences). The membranes were incubated with a specific primary antibody followed by incubation with the respective fluorophore-labeled secondary antibody (IRDye 680LT and IRDye 800CW). Images were acquired in the 700nm (IRDye 680LT) and 800nm (IRDye 800CW) channel. Images were quantified using the LI-COR Image Studio 2.1.15 software. Intensities were normalized against the loading control (β-Actin). Additional normalizations are indicated at the respective experiment.

2.9 Immunoprecipitation

To study posttranslational modifications of single proteins or the interaction of protein complexes, immunoprecipitations were carried out. Clarified lysates from Jurkat T cells or HEK293T cells were prepared as described before. As control of the input, 30µL of the lysate were transferred into a new microcentrifuge tube together with 7,5µL 5x reducing sample buffer and incubated for 5min at 99°C. For immunoprecipitations, 30µL of Protein G beads were added to the lysate together with 2µg of the antibody against the target protein. Samples were incubated for 12-16h in an endo-over-end rotator at 4°C. Beads were pelleted by centrifugation at 7500g for 2min and 4°C. Beads were washed four times with IP wash buffer. After the last washing step, the supernatant was aspirated completely. The beads were mixed with 2x reducing sample buffer and incubated for 5min at 99°C. Samples were forwarded to SDS-PAGE and Western blot.

2.10 DCP-Bio1 labeling

In order to study posttranslational modifications, in particular sulfenylation of cysteine residues, a trapping approach using the sulfenic acid reactive probe DCP-Bio1 (Poole et al. 2007) was used. DCP-Bio1 is a dimedone derivative exhibiting a high reactivity with sulfenylated cysteines (S-OH) which leads to alkylation of this residue (**Figure 2.1**). Since this probe is derivatized with biotin, it is suitable for the pull-down of labeled proteins or the detection by western blot, taking advantage of the biotin-streptavidin interaction.

For labeling of P116 cells, $10x10^6$ cells were incubated with 1mM DCP-Bio1 in RPMI for 1h at 37°C. Subsequently, cells were washed with PBS, lysed in 500µL lysis buffer and incubated on ice for 20min. Lysates were clarified by centrifugation (16,000g, 10min, 4°C) and forwarded to immunoprecipitation, SDS-PAGE and Western blot. Signal was detected using either Streptavidin-HRP for detection with ECL or Streptavidin IRDye 800CW for detection with fluorescence. Signals were normalized to the signal of the respective total antibody.

For labeling of HEK293T cells, cells were transfected in 24-well plates and the following day incubated with 0.5mM DCP-Bio1 for 1h at 37°C and 5% CO_2. Subsequently, cells were lysed in 200µL lysis buffer, transferred into a new microcentrifuge tube and incubated on ice for 20min. Lysates were clarified and forwarded to immunoprecipitation, SDS-PAGE and Western blot. Signal was detected using Streptavidin IRDye 800CW and the Odyssey system. Signals were normalized to the signal of the respective total antibody.

For both cell lines, the western blot analysis of Zap70 sulfenylation was done in two steps. First, ~10% of the IP-material was used to perform a first SDS-PAGE and western blot. This was used to quantify the amount of Zap70 immunoprecipitated. Next, a second SDS-PAGE and western blot was performed, and the amount loaded on the gel was normalized according to the quantification of the first western blot to achieve equal Zap70-loading. This procedure was necessary as the expression of Zap70wt and Zap70C575A differed considerably.

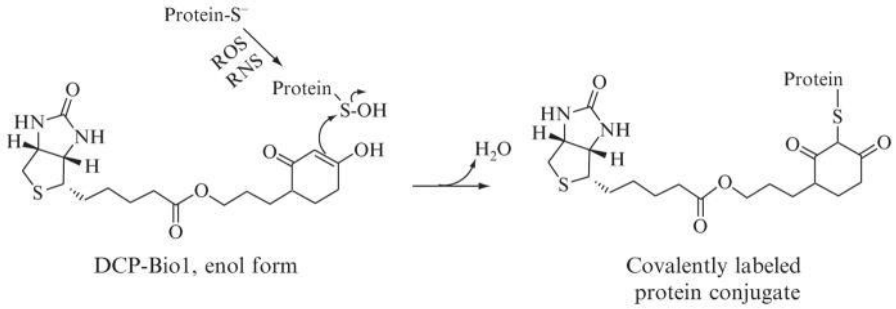


Figure 2.1: Labeling of sulfenylated cysteines with DCP-Bio1. (Nelson et al. 2010)

2.11 Biotin-switch technique

In order to analyze the nitrosylation of cysteine residues, the biotin-switch technique (BST) was used (Forrester et al. 2009). This method is based on the sequential labeling of cysteine residues depending on their modification state (**Figure 2.2**). In the first step, free thiols are blocked by reaction with the alkylating agent MMTS whereas nitrosylated cysteines are not reactive with this compound. In the second step, nitrosylated cysteines are reduced by ascorbate and forwarded to modification by HPDP-biotin which enables the detection of nitrosylated proteins by the biotin-streptavidin interaction. In the last step, biotinylated proteins are pulled down by streptavidin and can subsequently be analyzed by SDS-PAGE and western blot.

Briefly, HEK293T cells were plated onto 10cm culture plates (3 plates/transfection) followed by transfection of Zap70wt, Zap70C575A, or empty vector control. After 24h, one sample of each transfection was incubated with 400µM sodium nitroprusside (SNP) for 3h to induce nitrosylation whereas two samples remained untreated. Afterwards, cells were detached using a cell scraper, washed with 5mL PBS and collected by centrifugation (100g, 5min). Subsequently, cells were lysed in 2mL BST lysis buffer and incubated on ice for 20min. Lysates were clarified by centrifugation (16,000g, 4°C, 10min) and protein concentration was determined. In the following, the procedure for samples with total protein of ≤2mg is described. If more protein was used the respective amounts were doubled. All subsequent

steps were conducted under avoidance of light exposure. Samples were diluted to a volume of 1.8mL with HEN buffer and 200µL of 25% SDS was added. To initiate blocking, 20µL of 10% MMTS in DMF was added and cells were incubated for 20min at 50°C with repeated vortexing. For protein precipitation, 6mL of ice-cold acetone was added and samples were incubated at -20°C for 20min. Proteins were collected by centrifugation (2,000g, 5min) and washed with 70% acetone (4x5mL). Subsequently, samples were resuspended in 240µL HENS and 30µL HPDP-biotin (2.5mg/mL in DMSO) was added. To initiate labeling, 30µL of 200mM Ascorbate was added and the samples were incubated for 1h at room temperature under constant agitation. As negative control one sample was treated with 30µL 200mM NaCl instead of ascorbate. Next, proteins were precipitated by addition of 900µL ice-cold acetone and samples were incubated at -20°C over night. Proteins were collected by centrifugation (5000g, 5min) and samples were washed with 70% acetone (4x1mL). Subsequently, pellets were resuspended in 250µL HENS/10 and 750µL neutralization buffer was added. To quantify the relative Zap70 protein concentration in the different samples, 30µL of the samples were taken and forwarded to western blot analysis as described before. Next, Zap70 expression was determined by quantification of the band intensities in the western blots. Based on the quantifications the volumes of the different samples were adjusted and diluted with samples from cells transfected with empty control vector. The adjusted samples were forwarded to streptavidin pull-down. To do so, 40µL streptavidin beads/sample (High capacity streptavidin agarose) were washed with PBS, resuspended in 100µL PBS and added to the samples. 30µL of the samples were saved as input. Pull-down was carried out over night at 4°C under constant agitation. Subsequently, beads were washed with BST wash buffer (4x1mL). After the last washing step, the supernatant was removed completely and 40µL 2xSB was added and the samples were boiled at 99°C for 5min followed by SDS-PAGE and western blot.

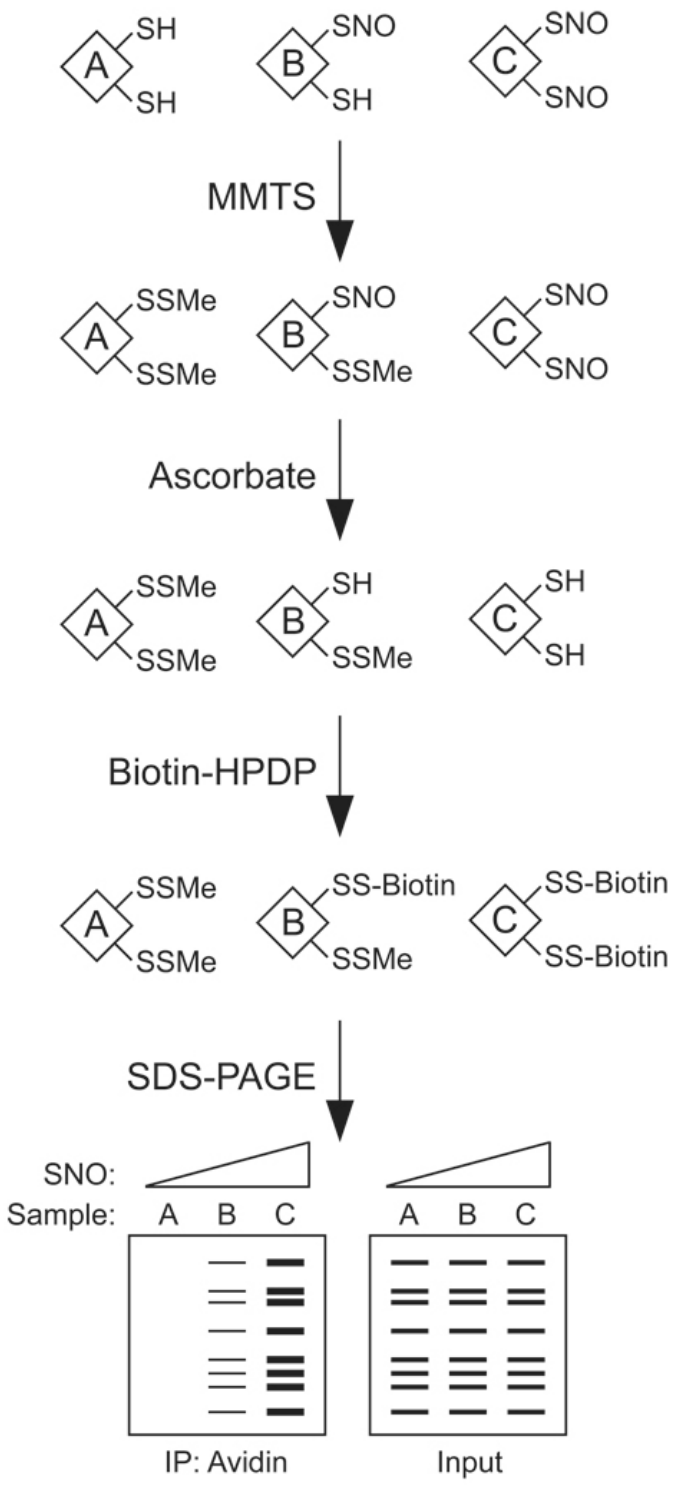


Figure 2.2: **Workflow of the analysis of nitrosylated proteins using the biotin-switch technique.** (Forrester et al. 2009)

2.12 Pulse-chase experiments

For pulse-chase experiments, HEK293T cells were transfected in 6-well plates one day before the experiment. On the day of the experiment, HEK293T cells were starved of cysteine and methionine for 40min followed by addition of 100µCi [S^{35}]methionine and cysteine in DMEM lacking cysteine and methionine supplemented with dialyzed FBS for 15min. Subsequently, cells were washed and incubated in normal DMEM supplemented with 10% FBS for the indicated time points. The chase was stopped by lysing the cells in 1mL RIPA buffer. Lysates were cleared by centrifugation (16,000g, 10min, 4°C) and normalized for the incorporation of radioactivity with [S^{35}] counts in trichloroacetic acid precipitates and forwarded to immunoprecipitation and SDS-PAGE. For pulse-chase experiments, beads were washed four times with RIPA buffer. Signals were detected using autoradiography and quantified using the LI-COR Image Studio 2.1.15 software.

2.13 Extraction of membrane proteins

For the extraction of membrane proteins, the Mem-PER™ Plus Membrane Protein Extraction Kit (ThermoFischer Scientific) was used according to the manufacturer´s instructions. Briefly, 10^6 HEK293T cells/well were plated onto 6-well plates and 4 wells/construct were transfected. 24h after transfection, cells were detached using a cell scraper, washed with 3 and 1.5mL wash solution, and collected by centrifugation (500g, 5min). Subsequently, cells were permeabilized by addition of 750µL permeabilization buffer (10min, 4°C, constant mixing) and centrifuged (16,000g, 15min). Supernatant, containing cytosolic proteins, was removed and stored on ice. The pellet was resuspended in 500µL solubilization buffer and incubated for 30min at 4°C under constant mixing. Next, samples were centrifuged (16,000g, 15min, 4°C) and the supernatant, containing solubilized membrane proteins, was transferred into a fresh tube and stored on ice. Afterwards, protein concentrations were determined, and samples were diluted to achieve equal protein concentration. For preparation of SDS-PAGE, 5x sample buffer was added to the samples and they were boiled 5min at 99°C. Finally, samples were forwarded to SDS-PAGE and western blot analysis.

2.14 Intracellular staining of pT202/pY204 Erk1/2

P116 cells expressing Zap70wt-eYFP or mutant proteins were stimulated as described before. After stimulation cells were fixed by incubation in 4% PFA for 10min at 37°C. For permeabilization cells were transferred into ice-cold 90% methanol and stored at -20°C for at least 30min. For intracellular staining of pT202/pY204 Erk1/2 cells were resuspended in 0.5% BSA in PBS and incubated with anti-pT202/pY204 Erk1/2 for 1h at room temperature.

Cells were washed in 0,5% BSA in PBS and incubated with APC-conjugated goat anti-rabbit secondary antibody for 1h in the dark. Cells were washed in 0.5% BSA in PBS and analyzed on a FACS LSRFortessa (BD Biosciences). Data were analyzed using FlowJo 7.6.5 (Tree Star).

2.15 Flow cytometry analysis

For the quantification of the protein expression of eYFP-tagged Zap70wt and mutant proteins in P116 cells, a FACS Calibur was used. First, viable cells were selected according to FSC/SSC parameters of untransfected P116 cells. Next, Zap70 expression was determined according to fluorescence of the eYFP-tag (green laser, FL1) and the mean fluorescence intensity of the eYFP positive cells was calculated.

2.16 Data analysis and statistics

Data analysis was performed with GraphPad Prism 7. All data are presented as mean ± SEM. Statistical significance was determined between groups using a Student's t-test. Asterisks indicate level of significance (* $P \leq 0,05$; ** $P \leq 0,01$; *** $P \leq 0,001$).

3. Results

3.1 Screening of Zap70 cysteine to alanine mutants

3.1.1 Conserved cysteines in Zap70

The oxidation of cysteine residues as a novel mechanism to regulate the function of tyrosine kinases is a field of research which gained a lot of attention in the last decades. The aim of this new avenue of research is the development of new therapeutic compounds to treat kinase-driven diseases. If and how Zap70 is regulated via cysteine oxidation is to date only a concept. There is only a small body of evidence indicating that oxidation could play a role in the regulation of this kinase. An early report from 1996 by Stefanová and colleagues showed that T cells from HIV patients express only low levels of Zap70 (Stefanová et al. 1996). Nevertheless, Zap70 expression was rescued upon treatment of cell lysates with the reducing agent dithiothreitol (DTT). This observation suggested that an alteration of the conformation of the kinase due to excessive ROS production can occur in the infected cells. However, how oxidation affects Zap70 conformation remains still unknown. A more recent work suggested that cysteine 39 in the N-terminal SH2-domain of Zap70 is accessible for redox modifications. It was shown that the binding of the tandem SH2-domains of Zap70 is abolished by treatment with H_2O_2, whereas treatment with a reducing agent protected against H_2O_2 treatment. Interestingly, substitution of cysteine 39 with a serine was protective against H_2O_2 (Visperas et al. 2015). Because of its location within the phosphotyrosine binding pocket of the N-terminal SH2-domain, the *in vitro* data described above suggest that the oxidation of this cysteine prevents binding to the phosphorylated ITAMs and thereby could regulate Zap70 recruitment to the TCR/CD3 complex. However, data demonstrating its functional importance in cells are missing.

Analysis of the amino acid sequence revealed that Zap70 possesses 17 cysteine residues (UniProtKB - P43403). Of these residues, 8 are conserved either in the highly homologous kinase Syk or in the prototype kinase of the Src-family kinases c-Src whereas the other residues did not show conservation. Three of these conserved cysteines are located within the N-terminal SH2-domain (C84, C96, C102) and 5 are located in the kinase domain (C405, C510, C560, C564, C575) (**Figure 3.1**). To analyze whether these cysteine residues are involved in the regulation of Zap70 function, I mutated them to alanine and the ability of the mutants to reconstitute T-cell receptor signaling was evaluated upon re-expression in the Zap70-deficient P116 T-cell line. Additionally, I included also the non-conserved C39 in the screening to test whether it is functionally important or not.

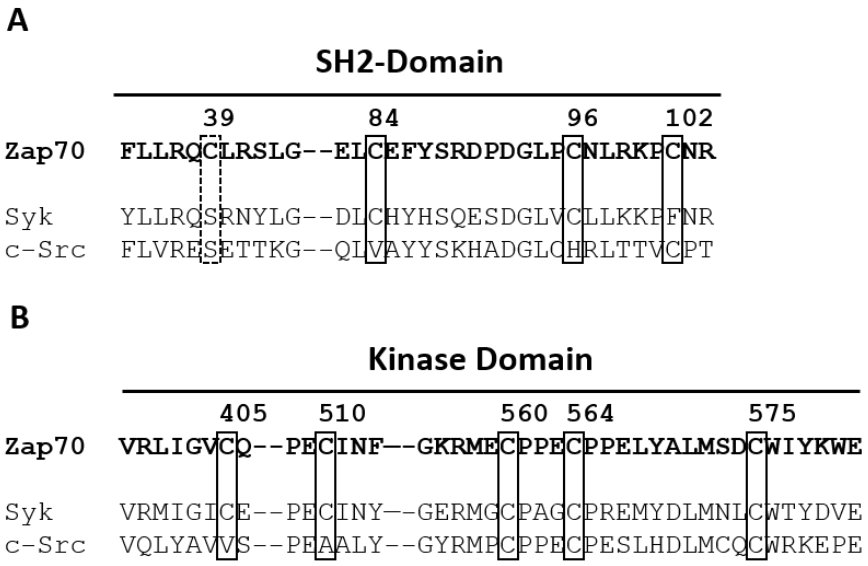


Figure 3.1: **Sequence alignment of Zap70, Syk and c-Src.** Boxes indicate conserved cysteines and numbers the position in Zap70. The box with the dashed line marks the not conserved cysteine 39.

(A) Sequence alignment of the SH2-domains.

(B) Sequence alignment of the kinase domains.

3.1.1.1 Cysteine residues in the SH2-domain

The first group of residues which are of potential interest for the regulation of Zap70 are located within the N-terminal SH2-domain at positions 39, 84, 96, and 102. Of these four cysteines, cysteine 39 is located within the phosphotyrosine binding pocket of this SH2-domain. As mentioned above, this cysteine could directly influence the binding properties of the N-SH2 domain (Visperas et al. 2015). In contrast, cysteines 84, 96, and 102 are located in the backbone of the N-SH2-domain and therefore are unlikely to directly affect the recognition of the phosphorylated tyrosine residue. However, it is possible that these cysteines are crucial for the structural integrity of the N-SH2-domain and hence could modulate its binding properties (**Figure 3.2**).

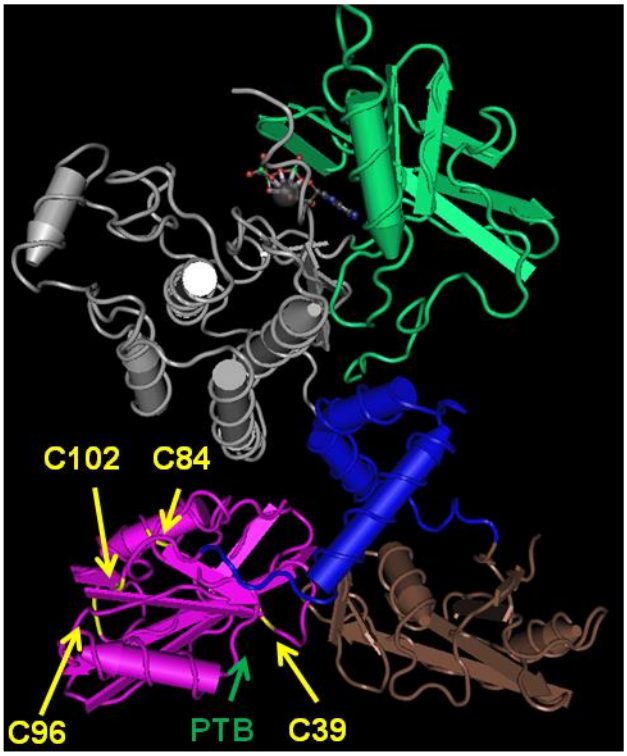


Figure 3.2: **Position of the cysteine residues within the N-terminal SH2-domain of Zap70.** Yellow arrows indicate the position of the investigated cysteines. The green arrow indicates the position of the phosphotyrosine-binding pocket (PTB) of the N-terminal SH2-domain. Colored structures indicate different domains: Pink - N-terminal SH2-domain; blue - InterdomainA; brown - C-terminal SH2-domain; grey - kinase domain N-lobe; green - kinase domain C-lobe (structure PDB: 2ozo)

3.1.1.2 Cysteine residues in the kinase domain

The second group of cysteines I have investigated are located within the kinase domain of Zap70 (**Figure 3.3**). One of these cysteines (C405) is located in the N-lobe of the kinase domain. This region of the kinase domain has been identified to be crucial for the activation of the catalytic domains of Src and Csk and could be therefore of particular interest for the regulation of Zap70 (Huang et al. 2009). The other four cysteines I investigated are located within the C-lobe of the kinase domain (**Figure 3.3**). Cysteine 510 is located in the αF-helix directly succeeding the highly flexible activation loop of the kinase, which bears the activatory tyrosine 493. Therefore, the oxidation of this cysteine could modulate the conformational dynamics crucial for activation of the kinase domain. Cysteines 560, 564, and 575 are clustered around the αI-helix, a region crucial for the stabilization of the kinase domain in the inactive conformation (Jin et al. 2004).

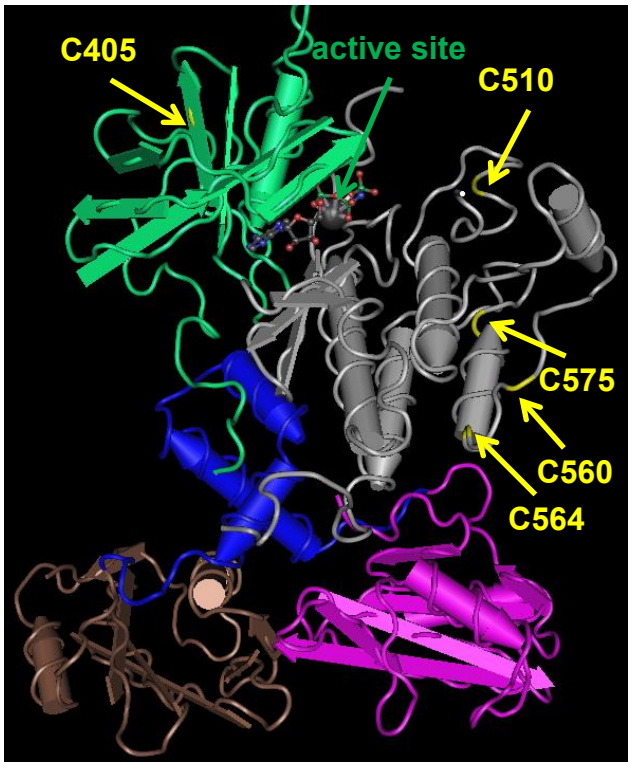


Figure 3.3: **Location of the cysteine residues in the kinase domain of Zap70.** Yellow arrows indicate the location investigated cysteines. The green arrow indicates the location of the active site. Colored structures indicate different domains: Pink - N-terminal SH2-domain; blue - InterdomainA; brown - C-terminal SH2-domain; grey - kinase domain N-lobe; green - kinase domain C-lobe (structure PDB: 2ozo)

3.1.2 Functional screening of the Zap70 Cys-to-Ala mutants

In order to evaluate the functional importance of the selected conserved cysteines, I introduced Cys-to-Ala substitutions using site-directed mutagenesis, which represents the conventional way to study the function of a given cysteine (Hänggi et al. 2006; Wang et al. 2014; Wei, Wu 2005). Subsequently, I expressed eYFP-tagged Zap70 either wt or Cys-to-Ala mutant in the Zap70-deficient Jurkat T-cell line P116, which has an impaired TCR signaling (**Figure 3.4**) (Williams et al. 1998). The efficiency of transfection was evaluated by analyzing the mean fluorescence intensity of the eYFP-tag. To assess the function of the cysteine residues, I used the following readouts:

I. Reconstitution of sustained TCR signaling by analyzing the activation of Erk1/2 using flow cytometry.
II. Reconstitution of transient TCR signaling by analyzing the activation of Erk1/2 using flow cytometry and Ca^{2+}-flux.

To investigate if the Zap70 cysteine mutants are able to reconstitute TCR signaling, I used two different methods of stimulation giving rise to different signaling kinetics (Arndt et al. 2013; Poltorak et al. 2014). Anti-CD3ε antibodies were used either immobilized on microbeads (iAbs) or crosslinked in solution (sAbs). iAbs induce a focal stimulation which mimics the contact an antigen-presenting cell and induce a weak but sustained signaling. Conversely, sAbs stimulation, which is widely used in the literature for signaling studies, induces a strong but transient TCR signal. Therefore, these stimulations enable the analysis of the function of the cysteine residues under different signal strengths and kinetics.

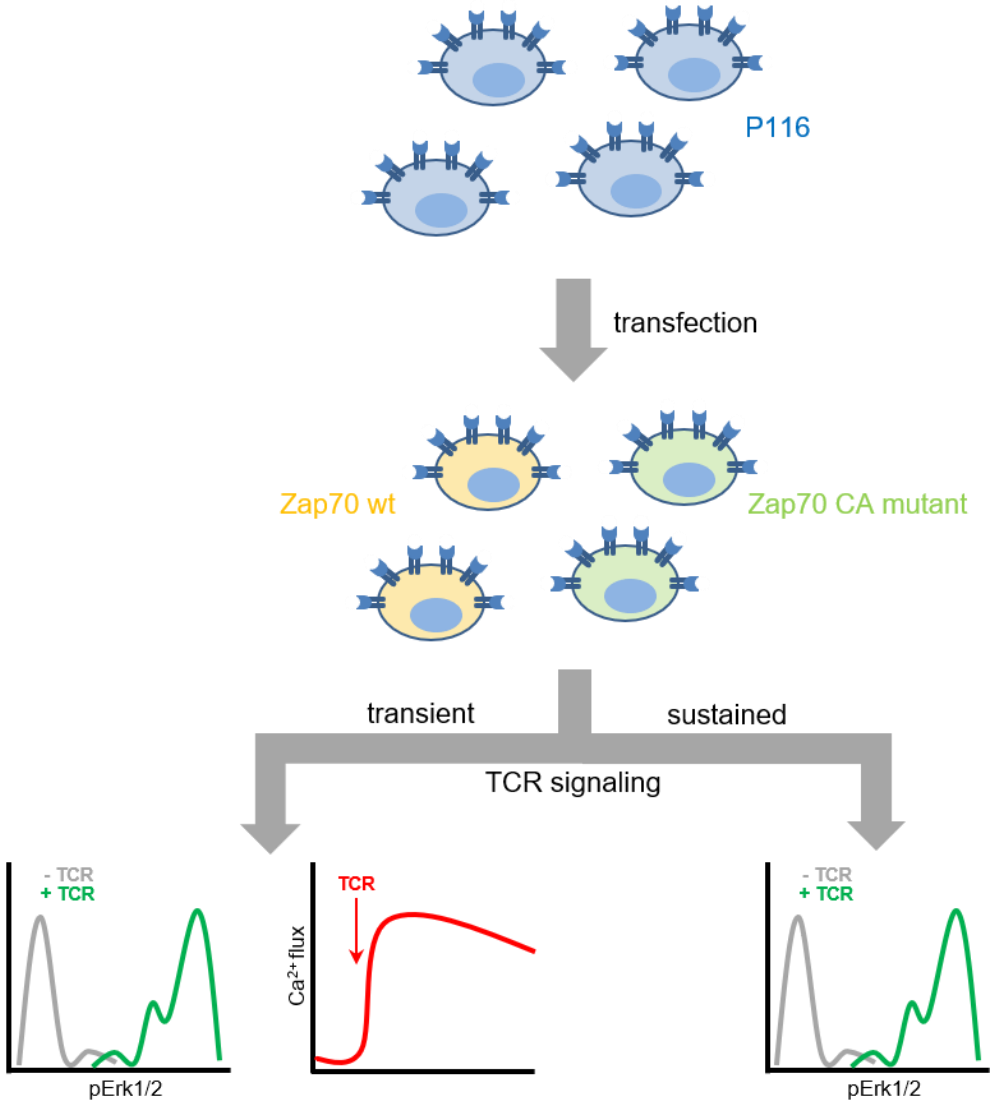


Figure 3.4: **Workflow for the functional screening of Zap70 Cys-to-Ala mutants.** Zap70 deficient Jurkat T cells (P116) were transfected with either Zap70wt or Cys-to-Ala mutants (Zap70 CA mutant). Subsequently, the ability to reconstitute TCR signaling by transient and sustained TCR stimulation was assessed.

3.1.2.1 Summary of the functional screening of the Zap70 Cys-to-Ala mutants

I analyzed the functional role of the conserved and non-conserved cysteine residues of Zap70 (**Figure 3.1**) using the described methods. These analyzes revealed that the cysteines at the positions 84, 102, 405, and 560 seem to be functionally dispensable as Cys-to-Ala mutants of these residues were able to reconstitute TCR-mediated Erk1/2 activation (**Table 3.1**). On the other hand, mutation of the cysteines 96 and 510 slightly affected TCR signaling under the used conditions. For both residues, substitution with alanine impaired the expression of the mutant proteins. In the case of the C96A mutant, reconstitution of pErk1/2 upon sustained TCR stimulation was increased despite its reduced expression. Conversely, Zap70C510A failed to fully reconstitute the induction of pErk1/2 upon transient TCR signaling (**Table 3.1**). Nevertheless, the most striking effects were observed upon mutation of C39, C564, and C575. The C39A mutant seems to be strongly impaired upon transient TCR stimulation (**Table 3.1** and **Figure 3.5**), whereas the C564A mutant exhibited increased TCR signaling upon both transient and sustained stimulation (**Table 3.1** and **Figure 3.6**). In the following section, I will describe in detail the results of the analysis of the C39A and C564A mutants. However, the main focus of this thesis will be on the in-depth analysis of the Zap70C575A mutant as this mutation resulted in the most severe functional impairment amongst the studied mutants (**Table 3.1**).

Table 3.1: **Summary of the functional screening of the Zap70 Cys-to-Ala mutants.** Arrows indicate the impact of the mutation on the respective readout. Arrows in brackets indicate a not significant tendency.

mutant	expression	iAbs - pErk1/2	sAbs - pErk1/2	sAbs - Ca^{2+}-flux
C39A	=	=	↓	↓
C84A	=	=	=	=
C96A	↓	↑	=	=
C102A	=	=	=	=
C405A	=	=	=	=
C510A	(↓)	=	↓	=
C560A	=	=	=	=
C564A	↓	(↑)	↑	(↑)
C575A	↓	↓	↓	↓

3.1.2.2 Functional role of Zap70 C39

As described above, the non-conserved C39 is located within the phosphotyrosine-binding pocket (PTB) of the N-terminal SH2-domain (**Figure 3.1A**). Posttranslational modifications within the PTB of SH2-domains have already been shown to modulate the binding for interacting molecules. For example, Src-family kinases possess a highly conserved tyrosine residue in the PTB of their SH2-domain which, upon phosphorylation, renders these SH2-domains insensitive to phosphopeptides and hence modulates the function of these kinases (Stover et al. 1996; Jin et al. 2015). Substitution of cysteine 39 with alanine (Zap70C39A) did not affect the expression of eYFP-tagged Zap70 as the mean fluorescence intensity of the wt and mutant protein are comparable (**Figure 3.5A**). To analyze if the Cys-to-Ala substitution has an impact on the reconstitution of sustained TCR signaling, I stimulated P116 T cells expressing either Zap70wt or Zap70C39A with iAbs. The kinetic of Erk1/2 phosphorylation was not different between Zap70wt and the C39A mutant. In both cases, the phosphorylation was rapidly induced upon stimulation and reached a plateau at 30min followed by a sustained Erk1/2 phosphorylation up to 60min (**Figure 3.5B**). In contrast, stimulation of T cells expressing Zap70C39A with sAbs shows a clear defect in the reconstitution of the phosphorylation of Erk1/2 3min upon stimulation compared to Zap70wt. The reduction in the activation of Erk1/2 was about 25% less than in cells expressing wt Zap70 (**Figure 3.5C**). In line with these observations, the mobilization of Ca^{2+} from intracellular stores upon stimulation with sAbs was also markedly defective in P116 T cells expressing the Zap70 mutant (**Figure 3.5D**) However, the response to stimulation with Ionomycin, a stimulus bypassing proximal TCR signaling, was comparable for both molecules, thus indicating that the cells were equally loaded with the calcium-sensitive dye. In conclusion, Zap70C39A is functionally defective in the reconstitution of transient but not sustained TCR signaling.

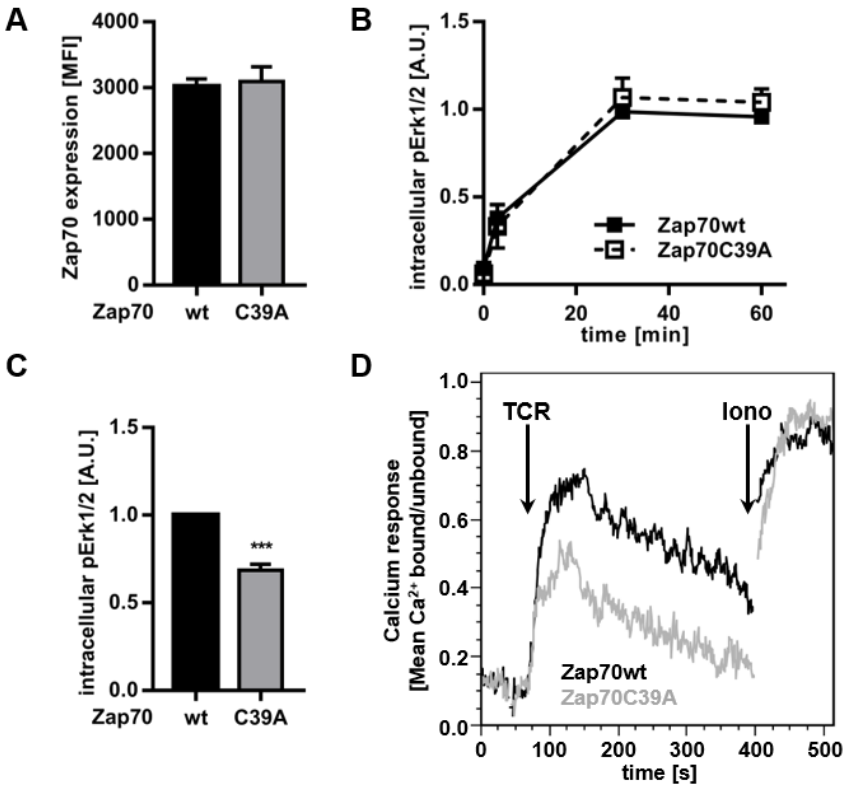


Figure 3.5: **Screening of the Zap70C39A mutant.** P116 T cells were transfected with eYFP-tagged Zap70 constructs coding for either wt or mutant proteins.

(A) eYFP mean fluorescence intensity (MFI) was measured using flow cytometry (n=3).

(B) Cells were either left unstimulated or stimulated with anti-CD3 antibodies immobilized on microbeads for 3, 30, and 60min and phosphorylation of Erk1/2 in $eYFP^+$ cells was evaluated by intracellular staining (n=3).

(C) Cells were either left unstimulated or stimulated with soluble anti-CD3 antibodies for 3min and the phosphorylation of Erk1/2 in $eYFP^+$ cells was evaluated by intracellular staining. (n=3) (***P<0.001)

(D) Cells were loaded with the calcium sensitive dye Indo1-AM. Subsequently, baseline fluorescence was recorded and cells were stimulated with soluble anti-TCR antibodies (first arrow). As a control for equal loading with Indo1-AM, cells were stimulated with Ionomycin (second arrow). One representative experiment of at least three independent experiments is shown.

3.1.2.3 Functional role of Zap70 C564

In contrast to C39, C564 is located within the kinase domain of Zap70. This cysteine marks the N-end of the αI-helix (**Figure 3.3**) and is part, together with C560, of a CPPECPPE sequence exhibiting a number of residues conserved also in other kinases. C564 is a highly conserved residue in the Syk-kinases and even in the majority of the Src-family kinases (**Figure 3.1B**). Due to this high degree of conservation this residue was particularly interesting for my studies. Already the analysis of the protein expression revealed an interesting result. The expression of the Zap70C564A mutant in P116 T cells was strongly reduced (~30%) compared to Zap70wt (**Figure 3.6A**). This indicates that C564 is crucial for the stability Zap70. Subsequently, I analyzed the ability of the C564A Zap70-mutant to reconstitute TCR signaling. Interestingly, the analysis of the reconstitution of the phosphorylation of Erk1/2 upon induction of sustained TCR signaling clearly showed that Zap70C564A was not functionally impaired. Rather, this mutant even showed a better reconstitution of TCR-mediated Erk1/2 activation compared to Zap70wt kinase (**Figure 3.6B**). In addition, this observation was further supported by the results using sAbs which induce a transient TCR signaling. Like the stimulation with iAbs, also upon stimulation with sAbs this mutant was able to reconstitute the phosphorylation of Erk1/2 stronger than Zap70wt (**Figure 3.6C**). Furthermore, the analysis of the mobilization of Ca^{2+}-flux also revealed the trend that the C564A mutant induces a stronger flux, albeit at a more modest level (**Figure 3.6D**). In summary, the analysis of the Zap70C564A mutant revealed that C564 seems to be structurally important, as the expression of the mutant protein was reduced. However, this mutation rendered the protein more active, as reconstitution of TCR signaling, especially upon sAbs stimulation, was increased compared to Zap70wt.

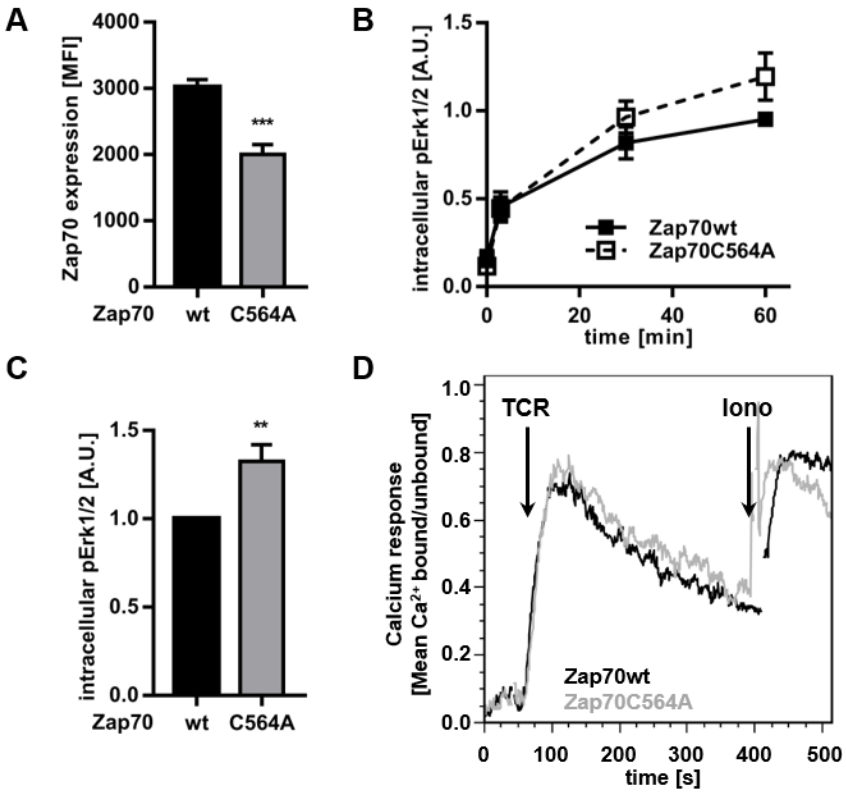


Figure 3.6: **Screening of the Zap70C564A mutant.** P116 T cells were transfected with eYFP-tagged Zap70 constructs coding for either wt or mutant proteins.

(A) eYFP mean fluorescence intensity (MFI) was measured using flow cytometry. (n=3) (***P<0.001)

(B) Cells were either left unstimulated or stimulated with anti-CD3 antibodies immobilized on microbeads for 3, 30, and 60min and phosphorylation of Erk1/2 in $eYFP^+$ cells was evaluated by intracellular staining (n=3).

(C) Cells were either left unstimulated or stimulated with soluble anti-TCR antibodies for 3min and the phosphorylation of Erk1/2 in $eYFP^+$ cells was evaluated by intracellular staining. (n=3) (**P<0.005)

(D) Cells were loaded with the calcium sensitive dye Indo1-AM. Subsequently, baseline fluorescence was recorded and cells were stimulated with soluble anti-CD3 antibodies (first arrow). As a control for equal loading with Indo1-AM, cells were treated with Ionomycin (second arrow). One representative experiment of at least three independent experiments is shown.

3.2 Analysis of the function of C575

In the following sections, I will describe in detail the characterization of the Zap70C575A mutant. From my screening of the cysteine to alanine mutants it has emerged that the C575A mutation strongly impaired Zap70 function. Therefore, I focused my experiments mostly on the analysis of this particular mutant. C575 is located in the C-lobe of the kinase domain and marks the end of the αI-helix (**Figure 3.3**). Interestingly, this residue is conserved in all, but two (i.e. Fgr and EphB6), human tyrosine kinases (**Figure 3.1B**).

3.2.1 C575 regulates Zap70 expression

I have initially investigated why the Zap70C575A mutant is not able to reconstitute TCR-mediated Erk1/2 activation upon re-expression in the Zap70-deficient P116 T-cell line (**Table 3.1**). I have formulated the following hypotheses: (i) the mutation affects the expression of Zap70 or (ii) the mutation impairs the kinase activity of Zap70. Therefore, I analyzed first the expression of the Zap70C575A mutant. I expressed Zap70wt and Zap70C575A fused to an eYFP-tag in P116 T cells and analyzed the mean fluorescence intensity of the eYFP-signal using flow cytometry. This analysis showed that the expression of the C575A mutant was strongly decreased compared to the wt (**Figure 3.7A** and **B**). However, reduced Zap70 expression was not due to reduced transfection efficiency, as both wt and C575A constructs yielded similar proportion of $eYFP^+$ cells (**Figure 3.7A**). Analysis of the mean fluorescence intensity (MFI) of the eYFP-tag revealed that the MFI values form $eYFP^+$ cells expressing Zap70C575A were lower compared to those from cells expressing Zap70wt (**Figure 3.7A**). Quantifications of MFI values from different experiments revealed that mutation of C575 results in a marked reduction (~70%) of Zap70 expression (**Figure 3.7B**). To confirm the effect of the mutation on Zap70 expression, I used another cell type. I took advantage of the widely used HEK293T cells. These cells are derived from the human embryonic kidney and are easy to transfect which makes them an excellent cellular system to study the expression of proteins. I transfected Zap70wt-Myc-DDK (a construct coding for Zap70 fused to a tandem Myc-FLAG-tag) and the C575A mutant in the HEK293T cells and analyzed the expression of both proteins using western blot analysis. These experiments confirmed the results obtained with P116 cells. Indeed, also in HEK293T cells the expression of the C575A mutant was strongly reduced compared to the wt. It appeared that the expression in the HEK293T cells is even lower than in the P116 cells (**Figure 3.7C**). This could be due to the different origin of the cell lines used and/or due to the smaller tag used for the experiments in the HEK293T cells. The Myc-DDK-tag has only a size of 3 kDa, whereas the eYFP-tag for the flow cytometry analysis has a size of ~27 kDa. Furthermore, it is well-known that GFP and its

derivatives are extraordinarily stable due to their special three-dimensional structure (Chalfie) which could additionally contribute to the observed differences.

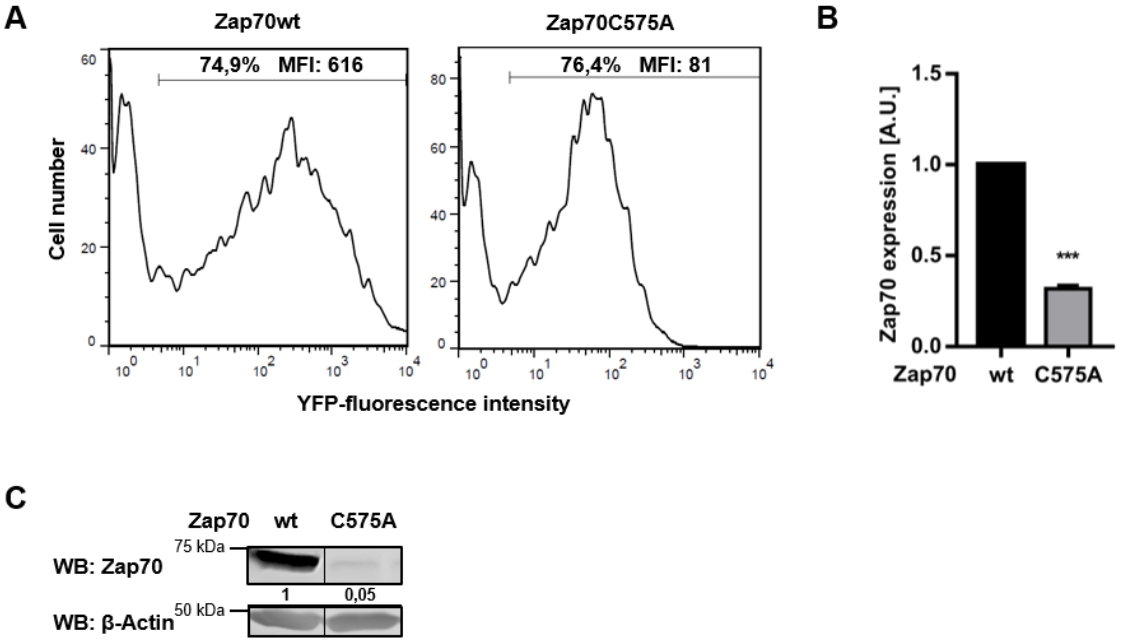


Figure 3.7: Analysis of the expression of Zap70C575A.

(A) Representative histogram of Zap70 expression in P116 cells. P116 cells were transfected with either Zap70wt-eYFP or Zap70C575A-eYFP. 18h after transfection cells were analyzed for eYFP-fluorescence using flow cytometry.

(B) Quantification of the Zap70 expression in P116 cells. Cells were transfected as in A and the MFI of the eYFP-fluorescence was quantified and normalized to the signal of the wt which was assigned to 1. (n=9) (***P<0.001)

(C) Representative western blot of the expression of Zap70wt and C575A in HEK293T cells. Cells were transfected with either Zap70wt-Myc-DDK or Zap70C575A-Myc-DDK. 24h after transfection cells were lysed and Zap70 expression was determined by western blot using an anti-Myc antibody. β-Actin served as control for equal loading.

3.2.2 Zap70C575A is instable

After having demonstrated that the C575A mutation results in a defective expression of Zap70, I investigated the cause of this phenomenon. I hypothesized that reduced protein stability could account for the reduced Zap70 expression. To assess this hypothesis, I analyzed expression kinetics of Zap70wt and Zap70C575A. I transfected the Zap70-eYFP fusion proteins in P116 cells and measured the MFI of the eYFP-tag using flow cytometry starting 16h after transfection up to 48h. Since the expression of the two proteins differs so much it is difficult to evaluate relative changes in protein expression based on the MFI values. Therefore, it was necessary to normalize the MFI of the different time points to the value at t=0h. Since these experiments were conducted with transiently transfected cells, the expression of both proteins strongly decreased during the experiment. The expression of Zap70wt almost linearly declined during the first 24h of the experiment and reached at this time point approximately 50% of the initial level (**Figure 3.8A**). After this initial phase, the expression of the wt protein declined slower. At the end of the experiment, the expression of Zap70wt was 40% of the protein expression at the beginning of the experiment. The expression of Zap70C575A showed a similar decline during the first 12h (**Figure 3.8A**). Both proteins lose ~35% of their initial expression. After 12h post-transfection, the expression of Zap70C575A declines more rapidly than the expression of Zap70wt. Thus, the expression between wt and C575A Zap70 after 24h was remarkably different (**Figure 3.8A**). At this time point, only 25% of the expression of Zap70C575A was retained, whereas the expression of the wt at this time point was still 50% compared to the starting value. This difference increased during the 2nd phase of the experiment even more. After 48h, the expression of Zap70C575A reached ~10% of the starting value giving rise to a fourfold higher expression of the wt relative to the C575A mutant (40% expression of Zap70wt vs. 10% expression of Zap70C575A after 48h) (**Figure 3.8A**).

These data support the idea of decreased protein stability upon introducing a C575A mutation in Zap70. However, simply tracking the MFI of the eYFP-tag to study the expression of the two proteins is not the optimal approach, as this only represents the sum of the newly synthesized Zap70 and Zap70 which is about to be degraded. Therefore, I decided to use two additional approaches to analyze the stability of Zap70C575A. A well-described and widely used method to examine the stability of proteins in living cells is the treatment with cycloheximide (CHX), a potent inhibitor of protein synthesis. Treatment of cells with CHX prevents the production of new proteins, and therefore allows to track the fate of the proteins present in the cell at the time of the addition of the compound (Gossan et al. 2014; Kaiser et al. 2014). To further study the stability of Zap70wt and C575A, I transfected P116 cells with the eYFP-tagged proteins. 16h post-transfection, I treated the cells with 20µg/mL CHX.

Subsequently, I measured the eYFP-fluorescence intensity every 2h and normalized it to the value at t=0h. Due to the toxicity of CHX the experiment was terminated after 8h. The CHX treatment had only a minor effect on the level of Zap70wt which stayed almost stable during the entire course of the experiment (**Figure 3.8B**) thus indicating that the protein has a half-life longer than 8h. In contrast, treatment with CHX showed a significant impact on the level of the C575A mutant (**Figure 3.8B**). The protein level stably decreased during the treatment with CHX giving rise to a significant reduction compared to Zap70wt at 6h and 8h. After 8h of CHX treatment, only ~75% of Zap70C575A was detectable.

In the second approach to study the stability of Zap70C575A, I conducted pulse-chase experiments. These experiments are based on the incorporation of radioactively [S35]-labeled amino acids (cysteine and methionine) into newly synthesized proteins and the subsequent analysis of the incorporated radioactivity. Conversely to CHX treatment, pulse-chase experiments do not significantly affect the viability of the cells. Additionally, the detection of the radiolabeled protein is highly sensitive with a high dynamic range. For these experiments, I transfected HEK293T cells with vectors encoding for either Zap70wt-Myc-DDK, the cysteine mutant, or an empty vector as control. The following day, cells were starved and pulsed for 15min with [S^{35}]-labeled amino acids. After washing, cells were incubated with standard growth medium until lysis (chase). During the chase the cells produce new, not labeled protein, whereas the labeled protein is slowly degraded. This allows to track the fate of the labeled proteins. For both proteins, a strong decrease in the incorporated radioactivity can be observed (**Figure 3.8C** and **D**). The signal of the wt protein decreases ~60% during the 6h of the experiment. However, the decrease in the incorporated radioactivity for the C575A mutant is clearly stronger leading to an 80% reduction after 6h. If only the 6h time points are compared the reduction of the C575A mutant relative to wt is 50% (**Figure 3.8C** and **D**).

In summary, all the approaches used in my study indicate that the C575A mutation results in a reduction in the half-life of Zap70. The differences in the extend of the reduction of the individual methods may be due to the different cell lines, the different expression constructs and the transient transfections. I tried to generate stably transfected cell lines expression either wt or C575A Zap70. Unfortunately, the expression C575A mutant in the stable transfectants was not detectable anymore.

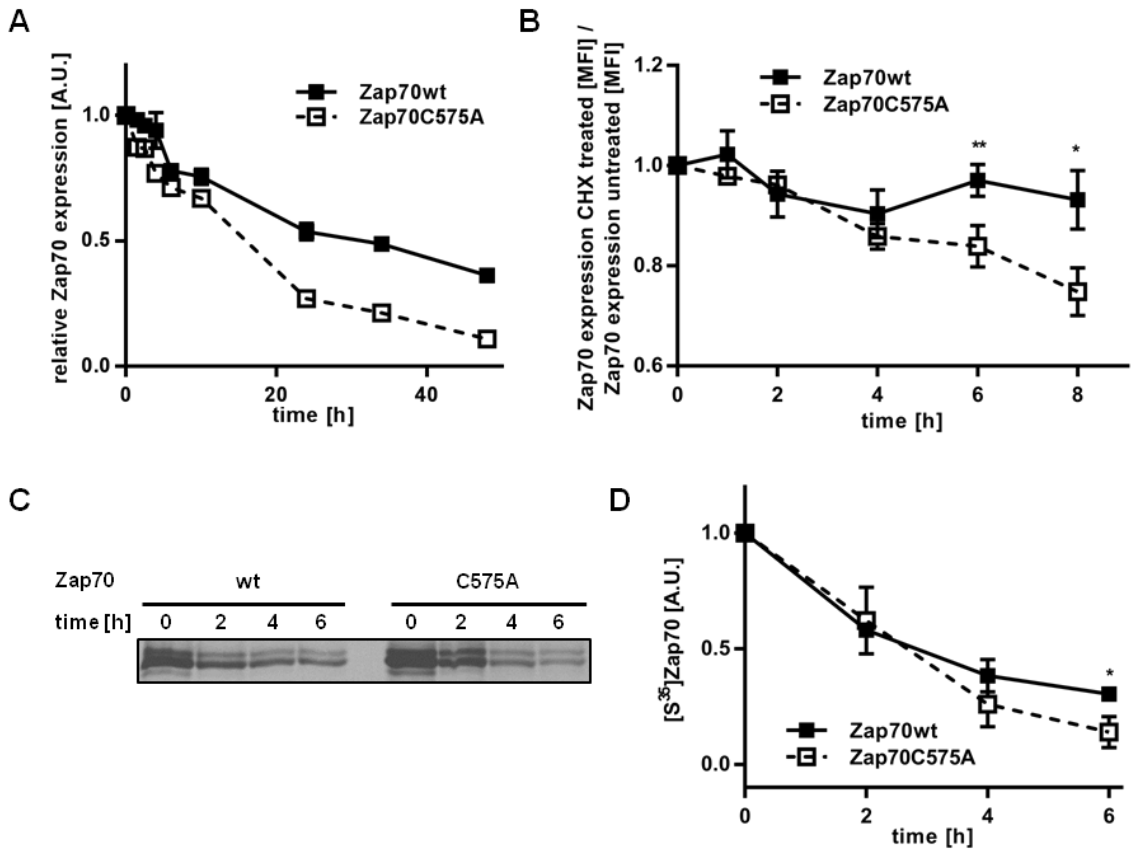


Figure 3.8: Analysis of the stability of Zap70C575A.

(A) P116 cells were transiently transfected with Zap70-eYFP constructs. Zap70wt or C575A expression was monitored using flow cytometry starting 16h after transfection over a 48h-long period and normalized to the value at t=0h which was assigned to 1.

(B) P116 cells were transiently transfected with Zap70-eYFP constructs. 16h after transfection Zap70wt or C575A expressing cells were treated with 20µg/mL cycloheximide (CHX) up to 8h. Zap70wt or C575A expression was monitored using flow cytometry. Values were normalized to t=0h which was assigned to 1. (n=3) (*P<0.05; **P<0.005)

(C) Representative autoradiogram of the pulse-chase experiments. HEK293T cells were transiently transfected with either Myc-DDK tagged wt or C575A Zap70. One day after transfection, proteins were labelled for 15min with [S^{35}]-cysteine and methionine, followed by incubation for 0, 2, 4, and 6h in standard growth medium. Subsequently, cells were lysed and Zap70 was immunoprecipitated. Finally, precipitates were loaded on a SDS-gel followed by autoradiography.

(D) Quantification of the incorporated radioactivity of the pulse chase experiments. Signal was normalized to t=0h which was assigned to 1. (n=2) (*P<0.05)

3.2.3 Analysis of the degradation pathways of Zap70C575A

The data on the stability of Zap70C575A clearly indicate a shorter protein half-life compared to the wt kinase. The half-life of a protein is strongly dependent on its degradation kinetics and a shorter half-life is usually associated with a fast degradation. To investigate the mechanisms leading to the degradation of Zap70C575A, I analyzed different degradation pathways. Eukaryotic cells possess two main routes for the degradation of proteins: (1) the ubiquitin-proteasome system and (2) the lysosomal degradation. In addition to the two main pathways, also cytosolic proteases can regulate protein degradation. To dissect whether these degradation pathways are responsible for the reduced protein expression of Zap70C575A, I performed additional experiments.

3.2.3.1 The Ubiquitin-Proteasome System

First, I assessed the question whether the Ubiquitin-Proteasome System (UPS) is responsible for the degradation of Zap70C575A. As for most other cellular proteins, the UPS is the main pathway for the degradation of Zap70 (Wang et al. 2010). Therefore, elevated degradation of the C575A is most likely due to increased degradation via this pathway. In this process, the target protein is marked for degradation by the addition of ubiquitin molecules to specific lysine residues. Usually, more ubiquitins are added to form multi-ubiquitin chains, thus marking the target proteins for degradation via a multi-subunit protease complex, the so-called proteasome (Sorokin et al. 2009).

It is well-described that the expression of Zap70 is controlled via critical amino acid residues regulating its ubiquitination. The best described negative regulatory residue mediating Zap70 ubiquitination and degradation is Y292 located in the InterdomainB of the kinase. Upon phosphorylation, this residue serves as a docking site for the E3-ubiquitin ligase c-Cbl, which ubiquitinates Zap70, thus leading to its degradation via the proteasome (Wang et al. 2010). To analyze if c-Cbl is involved in the degradation of Zap70C575A, I introduced a tyrosine-to-phenylalanine substitution in Zap70C575A. This represents the conventional approach to study the role of a tyrosine residue, as it makes phosphorylation at this site impossible but retains the structural features of the aromatic ring of the amino acid. This substitution has already used before to study the role of Y292 (Magnan et al. 2001). I transfected eYFP-tagged Zap70wt, Zap70C575A, and Zap70Y292F/C575A into P116 cells. 16h after transfection, I measured the expression of the different proteins by flow cytometry. These experiments clearly showed that the expression of Zap70C575A and of Zap70Y292F/C575A are similar (**Figure 3.9A**). This leads to the conclusion that the introduction of the Y292F

mutation does not rescue Zap70C575A expression, thus arguing against a possible role for c-Cbl in this process.

In addition to Y292, I investigated a possible role of the K579 residue. Recently, this residue has been shown to be involved in the regulation of Zap70 activity, as it becomes polyubiquitinated in $CD8^+$ T cells (Yang et al. 2015). Due to the close proximity of K579 to C575, I speculated that the C575A mutation could render the K579 residue more prone to ubiquitination and hence lead to an enhanced degradation of Zap70. Therefore, I introduced a K579R substitution, a conventional mutation to study the role of lysine residues, into Zap70C575A and analyzed protein expression (**Figure 3.9B**). The C575A/K579R double mutant showed only a slight increase in protein expression compared to the single C575A mutant. Therefore, also ubiquitination via K579 does not seem to play a major role in the regulation of the stability of Zap70C575A.

Since mutation of individual residues did not increase the stability of the C575A mutant, I wanted to examine if inhibition of the proteasome itself could rescue its expression. To do so, I used the specific inhibitor MG132 to prevent proteasomal degradation (Palombella et al. 1994). If the UPS is involved in the degradation of Zap70C575A, treatment with MG132 should increase the expression of the mutant. However, the data shown in **Figure 3.9C** indicate that treatment with MG132 only minimally enhances (~10%) the expression of Zap70C575A.

In summary, these experiments show that the reduced protein stability of Zap70C575A is not due to excessive degradation via the UPS. Therefore, alternative degradation pathways need to be involved.

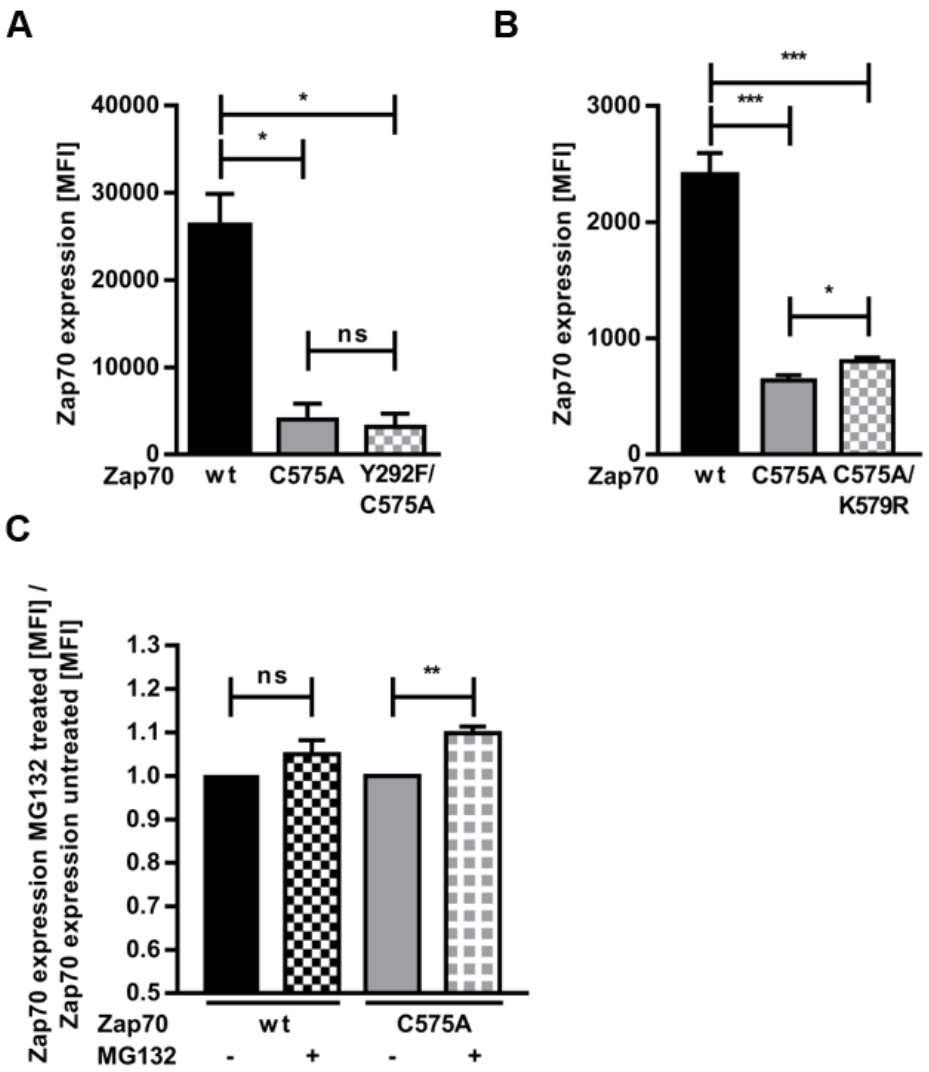


Figure 3.9: **Increased proteasomal degradations does not account for the reduced expression of Zap70C575A.** P116 cells were transfected with Zap70wt-eYFP, Zap70C575A-eYFP, the Y292F/C575A double mutant (A), or the C575A/K579R double mutant (B). 16h after transfection Zap70 expression was determined by the eYFP-fluorescence using flow cytometry.

(A) Analysis of the role of the Y292 residue. (n=3) (*P<0.05; ns=not statistically significant))

(B) Analysis of the role of the K579 residue. (n=3) (*P<0.05; ***P<0.001)

(C) Zap70 expression upon inhibition of the proteasome using 20mM MG132 for 4h. The values were normalized to the respective untreated controls which were assigned to 1. (n=4) (**P<0.005; ns=not statistically significant)

3.2.3.2 The lysosomal degradation pathway

Next, I analyzed the role of the lysosomes on the degradation of Zap70C575A. This degradation pathway is usually associated with the recycling of organelles, like mitochondria, or with the response to stressors (e.g. starvation) (Luzio et al. 2007). It involves the formation of the so-called autophagosomes. These consist of vesicles derived from the endoplasmic reticulum which contain small parts of the cytosol. Subsequently, these vesicles fuse with lysosomes which supply the proteins needed for degradation, like proteases or proton pumps. Inhibition of the fusion of the autophagosome with the lysosome with inhibitors like BafimlomycinA1 prevents the maturation of the autophagosome and thereby inhibits the degradation of proteins via the lysosomal pathway (Yamamoto et al. 1998). To examine the role of lysosomes in the degradation of Zap70C575A, I transfected eYFP-tagged Zap70wt of C575A mutant into P116 cells. 16h after transfection, I treated the cells with two different concentrations of BafilomycinA1 up to 5h. These treatments clearly show that the inhibition of the lysosomal degradation using both concentrations of BafilomycinA1 did not affect the expression of either Zap70wt or Zap70C575A (**Figure 3.10**). These results show that lysosomal degradation is not responsible for the reduced expression of Zap70C575A.

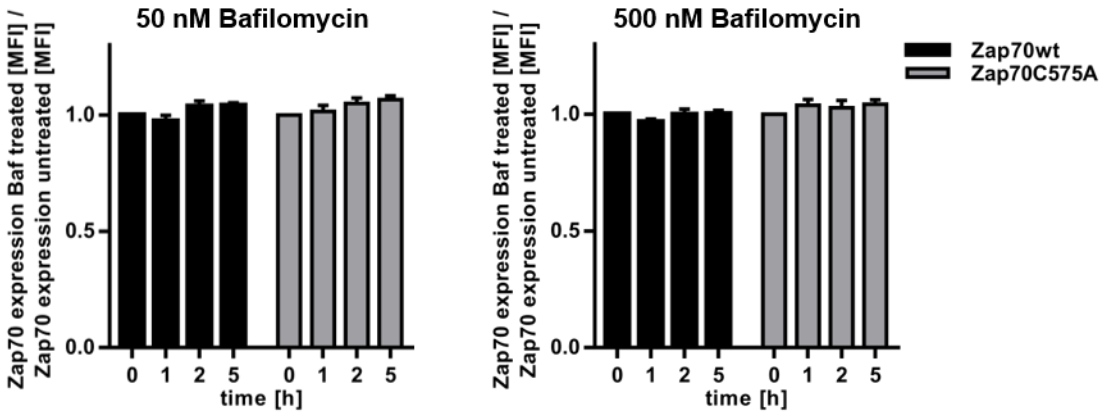


Figure 3.10: **The lysosome is not involved in the degradation of Zap70C575A.** P116 cells were transfected with either Zap70wt-eYFP or the C575A mutant. 16h after transfection, cells were treated with 50nM (left) or 500nM (right) Bafilomycin. Zap70 expression was determined by the eYFP-fluorescence using flow cytometry. The values were normalized to the respective untreated controls which were assigned to 1. (n=3)

3.2.3.3 The degradation via cytosolic proteases

Since neither of the two tested pathways seems to play a major role in the degradation of Zap70C575A, I decided to test if the inhibition of subclasses of proteases would give more clear results. To do so, I used three different inhibitors directed against different families of proteases. Pepstatin, a potent inhibitor of aspartyl peptidases, like Cathepsin D, did not increase the expression of Zap70C575A or Zap70wt (**Figure 3.11A**). Furthermore, the inhibition of cysteine proteases with the E64 inhibitor showed also no effect on the expression of both proteins (**Figure 3.11B**). Inhibition of cysteine and serine proteases using Leupeptin clearly increased the expression of Zap70C575A. However, also the expression of Zap70wt was increased to a similar extent. Therefore, also this pathway seems not to account for the reduced expression of Zap70C575A (**Figure 3.11C**).

Despite the fact that these experiments did not clearly revealed how Zap70C575A is degraded, some important conclusions can be made. The experiments using leupeptin to inhibit cysteine and serine proteases clearly indicated a role of these enzymes in the cellular homeostasis of Zap70. To my knowledge this is the first time that this mechanism has been reported and therefore, in-depth analysis of this phenomenon could contribute to the understanding of the regulation of Zap70 expression. Furthermore, the degradation via the UPS seems not to play a major role for the regulation of the expression of the C575A mutant as inhibition of proteasomal degradation only slightly increased the expression of the mutant. These results are in line with a publication describing a patient suffering from SCID in which Zap70 exhibited an amino acid substitution at position 572 (Zap70M572L). This Zap70 mutant was, similar to Zap70C575A, defective in the expression. In this work, the authors reported that the expression of the mutant could not be restored upon inhibition of the proteasome and they identified ATP-dependent proteolytic activity in the S500 fraction (which is devoid of the proteasome) to be responsible for Zap70 degradation. However, the identification of the degradation pathway was also in this case not possible (Matsuda et al. 1999). Therefore, it is possible that the C575A mutant is forwarded to a novel undescribed degradation pathway.

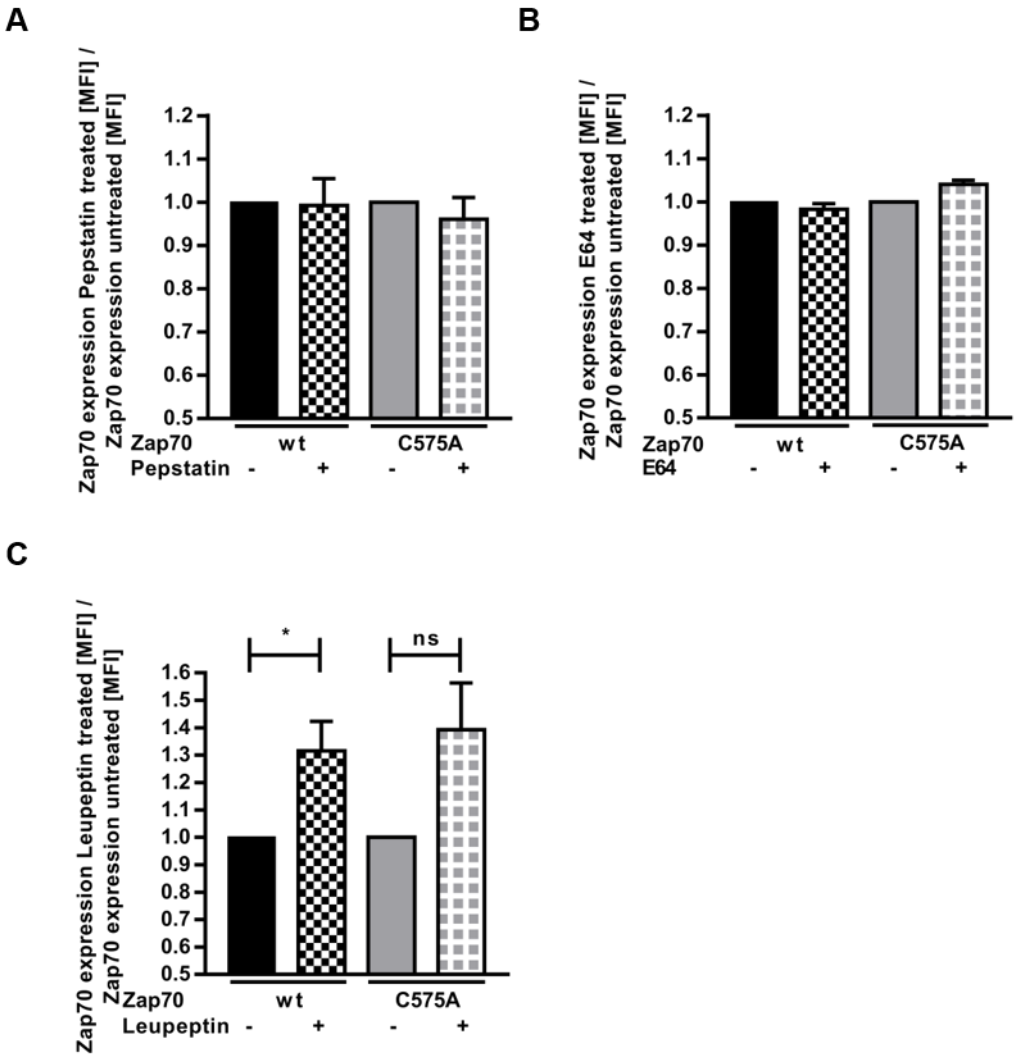


Figure 3.11: **Inhibition of cytosolic proteases does not rescue the expression of Zap70C575A.** P116 cells were transfected with either Zap70wt-eYFP or the C575A mutant. 16h after transfection, cells were treated with the indicated inhibitors. Zap70 expression was determined by the eYFP-fluorescence using flow cytometry. The values were normalized to the respective untreated controls which were assigned to 1.

(A) Zap70 expression upon treatment with 5µM Pepstatin for 4h. (n=3)

(B) Zap70 expression upon treatment with 10µM E64 for 4h. (n=3)

(C) Zap70 expression upon treatment with 100µM Leupeptin for 4h. (n=3) (*P<0.05; ns=not statistically significant)

3.2.4 The Hsp90/Cdc37 complex stabilizes Zap70C575A

The data presented above clearly demonstrate that the C575A mutation results in protein instability which in turn seems to forward this mutant for degradation.

In order to respond to a certain stimulus at a certain time point, eukaryotic cells need to assure that all required signaling molecules are functional and present in sufficient amounts. Due to their complex structure, kinases require sophisticated mechanisms for stabilization and functional support. This is achieved by the molecular chaperone heat shock protein 90 (Hsp90) and its co-chaperone cell division cycle 37 (Cdc37) (Young et al. 2004). Hsp90, a chaperone which has a plethora of client proteins, is responsible for the stabilization and refolding of mature proteins, like kinases (Li, Buchner 2013). Since Hsp90 lacks specificity, it cannot provide optimal support for all its clients. Therefore, Hsp90 acts in concert other co-chaperones such as Cdc37, which stabilizes in particular tyrosine kinases. Indeed, it is well-documented that the stability as well as the activity of kinases is strongly influenced by the Hsp90/Cdc37 complex (Verba, Agard 2017). Therefore, I investigated whether the Hsp90/Cdc37 complex is involved in the regulation of the stability of Zap70C575A.

3.2.4.1 Hsp90 activity is required for the expression of Zap70C575A

Previous works showed that Zap70 is a client protein of Hsp90 in Zap70^{pos} B-CLL and that Hsp90 inhibition leads to degradation of Zap70 (Castro et al. 2005). Therefore, I analyzed whether the expression of Zap70 relies on the activity of Hsp90 also in T cells. I transfected P116 cells with Zap70-eYFP constructs and analyzed Zap70 expression using flow cytometry in the presence or absence of the Hsp90 specific inhibitor Geldanamycin (GA) (Neckers 2003). GA treatment of cells expressing the wt kinase did not affect the expression of the protein (**Figure 3.12A**), thus indicating that, conversely to B-CLL, the stability of Zap70 does not strongly depend on Hsp90 in P116 T cells. In contrast, cells expressing the C575A mutant exhibited a clear additional decrease in Zap70 expression of ~60% (**Figure 3.12A**). These data indicate that the stability of Zap70C575A is more dependent on the activity of Hsp90 than Zap70wt.

I next investigated whether the association between Zap70C575A and Hsp90 is altered by performing co-immunoprecipitation studies. I expressed Zap70wt or Zap70C575A in HEK293T cells, precipitated Zap70, and analyzed the association with Hsp90 using immunoblot. I clearly found an interaction of Zap70wt with Hsp90. Surprisingly, I found that the association between Zap70C575A and Hsp90 was stronger (**Figure 3.12B**). Due to the great differences in protein expression between Zap70wt and Zap70C575A the amount of precipitated Zap70 is strongly reduced in the C575A sample. Nevertheless, the total amount

of Hsp90 associated to Zap70C575A is even higher than in the wt (**Figure 3.12B** left). Quantification of the Hsp90-Zap70 association finally revealed a 4-fold increased association of Zap70C575A with Hsp90 compared to the wt sample (**Figure 3.12B** right).

The enhanced association between Hsp90 and Zap70C575A may indicate an attempt of the chaperone system to stabilize mutant Zap70. Therefore, I tested whether overexpression of Hsp90 would rescue the defective expression of Zap70C575A. I expressed Zap70-eYFP constructs in P116 upon overexpression of Hsp90. The data presented in **Figure 3.12C** show that Hsp90 overexpression did not affect the expression of both wt and mutant Zap70. These experiments show that the instability of Zap70C575A is recognized by Hsp90 which results in increased association compared to Zap70wt. Interestingly, overexpression of Hsp90 did not increase the expression of Zap70C575A which indicates that the Hsp90-Zap70C575A association is already maximal without overexpression of Hsp90. Furthermore, this interaction seems to be required for the residual expression of the C575A-mutant as inhibition of Hsp90 further decreases the expression of the mutant.

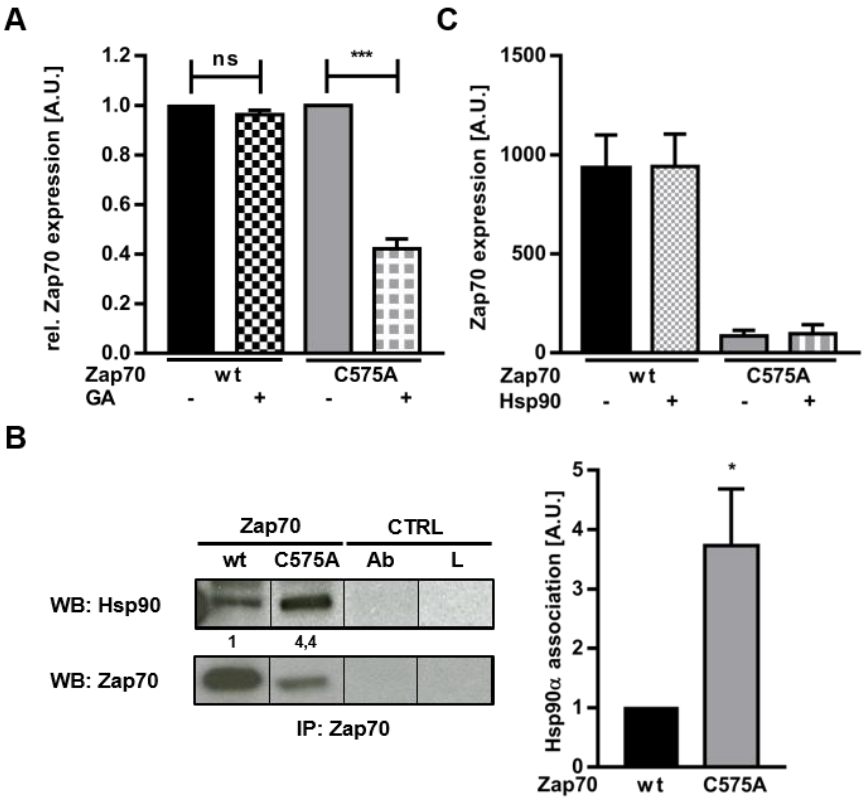


Figure 3.12: Regulation of Zap70C575A stability by Hsp90.

(A) Zap70wt-eYFP or Zap70C575A-eYFP were expressed in P116 cells. Cells were either treated for 4h with 1µM Geldanamycin (GA) or left untreated. Zap70 expression was determined by measuring the fluorescence intensity of the eYFP-tag. Values were normalized to the respective untreated sample which was assigned to 1. (n=3) (***P<0.001; ns=not statistically significant)

(B) Left: Zap70wt or the C575A mutant were expressed in HEK293T cells. The next day, cells were lysed and Zap70 was immunoprecipitated. Subsequently, proteins were separated by SDS-PAGE and Zap70-Hsp90 interaction was assessed by immunoblot. Representative western blot of at least three independent experiments. Right: Quantification of the Zap70-Hsp90 interaction from different experiments. Signal intensities were quantified and normalized to the amount of Zap70 in the immunoprecipitations. In a second step, the values were normalized to the Hsp90-Zap70 signal intensity of the wt sample which was assigned to 1. (n=3) (*P<0.05)

(C) Zap70wt-eYFP or Zap70C575A-eYFP were co-expressed with or without Hsp90 in P116 cells. After 24h, Zap70 expression was determined by measuring the fluorescence intensity of the eYFP-tag. (n=2)

3.2.4.2 Cdc37 overexpression partially rescues Zap70C575A protein levels

As mentioned above, the co-chaperone Cdc37 is highly specific for kinases. Cdc37 recognizes multiple areas in both the N- and C-lobe of the kinase domain (Verba, Agard 2017) and it can stabilize kinases even without the presence of Hsp90 (Lee et al. 2002). Therefore, I tested whether overexpression of Cdc37 can rescue Zap70C575A protein levels. I expressed Zap70wt or C575A in the presence or absence of Cdc37 in HEK293T cells and analyzed Zap70 expression using immunoblot. Overexpression of Cdc37 did not increase the expression of the wt kinase, whereas the expression of Zap70C575A was clearly increased (**Figure 3.13A**). I further confirmed western blot data using flow cytometry. I expressed eYFP-tagged Zap70wt or C575A mutant in the presence or absence of Cdc37 and quantified the eYFP fluorescence intensity using flow cytometry. I did not detect an increase in the expression of Zap70wt. In contrast, Cdc37 overexpression markedly increased the expression of Zap70C575A by 100% (**Figure 3.13B**). Therefore, I concluded that Cdc37 stabilizes the expression of Zap70C575A. However, attempts to directly demonstrate an interaction between Zap70 and Cdc37 failed, probably due to the weak interaction of Cdc37 with the client protein as previously reported (Sonamoto et al. 2015).

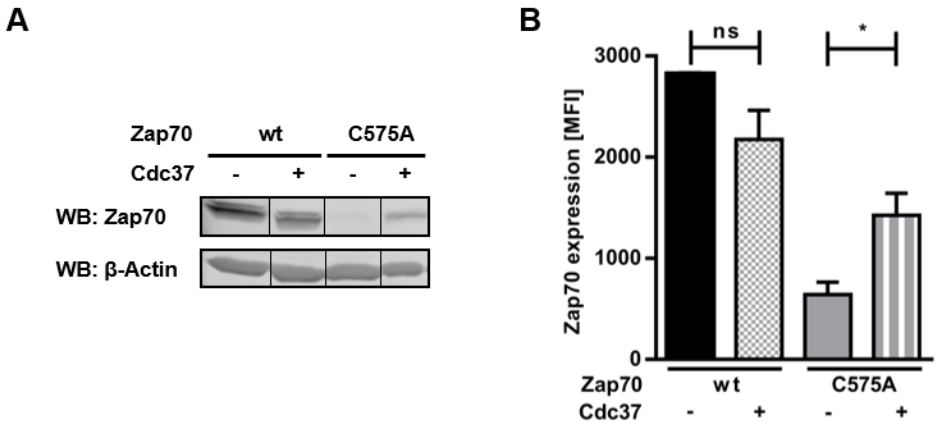


Figure 3.13: Cdc37 rescues Zap70C575A expression.

(A) HEK293T cells were co-transfected with either Zap70wt or C575A mutant in the presence or absence of Cdc37. After 24h, cells were lysed, proteins were separated using SDS-PAGE, and Zap70 expression was analyzed using immunoblot. β-Actin served as control for equal loading. One representative blot of at least three independent experiments.

(B) P116 cells were co-transfected with either eYFP-tagged Zap70wt or C575A mutant in the presence or absence of Cdc37. Subsequently, Zap70 expression was determined using flow cytometry. Values were normalized against the fluorescence intensity of Zap70wt in the absence of Cdc37 overexpression which was assigned to 1. (n=3) (*P<0.05; ns=not statistically significant)

3.2.5 Membrane targeting reconstitutes the expression of Zap70C575A

The previously described data clearly show that the C575A-mutant is instable and that the Hsp90/Cdc37-complex is partially able to stabilize the mutant. Unpublished data from our lab, focusing on the same conserved cysteine in Lck (C476), revealed that a Cys-to-Ala mutation at this position only mildly affected the protein stability of Lck. In contrast to Zap70, Lck is targeted to the plasma membrane via the myristylation and palmitoylation sites in its SH4 domain. Therefore, I asked the question whether the sub-cellular localization is the reason for these different effects. To assess this question, I designed a chimeric kinase consisting of the SH4-domain of Lck fused to the N-terminus of Zap70 (N-Lck-Zap70) (**Figure 3.14A**). To prove that the N-Lck-Zap70 chimera is indeed targeted to the plasma membrane, I separated the cytosolic and membrane proteins and analyzed Zap70 expression in these fractions. To do so, I transfected HEK293T cells with constructs coding for Zap70wt and N-Lck-Zap70wt followed by membrane extraction using the MemPer-Plus kit (Thermo Scientific). As expected, Zap70wt is localized in the cytosol of the cells (**Figure 3.14B**). In contrast, Zap70 fused to the SH4-domain of Lck is mainly localized at the cell membrane, thus demonstrating that the membrane targeting of the chimera was successful (**Figure 3.14B**). Next, to quantify the protein expression of Zap70 vs. N-Lck-Zap70, I performed western blot experiments. I transfected HEK293T cells with Zap70wt and Zap70C575A or the respective N-Lck-Zap70 constructs. For a more accurate quantification, both plasmids encoded also a GFP protein which was used to determine transfection efficiency. As expected, despite equal GFP expression the levels of Zap70C575A were strongly reduced compared to Zap70wt (**Figure 3.14C** and **D**). In clear contrast, this pronounced difference in the expression between wt and C575A mutant was abolished when Zap70 was targeted to the plasma membrane (**Figure 3.14C** and **D**). Indeed, both N-Lck-Zap70wt and the respective C575A mutant were equally expressed.

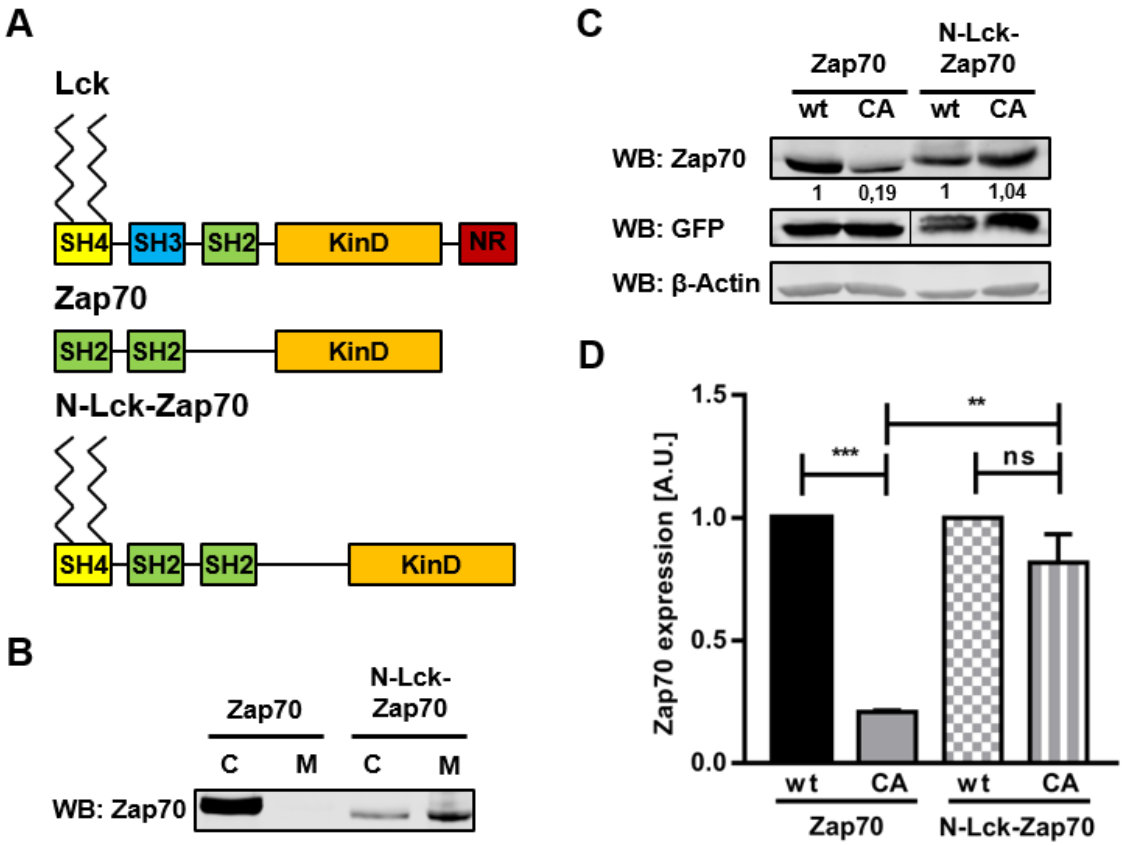


Figure 3.14: Forcing Zap70C575A to the plasma membrane reconstitutes its expression.

(A) Schematic representation of the structure of Lck, Zap70, and the N-Lck-Zap70 chimera. Top: Structure of Lck. Middle: Structure of Zap70. Bottom: Structure of N-Lck-Zap70. SH4 – SH4-domain with myristylation and palmitoylation sites; SH3 – SH3-domain; SH2 – SH2-domain; KinD – Kinase domain; NR – negative regulatory tale

(B) Analysis of the localization of N-Lck-Zap70. HEK293T cells were transfected with Zap70wt or N-Lck-Zap70wt. Next, membrane (M) and cytosolic (C) proteins were extracted and forwarded to SDS-PAGE and western blot analysis. Expression of Zap70 and N-Lck-Zap70 was determined by immunoblot analysis using an anti-Zap70 antibody.

(C) Representative western blot of the analysis of the expression of the N-Lck-Zap70 chimera. HEK293T cells were transfected with Zap70wt or N-Lck-Zap70wt or the respective C575A mutants (CA). After 24h, cells were lysed and proteins were separated by SDS-PAGE. Expression of Zap70 and N-Lck-Zap70 was determined by immunoblot analysis using an anti-Zap70 antibody. GFP served as normalization for the transfection efficiency.

(D) Quantification of the expression of the N-Lck-Zap70 chimera. Values were normalized against the respective wt sample which was assigned to 1. (n=3) (**P<0.005; ***P<0.001; ns=not statistically significant)

3.2.6 Zap70C575A is functionally defective

Zap70 is absolutely required for TCR signaling. Therefore, I was interested in the analysis of the effect of the C575A mutation on TCR signaling. For these studies P116 cells served as the cellular system as these are Zap70-deficient Jurkat T cells representing the best cell line to study Zap70 function. To stimulate these cells, I used two different stimuli (sAbs and iAbs) and two different readouts (phosphorylation of Erk1/2 and mobilization of Ca^{2+}-flux, see above). Because of the low expression of Zap70C575A, I performed flow cytometry analysis and investigated cells expressing comparable levels of Zap70. To do so, I gated on cells displaying comparable MFI of the eYFP-tag, which reflects similar Zap70 expression, and performed intracellular analyses (**Figure 3.15A**). For the analysis of the reconstitution of pErk1/2 upon transient (sAbs) stimulation, I transfected P116 cells with eYFP-tagged Zap70wt or C575A mutant. 16h after transfection the transfected cells were stimulated with sAbs for 3min and the phosphorylation of Erk1/2 and the mobilization of Ca^{2+}-flux was analyzed using flow cytometry. This revealed that Zap70C575A is defective in the reconstitution of TCR signaling as pErk1/2 was reduced by ~25% (**Figure 3.15B**). I obtained a similar result for the mobilization of Ca^{2+}-flux. Upon transient TCR stimulation Zap70wt as well as Zap70C575A can induce Ca^{2+}-flux (**Figure 3.15C**). However, the peak in the C575A transfected cells is lower than in the wt cells. After the maximal Ca^{2+}-response, the flux in both samples declines rapidly but in the C575A sample the decline is more pronounced than in the wt. Collectively, these data clearly show a defect of Zap70C575A in the reconstitution of TCR signaling upon transient stimulation. I next investigated the effect of the C575A mutation on sustained TCR signaling. Upon stimulation with iAbs, the Erk1/2 phosphorylation in the C575A expressing cells never reaches the level of the wt (**Figure 3.15D**). Furthermore, the signaling kinetics of this mutant do not resemble the expected sustained signaling as seen for the wt. The pErk1/2 signal already decreases after 60min giving rise to a reduction of 60% compared to the wt. This points at a severe functional defect of the C575A mutant upon stimulation with a stimulus inducing a signal strength similar to physiological conditions (Arndt et al. 2013).

In addition to the analyses I described above, I wanted to study the activation of downstream targets of Zap70. Unfortunately, due to the great differences in protein expression between Zap70wt and C575A it was not possible to conduct these experiments as reliable data interpretation was not possible.

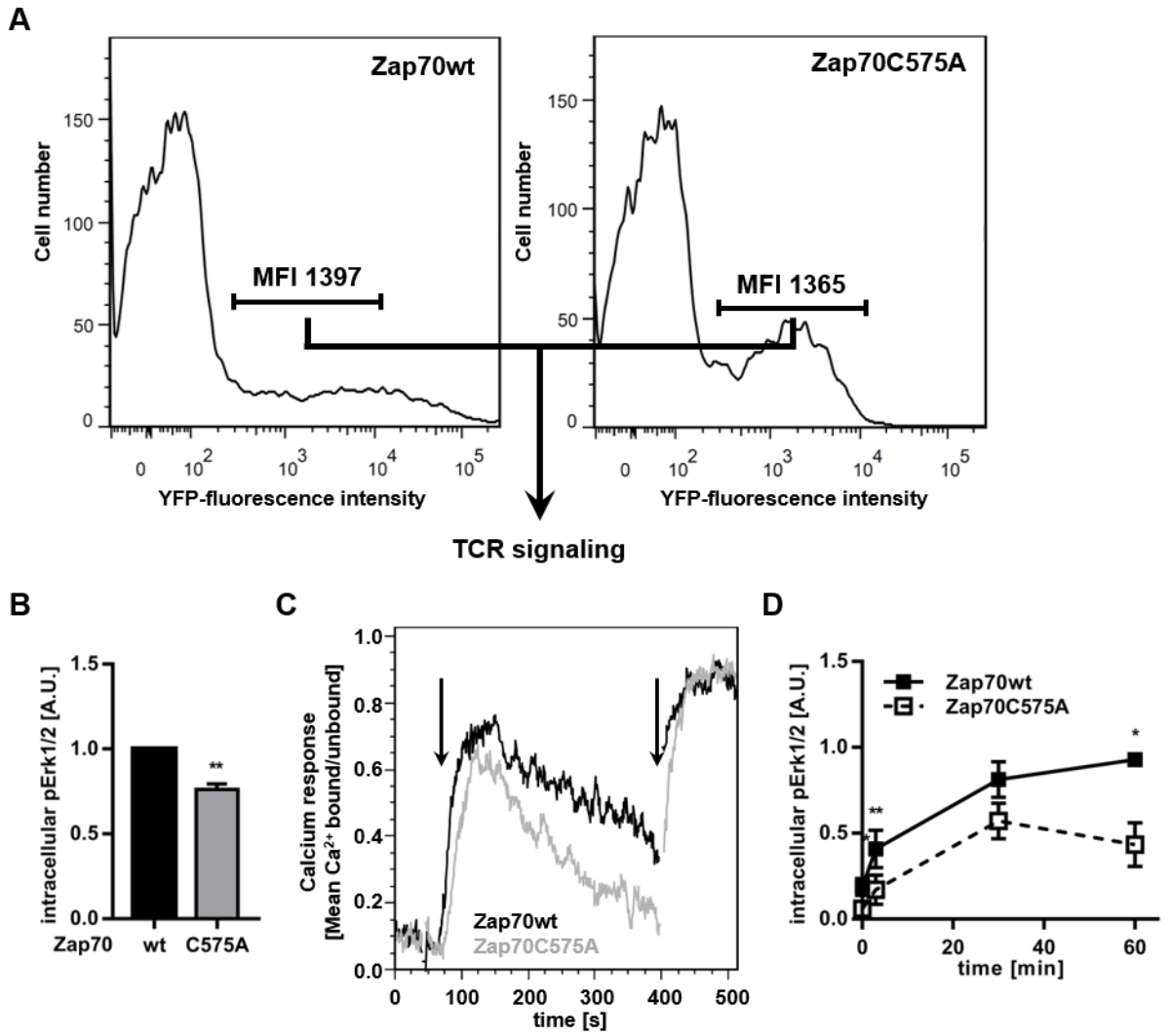


Figure 3.15: **Functional characterization of Zap70C575A.** Zap70wt or the C575A mutant were expressed in P116 cells. Cells were stimulated with iAbs or sAbs for the indicated time points.

(A) Gating strategy for the analysis of TCR signaling.

(B) Analysis of the reconstitution of pErk1/2 upon sAbs stimulation. Cells were transfected with Zap70-eYFP constructs and stimulated with sAbs for 3min. Phosphorylation of pErk1/2 was analyzed by flow cytometry. Only cells with comparable eYFP MFI were analyzed. Values were normalized to Zap70wt which was assigned to 1. (n=3) (**P<0.005)

(C) Analysis of the mobilization of Ca^{2+}-flux upon sAbs stimulation. Cells were transfected with Zap70-eYFP constructs and loaded with Indo1-AM. Subsequently, cells were stimulated with sAbs (first arrow) up to 8min. As control for equal loading, cells were treated with Ionomycin (second arrow). One representative histogram from at least three independent experiments is shown.

(D) Analysis of the reconstitution of pErk1/2 upon iAbs stimulation. Cells were transfected with Zap70-eYFP constructs and stimulated with iAbs for 0, 3, 30, and 60min. Phosphorylation of pErk1/2 was analyzed by flow cytometry. Only cells with comparable eYFP MFI were analyzed. Values were normalized to the highest value of the wt which was assigned to 1. (n=3) (*P<0.05; **P<0.005)

3.2.7 The overexpression of Cdc37 rescues the activity of Zap70C575A

The in-depth analysis of the Zap70C575A mutant revealed that this mutant is defective in both stability and activity. As I have shown that overexpression of Cdc37 leads to a partial rescue of the expression of Zap70C575A, I wanted to analyze whether Cdc37 also reconstitutes the function of this mutant. It is indeed known that Cdc37 also participates in the regulation of the activity of client kinases (MacLean, Picard 2003). To test this hypothesis, I analyzed the reconstitution of TCR signaling upon co-expression of Cdc37 with either wt or C575A Zap70. Like I already described above, stimulation of P116 cells expressing Zap70wt induced a strong and sustained phosphorylation of Erk1/2, whereas the C575A mutant was not able to fully reconstitute TCR signaling (**Figure 3.16A**). In contrast, Cdc37 overexpression fully reconstituted Zap70C575A activity (**Figure 3.16B**). Collectively, these observations together with the data presented in paragraph 3.2.4.2 indicate that the defect in the stability and the function of Zap70C575A can be reconstituted upon Cdc37.

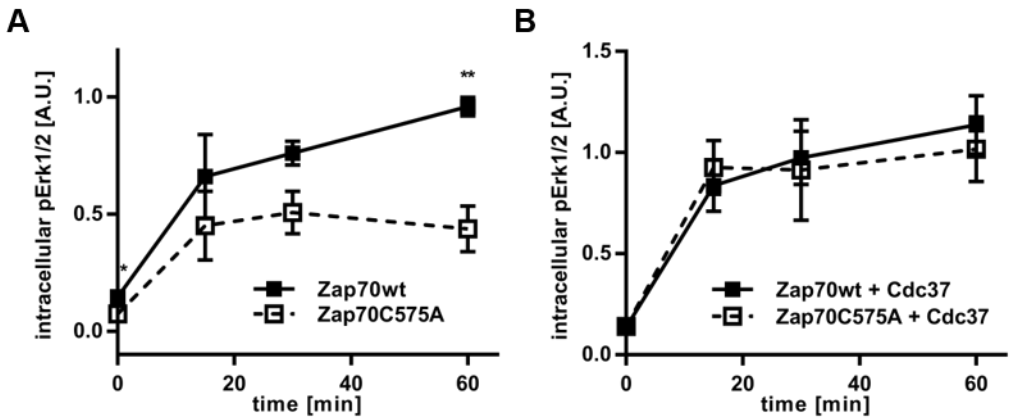


Figure 3.16: **Overexpression of Cdc37 fully rescues the function of Zap70C575A.** P116 cells were co-transfected with either Zap70wt-eYFP or Zap70C575A-eYFP in the presence (B) or absence (A) of Cdc37. Cells were stimulated with iAbs for 0, 15, 30, and 60min. Subsequently, cells were analyzed for the reconstitution of TCR-mediated Erk1/2 activation using intracellular staining. Only cells with similar Zap70 expression were gated and analyzed. Values were normalized against the highest value of the respective wt which was assigned to 1. (n=3) (*P<0.05; **P<0.005)

3.2.8 C575 in Zap70 is oxidized

The data on the stability and the activity of Zap70C575A indicate that C575 has a crucial role in the regulation of Zap70. However, it is so far unclear how this residue regulates these functions. In the last decades, modification of cysteines by reactive oxygen species (ROS) or reactive nitrogen species (RNS) has been proposed as a novel mechanism, akin to phosphorylation, to regulate kinase functions (Corcoran, Cotter 2013). How oxidation regulates kinases is not yet understood. However, the number of kinases which have been shown to function in a redox-dependent manner is constantly increasing. To date, oxidation has been shown to be critical for the regulation of kinases like c-Src, EGFR, or insulin receptor kinase (IRK) (Giannoni et al. 2005; Paulsen et al. 2011; Schmitt et al. 2005). The nature of the post-translational modification depends on the oxidizing agent. A potent and abundant cellular ROS is H_2O_2. Reaction of H_2O_2 with a cysteine leads to the formation of a sulfenic acid (-SOH). Detection of this modification is difficult due to its transient nature. However, to date specific probes to trap sulfenylated cysteines have been made available. Among them, the dimedone based probes are the most widely used. To explore the possibility that C575 is sulfenylated, I took advantage of the dimedone-derived probe DCP-Bio1. This probe possesses a biotin tag allowing the detection of the modification by the biotin-streptavidin interaction (**Figure 2.1**). I transfected Zap70wt or the cysteine mutant into P116 cells. The next day, I treated the transfected cells with 1mM DCP-Bio1 or with vehicle for 1h followed by immunoprecipitation of Zap70. Subsequently, samples were normalized for Zap70 expression. This step was necessary, as the expression of the C575A-mutant was so strongly reduced which made a comparison of the samples and the interpretation of the data difficult. Afterwards, proteins were separated using SDS-PAGE. Sulfenylation of Zap70 was analyzed by western blot using streptavidin-conjugated HRP. I detected sulfenylation of Zap70wt under these conditions (**Figure 3.17A**). In contrast, the sulfenylation of Zap70C575A was strongly reduced. As this mutant only lacks C575, this indicates that this residue is a major site for sulfenylation of Zap70. Quantification of several experiments revealed a ~50% reduction of Zap70 in the absence of C575 (**Figure 3.17B**).

Sulfenylation is not the only possible post-translational modification on cysteine residues. Upon reaction with nitric oxide (NO) cysteines can undergo nitrosylation. Like sulfenylation, this modification is well known for its impact on the regulation of many molecules. Among them, also kinases have been identified as susceptible for regulation by nitrosylation. Nitrosylation of cysteine 498, which is the equivalent of Zap70´s C575 in c-Src, enhances the kinase activity and in that way, modulates cellular responses (Rahman et al. 2010). To study nitrosylation in Zap70, I applied the well-known biotin-switch-technique (BST) (Jaffrey, Snyder 2001). In this assay, thiol groups are first blocked by reaction with an alkylating agent

which prevents false positive signals from these residues (see chapter 2.11). This is followed by the specific reduction of nitrosylated cysteines using ascorbate. The newly generated thiol groups are labelled with biotin in the last step. Upon streptavidin pull-down, the nitrosylated proteins are analyzed by SDS-PAGE and western blot. To study if C575 is also a target of nitrosylation, I expressed Zap70wt or C575A in HEK293T cells followed by treatment with the NO donor sodium nitroprusside (SNP) to induce nitrosylation or with medium to measure basal nitrosylation. Cells were lysed and assayed using BST. As a control, one sample for either wt or C575A Zap70 was treated with NaCl instead of ascorbate to prove the specificity of the assay. Using BST, I showed that Zap70 is constitutively nitrosylated in HEK293T cells (without SNP treatment) (**Figure 3.17C**). As expected, the nitrosylation of Zap70wt increases upon treatment with the NO donor (**Figure 3.17C**). These data indicate that Zap70 possesses nitrosylatable cysteines. The analysis of the nitrosylation of Zap70C575A revealed no difference in the level of nitrosylation compared to wt under both basal conditions and upon SNP treatment (**Figure 3.17C**).

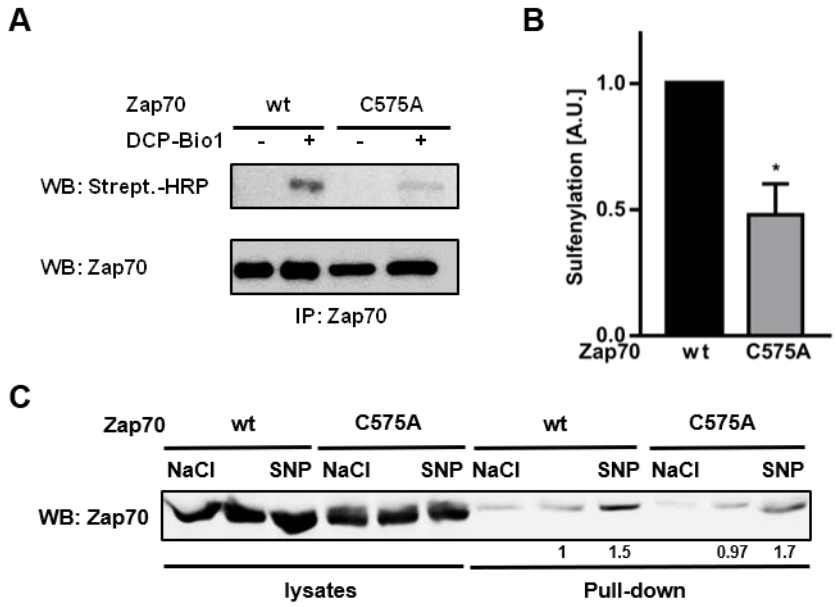


Figure 3.17 Analysis of Zap70 oxidation.

(A) P116 cells were transfected with either Zap70wt-eYFP or Zap70C575A-eYFP. Cells were treated with 1mM DCP-Bio1 or vehicle (DMSO) for 1h at 37°C. Subsequently, cells were lysed and Zap70 was immunoprecipitated. Due to the great differences in protein expression, samples were normalized for precipitated Zap70 by quantifying Zap70 levels in the immunoprecipitations using SDS-PAGE and western blot. Zap70 sulfenylation was detected by Streptavidin-HRP. Representative western blot of at least three independent experiments.

(B) Quantification of Zap70 sulfenylation. Intensities were first normalized according to immunoprecipitated Zap70 followed by normalization to the value for the wt sample which was assigned to 1. (n=3) (*P<0.05)

(C) HEK293T cells were transfected with either Zap70wt or Zap70C575A. Next, cells were lysed and forwarded to BST as described in the material and methods section. As positive control, one sample each was treated with 400µM sodium nitroprusside (SNP) for 3h to induce nitrosylation. For each sample one negative control which was treated with NaCl instead of ascorbate was included (NaCl). After pull-down of biotinylated proteins, samples were forwarded to SDS-PAGE and western blot. Pull-down of Zap70 was quantified by anti-Zap70 western blot. Samples were quantified against Zap70 expression in the lysates followed by normalization against Zap70wt which was assigned to 1. One representative experiment of at least three independent experiments is shown.

3.2.9 Long-term antioxidant-treatment leads to the destabilization of Zap70

The data presented above show that C575 is sulfenylated and that mutation of this residue results in decreased Zap70 stability. On the basis of these findings, I hypothesize that sulfenylation may stabilize Zap70. To test this hypothesis, I performed a long-term antioxidant treatment and assessed the effect on the stability of Zap70. A well-known antioxidant which is suitable for this study is N-acetyl-L-cysteine (NAC). NAC possesses free radical scavenging properties as it is a source of free sulfhydryl groups which can directly react with ROS (Zafarullah et al. 2003). To study the effect of NAC treatment on Zap70 expression, I used JE6 Jurkat T cells. JE6 T cells stably express Zap70 and do not require transfection. I incubated these cells with either different concentrations of NAC in growth medium or growth medium alone. 96h after treatment, I analyzed the expression of Zap70. As control, I tested the expression of PLCγ1 which is not a tyrosine kinase and therefore does not share the conserved cysteine. NAC treatment showed a clear dose-dependent effect on Zap70 expression (**Figure 3.18A** and **B**). Indeed, whereas treatment with 5µM NAC slightly reduced Zap70 expression, 10µM of NAC strongly reduced (about 45%) the levels of Zap70 compared to the untreated control sample (**Figure 3.18B**). In contrast, the NAC treatment did not affect the expression of the control molecule PLCγ1 (**Figure 3.18A** and **C**). These data support my hypothesis that Zap70 expression is regulated in a redox-dependent manner.

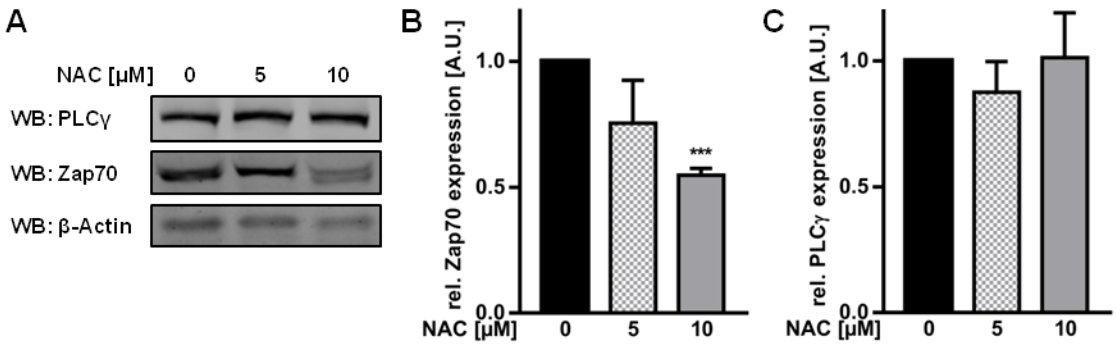


Figure 3.18: The expression of Zap70 is sensitive to NAC treatment.

(A) JE6 T cells were either left untreated or treated with either 5 or 10µM NAC for 96h. Subsequently, cells were lysed and proteins were separated by SDS-PAGE. Zap70 and PLCγ1 expression was assessed by immunoblot. β-Actin served as control for equal loading. Representative images of at least three independent experiments.

(B) Quantification of the Zap70 expression upon NAC treatment. Values were normalized to the loading control β-Actin followed by normalization to the untreated control which was assigned to 1. (n=3) (***P<0.001)

(C) Quantification of the PLCγ1 expression upon NAC treatment. Values were normalized to the loading control β-Actin followed by normalization to the untreated control which was assigned to 1. (n=3)

3.2.10 C575 is located within a highly conserved $Mx_{(2)}CWx_{(6)}R$ motif which also regulates Zap70 stability and sulfenylation

As shown in **Figure 3.1**, C575 is conserved in Syk- and Src-family kinases. In-depth analyses revealed that this cysteine is not only highly conserved in the Syk and most of the Src-kinases (Fgr, which possesses a threonine at this position, is the only exception), but also in the majority of the human tyrosine kinases (**Figure 3.19A**). Besides Fgr, also EphB6 possesses a threonine at the respective position. Moreover, I identified additional highly conserved residues surrounding the cysteine (**Figure 3.19A**) taking advantage of the motif search of the genome.jp database (http://www.genome.jp/tools/motif/). Besides the conserved cysteine this motif comprises a methionine residue three positions before the cysteine. Like cysteines, methionines are sulfur-containing amino acids which can be oxidized as well or can serve as nucleophiles due to the electron pairs of the sulfur. Furthermore, a tryptophan residue is highly conserved which succeeds the cysteine residue. Finally, separated by a flexible loop region of six amino acids, an arginine residue can be found which represents a charged basic amino acid. In summary, the conserved consensus amino acid sequence is $Mx_{(2)}CWx_{(6)}R$. This motif is also conserved in many several serine/threonine kinases (**Figure 3.19A**).

The observation that additional amino acids are conserved raised the question whether they are also involved in the regulation of Zap70 function. Interestingly, it has been shown that a spontaneous mutation of one of the conserved amino acid (M572L) also results in the loss of Zap70 expression and in severe immunodeficiency in humans (Matsuda et al. 1999). Therefore, I assessed whether mutations of the conserved amino acids in the motif affects Zap70 expression. I initially mutated methionine 572 to leucine in the attempt to recapitulate the phenomenon observed in the study of Matsuda and co-workers. The data shown in **Figure 3.19B** demonstrate that, indeed, a M572L mutation severely affected Zap70 expression in a manner comparable to the C575A mutation. I next tested the effects of the mutations of the two other conserved amino acids. I mutated the tryptophan 576 to leucine and the arginine 583 to alanine. Both mutations also strongly reduced Zap70 expression (**Figure 3.19B**). Interestingly, when I mutated the non-conserved aspartic acid 574, just preceding the conserved cysteine, to alanine the expression of Zap70 was not affected (**Figure 3.19B**). Collectively, These data indicate that all the conserved amino acids within the $Mx_{(2)}CWx_{(6)}R$ motif regulate the stability of Zap70.

In the above paragraph (**Figure 3.17A**), I demonstrated that C575 is a major target of sulfenylation in Zap70. This modification of cysteine residues does not occur spontaneously but rather requires certain conditions favoring the reaction of the residue with ROS. One

important prerequisite for the sulfenylation of a cysteine is the increase in its reactivity which is usually associated with deprotonation of the thiol and stabilization of the resulting thiolate (Lo Conte, Carroll 2013). This is typically achieved with the help of amino acids surrounding the cysteine. Therefore, I asked the question whether the motif could be required to increase the reactivity of the cysteine and therefore could be required for the regulation of Zap70 oxidation. To address this question, I analyzed the sulfenylation of the individual mutants and compared it to the wt kinase. To do so, I expressed Zap70wt or the different mutants in HEK293T cells. 24h after transfection the cells were treated with 0.5mM DCP-Bio1 for 1h followed by lysis and Zap70 immunoprecipitation. As expected, the sulfenylation of the C575A mutant was ~70% decreased compared to Zap70wt (**Figure 3.19C**). Interestingly, substitution of all the conserved amino acids M572, W576, and R583, but not of the non-conserved residue D574, led to a significant reduction of Zap70 sulfenylation despite the fact that C575 was still present in these mutants (**Figure 3.19C**). Taken together, these data indicate that M572, W576, and R583 orchestrate the sulfenylation of C575. Interestingly, the sulfenylation of the C575A mutant exhibits a tendency to be lower than that of the other mutants indicating a residual sulfenylation of C575 in the absence of M572, W576, and R583.

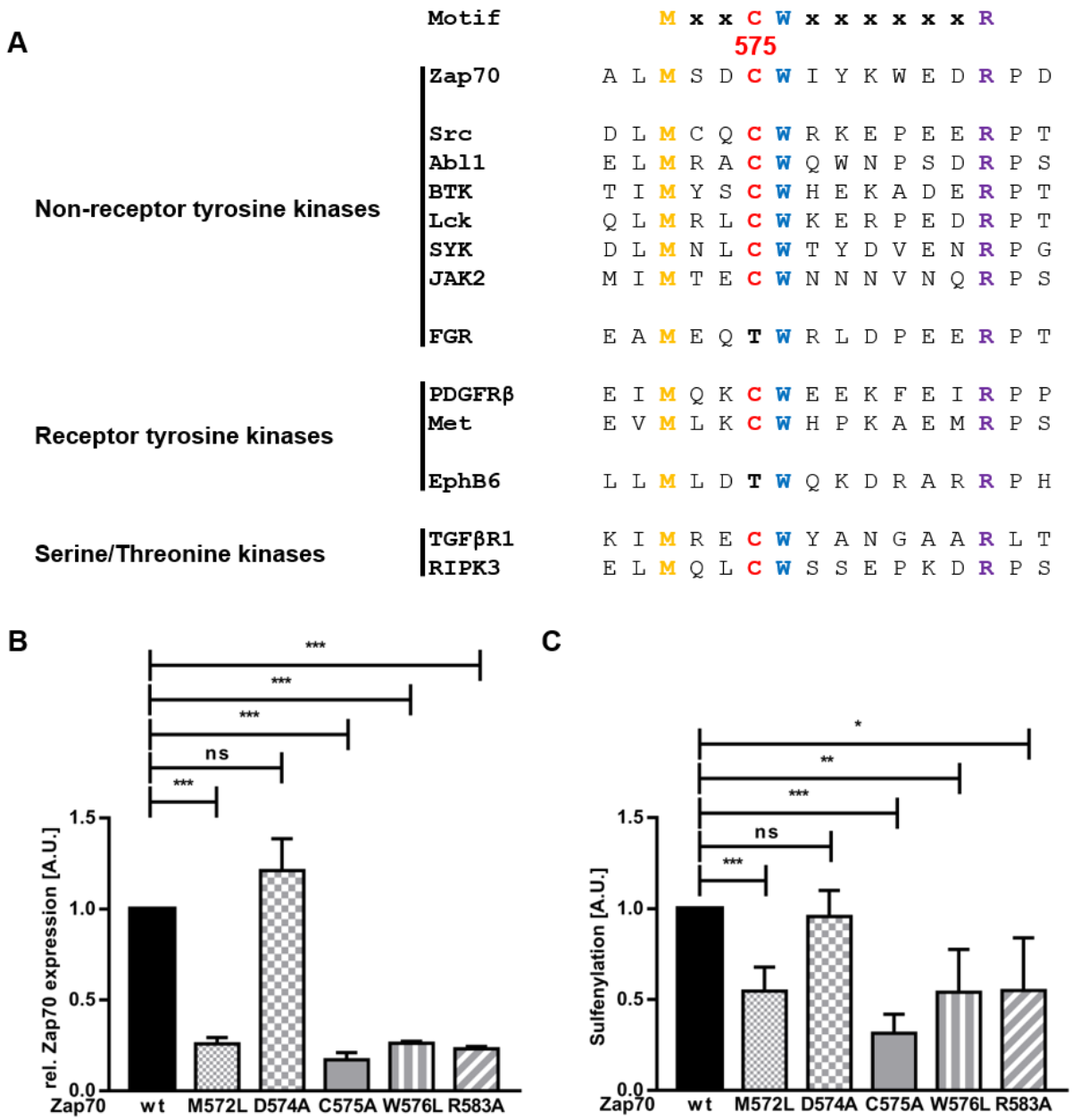


Figure 3.19: C575 in Zap70 is located within a highly conserved $Mx_{(2)}CWx_{(6)}R$-motif.

(A) Sequence alignment of several non-receptor and receptor tyrosine kinases as well as serine/threonine kinases. The $Mx_{(2)}CWx_{(6)}R$-motif is marked by colored amino acids. The 575 indicates the position of the cysteine residue in Zap70.

(B) P116 cells were transfected with eYFP-tagged Zap70wt or mutant proteins. The next day protein expression was measured using flow cytometry. Values were normalized to Zap70wt which was set to 1. (n=3) (***P<0.001); ns=not statistically significant)

(C) HEK293T cells were transfected as in (B). 24h after transfection cells were treated with 0.5mM DCP-Bio1 for 1h followed by immunoprecipitation of Zap70. Samples were analyzed by SDS-PAGE and western blot. Sulfenylation was quantified and normalized to the Zap70 signal. Values were normalized to the sulfenylation of Zap70wt which was assigned to 1. (n≥4) (*P<0.05; **P<0.005; ***P<0.001)

3.2.11 Systematic screening of the $Mx_{(2)}CW_{(6)}R$ motif does not create stable mutants

Substitution experiments presented above demonstrate that all highly conserved residues within the $Mx_{(2)}CWx_{(6)}R$ motif are indispensable for the stability of Zap70. However, not all the mutated amino acids possess similar chemical properties as the original amino acids and hence my results need to be further corroborated. Therefore, I decided to conduct a systematic screening of the motif by mutating the conserved amino acids either with residues with similar properties or in some cases with amino acids displaying other functional groups.

3.2.11.1 M572

Functional analysis of methionine residues is difficult as no other amino acid resembles both the structural and chemical features of this amino acid. The non-proteinogenic amino acid norleucine is described as a good substitution for methionine in terms of structure (Gilles et al. 1988). However, the usage of non-proteinogenic amino acids was for this screening not suitable as this requires the design and expression of tRNAs specific for the amino acid (Liu, Schultz 2010). Therefore, in addition to the M572L mutation, I decided to introduce five additional mutations: M572A, M572K, M572E, M572T, and M572C. I introduced the M572A mutation in accordance with the combinatorial alanine-scanning approach (Morrison, Weiss 2001) which suggests the substitution of amino acids of interest with alanine to exclude structural conflicts due to bulk side chains as introduced by the M572L mutation. However, the M572A substitution decreased the protein expression of Zap70 as the M572L mutation (**Figure 3.20A**). The mutation to hydroxyl- or sulfur-containing amino acids is a conventional approach for the substitution of methionine residues by keeping functional properties. Hence, I introduced the M572T and M572C mutations to study if the nucleophilic character of the methionine residue is mandatory. However, neither of the two substitutions reconstituted the expression of Zap70 (**Figure 3.20A**). Finally, to study if possible post-translational modifications, like oxidation of the M572 residue, and the associated introduction of charges account for the observed effects I substituted M572 with lysine (M572K) and glutamic acid (M572E). Also, these mutations impaired the expression of Zap70 (**Figure 3.20A**).

3.2.11.2 C575

Even though the Cys-to-Ala substitution is considered a conservative mutation to study the function of cysteine residues, I decided to substitute C575 with other amino acids. Besides mutation to alanine also the mutation to hydroxyl-containing amino acids, like serine or threonine, is considered to be a conservative substitution of cysteine residues and are therefore widely used (Smith, Marnett 1996). Here, especially the threonine substitution is of

major interest as Fgr and EphB6, the only human tyrosine kinases not possessing the cysteine within the $Mx_{(2)}CWx_{(6)}R$ motif, harbor a C-to-T substitution instead. Furthermore, previous publications showed that C-to-T substitutions are able to improve the stability of proteins (Cutler et al. 1987). Hence, I introduced the C575T and C575S mutations in Zap70. Both mutations also resulted in a loss of Zap70 expression. Interestingly, Zap70C575T displayed a slightly higher expression compared to C575A (**Figure 3.20B**). This indicates that also in Zap70 this substitution slightly reduced the defect in the expression. In order to retain the properties of a sulfur-containing amino acid, I additionally introduced the C575M mutation which also showed reduced expression. To mimic the oxidation state of C575, I mutated the cysteine to a glutamic acid, as previously described for other molecules (Permyakov et al. 2012). However, also the expression of Zap70C575E was defective. In conclusion, only the threonine substitution at position 575 showed a slightly higher expression. However, based on these data it is difficult to conclude certain features which are required at this position as substitutions which are formally closer to the cysteine than the threonine led to lower protein expression. Therefore, in-depth structural analyzes are required to gain detailed information about which chemical properties are needed for the side chain of the amino acid at this position.

3.2.11.3 W576

Apart from the conservative Trp-to-Leu mutation at position 576 shown above, I investigated other substitutions at this position. As the W576L mutant lacks the aromatic properties, I decided to introduce other three different aromatic amino acids - W576H, W576F, and W576Y. Although these substitutions are conservative mutations, each one has specific disadvantages. The F and Y substitutions could introduce a steric clash due to the introduction of six-membered at the position of a five-membered ring. In contrast, the mutation to H does not bear the risk of a steric clash but can create new hydrogen bonds (Moise et al. 2015) distinct from those built by the tryptophan which could lead to alterations in the structure of the protein. The analysis of the expression of all the mutants revealed that also in this case the expression of Zap70 was strongly reduced (**Figure 3.20C**).

3.2.11.4 R583

To further characterize the impact of mutations of R583 on the expression of Zap70, I introduced three different amino acids at this position. I substituted R583 with lysine as this is the widely used conservative mutation for this amino acid (Kuhlmann et al. 2014). Additionally, I replaced R583 with a glutamic acid, which harbors a negative charge instead of a positive one, and its respective amide glutamine. Both substitutions have been

previously used to study the functional role of arginines (Burgdorf et al. 2011; Sandberg et al. 2004). As shown in **Figure 3.20D**, all mutants showed a strongly reduced expression.

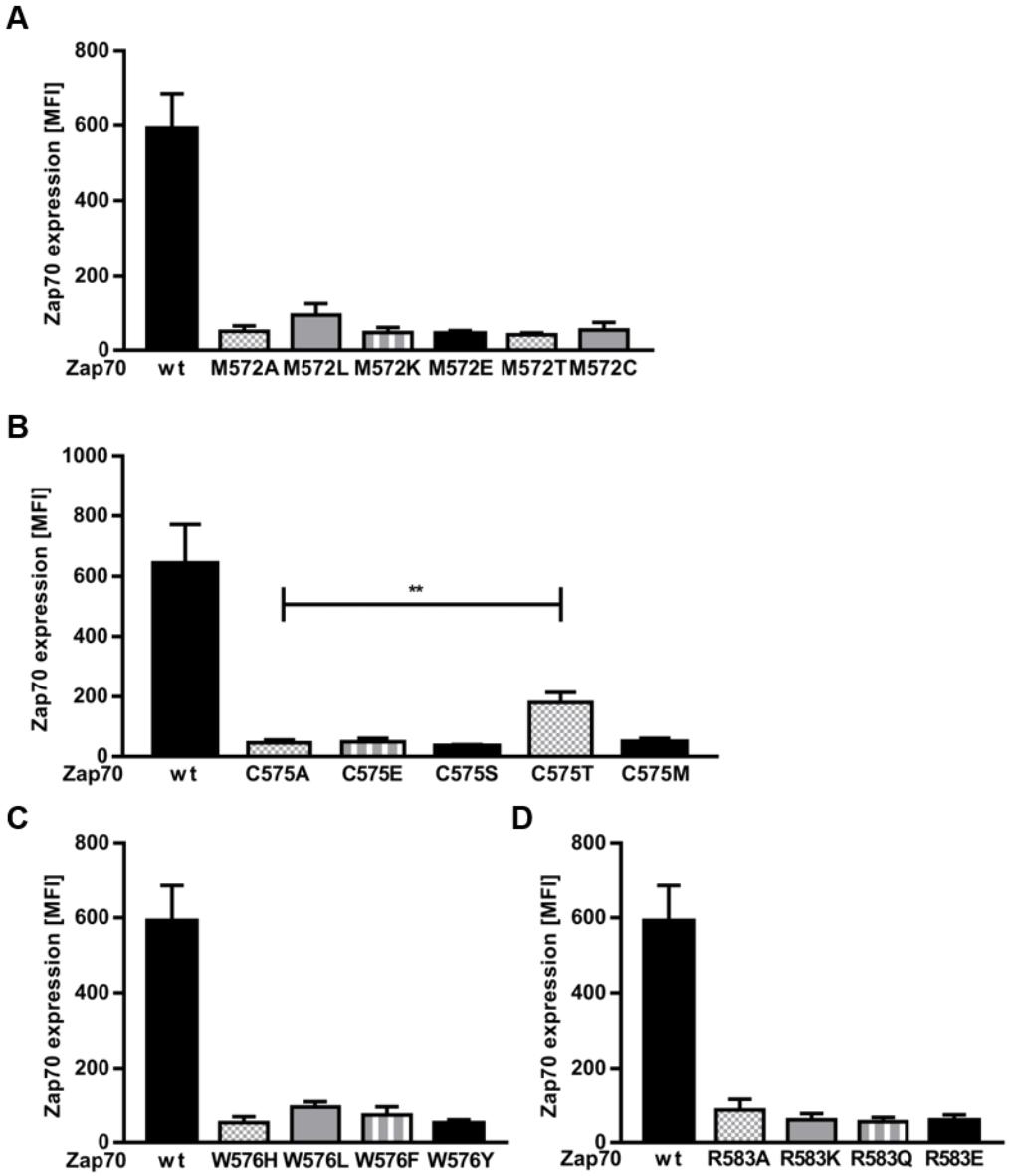


Figure 3.20: **Functional screening of mutants of the $Mx_{(2)}CWx_{(6)}R$ motif.** The indicated mutations have been introduced into eYFP-tagged Zap70 by site directed mutagenesis. Zap70 mutants were expressed in P116 cells and Zap70 expression was determined by the mean fluorescence intensity of the eYFP-tag by flow cytometry.

(A) Functional screening of M572 mutants. (n=3)

(B) Functional screening of C575 mutants. (n≥3) (**p<0.005)

(C) Functional screening of W576 mutants. (n=3)

(D) Functional screening of R583 mutants. (n=3)

3.2.12 The core cysteine within the $Mx_{(2)}CWx_{(6)}R$ motif regulates the stability of cytosolic tyrosine kinases

As described above (**Figure 3.19A**), the vast majority of human tyrosine kinases possess the $Mx_{(2)}CWx_{(6)}R$-motif. Therefore, I investigated whether this motif is important for the regulation of the expression of other tyrosine kinases. I selected six different kinases for my studies - Src, Syk, Jak2, BTK, Abl1, and c-Met. Of these kinases, one is a receptor tyrosine-kinase (c-Met) and one is associated to the cell membrane via myristylation (Src). The other four kinases are cytosolic. I expressed these kinases as either wt or cysteine mutants in HEK293T cells and assessed their expression. Analysis of the different mutants clearly showed a differential effect on their expression (**Figure 3.21**). Mutation of the cysteine in Syk, BTK, Jak2, and Abl1b resulted in a clear decrease in their expression. Mutation of C494 in Abl1b decreased the expression of ~50% compared to the wt protein (**Figure 3.21A**). Even more striking was the effect of Cys-to-Ala mutations in BTK, Jak2, and Syk. The protein levels of BTKC633A, Jak2C1105A, and SykC608A were ~10% of the respective wt (**Figure 3.21B-D**). In contrast, mutation of the cysteines in c-Met and c-Src did not affect the expression of these kinases (**Figure 3.21E** and **F**). Collectively, these results indicate a differential dependency of the expression of the tyrosine kinases on the conserved cysteine within the $Mx_{(2)}CWx_{(6)}R$ motif. Indeed, the stability of cytosolic kinases (Jak2, BTK, Syk, Abl1b, and Zap70) is strongly affected upon mutation of the conserved cysteine, whereas membrane-associated (Src) or transmembrane kinases (c-Met) do not lose their stability.

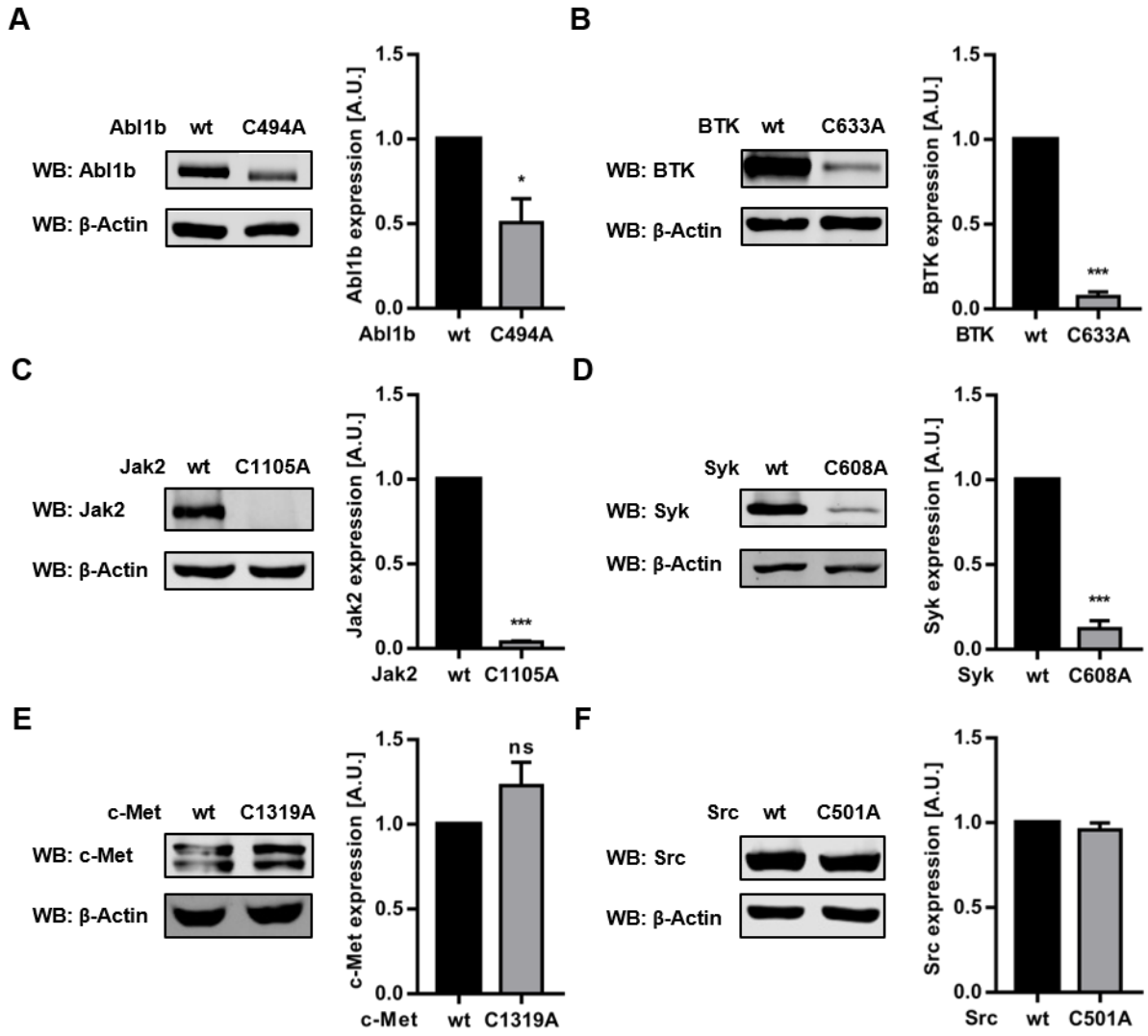


Figure 3.21: **Analysis of the expression of the Cys-to-Ala mutants of Abl1b, BTK, Jak2, Syk, c-Met, and Src.** The wt kinases and respective CA mutants were expressed in HEK293T cells. Cells were lysed and proteins were separated by SDS-PAGE. Expression of the kinases was determined by immunoblot analysis. β-Actin served as control for equal loading. For each kinase, a representative western blot and the quantification of the expression are shown. Expression of the individual proteins was quantified according to the signal intensities of the respective bands. Values were normalized against β-Actin followed by normalization against the respective wt which was assigned to 1.

(A) Analysis of Abl1bC494A. (n=3) (*P<0.05)

(B) Analysis of BTKC633A. (n=3) (***P<0.001)

(C) Analysis of Jak2C1105A. (n=3) (***P<0.001)

(D) Analysis of SykC608A. (n=3) (***P<0.001)

(E) Analysis of c-MetC1319A. (n=3) (ns=not statistically significant)

(F) Analysis of SrcC501A. (n=4)

4. Discussion

The characterization of the Zap70C575A mutant presented in this work revealed 5 main findings:

(1) The highly conserved C575 in Zap70 is crucial for both the stability and the activity of the kinase.
(2) C575 is likely constitutively sulfenylated.
(3) Overexpression of the co-chaperone Cdc37 partially rescues the stability but fully the activity of Zap70.
(4) C575 is localized within a highly conserved $Mx_{(2)}CWx_{(6)}R$ motif which regulates the stability of Zap70 and its oxidation.
(5) The destabilization upon substitution of the conserved cysteine with alanine is mainly restricted to cytosolic kinases.

These data lead to formulation of 3 main hypotheses:

I. The $Mx_{(2)}CWx_{(6)}R$ motif is a conserved mechanism for the regulation of tyrosine kinases with a dual role depending on the localization of the kinase. It regulates the stability of cytosolic tyrosine kinases and the activity of all human PTKs.
II. The $Mx_{(2)}CWx_{(6)}R$ motif is required to enable an efficient oxidation of the core cysteine residue.
III. The $Mx_{(2)}CWx_{(6)}R$ motif mediates protein-protein interactions likely with members of the chaperone system.

In the following sections, I will try to discuss these hypotheses and I will provide some possible explanations to the observed results. However, this study is the first to analyze the role of the $Mx_{(2)}CWx_{(6)}R$ motif in tyrosine kinases which leads to a general lack of data besides these presented in this work. Therefore, these explanations can only be considered as suggestions and need to be proven in subsequent studies.

4.1 Human tyrosine kinases with single mutations in the $Mx_{(2)}CWx_{(6)}R$ motif are not localized in the cytosol

The data presented in this thesis indicate an essential role of the $Mx_{(2)}CWx_{(6)}R$ motif not only in the regulation of Zap70 but also of other human tyrosine kinases. Sequence alignment studies revealed that the motif is a universal feature of human tyrosine kinases (**Figure 3.19A**). Nevertheless, of the 91 tyrosine kinases of the human kinome, four do not possess the full-length motif (**Figure 4.1**). In two of these exceptions, the core cysteine is replaced by a threonine (Fgr and EphB6), whereas in the other two kinases the methionine is replaced by a non-polar amino acid (M-to-I in BLK and M-to-A in TNK1). Detailed analysis of the sub-cellular localization of these four kinases reveals that all are localized at the plasma membrane. BLK and Fgr are both members of the SFKs and share an N-terminal myristylation site which anchors these kinases in the plasma membrane (Murray et al. 1997). EphB6 belongs to the family of ephrin receptors which are RTKs and therefore are per definition transmembrane molecules (Lisabeth et al. 2013). The last studied kinase not harboring the full-length motif is TNK1. The domain organization of this kinase does not bear a classical membrane-targeting module. However, one of the few reports about this kinase clearly shows that the majority of cellular TNK1 is targeted to the plasma membrane by an unknown mechanism (Felschow et al. 2000). These data in conjunction with the data obtained using Cys-to-Ala mutants of Jak2, Syk, BTK, Abl1, c-Src, and c-Met (**Figure 3.21**) clearly suggest that the localization of the kinase discriminates whether the $Mx_{(2)}CWx_{(6)}R$ motif regulates its stability or not (**Figure 4.2**). In fact, the stability of c-Src and c-Met was not affected by the Cys-to-Ala substitution. In contrast, the stability of cytosolic tyrosine kinases Zap70, Syk, BTK, Abl1, and Jak2 is strongly impaired upon mutation of the core cysteine. This explains why the four kinases which do not have a full-length motif are expressed. I hypothesize that this is due to their localization at the plasma membrane, which may protect them from degradation mechanisms which appear to be active in the cytosol. The data I obtained by targeting Zap70 to the plasma membrane are in favor of this hypothesis (**Figure 3.14**). The Zap70C575A mutant regained protein expression comparable to the wt kinase upon forcing it to the plasma membrane which indicates that the regulation of protein stability by the $Mx_{(2)}CWx_{(6)}R$ motif is solely dependent on the localization of the kinase and does not depend on the kinase itself. Thus, it is very likely that the mechanism by which the $Mx_{(2)}CWx_{(6)}R$ motif regulates protein stability is at least in part dependent on cytosolic factors probably interacting with the motif. Possible candidates for the interaction with the motif could be members of the chaperone system as these can directly influence the stability and activity of tyrosine kinases (discussed below). Additionally, changes in the redox state of the different compartments could explain the different role of the motif in the regulation of kinases stability

(**Figure 4.2**). Indeed, it is well described that the cellular compartments and organelles exhibit great differences in their respective redox states and therefore localization could modulate the oxidation state of the motif and hence its function (Go, Jones 2008).

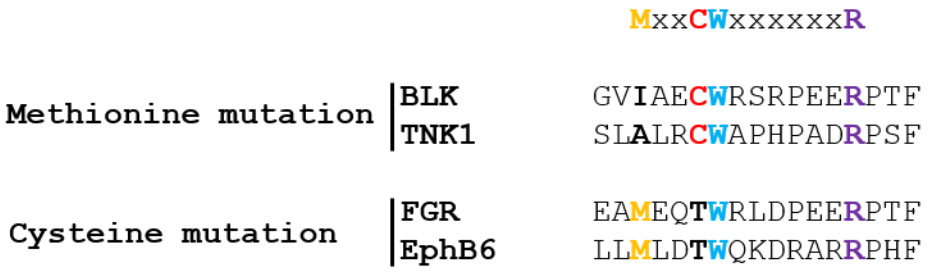


Figure 4.1: **Four human tyrosine kinases have single point mutations within the $Mx_{(2)}CWx_{(6)}R$ motif.**

Interestingly, it seems that kinases lacking the core cysteine have adapted to a new function or have developed alternative mechanisms to regulate their activity. In fact, EphB6 is catalytically inactive and hence does not function as a kinase but rather as a scaffold protein regulating other tyrosine kinases like the SFK Fyn (Matsuoka et al. 2005). In contrast, Fgr is still catalytically active but the Cys-to-Thr substitution has required the development of a new mechanism to regulate its kinase activity. This includes a PKC-mediated phosphorylation of the threonine (Takeda et al. 2006). It would be interesting to test whether substitution of the threonine residues in EphB6 "reactivates" the kinase activity. A similar approach would be required to test whether a T-to-C mutation could render Fgr sensitive for redox-dependent regulation of its kinase activity as it is known for c-Src (Rahman et al. 2010).

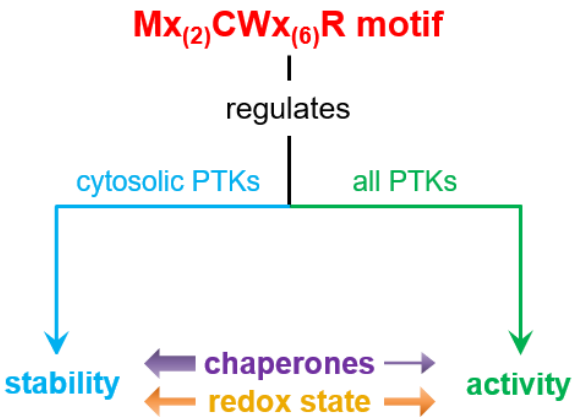


Figure 4.2: **The $Mx_{(2)}CWx_{(6)}R$ motif exerts different functions dependent on the localization of the kinase.**

4.2 The $Mx_{(2)}CWx_{(6)}R$ motif is evolutionary conserved

The presence of this conserved motif raises the question at which point during evolution this sequence appeared. The development of enzymes with tyrosine kinase activity in eukaryotes is from an evolutionary point of view a rather recent process. It is believed that the driving force in the establishment of these enzymes was the transition from uni- to multicellular organisms approx. 600 million years ago (Miller 2012). This is probably due to the fact that multicellularity requires sophisticated signaling networks for cell-cell communication. Consequently, a great variety of tyrosine kinases is found in metazoans (**Figure 4.3A**).

In the past decades, many efforts have been done in sequencing the whole genome of various organisms. This has made available the complete deciphering of the kinome of humans (Manning et al. 2002a), sea urchins (Bradham et al. 2006), fruit flies (Manning et al. 2002b), and Nematodes (Plowman et al. 1999). As Syk-family kinases (of which Zap70 is a member) are not present throughout all Metazoans, I have decided to look at the sequence of SFKs which are more ancient and hence allow me to follow their evolution throughout all Metazoan. As already mentioned above, the human kinome comprises 91 tyrosine kinases. The kinome of the sea urchin (*Strongylocentrotus purpuratus*) comprises in total 353 kinases of which 49 are classical tyrosine kinases (Bradham et al. 2006). Amongst these, 7 are members of the SFKs and 6 of them harbor the motif (**Figure 4.3B**). SFK1b, the only SFK without the motif, appears to be a truncated kinase lacking the whole C-terminal section of the kinase domain. Interestingly, of the 49 tyrosine kinases in *S. purpuratus*, 33 possess (~67%) the whole $Mx_{(2)}CWx_{(6)}R$ motif and additional 7 have one or two substitutions in the motif. The nine tyrosine kinases lacking the motif are characterized, like SFK1b, by a truncated kinase domain lacking the C-terminal end. Interestingly, these truncated kinase domains seem to have been established in a late phase of the evolution as, compared to the other organisms, this is a feature which can be found only in some Metazoans. This is further supported by the fact that amongst these truncated kinases are enzymes which are not truncated in humans and possess the motif, like TEC or RET.

Arthropods and Nematodes also belong to the Metazoans but are from a phylogenetic point of view more distant to vertebrates than *S. purpuratus*. Therefore, analysis of the kinome of members of these phyli allows to track the evolution of kinases within the Metazoans. Since the kinomes of *Drosophila melanogaster* (belonging to the Arthropods) and *Caenorhabditis elegans* (belonging to the Nematodes) have already been solved, analysis of the appearance of the $Mx_{(2)}CWx_{(6)}R$ motif is possible in these organisms. *D. melanogaster* possesses a total of 32 tyrosine kinases (Amit et al. 2007). All of these kinases harbor the motif either in full length (25 kinases = ~78%) or with single amino acid substitutions (7 kinases). In contrast, *C.*

elegans expresses 34 kinases (Amit et al. 2007) two of which are truncated and lack the C-terminal end of the kinase domain and therefore do not have the motif. The remaining 32 kinases, like for *D. melanogaster*, harbor either the full-length motif (16 kinases = ~47%) or the motif with one or two amino acid substitutions. In both organisms, members of the SFKs are expressed which possess the full-length motif (**Figure 4.3B**). Interestingly, all these analyzed Metazoans are characterized by the presence of the motif in the majority of their tyrosine kinases suggesting that this motif is not only conserved within human tyrosine kinases but also in other organisms. Furthermore, these examples nicely show the divergence in the evolution of tyrosine kinases within different phyli of Metazoans. In both *C. elegans* and *D. melanogaster*, more than 90% of the tyrosine kinases possess the motif either in full-length or with one or two substitutions (94% in *C. elegans* [16/34 harbor the full-length and 16/34 possess a single substitution] and 100% in *D. melanogaster* [25/32 harbor the full-length and 7/32 possess a single substitution]) a proportion comparable to human kinases. In contrast, despite its closer evolutionary relationship to humans, *S. purpuratus* has 9 kinases (~18%) lacking the motif indicating that these organisms developed these kinases after they have split in their evolution from the vertebrates.

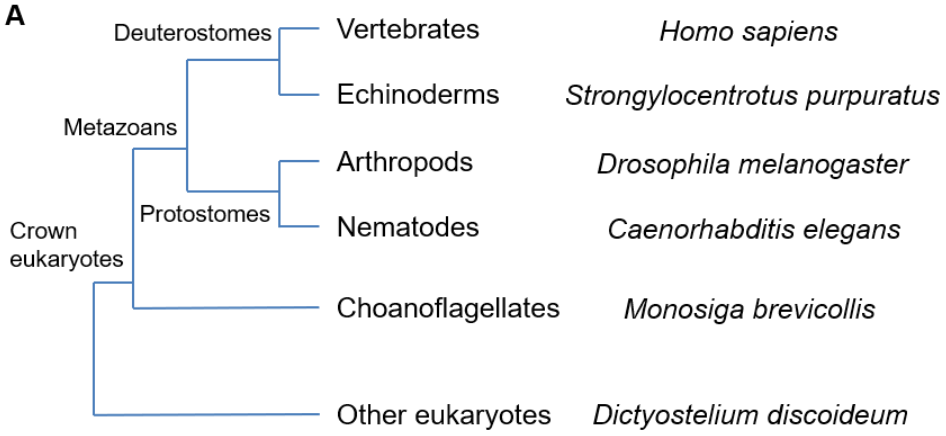
It is well known that tyrosine kinases can also be found outside of the Metazoans. Therefore, kinases from non-Metazoan organisms could indicate whether the $Mx_{(2)}CWx_{(6)}R$ motif emerged after or before the acquisition of multicellularity. The closest known relatives of the Metazoans are the Choanoflagellates (**Figure 4.3A**) (Lang et al. 2002; Steenkamp et al. 2006). It is believed that these organisms represent a transitional form between the uni- and multicellular life as these organisms are able to form colonies (Miller 2012). Most interestingly, it has been shown that the Choanoflagellates possess large numbers of tyrosine kinases. A well-studied member of the Choanoflagellates is *Monosiga brevicollis* which expresses 97 tyrosine kinases of which 85 possess the motif (**Figure 4.3B**). Also Src1, a member of the SFKs in this organism, already possesses the whole motif. The majority of the tyrosine kinases not possessing the motif are, like stated above, truncated versions. Interestingly, it seems that, while the other positions of the $Mx_{(2)}CWx_{(6)}R$ motif exhibit higher flexibility in their conservation, the arginine is conserved in all tyrosine kinases of *M. brevicollis* which are not truncated, indicating that at this point in evolution a general molecular mechanisms relying on the arginine residue seems to have been established already. In summary, this shows that even in the tyrosine kinases of the ancestors of the Metazoans the motif was present and likely of major importance.

Unfortunately, the exact evolution of the kinase domain of tyrosine kinases is not known. This makes it difficult to evolutionary follow the appearance of the $Mx_{(2)}CWx_{(6)}R$ motif. However, the presence of enzymes able to phosphorylate tyrosine residues has been also found in

organisms which are evolutionary far more distant from the Metazoans. The slime mold *Dictyostelium discoideum* does not possess classical tyrosine kinases. Yet, kinome analysis has revealed the expression of tyrosine kinase-like proteins in this organism (Goldberg et al. 2006). Interestingly, the motif is already present in the 63 members of this class of proteins. It seems that these enzymes do not possess the whole $Mx_{(2)}CWx_{(6)}R$ motif, as in the majority of the molecules the methionine residue is replaced by isoleucine or valine. However, also in some of the tyrosine kinase-like proteins the full motif can be found (**Figure 4.3B**).

Collectively, these analyses clearly reveal that the $Mx_{(2)}CWx_{(6)}R$ motif is evolutionary highly conserved and was even established before the classical tyrosine kinases have evolved. It seems that with the transition to multicellular organisms and the increase in tyrosine kinases also the motif in its final form was established as a fundamental part of the kinase domain.

A



B

H. sapiens	**Zap70**	567	LYALMSDCWIYKWEDRPDFL
	c-Src	493	LHDLMCQCWRKEPEERPTFE
S. purpuratus	**SFK1**	470	LYEIMLKCWHKDPQQRPTFE
D. melanogaster	**Src42A**	474	LYEIMLECWHKDPMRRPTFE
C. elegans	**Src-2**	463	LYDIMQQCWRSDPDKRPTFE
M. brevicollis	**Src1**	452	LYNIMLDCWKFKPDERPTFE
D. discoideum	**DPYK2**	1083	LKLLLTQCWEADPNDRPPFT

Figure 4.3: **Evolution of tyrosine kinases.**

(A) Phylogenetic tree of metazoans and other eukaryotes possessing tyrosine kinases or tyrosine kinase-like proteins in which the $Mx_{(2)}CWx_{(6)}R$ motif is conserved.

(B) Sequence alignment of tyrosine kinases and tyrosine kinase-like proteins from different organisms harboring the $Mx_{(2)}CWx_{(6)}R$ motif.

4.3 How does the $Mx_{(2)}CWx_{(6)}R$ motif regulates PTKs?

4.3.1 The $Mx_{(2)}CWx_{(6)}R$ motif could serve as a global switch for PTK activation

Since the emergence of ROS and RNS as mediators of signaling and the idea that PTKs could be regulated in a redox-dependent manner, a concept has been established suggesting that the regulation of tyrosine kinases may be organized by the action of two distinct switches. It has been proposed that one of these switches acts on a local level and is specific for the respective kinase. This local switch is thought to be mediated by auto- trans-phosphorylation, a feature which is common for most PTKs (Dennis, Bradshaw 2010). Additionally, this theory proposes the existence of a second switch that acts on a global level which is a prerequisite for the local switch to be functional. Since this switch is proposed to be universal for the class of PTKs, it should be based on a highly conserved structural feature. The $Mx_{(2)}CWx_{(6)}R$ motif might meet this criterium and it has been indeed proposed that the $Mx_{(2)}CWx_{(6)}R$ motif could serve as this kind of global switch (Nakashima et al. 2005). However, the concept of the $Mx_{(2)}CWx_{(6)}R$ motif as a global switch, which has been described before, is to date purely hypothetical and needs further investigations. Additionally, the hypothesis proposed by Nakashima and coworkers has not taken into account the presence of a highly conserved arginine residue and therefore only discussed the role of a $Mx_{(2)}CW$ motif. Furthermore, the duality in its actions (regulation of the stability and activity of kinases) has also not been taken into account. In the following chapters, I will further discuss the possible function of the previously described motif also in light of my recent data.

The function of the $Mx_{(2)}CWx_{(6)}R$ motif as a global switch for the regulation of kinases requires a switch like behavior which means the existence of two distinct states which would be considered as on and off (Sauvage et al. 2001), comparable to the two possible states of a tyrosine residue (phosphorylated and non-phosphorylated). Oxidation of cysteine residues is known to regulate the function of tyrosine kinases in various flavors (Corcoran, Cotter 2013) and is therefore also in the context of the $Mx_{(2)}CWx_{(6)}R$ motif as a putative switch regulating the activity of all human tyrosine kinases of interest. Various studies addressed the functional role of the core cysteine in different PTKs. To do so, different approaches were chosen which finally indicated a possible redox-switch-like behavior without proving the oxidation of the cysteine (indirect approach) or even have proven that the core cysteine is oxidized in certain kinases (direct approach).

4.3.1.1 Evidences that the $Mx_{(2)}CWx_{(6)}R$ motif is redox active

The functional relevance and the reactivity of the core cysteine has been shown so far only for a limited number of kinases including Lck (Trevillyan et al. 1999), v-Src (Senga et al.

2000), PDFGRβ (Lee et al. 2004), Yes, Lyn, and FAK (Rahman et al. 2008). In the majority of these studies, it has been demonstrated that the cysteine residue reacts with alkylating agents. Despite the fact that alkylation is no proof for oxidation, this observation suggests that the cysteine is reactive and hence that it has the potential to be also sulfenylated or nitrosylated. To assess whether modifications of the cysteine are also functionally relevant, cysteine to alanine or serine mutants were generated. For all kinases tested, substitution of the cysteine strongly impaired the activity of the kinase (**Table 4.1** and **Figure 4.2**) (Lee et al. 2004; Rahman et al. 2008; Veillette et al. 1993). Collectively, these data indicate that the core cysteine seems to be reactive and also crucial for proper kinase activity of Lck, v-Src, PDGFRβ, Yes, Lyn, and FAK, thus supporting the hypothesis that post-translational modifications of the core cysteine within the $Mx_{(2)}CWx_{(6)}R$ motif represent a general mechanism for the regulation of tyrosine kinases.

One of the major questions to be assessed is whether the cysteine is oxidized or not. Cysteine oxidation depends on many factors such as the oxygen species involved, the amino acid sequence of the protein, and cellular environment (e.g. subcellular localization) (Truong, Carroll 2013). In my work, I have described that C575 in Zap70 likely undergoes sulfenylation and therefore represents a thiol-sulfenic acid switch. This switch has been identified also in other kinases. For example, c-Src activity is regulated by a thiol-nitrosothiol switch upon NO production during cell invasion (Rahman et al. 2010). Conversely, cell adhesion seems not to induce oxidation of the core cysteine in c-Src (**Table 4.1**) (Giannoni et al. 2005). Unfortunately, in the latter study, an alkylating agent BIAM (biotinylated iodoacetamide), which labels reduced cysteines, was used for the detection of cysteine oxidation. Therefore, cysteine oxidation was quantified indirectly by detection of non-oxidized cysteines. Hence, these data could also be attributed to a lack of reactivity of the cysteine towards the alkylating agent which would also mask the detection of oxidation by this method. However, these studies suggest that these switches are context-dependent regulated and that not every stimulus triggers the switch to the same extent. Another form of thiol-switch, more precisely a thiol-disulfide bond switch, was identified in the RTK c-Ret. In this case, the core cysteine builds a disulfide bond with the core cysteine of another kinase upon UV or osmotic stress (Kato et al. 2000a). This homodimerization fosters transphosphorylation and increases the enzymatic activity of the kinases.

4.3.1.2 The role of the $Mx_{(2)}CWx_{(6)}R$ motif in the regulation of PTK stability is understudied

Just a few studies have assessed the role of the cysteine in the regulation of protein stability. Among them, one report has focused on Lck. The authors describe that the Cys-to-Ala

substitution in murine Lck (C475) results in an almost complete loss of protein expression due to decreased stability (**Table 4.1**) (Veillette et al. 1993). As Lck is localized at the plasma membrane, this studies could be considered as contradicting the hypothesis that the regulation of the protein stability by the $Mx_{(2)}CWx_{(6)}R$ motif depends on the subcellular localization of the respective kinase. Yet, this study has certain weaknesses which must be considered before to withdraw the hypothesis. These data are in contrast with results from our laboratory showing that the stability of an Lck-mutant harboring a Cys-to-Ala substitution is also reduced but to a lesser extent (about-30-40%) (unpublished data). The reason for this discrepancy is unclear. It is possible that the different cell lines used in our vs. Veillette´s study could account for the discrepant results. In fact, we express Lck in the Lck-deficient Jurkat T-cell line JLck in our study (Courtney et al. 2017), whereas Veillette and co-workers used the murine NIH3T3 fibroblast cell line. It is possible that the membrane localization of Lck at the plasma membrane is altered and hence Lck may be mostly expressed in the cytosol of NIHT3T cells. Unfortunately, this issue was not addressed in the publication. However, in another report, Bijlmakers and colleagues studied the localization of Lck in these cells (Bijlmakers et al. 1997). The group showed that the localization of wt Lck is distinct from that of Lck mutants deficient for membrane binding. Yet, also the localization of the wt molecule appeared not to be exclusively at the plasma membrane supporting the possible mislocalization of the LckC475A mutant in the Veillette study. Therefore, when considering these limitations of the study the available data still suggests that mainly the stability of cytosolic PTKs is regulated by the core cysteine (and probably the whole motif) (**Figure 4.2**).

Table 4.1: **Role of the cysteine within the $Mx_{(2)}CWx_{(6)}R$ motif in the regulation of the function of different kinases.** Kinases whose function is known to be regulated by the core cysteine of the $Mx_{(2)}CWx_{(6)}R$ motif are listed. The position of the core cysteine and its post-translational modification, the effects on the stability and activity are indicated. In brackets are the conditions described under which the effect on the activity was observed. ↓ - reduced; ↑ - increased;

Kinase	Cys	Modification	Stability	Activity	Ref.
Zap70	575	sulfenylation	↓	↓ (CA substitution)	(Thurm et al. 2017)
Lck	475	alkylation	↓*	↓ (CA substitution)	(Trevillyan et al. 1999; Veillette et al. 1993)
v-Src	498	ND	↓	↓ (CA substitution)	(Senga et al. 2000)
c-Src	498	ND	ND	= (upon Adhesion)	(Giannoni et al. 2005)
c-Src	498	nitrosylation	ND	↑ (upon Invasion)	(Rahman et al. 2010)
c-Ret	987	S-S bond	ND	↓ (CA substitution)	(Kato et al. 2000a)
PDGFRβ	940	ND	ND	↓ (CS substitution)	(Lee et al. 2004)
Yes	506	alkylation	ND	↓ (CA substitution)	(Rahman et al. 2008)
Lyn	479	alkylation	ND	↓ (CA substitution)	(Rahman et al. 2008)
FAK	658	alkylation	ND	↓ (CA substitution)	(Rahman et al. 2008)

4.3.1.3 Limitations of the available studies on the $Mx_{(2)}CWx_{(6)}R$ motif

As described above, the possible role of the core cysteine of the $Mx_{(2)}CWx_{(6)}R$ motif as the essential player in the regulation of PTKs has been reinforced by various reports. However, these studies share certain limitations. All of the described studies addressed the role of the core cysteine isolated from the motif and hence neglected the important role of the other residues. Furthermore, the majority of studies available only focus on kinases whose stabilities were not affected by mutation of the core cysteine. Therefore, the possible dual role of the motif has not been assessed in detail. The data presented in this thesis represent, to my knowledge, for the first time a study which focusses, on the one hand on a possible synergism of the whole motif and, on the other hand, on dissecting the effects on both protein activity and protein stability.

4.3.2 Is the $Mx_{(2)}CWx_{(6)}R$ motif required to modulate the reactivity of the core cysteine?

Based on the available data, the importance of the $Mx_{(2)}CWx_{(6)}R$ motif for the activity and stability of tyrosine kinases cannot be doubted. However, a major question in this regard has not been assessed so far. If the core cysteine represents a thiol-switch, what is the role of the other amino acids within the motif? In this paragraph, I will present a possible molecular mechanism which could explain why the whole motif is of importance. However, due to the general lack of data focusing on the molecular function of the $Mx_{(2)}CWx_{(6)}R$ motif this mechanism should only be considered as a possibility.

To understand the role of the motif we have to understand first what the requirements are for a cysteine to become reactive. To date, a number of computational approaches have been applied to estimate the susceptibility of certain cysteines to undergo oxidation. These studies have revealed several factors which are critical, like the distance to other cysteine residues, solvent accessibility, or pKa values (Sanchez et al. 2008). The quintessence of these studies is that, in order to serve as a regulatory switch, the reactivity of the cysteine needs to be increased. This is usually achieved upon deprotonation of the thiol (Truong, Carroll 2013). For many critical catalytically active cysteines, the molecular mechanism by which a thiol is deprotonated has been characterized like in the ROS-detoxifying enzyme peroxiredoxin (Wood et al. 2003). Its catalytic cycle requires the involvement of two critical amino acids which increase the reactivity of the cysteine. In the first step, the proton of the thiol group is attacked by a nucleophilic amino acid resulting in the formation of a highly reactive thiolate. This negative charge is then stabilized by a positively charged arginine enabling efficient reaction with the target molecule. In analogy to this reaction, the main function of the arginine residue of the $Mx_{(2)}CWx_{(6)}R$ motif might be the stabilization of the thiolate (**Figure 4.4**). The

methionine residue could serve as the equivalent nucleophilic amino acid enabling the deprotonation of the thiol group (Brotzel, Mayr 2007). An additional factor in the stabilization of the thiolate form is the tryptophan residue. Its extended aromatic ring could stabilize the thiolate form due to anion-π interactions (Schottel et al. 2008). In summary, the biochemical properties of the conserved amino acids could lead to the concerted deprotonation of the thiol followed by stabilization of the anion by electrostatic and anion-π interactions.

This described mechanism could be a novel way to regulate the oxidation of a crucial cysteine residue. However, to convincingly support this hypothesis based on the data presented in this thesis is difficult. My data show that the whole motif is involved in the regulation of Zap70 sulfenylation, likely via C575. In fact, substitution of the motif led to a significantly reduced total Zap70 sulfenylation regardless which amino acid was substituted. Therefore, it is highly likely that these amino acids act in concert to mediate Zap70 sulfenylation. However, identification of the chemical properties required to fulfill this task failed as all the mutants generated for the respective positions turned out to be instable. This effect was independent whether the obvious chemical properties of the amino acids were preserved in the substitution or not. These results formally do not support the suggested mechanism but are also unable to disprove it since no substitution was discovered which, on the one hand results in a stable kinase and, on the other hand has chemical properties distinct from those required for the described oxidation of the core cysteine. It is highly likely that besides the chemical properties, which can be deduced from the structure of the amino acid, additional factors are of importance. When it is taken into consideration that all human PTKs possess the motif and that the four kinases which bear point mutations are not located in the cytosol it can be speculated that it simply might be impossible to substitute single amino acids in a given cytosolic PTK without decreasing protein stability otherwise this substitution would likely be found in the human kinome. It might be of interest to study the structural adaptations of truncated kinases from other organisms and compare them with the respective human full-length counterpart. This could reveal additional mutations which are required to restore the expression of cytosolic PTKs. Furthermore, since protein expression served as the only readout in my work it is well possible that one of these mutants might restore Zap70 sulfenylation and be catalytically active like the wt kinase. Unfortunately, I was unable to address these points in my thesis.

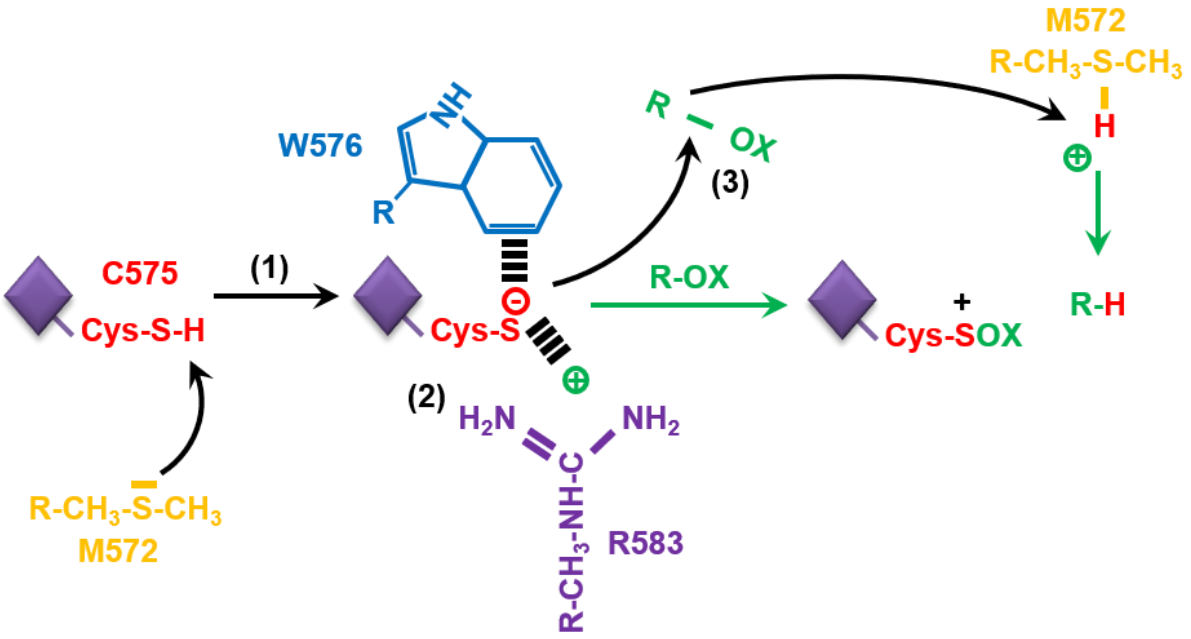


Figure 4.4: **Proposed mechanism of the oxidation of the core cysteine in the $Mx_{(2)}CWx_{(6)}R$ motif.** Molecular mechanism of the stabilization of the thiolate. (1) Nucleophilic attack of the methionine residue leads to the deprotonation of the thiol. (2) The thiolate is stabilized by electrostatic interactions with the arginine and by anion-π-interaction with the tryptophan. (3) The thiolate reacts with an oxidizing agent leading to the oxidation of the core cysteine and the recovery of the methionine residue. Indicated are the respective residues in Zap70.

4.4 The $Mx_{(2)}CWx_{(6)}R$ motif could regulate the interaction with the co-chaperone Cdc37

Structural changes in the $Mx_{(2)}CWx_{(6)}R$ motif by mutation appear to affect both kinase stability and activity. Molecular chaperones are required not only for the regulation of protein stability but also for kinase activity (Kim et al. 2013). Members of the heat-shock-proteins (HSPs) HSP40, HSC70, and HSP90 are well-known chaperones. These molecules bind nascent polypeptides dependent on their maturation state, with HSP40 recognizing the most immature peptides. HSP40 is followed by the binding of HSC70 and finally HSP90 which binds and stabilizes proteins throughout their final maturation (Young et al. 2004). Due to its broad substrate specificity the function of HSP90 is supported by several co-chaperones. Amongst them, Cdc37 is especially known to recognize and bind kinases. Unlike many other co-chaperones, Cdc37 is known to bind client proteins also in the absence of HSP90 and therefore can stabilize client proteins itself (MacLean, Picard 2003).

How the Cdc37-client interactions are regulated has been studied for many years. To date, it is evident that Cdc37 binds the kinase domain of client kinases. However, which parts of the kinase domain are recognized by Cdc37 is still a matter of debate. It is very likely that for proper function of Cdc37 multiple interactions with the kinase domain are required. It has been shown that Cdc37 can bind the N-terminal as well as the C-terminal lobes of the kinase domain (Terasawa, Minami 2005; Terasawa et al. 2006). Furthermore, specific sequences within these structures have been identified which are conserved and responsible for the interaction with Cdc37 (Zhao et al. 2004). However, experimental data suggest that to achieve a stable Cdc37-client interaction binding to the whole kinase domain is required (Prince, Matts 2004). Interestingly, despite the large number of interaction sites, Cdc37 binding seems to be sensitive even to point mutations within these conserved regions (Zhao et al. 2004).

Based on the data generated for my Ph.D. thesis, I propose that the $Mx_{(2)}CWx_{(6)}R$ motif could be an additional interacting sequence with Cdc37. Remarkably, I have shown that overexpression of Cdc37 rescues the stability (partially) and the activity of Zap70C575A.

Whether the interaction between Zap70 and Cdc37 is redox-dependent needs also to be further investigated. It is possible that the motif serves as a sensor for the redox-state of the cell which leads to deactivation and destabilization of kinases upon excessive production of ROS or RNS which could lead to hyper-oxidation of the core cysteine, conditions which are often associated to chronic inflammation or cancer.

With regard to the restored stability of Zap70 upon targeting to the plasma membrane, further studies are required to identify the factors which discriminate membrane-localized and cytosolic kinases. It is possible that other membrane-associated chaperones exist which are able to stabilize kinases independently of the motif and thereby protect membrane-bound kinases. In this case, the motif would only be required to serve as the global switch in the regulation of the activity of the kinase which is the reason why it is also present in RTKs.

4.5 Does the $Mx_{(2)}CWx_{(6)}R$ motif represent a target for the development of drugs modulating tyrosine kinase activity?

Deregulation of tyrosine kinases are the driving force for several human pathologies as cancer, immunodeficiency, or autoimmunity (Goodman et al. 2003; Do et al. 2004; Buckley et al. 1997; Siegel et al. 2002). For example, a single Val-to-Phe mutation at position 617 in Jak2 is known to induce myeloproliferative disease (McLornan et al. 2006). This mutation results in the constitutive activation of Jak2 and therefore chronic activation of the associated signaling pathways.

My data indicate that the $Mx_{(2)}CWx_{(6)}R$ motif influences also the stability of Jak2, Syk, Abl1, and BTK. These observations, together with published data showing that the cysteine is crucial for the kinase activity of different enzymes, raise the question whether targeting the motif could be a promising approach for the development of tyrosine kinase inhibitors. Of the "classical" tyrosine kinase inhibitors, the majority is directed against the ATP-binding site preventing binding of ATP and thereby phosphorylation of the target (Liu et al. 2013). The major challenge of this approach is the high degree of conservation of the ATP-binding site which renders most of these inhibitors unselective at high concentrations. Most likely, this would also be the case for inhibitors against the $Mx_{(2)}CWx_{(6)}R$ motif as it is also highly conserved among all human tyrosine kinases. However, since only the four amino acids of the motif are highly conserved it could be possible to design specific inhibitors based on structural features of non-conserved amino acids which are nearby. This could lead to the development of agents attacking the conserved cysteine, but which are dependent on residues which are specific for a single kinase.

4.6 Analysis of additional cysteine residues in Zap70

The screening of the functional role of cysteine residues in Zap70 revealed that not only C575 but also other cysteine residues appear to play a role in the regulation of Zap70 function. For example, substitutions of the cysteine residues at positions 39 and 564 appeared to influence the ability of the respective Zap70 mutants to reconstitute TCR signaling. The role of C39 in the regulation of Zap70 has already been assessed in a previous study. This work suggested that this residue is sensitive to oxidation which impairs Zap70-binding to the TCRζ-chains (Visperas et al. 2015). However, the data obtained in this thesis indicate that the non-oxidizable C39A-mutant is impaired in its ability to reconstitute TCR signaling which argues against this hypothesis (**Figure 3.5**). Also, the analysis of C564 could be of major interest in order to explore the regulation of Zap70. As C575, C564 is a cysteine residue which is highly conserved in various kinases (**Figure 3.1**). Interestingly, many tyrosine kinases possess a cluster of conserved cysteine residues in this area of the kinase domain (in Zap70 this cluster comprises C560, C564, and C575). The possible synergism of these cysteine clusters in the regulation of PTKs has already been studied for c-Src which revealed that these clusters take part in the regulation of c-Src activity (Oo et al. 2003). Therefore, also in the light of the fact that the C564A-mutant, in contrast to the C575A-mutant, reconstitutes TCR signaling stronger than the wt molecule (**Figure 3.6**) the synergism of these cysteine residues in Zap70 should be investigated in more detail. Unfortunately, these residues could not be forwarded to in-depth characterization in this work.

5. Bibliography

Akhand, A. A.; Pu, M.; Senga, T.; Kato, M.; Suzuki, H.; Miyata, T. et al. (1999): Nitric oxide controls src kinase activity through a sulfhydryl group modification-mediated Tyr-527-independent and Tyr-416-linked mechanism. In *The Journal of biological chemistry* 274 (36), pp. 25821–25826.

Amit, Ido; Wides, Ron; Yarden, Yosef (2007): Evolvable signaling networks of receptor tyrosine kinases. Relevance of robustness to malignancy and to cancer therapy. In *Molecular systems biology* 3, p. 151. DOI: 10.1038/msb4100195.

Arndt, Boerge; Poltorak, Mateusz; Kowtharapu, Bhavani S.; Reichardt, Peter; Philipsen, Lars; Lindquist, Jonathan A. et al. (2013): Analysis of TCR activation kinetics in primary human T cells upon focal or soluble stimulation. In *Journal of immunological methods* 387 (1-2), pp. 276–283. DOI: 10.1016/j.jim.2012.11.006.

Arpaia, E.; Shahar, M.; Dadi, H.; Cohen, A.; Roifman, C. M. (1994): Defective T cell receptor signaling and CD8+ thymic selection in humans lacking zap-70 kinase. In *Cell* 76 (5), pp. 947–958.

Au-Yeung, Byron B.; Deindl, Sebastian; Hsu, Lih-Yun; Palacios, Emil H.; Levin, Susan E.; Kuriyan, John; Weiss, Arthur (2009): The structure, regulation, and function of ZAP-70. In *Immunological reviews* 228 (1), pp. 41–57. DOI: 10.1111/j.1600-065X.2008.00753.x.

Ball, K. Aurelia; Johnson, Jeffrey R.; Lewinski, Mary K.; Guatelli, John; Verschueren, Erik; Krogan, Nevan J.; Jacobson, Matthew P. (2016): Non-degradative Ubiquitination of Protein Kinases. In *PLoS computational biology* 12 (6), e1004898. DOI: 10.1371/journal.pcbi.1004898.

Bijlmakers, M. J.; Isobe-Nakamura, M.; Ruddock, L. J.; Marsh, M. (1997): Intrinsic signals in the unique domain target p56(lck) to the plasma membrane independently of CD4. In *The Journal of cell biology* 137 (5), pp. 1029–1040.

Block, Karen; Eid, Assaad; Griendling, Kathy K.; Lee, Duck-Yoon; Wittrant, Yohann; Gorin, Yves (2008): Nox4 NAD(P)H oxidase mediates Src-dependent tyrosine phosphorylation of PDK-1 in response to angiotensin II. Role in mesangial cell hypertrophy and fibronectin expression. In *The Journal of biological chemistry* 283 (35), pp. 24061–24076. DOI: 10.1074/jbc.M803964200.

Bradham, Cynthia A.; Foltz, Kathy R.; Beane, Wendy S.; Arnone, Maria I.; Rizzo, Francesca; Coffman, James A. et al. (2006): The sea urchin kinome. A first look. In *Developmental biology* 300 (1), pp. 180–193. DOI: 10.1016/j.ydbio.2006.08.074.

Brotzel, Frank; Mayr, Herbert (2007): Nucleophilicities of amino acids and peptides. In *Organic & biomolecular chemistry* 5 (23), pp. 3814–3820. DOI: 10.1039/b713778h.

Brownlie, Rebecca J.; Zamoyska, Rose (2013): T cell receptor signalling networks. Branched, diversified and bounded. In *Nature reviews. Immunology* 13 (4), pp. 257–269. DOI: 10.1038/nri3403.

Buckley, R. H.; Schiff, R. I.; Schiff, S. E.; Markert, M. L.; Williams, L. W.; Harville, T. O. et al. (1997): Human severe combined immunodeficiency. Genetic, phenotypic, and functional diversity in one hundred eight infants. In *The Journal of pediatrics* 130 (3), pp. 378–387.

Burgdorf, Sarah W.; Clark, Charles L.; Burgdorf, James R.; Spector, Deborah H. (2011): Mutation of glutamine to arginine at position 548 of IE2 86 in human cytomegalovirus leads to decreased expression of IE2 40, IE2 60, UL83, and UL84 and increased transcription of US8-9 and US29-32. In *Journal of virology* 85 (21), pp. 11098–11110. DOI: 10.1128/JVI.05315-11.

Castro, Januario E.; Prada, Carlos E.; Loria, Olivier; Kamal, Adeela; Chen, Liguang; Burrows, Francis J.; Kipps, Thomas J. (2005): ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client. Inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. In *Blood* 106 (7), pp. 2506–2512. DOI: 10.1182/blood-2005-03-1099.

Chalfie, Martin (Ed.): Green fluorescent protein. Properties, applications, and protocols. 2. ed. Hoboken, NJ: Wiley-Interscience (Methods of biochemical analysis, 47). Available online at http://site.ebrary.com/lib/alltitles/docDetail.action?docID=10304679.

Chan, A. C.; Dalton, M.; Johnson, R.; Kong, G. H.; Wang, T.; Thoma, R.; Kurosaki, T. (1995): Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. In *The EMBO journal* 14 (11), pp. 2499–2508.

Chan, A. C.; Iwashima, M.; Turck, C. W.; Weiss, A. (1992): ZAP-70. A 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. In *Cell* 71 (4), pp. 649–662.

Chan, A. C.; Kadlecek, T. A.; Elder, M. E.; Filipovich, A. H.; Kuo, W. L.; Iwashima, M. et al. (1994): ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. In *Science (New York, N.Y.)* 264 (5165), pp. 1599–1601.

Chan, Alice Y.; Punwani, Divya; Kadlecek, Theresa A.; Cowan, Morton J.; Olson, Jean L.; Mathes, Erin F. et al. (2016): A novel human autoimmune syndrome caused by combined hypomorphic and activating mutations in ZAP-70. In *The Journal of experimental medicine* 213 (2), pp. 155–165. DOI: 10.1084/jem.20150888.

Chen, Cheng-Hsien; Cheng, Tzu-Hurng; Lin, Heng; Shih, Neng-Lang; Chen, Yen-Ling; Chen, Yee-Shiuan et al. (2006): Reactive oxygen species generation is involved in epidermal growth factor receptor transactivation through the transient oxidization of Src homology 2-containing tyrosine phosphatase in endothelin-1 signaling pathway in rat cardiac fibroblasts. In *Molecular pharmacology* 69 (4), pp. 1347–1355. DOI: 10.1124/mol.105.017558.

Chen, Liguang; Widhopf, George; Huynh, Lang; Rassenti, Laura; Rai, Kanti R.; Weiss, Arthur; Kipps, Thomas J. (2002): Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. In *Blood* 100 (13), pp. 4609–4614. DOI: 10.1182/blood-2002-06-1683.

Corcoran, Aoife; Cotter, Thomas G. (2013): Redox regulation of protein kinases. In *The FEBS journal* 280 (9), pp. 1944–1965. DOI: 10.1111/febs.12224.

Courtney, Adam H.; Amacher, Jeanine F.; Kadlecek, Theresa A.; Mollenauer, Marianne N.; Au-Yeung, Byron B.; Kuriyan, John; Weiss, Arthur (2017): A Phosphosite within the SH2 Domain of Lck Regulates Its Activation by CD45. In *Molecular cell* 67 (3), 498-511.e6. DOI: 10.1016/j.molcel.2017.06.024.

Cutler, R. L.; Pielak, G. J.; Mauk, A. G.; Smith, M. (1987): Replacement of cysteine-107 of Saccharomyces cerevisiae iso-1-cytochrome c with threonine. Improved stability of the mutant protein. In *Protein engineering* 1 (2), pp. 95–99.

Del Principe, Maria Ilaria; Del Poeta, Giovanni; Buccisano, Francesco; Maurillo, Luca; Venditti, Adriano; Zucchetto, Antonella et al. (2006): Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. In *Blood* 108 (3), pp. 853–861. DOI: 10.1182/blood-2005-12-4986.

Dennis, Edward A.; Bradshaw, Ralph A. (Eds.) (2010): Handbook of cell signaling. Volume 1. 2nd ed. Amsterdam, Boston: Elsevier/Academic Press. Available online at http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=351537.

Di Bartolo, V.; Mège, D.; Germain, V.; Pelosi, M.; Dufour, E.; Michel, F. et al. (1999): Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling. In *The Journal of biological chemistry* 274 (10), pp. 6285–6294.

Do, Nam-Yong; Lim, Sung-Chul; Im, Tae-Seung (2004): Expression of c-erbB receptors, MMPs and VEGF in squamous cell carcinoma of the head and neck. In *Oncology reports* 12 (2), pp. 229–237.

Elder, M. E. (1997): SCID due to ZAP-70 deficiency. In *Journal of pediatric hematology/oncology* 19 (6), pp. 546–550.

Elder, M. E.; Lin, D.; Clever, J.; Chan, A. C.; Hope, T. J.; Weiss, A.; Parslow, T. G. (1994): Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. In *Science (New York, N.Y.)* 264 (5165), pp. 1596–1599.

Engelman, Jeffrey A.; Zejnullahu, Kreshnik; Gale, Christopher-Michael; Lifshits, Eugene; Gonzales, Andrea J.; Shimamura, Takeshi et al. (2007): PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. In *Cancer research* 67 (24), pp. 11924–11932. DOI: 10.1158/0008-5472.CAN-07-1885.

Fabbro, Doriano (2015): 25 years of small molecular weight kinase inhibitors. Potentials and limitations. In *Molecular pharmacology* 87 (5), pp. 766–775. DOI: 10.1124/mol.114.095489.

Fagioli, F.; Biasin, E.; Berger, M.; Nesi, F.; Saroglia, E. H.; Miniero, R. et al. (2003): Successful unrelated cord blood transplantation in two children with severe combined immunodeficiency syndrome. In *Bone marrow transplantation* 31 (2), pp. 133–136. DOI: 10.1038/sj.bmt.1703800.

Felschow, D. M.; Civin, C. I.; Hoehn, G. T. (2000): Characterization of the tyrosine kinase Tnk1 and its binding with phospholipase C-gamma1. In *Biochemical and biophysical research communications* 273 (1), pp. 294–301. DOI: 10.1006/bbrc.2000.2887.

Fischer, Alain; Picard, Capucine; Chemin, Karine; Dogniaux, Stéphanie; Le Deist, Françoise; Hivroz, Claire (2010): ZAP70. A master regulator of adaptive immunity. In *Seminars in immunopathology* 32 (2), pp. 107–116. DOI: 10.1007/s00281-010-0196-x.

Forrester, Michael T.; Foster, Matthew W.; Benhar, Moran; Stamler, Jonathan S. (2009): Detection of protein S-nitrosylation with the biotin-switch technique. In *Free radical biology & medicine* 46 (2), pp. 119–126. DOI: 10.1016/j.freeradbiomed.2008.09.034.

Gavino, Christina; Landekic, Marija; Zeng, Jibin; Wu, Ning; Jung, Sungmi; Zhong, Ming-Chao et al. (2017): Morpholino-based correction of hypomorphic ZAP70 mutation in an adult with combined immunodeficiency. In *The Journal of allergy and clinical immunology* 139 (5), 1688-1692.e10. DOI: 10.1016/j.jaci.2017.02.002.

Gelkop, Sigal; Gish, Gerrald D.; Babichev, Yael; Pawson, Tony; Isakov, Noah (2005): T cell activation-induced CrkII binding to the Zap70 protein tyrosine kinase is mediated by Lck-dependent phosphorylation of Zap70 tyrosine 315. In *Journal of immunology (Baltimore, Md. : 1950)* 175 (12), pp. 8123–8132.

Giannoni, E.; Buricchi, F.; Grimaldi, G.; Parri, M.; Cialdai, F.; Taddei, M. L. et al. (2008): Redox regulation of anoikis. Reactive oxygen species as essential mediators of cell survival. In *Cell death and differentiation* 15 (5), pp. 867–878. DOI: 10.1038/cdd.2008.3.

Giannoni, Elisa; Buricchi, Francesca; Raugei, Giovanni; Ramponi, Giampietro; Chiarugi, Paola (2005): Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. In *Molecular and cellular biology* 25 (15), pp. 6391–6403. DOI: 10.1128/MCB.25.15.6391-6403.2005.

Gilles, A. M.; Marlière, P.; Rose, T.; Sarfati, R.; Longin, R.; Meier, A. et al. (1988): Conservative replacement of methionine by norleucine in Escherichia coli adenylate kinase. In *The Journal of biological chemistry* 263 (17), pp. 8204–8209.

Giorgio, Marco; Migliaccio, Enrica; Orsini, Francesca; Paolucci, Demis; Moroni, Maurizio; Contursi, Cristina et al. (2005): Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. In *Cell* 122 (2), pp. 221–233. DOI: 10.1016/j.cell.2005.05.011.

Go, Young-Mi; Jones, Dean P. (2008): Redox compartmentalization in eukaryotic cells. In *Biochimica et biophysica acta* 1780 (11), pp. 1273–1290. DOI: 10.1016/j.bbagen.2008.01.011.

Goldberg, Jonathan M.; Manning, Gerard; Liu, Allen; Fey, Petra; Pilcher, Karen E.; Xu, Yanji; Smith, Janet L. (2006): The dictyostelium kinome--analysis of the protein kinases from a simple model organism. In *PLoS genetics* 2 (3), e38. DOI: 10.1371/journal.pgen.0020038.

Gong, Q.; Jin, X.; Akk, A. M.; Foger, N.; White, M.; Gong, G. et al. (2001): Requirement for tyrosine residues 315 and 319 within zeta chain-associated protein 70 for T cell development. In *The Journal of experimental medicine* 194 (4), pp. 507–518.

Goodman, Patricia A.; Wood, Carla M.; Vassilev, Alexei O.; Mao, Chen; Uckun, Fatih M. (2003): Defective expression of Bruton's tyrosine kinase in acute lymphoblastic leukemia. In *Leukemia & lymphoma* 44 (6), pp. 1011–1018. DOI: 10.1080/1042819031000067576.

Gossan, Nicole C.; Zhang, Feng; Guo, Baoqiang; Jin, Ding; Yoshitane, Hikari; Yao, Aiyu et al. (2014): The E3 ubiquitin ligase UBE3A is an integral component of the molecular circadian clock through regulating the BMAL1 transcription factor. In *Nucleic acids research* 42 (9), pp. 5765–5775. DOI: 10.1093/nar/gku225.

Hampton, M. B.; Kettle, A. J.; Winterbourn, C. C. (1998): Inside the neutrophil phagosome. Oxidants, myeloperoxidase, and bacterial killing. In *Blood* 92 (9), pp. 3007–3017.

Hänggi, Emanuel; Grundschober, Anne Freimoser; Leuthold, Simone; Meier, Peter J.; St-Pierre, Marie V. (2006): Functional analysis of the extracellular cysteine residues in the human organic anion transporting polypeptide, OATP2B1. In *Molecular pharmacology* 70 (3), pp. 806–817. DOI: 10.1124/mol.105.019547.

Hata, Hiroshi; Sakaguchi, Noriko; Yoshitomi, Hiroyuki; Iwakura, Yoichiroh; Sekikawa, Kenji; Azuma, Yoshiaki et al. (2004): Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. In *The Journal of clinical investigation* 114 (4), pp. 582–588. DOI: 10.1172/JCI21795.

Hatada, M. H.; Lu, X.; Laird, E. R.; Green, J.; Morgenstern, J. P.; Lou, M. et al. (1995): Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. In *Nature* 377 (6544), pp. 32–38. DOI: 10.1038/377032a0.

Hauck, Fabian; Blumenthal, Britta; Fuchs, Sebastian; Lenoir, Christelle; Martin, Emmanuel; Speckmann, Carsten et al. (2015): SYK expression endows human ZAP70-deficient CD8 T cells with residual TCR signaling. In *Clinical immunology (Orlando, Fla.)* 161 (2), pp. 103–109. DOI: 10.1016/j.clim.2015.07.002.

Hess, Douglas T.; Matsumoto, Akio; Kim, Sung-Oog; Marshall, Harvey E.; Stamler, Jonathan S. (2005): Protein S-nitrosylation. Purview and parameters. In *Nature reviews. Molecular cell biology* 6 (2), pp. 150–166. DOI: 10.1038/nrm1569.

Hess, Douglas T.; Stamler, Jonathan S. (2012): Regulation by S-nitrosylation of protein post-translational modification. In *The Journal of biological chemistry* 287 (7), pp. 4411–4418. DOI: 10.1074/jbc.R111.285742.

Holmström, Kira M.; Finkel, Toren (2014): Cellular mechanisms and physiological consequences of redox-dependent signalling. In *Nature reviews. Molecular cell biology* 15 (6), pp. 411–421. DOI: 10.1038/nrm3801.

Honigberg, Lee A.; Smith, Ashley M.; Sirisawad, Mint; Verner, Erik; Loury, David; Chang, Betty et al. (2010): The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. In *Proceedings of the National Academy of Sciences of the United States of America* 107 (29), pp. 13075–13080. DOI: 10.1073/pnas.1004594107.

Hu, Hongbo; Wang, Hui; Xiao, Yichuan; Jin, Jin; Chang, Jae-Hoon; Zou, Qiang et al. (2016): Otud7b facilitates T cell activation and inflammatory responses by regulating Zap70 ubiquitination. In *The Journal of experimental medicine* 213 (3), pp. 399–414. DOI: 10.1084/jem.20151426.

Huang, Kezhen; Wang, Yue-Hao; Brown, Alex; Sun, Gongqin (2009): Identification of N-terminal lobe motifs that determine the kinase activity of the catalytic domains and regulatory strategies of Src and Csk protein tyrosine kinases. In *Journal of molecular biology* 386 (4), pp. 1066–1077.

Hubbard, S. R.; Till, J. H. (2000): Protein tyrosine kinase structure and function. In *Annual review of biochemistry* 69, pp. 373–398. DOI: 10.1146/annurev.biochem.69.1.373.

Jaffrey, S. R.; Snyder, S. H. (2001): The biotin switch method for the detection of S-nitrosylated proteins. In *Science's STKE : signal transduction knowledge environment* 2001 (86), pl1. DOI: 10.1126/stke.2001.86.pl1.

Jin, Lei; Pluskey, Scott; Petrella, Eugene C.; Cantin, Susan M.; Gorga, Joan C.; Rynkiewicz, Michael J. et al. (2004): The three-dimensional structure of the ZAP-70 kinase domain in complex with staurosporine. Implications for the design of selective inhibitors. In *The Journal of biological chemistry* 279 (41), pp. 42818–42825. DOI: 10.1074/jbc.M407096200.

Jin, Lily L.; Wybenga-Groot, Leanne E.; Tong, Jiefei; Taylor, Paul; Minden, Mark D.; Trudel, Suzanne et al. (2015): Tyrosine phosphorylation of the Lyn Src homology 2 (SH2) domain modulates its binding affinity and specificity. In *Molecular & cellular proteomics : MCP* 14 (3), pp. 695–706. DOI: 10.1074/mcp.M114.044404.

Juarez, Jose C.; Manuia, Mari; Burnett, Mark E.; Betancourt, Oscar; Boivin, Benoit; Shaw, David E. et al. (2008): Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling. In *Proceedings of the National Academy of Sciences of the United States of America* 105 (20), pp. 7147–7152. DOI: 10.1073/pnas.0709451105.

Kaiser, Frank J.; Ansari, Morad; Braunholz, Diana; Concepción Gil-Rodríguez, María; Decroos, Christophe; Wilde, Jonathan J. et al. (2014): Loss-of-function HDAC8 mutations cause a phenotypic spectrum of Cornelia de Lange syndrome-like features, ocular hypertelorism, large fontanelle and X-linked inheritance. In *Human molecular genetics* 23 (11), pp. 2888–2900. DOI: 10.1093/hmg/ddu002.

Kato, M.; Iwashita, T.; Akhand, A. A.; Liu, W.; Takeda, K.; Takeuchi, K. et al. (2000a): Molecular mechanism of activation and superactivation of Ret tyrosine kinases by ultraviolet light irradiation. In *Antioxidants & redox signaling* 2 (4), pp. 841–849. DOI: 10.1089/ars.2000.2.4-841.

Kato, M.; Iwashita, T.; Takeda, K.; Akhand, A. A.; Liu, W.; Yoshihara, M. et al. (2000b): Ultraviolet light induces redox reaction-mediated dimerization and superactivation of oncogenic Ret tyrosine kinases. In *Molecular biology of the cell* 11 (1), pp. 93–101.

Kemble, David J.; Sun, Gongqin (2009): Direct and specific inactivation of protein tyrosine kinases in the Src and FGFR families by reversible cysteine oxidation. In *Proceedings of the*

National Academy of Sciences of the United States of America 106 (13), pp. 5070–5075. DOI: 10.1073/pnas.0806117106.

Kim, Yujin E.; Hipp, Mark S.; Bracher, Andreas; Hayer-Hartl, Manajit; Hartl, F. Ulrich (2013): Molecular chaperone functions in protein folding and proteostasis. In *Annual review of biochemistry* 82, pp. 323–355. DOI: 10.1146/annurev-biochem-060208-092442.

Kong, G.; Dalton, M.; Bubeck Wardenburg, J.; Straus, D.; Kurosaki, T.; Chan, A. C. (1996): Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. In *Molecular and cellular biology* 16 (9), pp. 5026–5035.

Krauss, Gerhard (2014): Biochemistry of Signal Transduction and Regulation. 5. ed. s.l.: Wiley-VCH. Available online at http://onlinelibrary.wiley.com/book/10.1002/9783527667475.

Kuhlmann, Anne-Sophie; Steckbeck, Jonathan D.; Sturgeon, Timothy J.; Craigo, Jodi K.; Montelaro, Ronald C. (2014): Unique functional properties of conserved arginine residues in the lentivirus lytic peptide domains of the C-terminal tail of HIV-1 gp41. In *The Journal of biological chemistry* 289 (11), pp. 7630–7640. DOI: 10.1074/jbc.M113.529339.

Lander, H. M.; Sehajpal, P.; Levine, D. M.; Novogrodsky, A. (1993): Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. In *Journal of immunology (Baltimore, Md. : 1950)* 150 (4), pp. 1509–1516.

Lang, B. F.; O'Kelly, C.; Nerad, T.; Gray, M. W.; Burger, G. (2002): The closest unicellular relatives of animals. In *Current biology : CB* 12 (20), pp. 1773–1778.

Lee, Joon-Won; Kim, Jee-Eun; Park, Eun-Jung; Kim, Jin-Hyun; Lee, Chang-Hun; Lee, Seung-Rock; Kwon, Jongbum (2004): Two conserved cysteine residues are critical for the enzymic function of the human platelet-derived growth factor receptor-beta. Evidence for different roles of Cys-822 and Cys-940 in the kinase activity. In *The Biochemical journal* 382 (Pt 2), pp. 631–639. DOI: 10.1042/BJ20040624.

Lee, Paul; Rao, Jie; Fliss, Albert; Yang, Emy; Garrett, Stephen; Caplan, Avrom J. (2002): The Cdc37 protein kinase-binding domain is sufficient for protein kinase activity and cell viability. In *The Journal of cell biology* 159 (6), pp. 1051–1059. DOI: 10.1083/jcb.200210121.

Leonberg, Amanda Kae; Chai, Yuh-Cherng (2007): The functional role of cysteine residues for c-Abl kinase activity. In *Molecular and cellular biochemistry* 304 (1-2), pp. 207–212. DOI: 10.1007/s11010-007-9501-y.

Li, D.; Ambrogio, L.; Shimamura, T.; Kubo, S.; Takahashi, M.; Chirieac, L. R. et al. (2008): BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. In *Oncogene* 27 (34), pp. 4702–4711. DOI: 10.1038/onc.2008.109.

Li, Edwin; Hristova, Kalina (2006): Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies. In *Biochemistry* 45 (20), pp. 6241–6251. DOI: 10.1021/bi060609y.

Li, Jing; Buchner, Johannes (2013): Structure, function and regulation of the hsp90 machinery. In *Biomedical journal* 36 (3), pp. 106–117. DOI: 10.4103/2319-4170.113230.

Lisabeth, Erika M.; Falivelli, Giulia; Pasquale, Elena B. (2013): Eph receptor signaling and ephrins. In *Cold Spring Harbor perspectives in biology* 5 (9). DOI: 10.1101/cshperspect.a009159.

Liu, Chang C.; Schultz, Peter G. (2010): Adding new chemistries to the genetic code. In *Annual review of biochemistry* 79, pp. 413–444. DOI: 10.1146/annurev.biochem.052308.105824.

Liu, Qingsong; Sabnis, Yogesh; Zhao, Zheng; Zhang, Tinghu; Buhrlage, Sara J.; Jones, Lyn H.; Gray, Nathanael S. (2013): Developing irreversible inhibitors of the protein kinase

cysteinome. In *Chemistry & biology* 20 (2), pp. 146–159. DOI: 10.1016/j.chembiol.2012.12.006.

Lo Conte, Mauro; Carroll, Kate S. (2013): The redox biochemistry of protein sulfenylation and sulfinylation. In *The Journal of biological chemistry* 288 (37), pp. 26480–26488. DOI: 10.1074/jbc.R113.467738.

Lupher, M. L.; Songyang, Z.; Shoelson, S. E.; Cantley, L. C.; Band, H. (1997): The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr292 negative regulatory phosphorylation site of ZAP-70. In *The Journal of biological chemistry* 272 (52), pp. 33140–33144.

Luzio, J. Paul; Pryor, Paul R.; Bright, Nicholas A. (2007): Lysosomes. Fusion and function. In *Nature reviews. Molecular cell biology* 8 (8), pp. 622–632. DOI: 10.1038/nrm2217.

MacLean, Morag; Picard, Didier (2003): Cdc37 goes beyond Hsp90 and kinases. In *Cell stress & chaperones* 8 (2), pp. 114–119.

Magnan, A.; Di Bartolo, V.; Mura, A. M.; Boyer, C.; Richelme, M.; Lin, Y. L. et al. (2001): T cell development and T cell responses in mice with mutations affecting tyrosines 292 or 315 of the ZAP-70 protein tyrosine kinase. In *The Journal of experimental medicine* 194 (4), pp. 491–505.

Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. (2002a): The protein kinase complement of the human genome. In *Science (New York, N.Y.)* 298 (5600), pp. 1912–1934. DOI: 10.1126/science.1075762.

Manning, Gerard; Plowman, Gregory D.; Hunter, Tony; Sudarsanam, Sucha (2002b): Evolution of protein kinase signaling from yeast to man. In *Trends in biochemical sciences* 27 (10), pp. 514–520.

Matsuda, S.; Suzuki-Fujimoto, T.; Minowa, A.; Ueno, H.; Katamura, K.; Koyasu, S. (1999): Temperature-sensitive ZAP70 mutants degrading through a proteasome-independent pathway. Restoration of a kinase domain mutant by Cdc37. In *The Journal of biological chemistry* 274 (49), pp. 34515–34518.

Matsuoka, Hiroshi; Obama, Hiroya; Kelly, Meghan L.; Matsui, Toshimitsu; Nakamoto, Masaru (2005): Biphasic functions of the kinase-defective Ephb6 receptor in cell adhesion and migration. In *The Journal of biological chemistry* 280 (32), pp. 29355–29363. DOI: 10.1074/jbc.M500010200.

McLornan, Donal; Percy, Melanie; McMullin, Mary Frances (2006): JAK2 V617F. A single mutation in the myeloproliferative group of disorders. In *The Ulster medical journal* 75 (2), pp. 112–119.

Miller, Evan W.; Dickinson, Bryan C.; Chang, Christopher J. (2010): Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. In *Proceedings of the National Academy of Sciences of the United States of America* 107 (36), pp. 15681–15686. DOI: 10.1073/pnas.1005776107.

Miller, W. Todd (2012): Tyrosine kinase signaling and the emergence of multicellularity. In *Biochimica et biophysica acta* 1823 (6), pp. 1053–1057. DOI: 10.1016/j.bbamcr.2012.03.009.

Moise, Gwendolyn; Gallup, Nathan M.; Alexandrova, Anastassia N.; Hengge, Alvan C.; Johnson, Sean J. (2015): Conservative tryptophan mutants of the protein tyrosine phosphatase YopH exhibit impaired WPD-loop function and crystallize with divanadate esters in their active sites. In *Biochemistry* 54 (42), pp. 6490–6500. DOI: 10.1021/acs.biochem.5b00496.

Monteiro, Hugo P.; Costa, Paulo E.; Reis, Adriana K. C. A.; Stern, Arnold (2015): Nitric oxide. Protein tyrosine phosphorylation and protein S-nitrosylation in cancer. In *Biomedical journal* 38 (5), pp. 380–388. DOI: 10.4103/2319-4170.158624.

Montezano, A. C.; Callera, G. E.; Yogi, A.; He, Y.; Tostes, R. C.; He, G. et al. (2008): Aldosterone and angiotensin II synergistically stimulate migration in vascular smooth muscle cells through c-Src-regulated redox-sensitive RhoA pathways. In *Arteriosclerosis, thrombosis, and vascular biology* 28 (8), pp. 1511–1518. DOI: 10.1161/ATVBAHA.108.168021.

Morrison, K. L.; Weiss, G. A. (2001): Combinatorial alanine-scanning. In *Current opinion in chemical biology* 5 (3), pp. 302–307.

Murray, D.; Ben-Tal, N.; Honig, B.; McLaughlin, S. (1997): Electrostatic interaction of myristoylated proteins with membranes. Simple physics, complicated biology. In *Structure (London, England : 1993)* 5 (8), pp. 985–989.

Naik, Edwina; Dixit, Vishva M. (2016): Usp9X Is Required for Lymphocyte Activation and Homeostasis through Its Control of ZAP70 Ubiquitination and PKCβ Kinase Activity. In *Journal of immunology (Baltimore, Md. : 1950)* 196 (8), pp. 3438–3451. DOI: 10.4049/jimmunol.1403165.

Nakashima, Izumi; Takeda, Kozue; Kawamoto, Yoshiyuki; Okuno, Yusuke; Kato, Masashi; Suzuki, Haruhiko (2005): Redox control of catalytic activities of membrane-associated protein tyrosine kinases. In *Archives of biochemistry and biophysics* 434 (1), pp. 3–10. DOI: 10.1016/j.abb.2004.06.016.

Neckers, Len (2003): Development of small molecule Hsp90 inhibitors. Utilizing both forward and reverse chemical genomics for drug identification. In *Current medicinal chemistry* 10 (9), pp. 733–739.

Nelson, Kimberly J.; Klomsiri, Chananat; Codreanu, Simona G.; Soito, Laura; Liebler, Daniel C.; Rogers, Leann C. et al. (2010): Use of dimedone-based chemical probes for sulfenic acid detection methods to visualize and identify labeled proteins. In *Methods in enzymology* 473, pp. 95–115. DOI: 10.1016/S0076-6879(10)73004-4.

Noraz, N.; Schwarz, K.; Steinberg, M.; Dardalhon, V.; Rebouissou, C.; Hipskind, R. et al. (2000): Alternative antigen receptor (TCR) signaling in T cells derived from ZAP-70-deficient patients expressing high levels of Syk. In *The Journal of biological chemistry* 275 (21), pp. 15832–15838. DOI: 10.1074/jbc.M908568199.

Oo, Myat Lin; Senga, Takeshi; Thant, Aye Aye; Amin, A. R. M. Ruhul; Huang, Pengyu; Mon, Naing Naing; Hamaguchi, Michinari (2003): Cysteine residues in the C-terminal lobe of Src. Their role in the suppression of the Src kinase. In *Oncogene* 22 (9), pp. 1411–1417. DOI: 10.1038/sj.onc.1206286.

Orchard, Jenny A.; Ibbotson, Rachel E.; Davis, Zadie; Wiestner, Adrian; Rosenwald, Andreas; Thomas, Peter W. et al. (2004): ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. In *Lancet (London, England)* 363 (9403), pp. 105–111. DOI: 10.1016/S0140-6736(03)15260-9.

Palacios, Emil H.; Weiss, Arthur (2007): Distinct roles for Syk and ZAP-70 during early thymocyte development. In *The Journal of experimental medicine* 204 (7), pp. 1703–1715. DOI: 10.1084/jem.20070405.

Palombella, V. J.; Rando, O. J.; Goldberg, A. L.; Maniatis, T. (1994): The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. In *Cell* 78 (5), pp. 773–785.

Paulsen, Candice E.; Truong, Thu H.; Garcia, Francisco J.; Homann, Arne; Gupta, Vinayak; Leonard, Stephen E.; Carroll, Kate S. (2011): Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. In *Nature chemical biology* 8 (1), pp. 57–64. DOI: 10.1038/nchembio.736.

Permyakov, Sergei E.; Zernii, Evgeni Yu; Knyazeva, Ekaterina L.; Denesyuk, Alexander I.; Nazipova, Aliya A.; Kolpakova, Tatiana V. et al. (2012): Oxidation mimicking substitution of conservative cysteine in recoverin suppresses its membrane association. In *Amino acids* 42 (4), pp. 1435–1442. DOI: 10.1007/s00726-011-0843-0.

Plowman, G. D.; Sudarsanam, S.; Bingham, J.; Whyte, D.; Hunter, T. (1999): The protein kinases of Caenorhabditis elegans. A model for signal transduction in multicellular organisms. In *Proceedings of the National Academy of Sciences of the United States of America* 96 (24), pp. 13603–13610.

Poltorak, Mateusz; Arndt, Boerge; Kowtharapu, Bhavani S.; Reddycherla, Amarendra V.; Witte, Vanessa; Lindquist, Jonathan A. et al. (2013): TCR activation kinetics and feedback regulation in primary human T cells. In *Cell communication and signaling : CCS* 11, p. 4. DOI: 10.1186/1478-811X-11-4.

Poltorak, Mateusz; Meinert, Ines; Stone, James C.; Schraven, Burkhart; Simeoni, Luca (2014): Sos1 regulates sustained TCR-mediated Erk activation. In *European journal of immunology* 44 (5), pp. 1535–1540. DOI: 10.1002/eji.201344046.

Poole, Leslie B.; Klomsiri, Chananat; Knaggs, Sarah A.; Furdui, Cristina M.; Nelson, Kimberly J.; Thomas, Michael J. et al. (2007): Fluorescent and affinity-based tools to detect cysteine sulfenic acid formation in proteins. In *Bioconjugate chemistry* 18 (6), pp. 2004–2017. DOI: 10.1021/bc700257a.

Prince, Thomas; Matts, Robert L. (2004): Definition of protein kinase sequence motifs that trigger high affinity binding of Hsp90 and Cdc37. In *The Journal of biological chemistry* 279 (38), pp. 39975–39981. DOI: 10.1074/jbc.M406882200.

Rahman, Mohammad Aminur; Senga, Takeshi; Ito, Satoko; Hyodo, Toshinori; Hasegawa, Hitoki; Hamaguchi, Michinari (2010): S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. In *The Journal of biological chemistry* 285 (6), pp. 3806–3814. DOI: 10.1074/jbc.M109.059782.

Rahman, Mohammad Aminur; Senga, Takeshi; Oo, Myat Lin; Hasegawa, Hitoki; Biswas, Md Helal Uddin; Mon, Naing Naing et al. (2008): The cysteine-cluster motif of c-Yes, Lyn and FAK as a suppressive module for the kinases. In *Oncology reports* 19 (4), pp. 975–980.

Roifman, Chaim M.; Dadi, Harjit; Somech, Raz; Nahum, Amit; Sharfe, Nigel (2010): Characterization of ζ-associated protein, 70 kd (ZAP70)-deficient human lymphocytes. In *The Journal of allergy and clinical immunology* 126 (6), 1226-33.e1. DOI: 10.1016/j.jaci.2010.07.029.

Roifman, Chaim M.; Somech, Raz; Kavadas, Fotini; Pires, Linda; Nahum, Amit; Dalal, Ilan; Grunebaum, Eyal (2012): Defining combined immunodeficiency. In *The Journal of allergy and clinical immunology* 130 (1), pp. 177–183. DOI: 10.1016/j.jaci.2012.04.029.

Rosenwald, A.; Alizadeh, A. A.; Widhopf, G.; Simon, R.; Davis, R. E.; Yu, X. et al. (2001): Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. In *The Journal of experimental medicine* 194 (11), pp. 1639–1647.

Sakaguchi, Noriko; Takahashi, Takeshi; Hata, Hiroshi; Nomura, Takashi; Tagami, Tomoyuki; Yamazaki, Sayuri et al. (2003): Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. In *Nature* 426 (6965), pp. 454–460. DOI: 10.1038/nature02119.

Sakaguchi, Shimon; Sakaguchi, Noriko; Yoshitomi, Hiroyuki; Hata, Hiroshi; Takahashi, Takeshi; Nomura, Takashi (2006): Spontaneous development of autoimmune arthritis due to genetic anomaly of T cell signal transduction. Part 1. In *Seminars in immunology* 18 (4), pp. 199–206. DOI: 10.1016/j.smim.2006.03.007.

Salojin, K. V.; Zhang, J.; Meagher, C.; Delovitch, T. L. (2000): ZAP-70 is essential for the T cell antigen receptor-induced plasma membrane targeting of SOS and Vav in T cells. In *The Journal of biological chemistry* 275 (8), pp. 5966–5975.

Sanchez, Ricardo; Riddle, Megan; Woo, Jongwook; Momand, Jamil (2008): Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. In *Protein science : a publication of the Protein Society* 17 (3), pp. 473–481. DOI: 10.1110/ps.073252408.

Sandberg, Eric M.; VonDerLinden, Dannielle; Ostrov, David A.; Sayeski, Peter P. (2004): Jak2 tyrosine kinase residues glutamic acid 1024 and arginine 1113 form a hydrogen bond interaction that is essential for Jak-STAT signal transduction. In *Molecular and cellular biochemistry* 265 (1-2), pp. 161–169.

Sauvage, Jean-Pierre; Amendola, V.; Ballardini, R.; Balzani, V.; Credi, A.; Fabbrizzi, L. et al. (Eds.) (2001): Molecular Machines and Motors. Berlin, Heidelberg: Springer Berlin Heidelberg (Structure and Bonding, 99). Available online at http://dx.doi.org/10.1007/3-540-44421-1.

Schmitt, Thomas L.; Hotz-Wagenblatt, Agnes; Klein, Helmut; Dröge, Wulf (2005): Interdependent regulation of insulin receptor kinase activity by ADP and hydrogen peroxide. In *The Journal of biological chemistry* 280 (5), pp. 3795–3801. DOI: 10.1074/jbc.M410352200.

Schottel, Brandi L.; Chifotides, Helen T.; Dunbar, Kim R. (2008): Anion-pi interactions. In *Chemical Society reviews* 37 (1), pp. 68–83. DOI: 10.1039/b614208g.

Senga, T.; Miyazaki, K.; Machida, K.; Iwata, H.; Matsuda, S.; Nakashima, I.; Hamaguchi, M. (2000): Clustered cysteine residues in the kinase domain of v-Src. Critical role for protein stability, cell transformation and sensitivity to herbimycin A. In *Oncogene* 19 (2), pp. 273–279. DOI: 10.1038/sj.onc.1203296.

Shah, Neel H.; Wang, Qi; Yan, Qingrong; Karandur, Deepti; Kadlecek, Theresa A.; Fallahee, Ian R. et al. (2016): An electrostatic selection mechanism controls sequential kinase signaling downstream of the T cell receptor. In *eLife* 5. DOI: 10.7554/eLife.20105.

Shirkani, Afshin; Shahrooei, Mohammad; Azizi, Gholamreza; Rokni-Zadeh, Hassan; Abolhassani, Hassan; Farrokhi, Shokrollah et al. (2017): Novel Mutation of ZAP-70-related Combined Immunodeficiency. First Case from the National Iranian Registry and Review of the Literature. In *Immunological investigations* 46 (1), pp. 70–79. DOI: 10.1080/08820139.2016.1214962.

Siegel, Sheera; Futterweit, Walter; Davies, Terry F.; Concepcion, Erlinda S.; Greenberg, David A.; Villanueva, Ronald; Tomer, Yaron (2002): A C/T single nucleotide polymorphism at the tyrosine kinase domain of the insulin receptor gene is associated with polycystic ovary syndrome. In *Fertility and sterility* 78 (6), pp. 1240–1243.

Simeoni, Luca; Thurm, Christoph; Kritikos, Andreas; Linkermann, Andreas (2016): Redox homeostasis, T cells and kidney diseases. Three faces in the dark. In *Clinical kidney journal* 9 (1), pp. 1–10. DOI: 10.1093/ckj/sfv135.

Smaill, J. B.; Rewcastle, G. W.; Loo, J. A.; Greis, K. D.; Chan, O. H.; Reyner, E. L. et al. (2000): Tyrosine kinase inhibitors. 17. Irreversible inhibitors of the epidermal growth factor receptor. 4-(phenylamino)quinazoline- and 4-(phenylamino)pyrido3,2-dpyrimidine-6-acrylamides bearing additional solubilizing functions. In *Journal of medicinal chemistry* 43 (7), pp. 1380–1397.

Smith, C. J.; Marnett, L. J. (1996): Effects of cysteine-to-serine mutations on structural and functional properties of prostaglandin endoperoxide synthase. In *Archives of biochemistry and biophysics* 335 (2), pp. 342–350. DOI: 10.1006/abbi.1996.0515.

Sonamoto, Rie; Kii, Isao; Koike, Yuka; Sumida, Yuto; Kato-Sumida, Tomoe; Okuno, Yukiko et al. (2015): Identification of a DYRK1A Inhibitor that Induces Degradation of the Target Kinase using Co-chaperone CDC37 fused with Luciferase nanoKAZ. In *Scientific reports* 5, p. 12728. DOI: 10.1038/srep12728.

Sorokin, A. V.; Kim, E. R.; Ovchinnikov, L. P. (2009): Proteasome system of protein degradation and processing. In *Biochemistry. Biokhimiia* 74 (13), pp. 1411–1442.

Steenkamp, Emma T.; Wright, Jane; Baldauf, Sandra L. (2006): The protistan origins of animals and fungi. In *Molecular biology and evolution* 23 (1), pp. 93–106. DOI: 10.1093/molbev/msj011.

Stefanová, I.; Saville, M. W.; Peters, C.; Cleghorn, F. R.; Schwartz, D.; Venzon, D. J. et al. (1996): HIV infection--induced posttranslational modification of T cell signaling molecules associated with disease progression. In *The Journal of clinical investigation* 98 (6), pp. 1290–1297. DOI: 10.1172/JCI118915.

Stover, D. R.; Furet, P.; Lydon, N. B. (1996): Modulation of the SH2 binding specificity and kinase activity of Src by tyrosine phosphorylation within its SH2 domain. In *The Journal of biological chemistry* 271 (21), pp. 12481–12487.

St-Pierre, Julie; Buckingham, Julie A.; Roebuck, Stephen J.; Brand, Martin D. (2002): Topology of superoxide production from different sites in the mitochondrial electron transport chain. In *The Journal of biological chemistry* 277 (47), pp. 44784–44790. DOI: 10.1074/jbc.M207217200.

Stuehr, D. J. (1999): Mammalian nitric oxide synthases. In *Biochimica et biophysica acta* 1411 (2-3), pp. 217–230.

Tabet, Fatiha; Schiffrin, Ernesto L.; Callera, Glaucia E.; He, Ying; Yao, Guoying; Ostman, Arne et al. (2008): Redox-sensitive signaling by angiotensin II involves oxidative inactivation and blunted phosphorylation of protein tyrosine phosphatase SHP-2 in vascular smooth muscle cells from SHR. In *Circulation research* 103 (2), pp. 149–158. DOI: 10.1161/CIRCRESAHA.108.178608.

Takeda, Kozue; Kawamoto, Yoshiyuki; Okuno, Yusuke; Kato, Masashi; Takahashi, Masahide; Suzuki, Haruhiko et al. (2006): A PKC-mediated backup mechanism of the MXXCW motif-linked switch for initiating tyrosine kinase activities. In *FEBS letters* 580 (3), pp. 839–843. DOI: 10.1016/j.febslet.2006.01.005.

Terasawa, Kazuya; Minami, Yasufumi (2005): A client-binding site of Cdc37. In *The FEBS journal* 272 (18), pp. 4684–4690. DOI: 10.1111/j.1742-4658.2005.04884.x.

Terasawa, Kazuya; Shinozaki, Fumika; Minami, Michiko; Minami, Yasufumi (2006): Client binding of Cdc37 is regulated intramolecularly and intermolecularly. In *Bioscience, biotechnology, and biochemistry* 70 (6), pp. 1542–1546.

Thurm, Christoph; Poltorak, Mateusz P.; Reimer, Elisa; Brinkmann, Melanie M.; Leichert, Lars; Schraven, Burkhart; Simeoni, Luca (2017): A highly conserved redox-active Mx(2)CWx(6)R motif regulates Zap70 stability and activity. In *Oncotarget* 8 (19), pp. 30805–30816. DOI: 10.18632/oncotarget.16486.

Trevillyan, J. M.; Chiou, X. G.; Ballaron, S. J.; Tang, Q. M.; Buko, A.; Sheets, M. P. et al. (1999): Inhibition of p56(lck) tyrosine kinase by isothiazolones. In *Archives of biochemistry and biophysics* 364 (1), pp. 19–29. DOI: 10.1006/abbi.1999.1099.

Truong, Thu H.; Carroll, Kate S. (2013): Redox regulation of protein kinases. In *Critical reviews in biochemistry and molecular biology* 48 (4), pp. 332–356. DOI: 10.3109/10409238.2013.790873.

Veillette, A.; Dumont, S.; Fournel, M. (1993): Conserved cysteine residues are critical for the enzymatic function of the lymphocyte-specific tyrosine protein kinase p56lck. In *The Journal of biological chemistry* 268 (23), pp. 17547–17553.

Verba, Kliment A.; Agard, David A. (2017): How Hsp90 and Cdc37 Lubricate Kinase Molecular Switches. In *Trends in biochemical sciences* 42 (10), pp. 799–811. DOI: 10.1016/j.tibs.2017.07.002.

Visperas, Patrick R.; Winger, Jonathan A.; Horton, Timothy M.; Shah, Neel H.; Aum, Diane J.; Tao, Alyssa et al. (2015): Modification by covalent reaction or oxidation of cysteine residues in the tandem-SH2 domains of ZAP-70 and Syk can block phosphopeptide binding. In *The Biochemical journal* 465 (1), pp. 149–161. DOI: 10.1042/BJ20140793.

Wang, Chunling; Ma, Hsin-Chieh; Wimmer, Eckard; Jiang, Ping; Paul, Aniko V. (2014): A C-terminal, cysteine-rich site in poliovirus 2C(ATPase) is required for morphogenesis. In *The Journal of general virology* 95 (Pt 6), pp. 1255–1265. DOI: 10.1099/vir.0.062497-0.

Wang, H. Y.; Altman, Y.; Fang, D.; Elly, C.; Dai, Y.; Shao, Y.; Liu, Y. C. (2001): Cbl promotes ubiquitination of the T cell receptor zeta through an adaptor function of Zap-70. In *The Journal of biological chemistry* 276 (28), pp. 26004–26011. DOI: 10.1074/jbc.M010738200.

Wang, Haopeng; Kadlecek, Theresa A.; Au-Yeung, Byron B.; Goodfellow, Hanna E. Sjölin; Hsu, Lih-Yun; Freedman, Tanya S.; Weiss, Arthur (2010): ZAP-70. An essential kinase in T-cell signaling. In *Cold Spring Harbor perspectives in biology* 2 (5), a002279. DOI: 10.1101/cshperspect.a002279.

Wang, Xiaoqian; Simeoni, Luca; Lindquist, Jonathan A.; Saez-Rodriguez, Julio; Ambach, Andreas; Gilles, Ernst D. et al. (2008): Dynamics of proximal signaling events after TCR/CD8-mediated induction of proliferation or apoptosis in mature CD8+ T cells. In *Journal of immunology (Baltimore, Md. : 1950)* 180 (10), pp. 6703–6712.

Watts, J. D.; Affolter, M.; Krebs, D. L.; Wange, R. L.; Samelson, L. E.; Aebersold, R. (1994): Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. In *The Journal of biological chemistry* 269 (47), pp. 29520–29529.

Wei, Jianning; Wu, Jang-Yen (2005): Structural and functional analysis of cysteine residues in human glutamate decarboxylase 65 (GAD65) and GAD67. In *Journal of neurochemistry* 93 (3), pp. 624–633. DOI: 10.1111/j.1471-4159.2005.03046.x.

Williams, B. L.; Irvin, B. J.; Sutor, S. L.; Chini, C. C.; Yacyshyn, E.; Bubeck Wardenburg, J. et al. (1999): Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. In *The EMBO journal* 18 (7), pp. 1832–1844. DOI: 10.1093/emboj/18.7.1832.

Williams, B. L.; Schreiber, K. L.; Zhang, W.; Wange, R. L.; Samelson, L. E.; Leibson, P. J.; Abraham, R. T. (1998): Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor. Reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. In *Molecular and cellular biology* 18 (3), pp. 1388–1399.

Wood, Zachary A.; Schröder, Ewald; Robin Harris, J.; Poole, Leslie B. (2003): Structure, mechanism and regulation of peroxiredoxins. In *Trends in biochemical sciences* 28 (1), pp. 32–40.

Yamamoto, A.; Tagawa, Y.; Yoshimori, T.; Moriyama, Y.; Masaki, R.; Tashiro, Y. (1998): Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. In *Cell structure and function* 23 (1), pp. 33–42.

Yan, Qingrong; Barros, Tiago; Visperas, Patrick R.; Deindl, Sebastian; Kadlecek, Theresa A.; Weiss, Arthur; Kuriyan, John (2013): Structural basis for activation of ZAP-70 by

phosphorylation of the SH2-kinase linker. In *Molecular and cellular biology* 33 (11), pp. 2188–2201. DOI: 10.1128/MCB.01637-12.

Yang, Mingjin; Chen, Taoyong; Li, Xuelian; Yu, Zhou; Tang, Songqing; Wang, Chen et al. (2015): K33-linked polyubiquitination of Zap70 by Nrdp1 controls CD8(+) T cell activation. In *Nature immunology* 16 (12), pp. 1253–1262. DOI: 10.1038/ni.3258.

Yoo, Sa Kan; Starnes, Taylor W.; Deng, Qing; Huttenlocher, Anna (2011): Lyn is a redox sensor that mediates leukocyte wound attraction in vivo. In *Nature* 480 (7375), pp. 109–112. DOI: 10.1038/nature10632.

Yoshimura, Naruo; Kudoh, Shinzoh; Kimura, Tatsuo; Mitsuoka, Shigeki; Matsuura, Kuniomi; Hirata, Kazuto et al. (2006): EKB-569, a new irreversible epidermal growth factor receptor tyrosine kinase inhibitor, with clinical activity in patients with non-small cell lung cancer with acquired resistance to gefitinib. In *Lung cancer (Amsterdam, Netherlands)* 51 (3), pp. 363–368. DOI: 10.1016/j.lungcan.2005.10.006.

Young, Jason C.; Agashe, Vishwas R.; Siegers, Katja; Hartl, F. Ulrich (2004): Pathways of chaperone-mediated protein folding in the cytosol. In *Nature reviews. Molecular cell biology* 5 (10), pp. 781–791. DOI: 10.1038/nrm1492.

Zafarullah, M.; Li, W. Q.; Sylvester, J.; Ahmad, M. (2003): Molecular mechanisms of N-acetylcysteine actions. In *Cellular and molecular life sciences : CMLS* 60 (1), pp. 6–20.

Zhao, Q.; Weiss, A. (1996): Enhancement of lymphocyte responsiveness by a gain-of-function mutation of ZAP-70. In *Molecular and cellular biology* 16 (12), pp. 6765–6774.

Zhao, Qiang; Boschelli, Frank; Caplan, Avrom J.; Arndt, Kim T. (2004): Identification of a conserved sequence motif that promotes Cdc37 and cyclin D1 binding to Cdk4. In *The Journal of biological chemistry* 279 (13), pp. 12560–12564. DOI: 10.1074/jbc.M308242200.

Curriculum vitae

PERSONAL INFORMATION

Name	Christoph Thurm
Home Address	Halberstädter Straße 29 39112 Magdeburg Germany
Date of birth	27.06.1988
Place of birth	Borna, Germany
Nationality	German
E-mail	christoph.thurm@googlemail.com

EDUCATION

01/2015-date	PhD studies (Immunology/Biochemistry), Otto-von-Guericke University Magdeburg, Germany
10/2010-09/2012	Master of Science (Biochemistry), University Leipzig, Germany Concentration: Molecular Biochemistry
10/2007-09/2010	Bachelor of Science (Biochemistry), University Leipzig, Germany

RESEARCH EXPERIENCE

01/2015-date	PhD student, Otto-von-Guericke University Magdeburg, Institute for molecular and clinical immunology, Germany
01/2015-date	MGK854 "Molecular organization of cellular communication within the immune system" (SFB854), Otto-von-Guericke University Magdeburg, Germany
01/2013-12/2014	Research Assistant, Otto-von-Guericke University Magdeburg, Department of Neurology, Neurodegeneration Research Laboratory (NRL), Germany
2011	Student Assistant, Institute of Biochemistry, Dept. Biophysical Chemistry, University Leipzig, Germany
2010	Student Assistant, Institute of Biochemistry, Dept. Metabolic Biochemistry and Enzymology, University Leipzig, Germany

List of publications

1. Thurm, Christoph; Poltorak, Mateusz P.; Reimer, Elisa; Brinkmann, Melanie M.; Leichert, Lars; Schraven, Burkhart; Simeoni, Luca (2017): A highly conserved redox-active $Mx_{(2)}CWx_{(6)}R$ motif regulates Zap70 stability and activity. In *Oncotarget* 8 (19), pp. 30805–30816.
2. Fröhlich, Christina; Zschiebsch, Katja; Gröger, Victoria; Paarmann, Kristin; Steffen, Johannes; Thurm, Christoph; Schropp, Eva-Maria; Brüning, Thomas; Gellerich, Frank; Radloff, Martin; Schwabe, Rainer; Lachmann, Ingolf; Krohn, Markus; Ibrahim, Saleh; Pahnke, Jens (2016): Activation of Mitochondrial Complex II-Dependent Respiration Is Beneficial for α-Synucleinopathies. In *Molecular Neurobiology* (53), pp. 4728-4744.
3. Simeoni, Luca; Thurm, Christoph; Kritikos, Andreas; Linkermann, Andreas (2016): Redox homeostasis, T cells and kidney diseases. Three faces in the dark. In *Clinical kidney journal* 9 (1), pp. 1–10.