THE ROLE OF CD3 DELTA INTERACTING PROTEINS IN T-CELL RECEPTOR ASSEMBLY AND SIGNALING

by

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ABSTRACT

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The signals initiated from the T cell receptor (TCR)-CD3 complex receptors are not only important for the correct functioning but also for the differentiation and survival of T cells. While CD3 chains play an essential role in the assembly of and signaling by the TCR and preTCR complexes, the logic behind the redundancy of these signaling subunits has not been addressed. In this study, we aimed to identify novel proteins that interact with the CD3delta subunit of the TCR complex. We performed a membrane based split ubiquitin yeast two hybrid screen. This novel genetic screening system allows the identification of interaction partners of membrane-associated proteins. We used the CD3delta chain as bait and screened a Jurkat cell cDNA library to identify CD3δ interacting proteins. We confirmed the in-vivo relevance of these interactions by co-immunoprecipitation in human tissue culture cell lines. We further examined the functional significance of two CD3delta interacting proteins: BCAP31 and CD81.We demonstrate that BCAP31, previously shown to associate with B cell receptor, interacts with CD3delta in the endoplasmic reticulum (ER) and plays a role in TCR assembly. We also identified the tetraspanin superfamily member CD81, previously shown to have a role in early T cell development, as a CD3delta interaction partner. In order to investigate the functional significance of this interaction, we silenced CD81 expression in T lymphocyte cell lines by shRNA mediated expression arrest and performed functional assays. We demonstrate that CD81 is important for TCR/CD4 co-receptor mediated signaling of T lymphocytes, possibly inhibiting the initiation of TCR signals.

ÖZET

CD3 DELTA İLE ETKİLEŞİMDE BULUNAN PROTEİNLERİN T HÜCRESİ RESEPTÖRÜ OLUŞUMU VE SİNYALLEMESİNDEKİ ROLÜ

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Anahtar Kelimeler: TCR, CD3delta, Maya ikili hibrid, CD81, BCAP31

T hücresi reseptör (TCR-CD3) kompleksinden başlayan sinyaller, hem T hücrelerinin düzgün işleyişi hem de farklılaşması ve hayatta kalması için önemlidir. CD3 zincirleri, TCR ve preTCR komplekslerinin oluşmasında ve sinyal iletiminde önemli rol oynarlar, ancak bu kadar çeşitli CD3 zinciri varlığının ardındaki mantık günümüze değin tam olarak açıklanabilmiş değildir. Biz bu çalışmada, CD3delta zinciriyle etkileşime giren yeni proteinler bulmayı hedefledik. Bu amaçla Dual membran sistemi olarak adlandırılan, split-ubiquitin prensibine dayanan maya ikili hibrid sisteminden faydalandık. Bu yeni sistem, hücre membranında bulunan proteinlerin etkileşimde bulundukları diğer proteinleri belirlemeye olanak vermektedir. Araştırmamızda, bu yeni sistem yardımıyla, CD3delta ile etkileşime giren proteinleri bulmak amacıyla, CD3delta zincirini olta olarak kullanarak, Jurkat hücresi cDNA kütüphanesi taranmıştır. Belirlenen etkileşimlerin in-vivo önemini insan hücre kültürü hatlarında immun çökeltme metodu ile doğrulanmıştır. CD3delta ile etkileşime giren proteinlerden iki tanesi BCAP31 ve CD81 detaylı olarak incelenmiştir. Daha önceden B hücresi reseptörüyle etkileşimi gösterilmiş olan BCAP31'in, CD3delta ile endoplazmik retikulumda etkileşmekte olduğu ve TCR oluşumunda olası bir rol oynadığı gösterilmiştir. Tetraspanin ailesine ait olan CD81'in de daha önceden yapılmış olan araştırmalarda T hücresi gelişiminde rol oynadığı belirtilmiştir. Bu çalışmada CD3delta ile etkileşime girdiği gösterilen CD81'in işlevsel rolünü araştırmak için, T lenfosit hücre hatlarında shRNA kullanarak CD81 ifadesini susturduk. Yapılan işlevsel çalışmalar sonucunda T lenfositlerinde, CD81'in TCR/CD4 coreseptör bağımlı sinyal iletiminde önemli olduğu ve bu etkinin TCR sinyallerinin başlamasında muhtemel engelleyici bir rolü olduğu sonucuna varılmıştır.

To my family…

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α	Alpha
β	Beta
δ	Delta
γ	Gamma
ε	Epsilon
ADE	Adenine
APC	Antigen Presenting Cell
bp	Basepair
BLAST	Basic Local Alignment Search Tool
CD	Cluster of differentiation
cDNA	Complementary DNA
CIAP	Calf Intestinal Alkaline Phosphatase
CMV	Cytomegalovirus
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DN	Double Negative
DNA	Deoxyribonucleic Acid
DP	Double Positive
EDTA	Ethylenediaminetetraaceticacid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
HEK	Human Embriyonic Kidney
HIS	Histidine
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
LB	Luria Broth

Lck	Lymphocyte-Specific Protein-Tyrosine Kinase
Lef	Lymphoid Enhancer Factor
LEU	Leucine
MHC	Major Histocompatibility Complex
NCBI	National Center for Biotechnology Information
NK	Natural Killer Cells
OD	Optimal Density
PEG	Polyethylenglycol
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
RNA	Ribonucleic Acid
SC	Synthetic Complete
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP	Single Positive
SV40	Simian Virus 40
STAT	Signal Transducer and Activator of Transcription
TBE	Tris Borate EDTA
TCF	T cell factor
TCR	T Cell Receptor
Th	T Helper Cell
TRP	Tryptophan
URA	Uracil
USCS	University of California at Santa Cruz
YPD	Yeast Extract-Peptone-Dextrose

1. INTRODUCTION

SECTION I: SCIENTIFIC BACKGROUD

1.1 T CELLS IN THE IMMUNE SYSTEM

The immune system defends the host against different kinds of infection. Innate immunity serves as a first line of defense but lacks the ability to recognize certain pathogens and to provide the specific protective immunity that prevents reinfection. Adaptive immunity is based on clonal selection from a repertoire of lymphocytes bearing highly diverse antigen specific receptors that enable the immune system to recognize any foreign antigen. The destruction of invaders is the function of the T lymphocytes, or T cells, which are responsible for the cell mediated immune responses of adaptive immunity. Cell-mediated reactions depend on direct interactions between T lymphocytes and cells bearing the antigen that the T cells recognize. There are two different classes of T cells; the ones that bear CD8 as the coreceptor are called cytotoxic T cells. Cytotoxic T cells recognize the cells that are infected by viruses by the aid of displayed antigen of the viruses on the cell surface and directly kill the infected cell. The latter class of T cells is helper T cells that have the CD4 as the coreceptor on their surface. CD4 T lymphocytes can be divided into two subsets, which carry out different functions in defending the body, in particular from bacterial infections. The first subset of CD4 T lymphocytes, known as TH1 cells, is important in the control of intracellular bacterial infections. They activate macrophages, inducing the fusion of their lysosomes with the vesicles containing the bacteria and at the same time stimulating other antibacterial mechanisms of the phagocyte. TH1 cells also release cytokines and chemokines that attract macrophages to the site of infection. The second subset of CD4 T cells, called TH2 cells, have a central role in the

destruction of extracellular pathogens by activating B cells. There are only a few antigens with special properties that can activate naive B lymphocytes on their own. Most antigens require an accompanying signal from helper T cells before they can stimulate B cells to proliferate and differentiate into cells secreting antibody.[1-3]

All the effects of T lymphocytes depend upon interactions with target cells containing foreign proteins. The only way that T cells recognize their targets is when they are displayed on the cell surface by specialized molecules called major histocompatibility complex (MHC), in the host cell. There are two types of MHC molecules MHCI and MHCII, which differ in the source of the peptide that they trap and carry to the cell surface. MHCI displays the peptides derived from proteins synthesized in the cytosol, and thus able to display fragments of viral proteins on the cell surface, whereas MHCII displays the peptides derived from proteins in intracellular vesicles, and thus display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytic cells and B cells. The two classes of MHC with the peptide they display on the cell surface are recognized by different functional classes of T cells. Cytotoxic T cells with CD8 coreceptor recognize MHCI molecules bearing viral peptides, TH1 or TH2 cells recognize MHCII bearing peptides derived from pathogens taken up into vesicles.[2, 3]

1.2 T CELL RECEPTOR –TCR-

The signal transduction cascades starting from the specialized receptors on the cell membrane going through the cell is responsible for a cell's ability to communicate with its environment. As lymphocyte maturation and survival are regulated by signals received through their antigen receptors, there is an intense biomedical research effort dedicated to understand the signaling mechanism of immune system cells. T cells sense their environment through the T cell receptor (TCR/CD3) complex on their surface. TCR is a complex of integral membrane proteins, composed of 8 highly conserved, potentially charged residues : TCR alpha (TCRα), TCR beta (TCRβ), CD3 delta (CD3δ), CD3 gamma (CD3γ) , 2 CD3 epsilon (CD3ε) and 2 TCR zeta(TCRζ) (Figure 1.1)[1, 4]

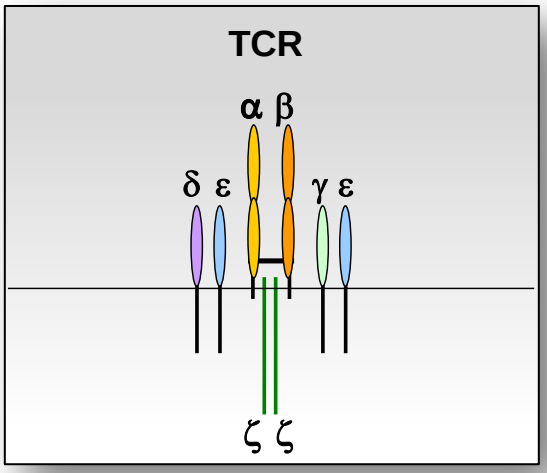

Figure 1.1: The T cell receptor complex

Elucidating the stoichiometry of TCR complex is crucial in understanding the mechanism of TCR signal initiation as the receptor activation is thought to result from a conformational change in the TCR complex. The signal transduction through the TCR complex is not only important for the proper functioning, but also for the differentiation and survival of T cells.

1.3 THE ASSEMBLY OF THE TCR

Highly conserved charged regions found both in the transmembrane helices of the TCR and the CD3 subunits have an essential role in the assembly of TCR- CD3 complex. Any mutation introduced to one of these highly conserved polar residues was shown to impair the complex formation, leading to the conclusion that these residues have an essential role in the steps of assembly.[5] The assembly of TCR complex involves three steps that take place in the endoplasmic reticulum: generation of CD3δε , CD3γε and TCRζζ dimers is followed by the formation TCRα- CD3δε and TCRβ- CD3γε trimers. These trimers cannot be exported to the cell surface unless they complete the final assembly step forming a hexa-polypeptide that assembles with a dimer of TCRζζ, forming the fully competent TCR complex.[4, 6] (Figure1.2) The significance of the other portions of

TCR complex, including the transmembrane regions, the connecting peptides and the constant regions are poorly understood in terms of their organization or how they pass information.[6]

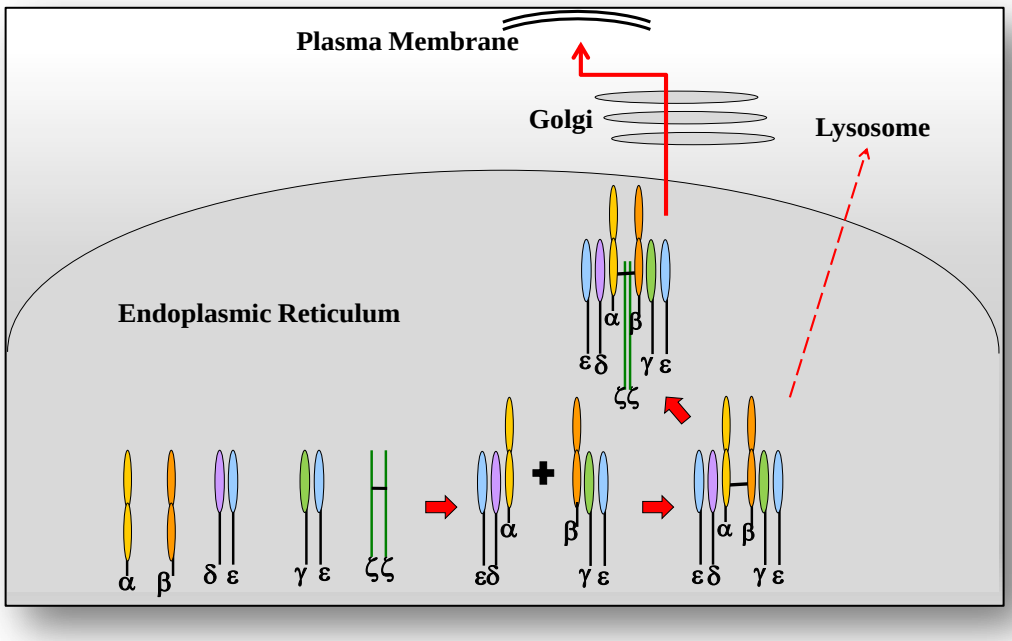

Figure 1.2: T cell receptor assembly in endoplasmic reticulum.

1.4 TCR SIGNALING AND T CELL ACTIVATION

T cells are stimulated by antigenic peptides that are presented by the MHC class I or class II molecules expressed on the surface of antigen presenting cells (APCs).[1] The complex receptor structure enables T cells to differentially respond to different ligands, peptides presented on MHC molecules on the surface of the APCs. The TCR can even differentiate between peptides differing only in one aminoacid.[4, 6] This ability arises from the precise ligand binding specificities of TCR and the distinct intracellular signals it transmits.

In resting T cells TCR partitions into detergent soluble fractions whereas in activated T cells TCR moves into detergent-insoluble fractions.[7] The T-cell immune response is initiated upon engagement of the TCR and coreceptor, CD4 or CD8, by cognate antigen bound MHC complexes presented by antigen-presenting cells. TCR-coreceptor engagement induces the activation of biochemical signaling pathways that, in combination with signals from costimulator molecules and cytokine receptors, direct the outcome of the response. Following the interaction of TCRα and TCRβ with the cognate ligand, CD3 chains function in transmission of the incoming signal. The molecular events taking place inside the cell involves phosphorylation of tyrosine residues in the cytoplasmic tail regions of the CD3 chains (Immunoreceptor tyrosine-based activation motifs-ITAMs) and binding of adaptor proteins to these ITAMs of the TCR. ITAMs contain two YXXL (Y:tyrosine,L:leucine, X: any amino acid) sequences separated by a 6-8 amino acid long spacer.[8] Two Src-family kinases Lck (56kDa) and Fyn (59KDa) specifically phosphorylate the Tyr residues in the ITAMs of CD3 and TCRζ chains, which then serve as docking sites for Syk family kinase ZAP-70.[9-11] Src family kinases then phosphorylate and activate the recruited ZAP-70.[12] Trans- and autophosphorylation of ZAP-70 results in the phosphorylation of key adaptor proteins like LAT[13], leading to the recruitment and activation of other kinase families and enzymes, resulting in secondary messenger generation. The coordinated action of secondary messengers, enzymes and scaffold molecules results in activation of diverse families of transcription factors as NFκB, NFAT and AP-1. As a result of the activity of these transcription factors new proteins are produced that functions in T cell differentiation, proliferation and/or acquisition of effector functions.[1, 14] (Figure 1.3)

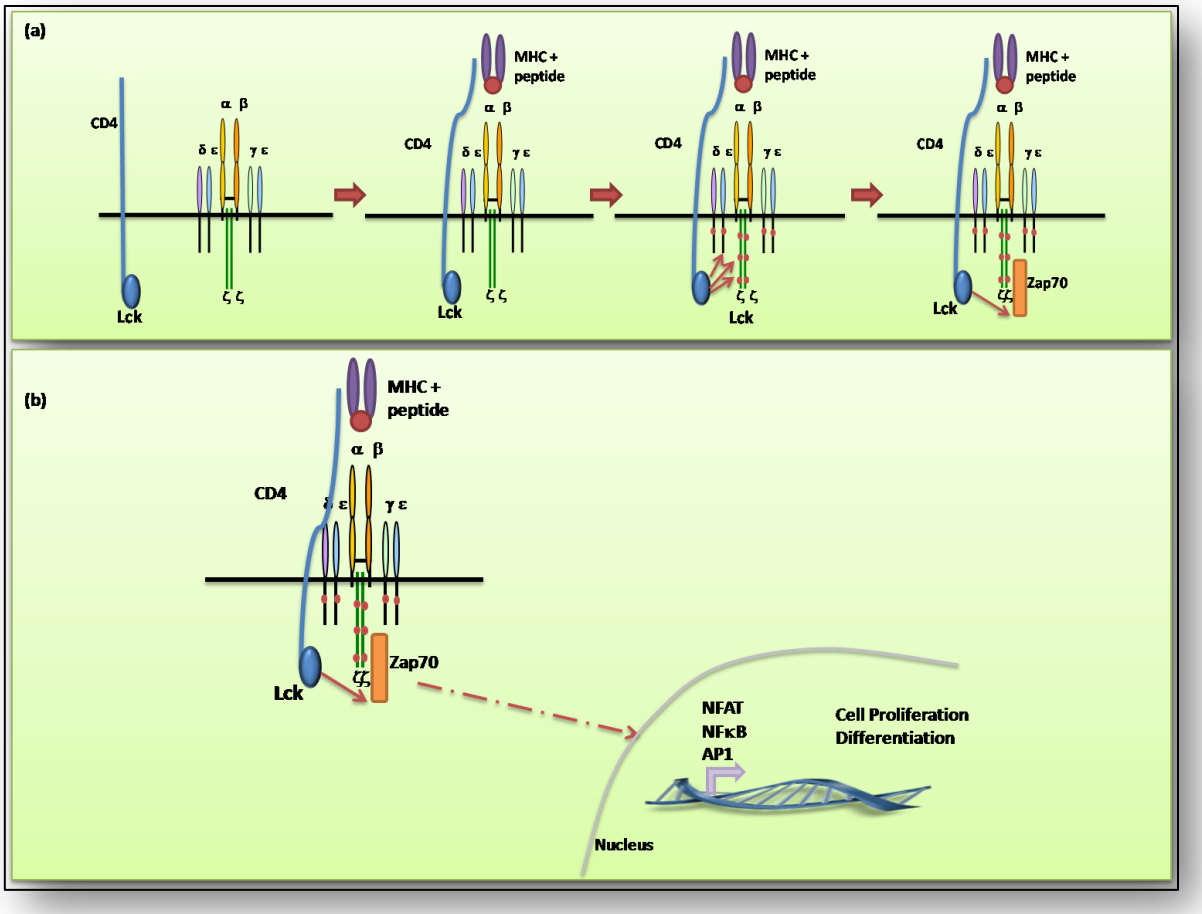

Figure 1.3: TCR mediated T lymphocyte stimulation and activation. In (a) the initial signaling events initiated by the binding of TCR to MHC presented peptide leading to phosphorylation of ITAMs of CD3 and TCRζ chains, resulting in Lck and ZAP70 activation are shown. In (b) ZAP70 phosphorylation initiates downstream signal transduction cascades that results in activation of three transcription factors NFAT, NFκB and AP1. Initiation of transcription of diverse set of gene leads to cell proliferation and differentiation.

Lck, like other Syk family kinases, have N terminal attachment sites for saturated fatty acid addition besides the SH2, SH3 and tyrosine kinase domains. This N terminal attachment sites allow palmitoylation and myristoylation that results in the ability of Lck to attach to cellular membranes.[15, 16] Another unique N terminal domain of Lck is required for its association with the coreceptors CD4 and CD8.[17] Therefore, the inner leaf of the T cell plasma membrane contains varying amounts of coreceptor-bound Lck and coreceptor-unbound Lck.[5, 17] Coreceptor dependent TCR signaling requires engagement of antigen bound MHC by TCR with CD4 or CD8 as the coreceptor. This engagement leads to

intracellular juxtaposition of coreceptor bound Lck with CD3 signaling motifs and facilitates its participation in the initiation of TCR signal transduction. [18, 19] In contrast, coreceptor independent TCR signaling is thought to be mediated by the free Lck that is cytosolic and not associated with coreceptors. [20]

1.4.1 The Role of Lipid Rafts in TCR Signaling

The precise order and regulation of the initial phosphorylation events at the plasma membrane after TCR stimulation remains to be defined. A current theory that describes this early signaling events involves the microdomains at the plasma membrane with distinct lipid and protein composition, called lipid rafts.[21, 22] Lipid rafts are dynamic formations rather than rigid structures, with a rapidly changing size and organization.[21] It is found that in T lymphocytes, molecules with a key role in signaling, including Lck, localize to these domains. The raft and the actin cytoskeleton play important roles in facilitating accumulation of Lck at the stimulated TCR clusters.[23] The mutant versions of the Lck and LAT which fail to localize to the lipid rafts cannot function in TCR signaling, showing the importance of this microdomains in TCR mediated signaling.[13, 24] Diverse studies suggested that upon stimulation, the TCR translocates to lipid rafts and it is phosphorylated by the action of the lipid raft resident Lck. [25, 26] An alternative theory about localization of the TCR to lipid rafts has also been proposed, suggesting that the TCR may constitutively associate with lipid rafts in the absence of stimulation.[27, 28] However, in this model of localization it is not clear what prevents the phosphorylation of TCR with Lck in unstimulted cells, if both TCR and Lck are localized to the same microdomains in the cell membrane in close proximity. The activity of Lck that plays a more major role in this alternative theory can be explained in part by the localization of CD4 coreceptor to lipid rafts. The fact that CD4 stimulation enhances the signaling by TCR can therefore be explained in part as it induces activation of lipid rafts and formation of molecular assemblies at the site of immunological synapse. [29]

1.5 T CELL DEVELOPMENT

T lymphocytes originate from bone marrow derived hematopoietic stem cells. T lymphocyte precursors formed in the bone marrow migrate to the thymus where they pass through series of distinct phases depending on the changes in the status of T cell receptor genes, expression of TCR on the cell surface and by changes in expression of cell surface proteins and the co receptor proteins CD4, CD8. The supporting dendritic and epithelial cells within the thymus direct T cell differentiation which can be subdivided into three phases.[1] The first phase is the Double Negative (DN) phase of T cells in which they do not express any mature T cell markers: TCR-CD3 complex or co-receptors CD4 and CD8. As these immature, double negative thymocytes rearrange their T cell receptor β-chain locus so produce a TCRβ protein that assembles into a multi-subunit receptor complex named preTCR. In addition to TCRβ the pre-TCR complex contains the surrogate preTCRα molecule along with the CD3 molecules[30, 31]. Proliferation is triggered by the presence of this surrogate receptor expression.

The second developmental stage of thymocytes is called double positive (DP) because of the surface expression of CD4 and CD8 co-receptors. Rearrangement of the TCR alpha chain occurs in this DP stage. These small double positive thymocytes initially express low levels of TCR. Depending on the ability of the TCR to recognize MHC-self peptides on the surface of thymic epithelium, thymocytes undergo positive selection and negative selection. Only those thymocytes which bind the MHC/antigen complex with adequate affinity receive a vital "survival signal" and will be positively selected. Developing thymocytes which do not have adequate affinity cannot not serve useful functions in the body; the cells must be able to interact with MHC and peptide complexes in order to affect immune responses. Therefore, thymocytes with low affinity to peptide-MHC die by apoptosis. This positive selection ensures that useful TCR specificities are preserved. T cells that survive positive selection migrate further into the cortico-medullary junction of the thymus where they encounter macrophages and dendritic cells, bone-marrow derived antigen presenting cells (APCs) with high expression of MHC-self peptide

complexes. T cells which bind self peptide-MHC with too high affinity at this stage undergo negative selection and die by apoptosis[1, 32].

The survivors of positive and negative selection go on to mature into the third stage of thymocytes, which express higher levels of TCR on their surface. These mature thymocytes cease to express one or other of the two co-receptor molecules, becoming either CD4 or CD8 single positive(SP) thymocytes[1, 33]. Mature SP T cells are ready to leave the thymus and migrate to periphery (lymph nodes) to function in the immune response. (Figure 1.4)

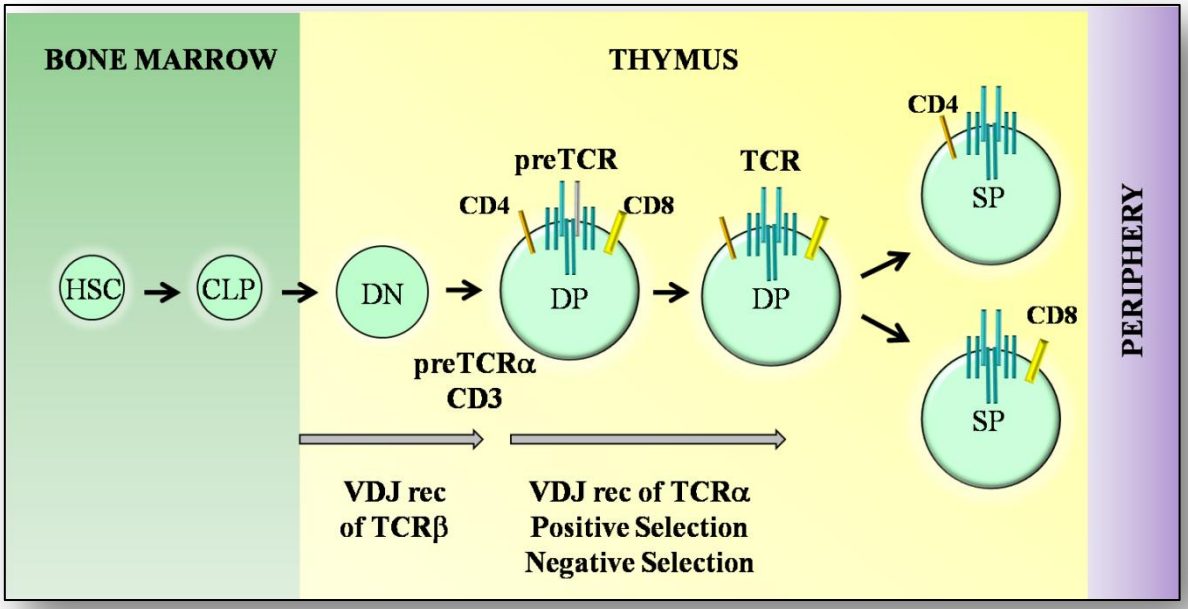

Figure 1.4: T lymphocyte development starting from hematopoietic stem cells in bone marrow to the single positive T cells in the periphery. Hematopoietic stem cells (HSC) are differentiated into common lymphoid progenitor (CLP) and leaves bone marrow to go to the thymus for developing T cells. Double Negative (DN) T cells are the first population of cells in thymus that start the VDJ recombination of the TCRβ chain, functional TCRβ along with preTCRα and CD3s are expressed on the surface of T cells and with the presence of CD4 and CD8 coreceptors, they are called Double Positive (DP) cells. Following the VDJ recombination of TCRα chain, positive and negative selection processed leads to TCR expressing DP cells which then further differentiate into either CD4 or CD8 positive Single Positive(SP)T cells. These fully maturated SP T cells are the population of T cells that go to periphery to function in the immune system.

Thymus derived γ/δ T lymphocytes differentiate from CD4-CD8- DN T cell precursors forming a separate lineage of cells from α/β T cells. Expression of γ/δ TCR usually occurs without the typical co-receptors and the details of positive and negative selection patterns are still unclear.[1]

1.5.1 The Role of CD3delta in T Cell Development

PreT cell receptor (preTCR) and TCR complex play a critical role in thymocyte development. PreTCR complex consisting of pTα/TCR β heterodimer and CD3chains and plays an essential role in the DN to DP transition. PreTCR-mediated signal transduction results in survival of DN thymocytes and further differentiation into DP cells. This is accompanied by an extensive proliferation resulting in an increase in thymic cellularity. In contrast, TCR complex consisting of TCRαβ heterodimer and CD3 chains is mainly required for the DP to SP differentiation.

Genes encoding CD3γ, δ, and ε chains are tightly linked and map to a 50 kb region on chromosome 11 in human and chromosome 9 in mice[34]. Among the CD3 chains CD3γ and CD3δ show considerable homology to each other and suggested to have evolved by gene duplication[35]. Cell line studies made so far suggest a cumulative role for CD3 chains in amplifying weak signals whereas the specialized roles of each chains at least during T cell development is shown via studies in chain deficient mice. It has been shown that the lack of CD3ε invariably leads to the absence of TCR expression. Similarly,CD3γ or CD3ζ deficiency show blockade in thymocyte development at the DN stage and reduced thymic cellularity although a small number of DP and SP thymocytes could be detected in these mice[34, 36, 37]. CD3γ or CD3ζ deficient mice impair the development of γδ T cells as well. In contrast, CD3δ-deficient mice show an impaired thymic selection of αβT cells at the DP stage, whereas γδ T cell development is normal indicating a dispensable nature of CD3δ in the pre-TCR function in mice.[36] However, there is recent data suggesting that a defect in human CD3δ results in impaired development at the DN stage indicating a role for hCD3δ in preTCR-mediated DN to DP transition[37].

In human, defect in CD3δ gene leads to Severe Combined Immunodeficiecy (SCID) characterized by the absence of T cells but normal numbers of B cells (T-B+ SCID). These patients had an early arrest in T-cell development, with a nearly complete absence of circulating mature T cells and a complete lack of γ/δ T cells. CD3δ lacking human phenotype does not resemble CD3δ knock out mouse, as humans but not mice lacking CD3 δ have no double-positive thymocytes and no γ/δT cells.[38] The levels of CD3γ and CD3ε proteins were lower in the patient's thymocytes lacking CD3δ than in normal control samples, possibly because CD3 complexes lacking CD3δ are rapidly degraded.[39]

1.6 THE TETRASPANIN PROTEIN CD81

CD81 also known as TAPA-1 is a 26kDa transmembrane protein that belongs to tetraspanin superfamily with four transmembrane domains, aminoterminal and carboxyterminal cytoplasmic domains, and two extracellular loops[40, 41]. (Figure 1.5) The members of tetraspanin family are involved in diverse cellular processes and their activities are due to the association of different tetraspanins with each other and with other lineage specific proteins in cell membrane in the form of microdomains known as tetraspanin web.[42] This web is shown to be composed of several tetraspanin molecules that associate laterally with each other and with nontetraspanin partners such as cell surface receptors, adhesion molecules and transmembrane signaling proteins.[42-45]

CD81 is expressed on the cell surface of most cells of the body throughout their cellular differentiation including lymphocytes and has been suggested to have diverse roles in lymphocyte development and activation regulation. CD81 is believed to be essential for B cell development and regulating activation as it is expressed early and associates with CD19 and CD21 to form a signal transduction complex.[46, 47] Co-ligation of this signal transduction complex and surface B cell receptor (BCR) resulted in enhanced proliferation of B cells.[48] CD81 is moreover required for normal expression of CD19, post transcriptionally effecting CD19 expression during B cell development. [49]

After the role of CD81 in B cells in shown, studies to asses any possible significance of CD81 on T lymphocytes were more intense. CD81 is found to be expressed on human T lymphocytes.[50] There are only few studies on interaction partners of CD81 on T lymphocytes and these studies indicate that CD81 is prominently associated with CD4 and CD8.[50, 51] In effort to define the CD4 domain needed for association with CD81, it was found that cytoplasmic region of CD4 was sufficient for the association with CD81. This interaction did not reduce when Lck binding domain of CD4 is deleted, however interestingly CD4 engaged with Lck could not associate with CD81.[51] On the other hand, in one particular study CD81 is shown to associate weakly with CD3.[50] But there is no further study that confirms or examines this interaction between CD3 and CD81.

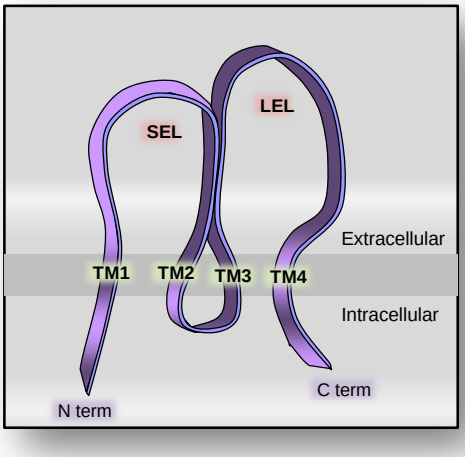

Figure 1.5: Secondary structure of CD81. (TM: transmembrane region, SEL: small extracellular loop, LEL: large extracellular loop)

1.6.1 The Role of CD81 in T Cell Development.

CD81 knock out ($CD81^{-/-}$) mice lacking expression of CD81 have been generated in 1997 by Campbell and Muller [52]. Total thymocyte cell numbers were slightly increased in mutant animals as compared with those of wild type animals whereas the CD4/CD8 profiles of $CD81^{-/-}$ thymus were indistinguishable from those of $CD81^{+/+}$ thymus as well as the TCR expression. Positive and negative T cell selection processes occur normally in the absence of CD81. [52] Moreover, Maecker et al have reported that CD81 expression is

developmentally controlled during thymocyte maturation, being upregulated on $CD4^+$ $CD8^+$ DP thymocytes, then down-regulated again on mature single-positive thymocytes and is low to negative on peripheral T-cells.[53] CD81 expression can also be induced in mature T cells upon activation.[54] Data from different groups suggest that interactions between immature thymocytes and stromal cells expressing CD81 are required and may be sufficient to induce early events associated with T cell development as T-cell development at the DN stage is blocked by treatment with an antibody directed against CD81 (2F7).[55, 56]

1.6.2 The Role of CD81 In T Cell Activation and T Cell Mediated Immune Response.

When peripheral T cell functions were analyzed, T cells from $CD81^{-/-}$mice proliferated much better in response to various stimuli including anti-CD3 monoclonal antibody (mAb). When T cells were co-stimulated with anti-CD28 mAb and anti-CD3 mAb, proliferation of $CD81^{-/-}$ cells were increased even better than $CD81^{+/+}$ cells. [52] In different studies, treatment of human thymocytes or mature T-cells with an antibody directed against CD81 (5A6) has been shown to activate the integrin LFA-1 [50, 56] In humans, CD81 has been implicated in the induction of IL-4 secretion from T cells during Th2 immune responses when anti-CD81 monoclonal antibody is added to the cultures of lymphocytes from allergic individuals.[57] In mice lacking CD81 on the other hand , there was less IL-4 and less IgG1 antibody production during Th2 responses and CD81 on B cells induced IL-4 secretion from T cells during Th2 responses.[53, 58] Although both proliferation responses following either TCR or BCR were affected in $CD81^{-/-}$ mice, both tyrosine phosphorylation and intracellular calcium flux in lymphocytes following the engagement of these cell surface receptors were entirely comparable with those in wild type mice.[52]

The functionality of CD81 on thymocytes was further investigated by assessing its ability to provide costimulation in concert with CD3 cross linking. The result did not only show that cross linking of CD3 and CD81 promoted powerful proliferative response but also the physical proximity of CD81 and CD3 on the same cell surface is required for

stimulation.[50] Moreover, co-engagement of CD81 and CD3 on the surface of murine single positive T cells induces CD28 independent stimulation.[59] The exact detailed mechanism(s) involved in CD81 costimulatory functions in T cells is currently unknown. The association of CD81 with CD4 and CD8 suggests that CD81 could be regulating T cell activation by these important T cell coreceptors.

1.6.3 The Role of CD81 in the Immune Synapse

When immune response is initiated by specific interaction of T cells and corresponding APCs, immune synapse (IS) is formed at the interface of both cells. The central zone of IS contains the TCR, co-stimulatory molecules and signaling molecules. As CD81 was found to be involved in T cell dependent B cell mediated immune responses, behavior of CD81 during immune synapse formation was studied and CD81 was found to co-localize with CD3 in the central zone of IS.[60] The exact role of CD81 in the immune synapse is however difficult to study because of its presence at the surface of both T and B cells and its accumulation in the cell-contact area. (Figure 1.6)

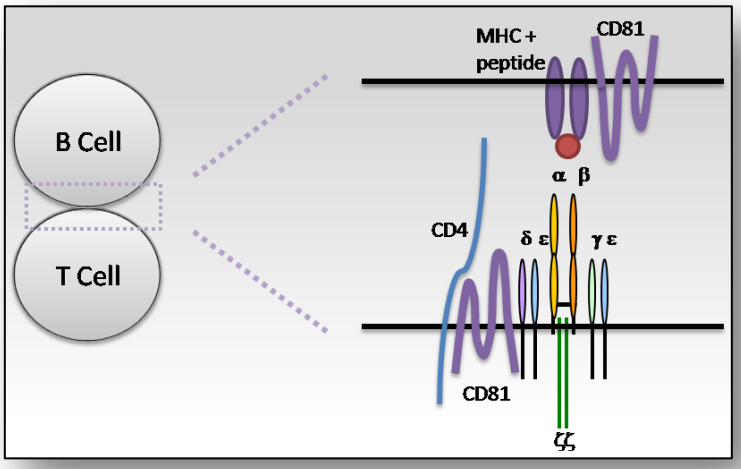

Figure 1.6: Proposed role of CD81 in T -B lymphocyte interface.

1.7 THE BCAP31 PROTEIN

BCAP31 that is also known as BAP31 is a component of the ER quality control compartment. It associates with nascent membrane proteins that are in transit between ER and cis-Golgi. This membrane protein with three transmembrane regions identified, has a C terminal KKEE motif that serves as an ER retrieval and retention signal.[61] In the C-terminal domain of BCAP31 there are also two identical recognition sites for initiator caspases 8 and 1 of the programmed cell death cascade.[62] Cleavage at the first site results in a 20-kD cleavage product that remains integrated in the ER membrane (residues 1–164 of BAP31), which has been implicated as a mediator of cytoplasmic membrane fragmentation events associated with apoptosis.

1.7.1 The Role of BCAP31 in the Immune System

Interaction with BCAP31 in the ER makes the assembly of partner proteins easier, leading to facilitated fabrication of macromolecular signaling complexes.[63] BCAP31 was originally discovered as a B cell Receptor associated protein and therefore named BCAP.[54, 64] It was shown to modulate surface expression levels of several proteins involving the ones important in immune system as MHCI and IgD.[61, 65-67] In a study to investigate BAP31s role in MHC class I expression on surface, there happens a moderately increased export rate and increased stability of exported MHCI, when BCAP31 was overexpressed. However, neither small interfering RNA (siRNA) -mediated reduction of BCAP31 levels, nor the loss of a functional *BCAP31* gene, affected MHCI levels or stability.[67] In a more recent study it is shown that BCAP31 is a component of the ER quality control compartment and that it cycles between the peripheral ER and a juxtanuclear ER, that correlates with the previously identified interactions.[68] BCAP31 has also been suggested to play a role in cytoplasmic membrane fragmentation events in cells undergoing apoptotic death. Its abundance in the cytoplasm of mature B-lymphocytes and in mature and immature T-lymphocytes together with the identified caspase cleavage sites suggests a role of apoptosis in the life cycle of lymphocytes.[69]

SECTION II: NOVEL TECHNOLOGIES

1.8 MEMBRANE BASED YEAST TWO HYBRID SYSTEM

Classical yeast two hybrid system is a powerful method for the in-vivo analysis of protein-protein interactions. Over 50% of known protein interactions in scientific literature were identified using yeast two hybrid system. Although being so powerful this system has some drawbacks as it is limited only to the soluble proteins or the soluble portions of the membrane proteins as the interactions are detected in the nucleus.[70, 71] It is assumed that almost one third of all proteins in eukaryotic cells are membrane associated.[72] As the membrane associated proteins have hydrophobic nature, conventional yeast two hybrid techniques cannot be employed to screen for interacting partners. There is a new genetic method for in-vivo detection of membrane protein interactions in yeast, that relies on split ubiquitin technology.[73] The main difference and therefore the convenience of this system lies beneath the ability to report interactions outside the nucleus by localizing the activator of the reporters out the nucleus. In this system ubiquitin, a highly conserved 76 amino acid long protein, serves as the first line of reporter of the interaction. Ubiquitin is the protein that marks the proteins for degradation when bound to the N terminal of the protein. After targeting the protein to degradation ubiquitin is cleaved from the protein by the action of ubiquitin specific proteases (UBPs) and recycled in this manner to be reused. (Figure1.7)

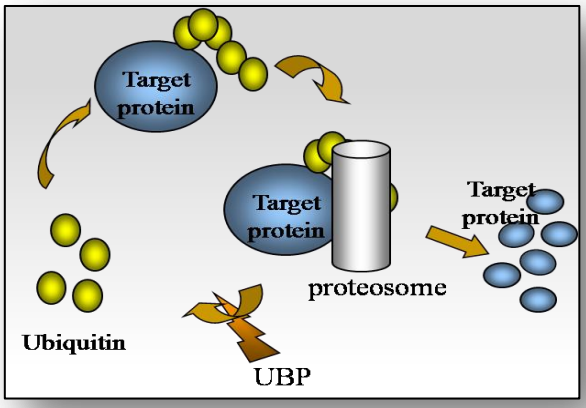

Figure 1.7: The mechanism of protein degradation by polyubiquitinylation and ubiquitin recycling by UBPs.

In the split-ubiquitin system, ubiquitin protein is divided into two halves N terminal ubiquitin (Nub) and C terminal (Cub). When these two portions are together the functional ubiquitin is formed. To use this fact as the screening system, a point mutation (isoleucine to glycine at position 13) is introduced in Nub and the mutated form is denoted as NubG. This single point mutation leads to a decrease in the affinity of Nub towards Cub and the functional ubiquitin is formed only when these Cub and NubG are brought into close proximity[72, 74]. (Figure1.8)

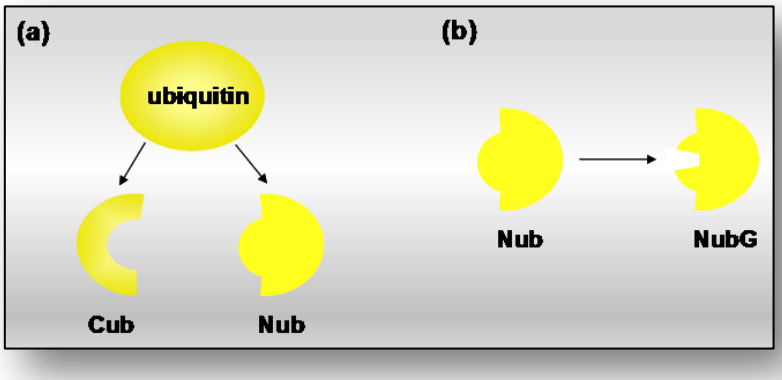

Figure 1.8: The split ubiquitin system: Ubiquitin can be divided into two halves, C terminal (Cub) and N terminal (Nub) (a). Introducing single point mutation to Nub, NubG is formed that has a decreased affinity towards Nub. (b)

In the dual membrane yeast two hybrid system this split ubiquitin is used as the screening system that makes it possible to detect the protein interactions outside the nucleus. There are membrane targeting signals in the bait and prey plasmids of the systems so that you can target your bait and/or prey protein of interest to the membrane bound to Cub and NubG respectively. As seen in figure 1.9, the bait protein is fused to Cub and prey protein is fused to NubG and only in the presence of an interaction between the bait and the prey the Cub and NubG come into close proximity and functional ubiquitin is formed. Then the action of the UBPSs recognize and cleave the ubiquitin from the complex and the LexA-VP16 transcription activator bound to ubiquitin is set free to go into the nucleus and activate the receptor genes. The receptor genes in the yeast strain used are auxotrophic markers as HIS and ADE and LacZ. Assaying the growth ability of yeast colonies in the HIS and ADE lacking medium and also β-galactosidase activity, any possible interaction between the bait and prey is possible to detect.[75]

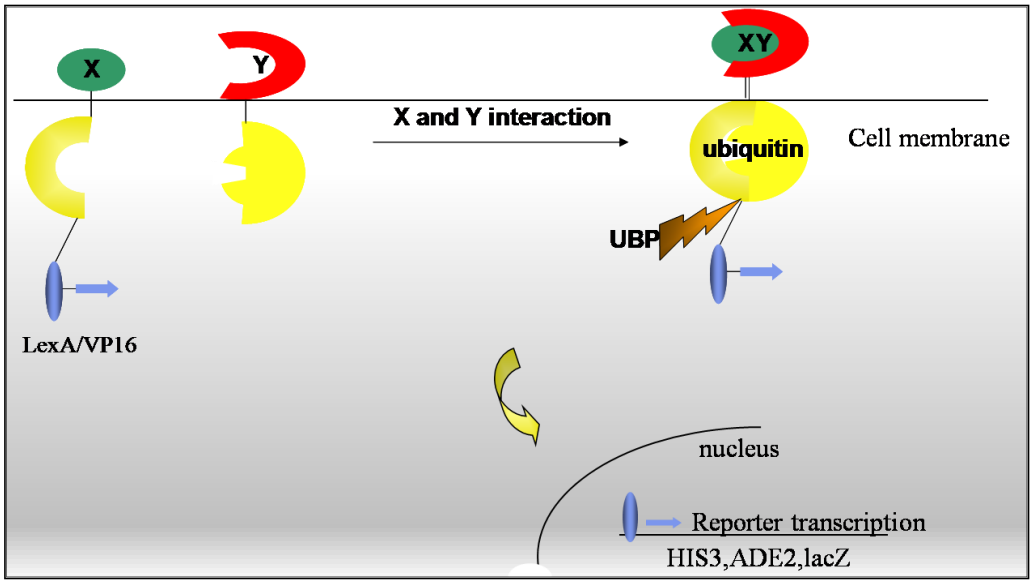

Figure 1.9: The split ubiquitin membrane yeast two hybrid system to detect protein interactions. Protein X that is fused to Cub and LexA/VP16 transcription factor, interacts with Protein Y that is fused to NubG. In case of interaction between X and Y proteins, Cub and NubG comes to close proximity forming the functional ubiquitin that is recognized by UBPs. Following UBP action LexA/VP16 is set free and translocated to nucleus to drive the expression of downstream reporter genes. There are three different reporters: HIS3 and ADE2 auxotrophic markers and LacZ.

1.9 shRNA MEDIATED EXPRESSION ARREST

Sequence specific gene silencing mediated by RNA interference (RNAi) was first recognized in *C.elegans* as a biological response to exogenous double stranded RNA (dsRNA)[76] From the day it was discovered, RNA mediated gene silencing is employed with diverse set of groups to silence and study functions of diverse set of genes. This powerful tool was ,at first, limited to specific cell types excluding mammalian cells due to the presence of dsRNA triggered antiviral response present in mammalian cells. Advances in RNA mediated gene silencing showed that synthetic 21 nucleotide long small interfering RNAs (siRNAs) instead of dsRNAs can be used efficiently to induce gene silencing in a wide range of mammalian cell lines.[77]

Recently, it was shown that there exist endogenously encoded triggers of gene silencing that act through elements of the RNAi machinery to regulate the expression of protein coding genes, called microRNAs (miRNAs).[78] miRNAs are a family of small non-protein coding regulatory genes found in many eukaryotic organisms and some have been shown to regulate the expression levels of homologous target gene transcripts.[79] These small RNAs are transcribed as short hairpin precursors, pri-miRNAs and they are processed in the nucleus by the action of an RNaseIII family nuclease, Drosha into about 70nt long pre-miRNAs.[80] Pre-miRNAs exported to the cytoplasm are then processed into the active 21 nucleotide long RNAs by the action of another RNaseIII family nuclease, called DICER, in the cytoplasm. DICER products are called mature miRNA. These mature miRNAs are incorporated into a multicomponent nuclease complex, RISC, which identifies substrates and mediates recognition of target mRNAs by base-pairing interactions. These cognate mRNAs are then targeted for destruction that results in the expression arrest of the target gene.[79] (Figure 1.10)

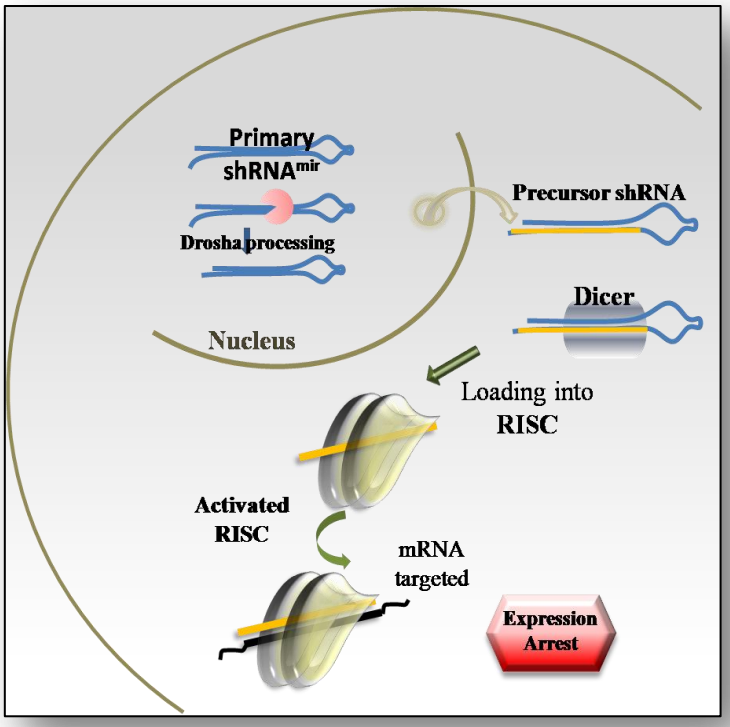

Figure 1.10: miRNA processing and miRNA based expression arrest: Pri-mRNA in nucleus is recognized and processed by Drosha forming Pre-miRNA. Pre-miRNA is exported to cytoplasm, where it is recognized by DICER and loaded to RISC complex. Activated RISC loaded with target mRNA and leads to expression arrest.

It is shown that short hairpin RNAs (shRNA) can be engineered to suppress the expression of desired genes in cultured mammalian cells.[78, 81] shRNAs can be transferred into cell lines of interest or endogenously expressed and cell lines with stable gene silencing can be constructed. shRNAs expressed from an RNA polymerase III vector, based on the mouse U6 RNA promoter can effectively inhibit gene expression in mammalian cells and retroviral plasmids can be efficiently used to deliver shRNAs into the nucleus of even hard to transfect mammalian cell lines and be effective both in vitro and in vivo.[81, 82] These retroviral plasmids used either for infecting or transfecting the cell line of interest can be efficiently used to silence gene expression and obtain functional data in vitro.

One such retroviral plasmid that express shRNA constructs as human microRNA30 (mir30) primary transcripts, is pSM2 plasmid of Open Biosystems. When the mir30 based shRNAmir is expressed in the nucleus its maturation occurs through sequential processing events as in case of miRNAs. [83](Figure 1.11)

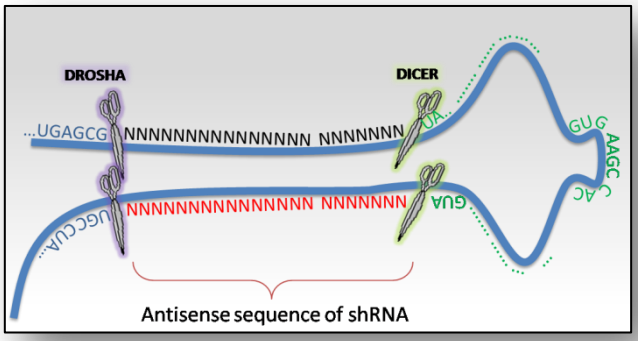

Figure 1.11: mir-30 based shRNAmir design: shRNAs are expressed as miR30 primary transcripts from the pSM2 retroviral plasmid. Blue sequences are the human mir30 based sequences, the black and the red sequences are the sequence specific sense and antisense strands of shRNA, the green sequences are the loop sequences. Drosha and DICER recognition and processing sites are shown as purple and green scissors respectively.

Another retroviral plasmid that efficiently expresses shRNAmir constructs this time from RNA polymerase II (PolII) promoter is MSCV-LTRmiR30-PIG (LMP) plasmid. This MSCV based retroviral plasmid can be used to express shRNAmir of interest ,which can be designed and cloned directly into the Xho1 and EcoR1 sites, flanked by 5' and 3' mir30 sequences. (Figure 1.12)

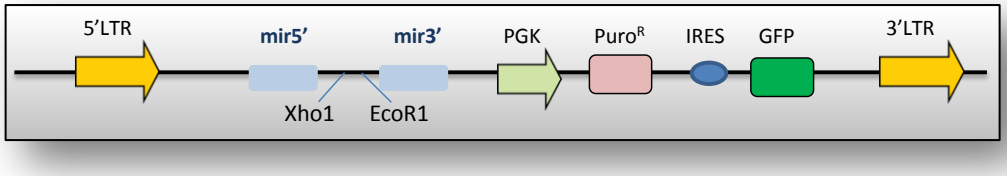

Figure 1.12: Map of the LMP plasmid: LMP drives the expression of shRNAs cloned in Xho1 EcoR1 restriction sites between humanmir30 sequences(mir5' and mir3') from the LTR promoter(5'LTR). Stable expression of the retroviral plasmid can be selected by puromycin resistance (Puro^R) driven by PGK promoter and visualization of successful infection or transfection is possible by the aid of IRES GFP coding sequence in the retroviral plasmid.

2. AIM OF THE STUDY

The TCR is made up of 2 ligand recognition and 6 signal transducing subunits. All signal transducing subunits contain ITAM domains with tyrosine residues that can be phosphorylated. These signal transducing subunits do not have overlapping functions. We have focused on the CD3δ subunit because this subunit has some differences when compared to the other signaling CD3 subunits of TCR.[36] The purpose of this study was to understand the mechanism of TCR signal transduction by identifying proteins that interact with the CD3δ subunit of this receptor. The membrane based yeast two hybrid method allows the identification of protein-protein interactions of the transmembrane proteins. Using this screening method we aimed to identify both transmembrane and cytoplasmic proteins that interact with the CD3δ subunit of the TCR.

By screening a Jurkat cDNA library we wanted to identify any interacting protein with CD3δ in the ER or on the cell membrane and we confirmed the specificity of these protein-protein interactions by expressing these cDNAs in mammalian tissue culture cells and co-immunoprecipitating their products in vitro. Among the putative proteins identified so far there are the ones which can function in TCR signaling along with the ones with unknown function. Elucidating the interaction pattern of these proteins with CD3δ and therefore the TCR complex will enlarge our perspective on TCR signaling and stoichiometry.

The reason to focus on the CD3δ subunit of the TCR is because CD3δ knockout mice do not have any peripheral T lymphocytes.[36] These mice have arrested development of at the positive selection stage of T lymphocytes in the thymus. This phenotype indicates that the CD3δ subunit is either required for signaling by, or the expression of the TCR on the surface of developing thymocytes. We aimed to identify 2 classes of cellular proteins.

The first group may play a role in the expression of TCR on the surface of T lymphocytes by interacting with the CD3δ subunit. The second group may play a role in the transduction of signals to the cytoplasm once the TCR is engaged.

Understanding and analyzing the TCR signaling is crucial as the problems in TCR signaling leads to problems in immune system of the organism as Severe Combined Immunodeficiency (SCID). Mature and functional T lymphocytes are the most important line of defense against cancer cells and bacteria or virus infected cells. In this study we would also like to test whether CD3δ or the identified interactors play a specific role in TCR or TCR-CD4 mediated signaling events that take place. Our studies will aid the identification of factors underlying immunodeficiencies and cancer. The very long term aim of this project is the identification of novel drugs to treat these diseases.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All the chemicals used in this project are listed in the Appendix A.

3.1.2 Equipment

All the equipment used in this project are listed in the Appendix B.

3.1.3 Buffers And Solutions

Standard buffers and solutions used in the project were prepared according to the protocols in Sambrook *et al*., 2001.[84]

3.1.3.1 Gel Electrophoresis:

10X Tris-Borate-EDTA (TBE) Buffer: 104 g Tris base, 55 g boric acid and 40 ml 0.5M EDTA at pH 8.0 were dissolved in 1 L of distilled H_2O.

Agarose gel: For 1% w/v agarose gel preparation, 1 g of agarose was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

SDS Separating Gel (13%): For 10 ml gel, 2.5 ml Tris 1.5M pH 8.8, 3 ml H_2O, 4.34 ml Acryl: Bisacryl (30%), 100 µl 10% SDS, 100 µl 10% APS, and 10 µl TEMED.

SDS Stacking Gel (4%): For 5mL gel, 1.25 ml Tris 0.5 M pH 6.8, 2.70 ml H2O, 1 ml Acryl: Bisacryl (30%), 50 µl 10% SDS, 15 µl 10% APS, and 7.5 µl TEMED.

5X Protein Loading Buffer Pack: Fermentas pack (#R0891) that ıncludes 5XLoadıng Dye (0.313 M Tris HCl (pH 6.8 at 25°C), 10% SDS, 0.05% bromophenol blue, 50% glycerol) and 20X Reducing agent (2M DTT) was used.

10X SDS Running Buffer: 30.3 g Tris base, 144 g Glycine, 10 g SDS were dissolved in 1 L ddH_20.

1X Transfer Buffer: 80 ml 10X Transfer Buffer , 160 ml MeOH, and 560 ml ddH_20.

Blocking Buffer: For 10ml preparation, 10 ml PBST and 0.5 g Milk powder/BSA.

3.1.3.2 Bacterial transformation:

Calcium Chloride ($CaCl_2$) Solution: 60mM $CaCl_2$ (diluted from 1M stock), 15% Glycerol ,10mM PIPES (pH=7) and the solution prepared was autoclaved at 121 °C for 15 min and stored at 4 °C.

3.1.3.3 Yeast Transformation

50% PEG : For 100ml solution 50g PEG4000 was dissolved in 80ml distilled water, the solution was stirred until completely dissolved and the total volume is then adjusted to 100ml. Solution was sterilized by filtering through a 0.22µm pore filter. As it is cruical to avoid evaporation the solution cap was sealed with parafilm.

10XTE: For 1L of solution , 100ml from 1M Tris-Cl(pH7.5) and 20ml from 0.5M EDTA(pH8.0) were mixed and volume is completed to 1L with distilled water. Solution was sterilized by filtering through a 0.22µm pore filter.

1MLiOAc (Lithium acetate) : For 100 ml of solution 10.2 g of LiOAc x $2H_2O$ was dissolved in 100 ml distilled water. Solution was sterilized by filtering through a 0.22µm pore filter.

Single stranded carrier DNA from salmon sperm: 200mg salmon sperm DNA typeIII sodium salt (Sigma D1636) was weigh into sterile glass beaker with the stirring bar. After adding 100ml sterile water all the DNA was dissolved by stirring for 2-3 hours. Alliquots into sterile eppendorf tubes were prepared and boiled in water bath for 5 minutes. Boiled DNA was chilled immediately in ice and stored at -20°C.

PEG/LiOAc master mix: 2.4 ml from 50%PEG and 360µl 1M LiOAc were mixed with 250 µl ssDNA (boiled two times prior to use)

3.1.3.4 Mammalian cell culture:

Phosphate-buffered saline (PBS): 9.88g of DPBS powder (Cellgro,Mediatech #55-031-PB) is dissolved in1 L distilled H_2O.

2X HEPES-buffered saline(2XHBS): 0.8 g NaCl, 0.027 g $Na_2HPO_4.2H_2O$ and 1.2 g HEPES were dissolved in 90 ml of distilled H_2O. pH was adjusted to 7.05 with 0.5 M NaOH and the solution was completed to 100 ml with distilled water. The buffer was filter-sterilized and stored at -20 °C.

Trypan blue dye (0.4% w/v): 40 µg of trypan blue was dissolved in 10 ml PBS.

TX100 Lysis Buffer: For 10 ml buffer, 0.5ml 20% TX100, 0.5 ml 1 M Tris (pH7.4), 0.3 ml 5M NaCl, 0.04 ml 0.5M EDTA, 8.7 ml water, and 1 tablet protease inhibitor (Complete mini EDTA free) were used.

1X PBS-Tween20 (PBST) Solution: 2 mL of Tween20 in 1L of 1XPBS.

3.1.4 Growth Media

3.1.4.1 Bacterial Growth Media:

Luria Broth from BD was used for liquid culture of bacteria. 20 g of LB Broth was dissolved in 1 L of distilled water and autoclaved at 121^{o}C for 20 min. For selection, kanamycin at a final concentration of 50 μg/ml and ampicillin at a final concentration of 100 μg/ml were added to liquid medium after autoclave .LB Agar from BD was used for preparation of solid medium for the growth of bacteria. 40 g of LB Agar was dissolved in 1 L distilled water and autoclaved at 121^{o}C for 15 min. Autoclaved medium was poured onto sterile Petri dishes (~20 ml/plate) after cooling down to 50^{o}C. For selection, kanamycin at a final concentration of 50 μg/ml and ampicillin at a final concentration of 100 μg/ml were added to the medium before pouring onto petri dishes. Sterile solid agar plates were kept at 4^{o}C.

3.1.4.2 Yeast Growth Media:

Yeast extract-Peptone-Dextrose (YPD) Broth from BD was used for liquid culture of wild type yeast strain NMY51. 50g of YPD Broth was dissolved in 1 L of distilled water and autoclaved at 121^{o}C for 15 min. YPD Agar from BD was used for preparation of solid medium for the growth of yeast. 65g of YPD Agar was dissolved in 1 L of distilled water and autoclaved at 121^{o}C for 15 min. Autoclaved medium was poured onto sterile Petri dishes (~20 ml/plate) after cooling down to 50^{o}C. SD medium and plates were prepared according to table 3.1, autoclaved for 15min at 121°C.

Supplement	**Amount/ liter**	**Final**
Yeast nitrogen base without aminoacids (Difco)	6.7g	0.7%
Synthetic Complete Drop-out Medium (CSM-Ade-His-Leu-Trp) ,Q-Biogene	0.6- 0.7g	0.1%
Glucose Monohydrate (Sigma)	20g	2%
Bacto Agar (Biolab) - For plates only-	20g	2%
Water	To 1 liter	

Table 3.1: The composition of SD medium

For selection, the aminoacid stocks and amounts used were as in Table 3.2 . Prepared aminoacid stocks were sterilized by filtering through a 0.22μm pore filter and the ones except adenine were stored at 4^{o}C, adenine was stored at room temperature. For pouring different amino acid selective medium, appropriate amino acid solutions were added to media before pouring onto Petri dishes. Sterile solid agar plates were kept at 4^{o}C

Amino acid	**Stock**	**Amount/ liter**	**Final concentration**
Tryptophan	10g/liter	2ml	0.02 g / liter
Histidine	10g/liter	2ml	0.02 g / liter
Leucine	10g/liter	10ml	0.1 g / liter
Adenine	2g/liter	10ml	0.02 g / liter

Table 3.2: Concentrations of aminoacids stocks.

3.1.4.3 Tissue Culture Growth Media:

Adherent cell lines: HEK 293T, NIH3T3,Hela and Phoenix cell lines were grown in filter-sterilized DMEM that is supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-Glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin.

Suspension cell lines: 3B4.15, VL3.3M2 and Jurkat cell lines were grown in filter-sterilized RPMI1640 that is supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-Glutamine, 100 unit/ml penicillin, 100 unit/ml streptomycin and 50μM beta mercaptoethanol.

Freezing medium: All the cell lines were frozen in medium containing DMSO added into fetal bovine serum (FBS) at a final concentration of 10% (v/v) and stored at 4^{o}C.

3.1.5 Molecular Biology Kits

- QIAGEN Plasmid Midi Kit, 12145, QIAGEN,Germany
- QIAGEN Plasmid Maxi Kit, 12163, QIAGEN, Germany
- Qiaprep Spin Miniprep Kit, QIAGEN,Germany
- Qiaquick Gel Extraction Kit,28706, QIAGEN,Germany
- Qiaquick PCR Purification Kit,28106, QIAGEN,Germany
- ROCHE Plasmid Miniprep Kit, ROCHE, Germany
- DUALmembrane kit 3, P01001,Dualsystems Biotech, Switzerland
- DSY Yeast Transformation Kit,P01003, Dualsystems Biotech, Switzerland
- DNA Blunting and Ligation Kit, K1512, Fermentas
- ImPromII Reverse Transcription System, A3800,Promega,USA

3.1.6 Enzymes

All the restriction enzymes and their corresponding 10X reaction buffers, DNA modifying enzymes and polymerases used in this study were from Fermentas.

3.1.7 Cell Types

3.1.7.1 Bacterial cells:

E. coli DH-5α (F- endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZdeltaM15 hsdR17) competent cells were used for bacterial transformation of plasmids.

3.1.7.2 Yeast cells:

The yeast strain NMY51 ((MATa his3Δ200 trp1–901 leu2–3,112 ade2 LYS2::(lexAop)-HIS3 ura3::$(lexAop)_8$-LacZ ade2::$(lexAop)_8$-ADE2GAL4) from Dualsystems Biotech was used for yeast two hybrid experiments.

3.1.7.3 Tissue culture cell lines:

HEK293T (derivative of human embryonic kidney 293 cell line that stably express large T antigen of SV40 and plasmids containing the SV40 origin are replicated to a copy number of between 400 - 1000 plasmids per cell within 293T, and therefore express at a higher level) was used for the transfection and immunoprecipitation experiments.[85] (ATCC: CRL-1573™)

NIH 3T3 is a mouse embryonic fibroblast cell line. This adherent cell line was used for the optimization of retroviral infection of mouse cell lines. (ATCC: CRL-1658™)

Phoenix – Eco cell line is a second-generation retrovirus producer cell line based on HEK293T cell line that express gag-pol-env proteins for the generation of helper free ecotropic retroviruses. [86]

VL3-3M2 is a double positive thymic lymphoma cell line. [87]

3B4.15 is a CD4 single positive T cell hybridoma that is specific pigeon cytochrome C in association with I-E^k .[88]

HeLa is a human epithelial carcinoma cell line. This cell line with its large cell size used fof the subcellular localization studies. (ATCC: CCL-2)

3.1.8 Vectors and Primers

Vectors and primers used in this project are listed in the Table 3.3 and Table 3.4.

Vector	Use	Yeast Auxotropic marker	E.coli resistance marker	Mammalian Resistance marker	Company
pBT3-SUC	Yeast two hybrid bait vector	LEU2	Kan	N/A	DualSystems Dual Membrane Kit3
pPR3-SUC	Yeast two hybrid prey vector	TRP1	Amp	N/A	DualSystems Dual Membrane Kit3
pDSL-Nx	Yeast two hybrid library vector	TRP1	Amp	N/A	DualSystems Dual Membrane Kit3
pCCW-Alg5	Yeast two hybrid positive control bait vector	LEU2	Kan	N/A	DualSystems Dual Membrane Kit3
pAI-Alg5	Yeast two hybrid positive control prey vector	TRP1	Amp	N/A	DualSystems Dual Membrane Kit3
pDL2-Alg5	Yeast two hybrid negative control prey vector	TRP1	Amp	N/A	DualSystems Dual Membrane Kit3
pcDNA-GFP	Transfection control vector	N/A	Amp	N/A	Constructed
pHA-Mex	Mammalian expression vector with N terminal HA tag	N/A	Kan	Neo	DualSystems P03401
pcDNA3.1Myc HIS (-)A	Mammalian expression vector with C terminal Myc tag	N/A	Amp /Kan	Neo	Invitrogen
LMP	MSCV based retroviral vector with GFP	N/A	Amp	Puro	Open Biosystems
pSM2-CD3δ	MSCV based retroviral vector expressing shRNAs against CD3δ	N/A	Kan	Puro	Open Biosystems

Table 3.3: The list of vectors used in this study.

Primer	Sequence	Use
Sfi-d-F1	CCGGGCCATTACGGCCTTCA AGGTACAAGTGACCG	Forward primer of CD3δ to clone into bait /prey plasmid without the leader peptide
Sfi-d-R	GACGGCCGAGCCGGCCTTAG ATTTCTTGTTCCGGGG	Reverse primer of CD3δ to clone into bait/prey plasmid
Sfi-d-F2	CCGGGCCATTACGGCCATGG AACACAGCGGGATT	Forward primer of CD3δ to clone into bait /prey plasmid with the leader peptide
Sfi-g-F1	CCGGGCCATTACGGCCCAGA CAAATAAAGCAAATTTG	Forward primer of CD3γ to clone into bait/prey plasmid without the leader peptide
Sfi-g-R	GACGGCCGAGCCGGCCTTCT TCTTCCTCAGTTGGTTTC	Reverse primer of CD3 γ to clone into bait/prey plasmid
Sfi-g-F2	CCGGGCCATTACGGCCATGG AGCAGAGGGGTCT	Forward primer of CD3γ to clone into bait/prey plasmid with the leader peptide
Sfi-e-F1	CCGGGCCATTACGGCCCAGG ACGATGCCGAGAAC	Forward primer of CD3ε to clone into bait/prey plasmid without the leader peptide
Sfi-e-R	GACGGCCGAGCCGGCCTTGA CTGCTCTCTGATTCAGATTG	Reverse primer of CD3ε to clone into bait/prey plasmid
Sfi-e-F2	CCGGGCCATTACGGCCATGC GGTGGAACACTTTC	Forward primer of CD3ε to clone into bait/prey plasmid with the leader peptide
Sfi-z-F1	CCGGGCCATTACGGCCCAGA GCTTTGGTCTGCTG	Forward primer of TCRζ to clone into bait/prey plasmid without the leader peptide
Sfi-z-R	GACGGCCGAGCCGGCCTTGA GCTGGGAGCTGCTACTG	Reverse primer of TCRζ to clone into bait/prey plasmid
Sfi-z-F2	CCGGGCCATTACGGCCATGA AGTGGAAAGTGTCTGTTC	Forward primer of TCRζ to clone into bait /prey plasmid with the leader peptide
Sfi-Ta-F1	CCGGGCCATTACGGCCGATC AGGTGGAGCAGAGTC	Forward primer of TCRα to clone into bait/prey plasmid without the leader peptide
Sfi-Ta-R	GACGGCCGAGCCGGCCTTAC TGGACCACAGCCTCAG	Reverse primer of TCRα to clone into bait/prey plasmid
Sfi-Ta-F2	CCGGGCCATTACGGCCATGC AGAGGAACCTGGG	Forward primer of TCRα to clone into bait/prey plasmid with the leader peptide
Sfi-pTa-F1	CCGGGCCATTACGGCCCTAC CATCAGGCATCGCTG	Forward primer of preTCRα to clone into bait/prey plasmid without the leader peptide
Sfi-pTa-R	GACGGCCGAGCCGGCCTTTG TCCAAATTCTGTGGGTG	Reverse primer of preTCRα to clone into bait/prey plasmid

Primer	Sequence	Use
Sfi-pTa-F2	CCGGGCCATTACGGCCATGC TTCTCCACGAGTGG	Forward primer of preTCRa to clone into bait/prey plasmid with the leader peptide
Sfi-Tb-F1	CCGGGCCATTACGGCCAAAG TCATTCAGACTCCAAGA	Forward primer of TCRβ to clone into bait/prey plasmid without the leader peptide
Sfi-Tb-1R	GACGGCCGAGCCGGCCTTGG AATTTTTTTTCTTGACCATG	Reverse primer of TCRβ to clone into bait/prey plasmid
Sfi-Tb-F2	CCGGGCCATTACGGCCATGG CTACAAGGCTCCTC	Forward primer of TCRβ to clone into bait/prey plasmid with the leader peptide
pDSL-Nx-Seq-F	GTCGAAAATTCAAGACAAGG	Forward sequencing primer for the cDNAs in pDSLNx vector.
pDSL-Nx-Seq-R	AAGCGTGACATAACTAATTAC	Reverse sequencing primer for the cDNAs in pDSLNx vector.
CD81-For	CCGGGCCATTACGGCCGGAG TGGAGGGCTGCACC	Forward primer to clone CD81/CD9 chimeras into pHAMex vector.
CD81-Rev	GACGGCCGAGGCGGCCTCAG TACACGGAGCTG	Reverse primer to clone CD81/CD9 chimeras into pHAMex vector.
CD9-For	CCGGGCCATTACGGCCCCGG TCAAAGGAGGCACC	Forward primer to clone CD81/CD9 chimeras into pHAMex vector.
CD9-Rev	GACGGCCGAGGCGGCCCTAG ACCATCTCGCGGTTC	Reverse primer to clone CD81/CD9 chimeras into pHAMex vector.
CD81Exon1For	GAGCGAGCGCGCAACG	Primers to test alternative splice variant of CD81.
CD81Exon1Rev	CCAGAAGACGAAATTGAAGAC G	Primers to test alternative splice variant of CD81.
CD81putExonFor	GGGGCATCTCAGAGGGCGC	Primers to test alternative splice variant of CD81.
CD81putExonRev	CTGTGGGAAGCCTCTCTGCC ACCTGC	Primers to test alternative splice variant of CD81.
CD81Exon2Rev	CTACATAGAAGGTGTTGGGC GC	Primers to test alternative splice variant of CD81.
BCAP31TrunFor1	CCGGGCCATTACGGCCAGTC TGCAGTGGACTGCAGTTGC	Forward primer for constructing BCAP31 Truncation 1and 5.
BCAP31TrunFor2	CCGGGCCATTACGGCCGAGT TGTTAGTGTCCTATGGC	Forward primer for constructing BCAP31 Truncation 2.

Primer	Sequence	Use
BCAP31TrunFor3	CCGGGCCATTACGGCCATTG CTGGCTTTTCCTTGCTGC	Forward primer for constructing BCAP31 Truncation 3.
BCAP31TrunFor4-164D	CCGGGCCATTACGGCCGGAG GCAAGTTGGATGTCGG	Forward primer for constructing BCAP31 Truncation 4.
BCAP31TrunRev-164D	GACGGCCGAGGCGGCCTCAG TCAACAGCAGCTCCCTTC	Reverse primer for constructing BCAP31 Truncation 5.
G1.2	CAATGAAGATGACACCGGATA TGGTGCCTATG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
G3.1	CACGGGCACCATGGCTGGCT TTATCTTCGCTG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
G3.2	CTTCCGGTCTCATGTCCCGCA ATGAGATATAC	Primers of fusion PCR to construct CD3 delta gamma chimeras.
G2.1	CGTCTACTGCTTTGCAGGACA GGATGGAGTTC	Primers of fusion PCR to construct CD3 delta gamma chimeras.
D3.2	CAGCGAAGATAAAGCCAGCC ATGGTGCCCGAG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
D1.1	CATAGGCACCATATCCGGTGT CATCTTCATTG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
D2.2	GAACTCCATCCTGTCCTGCAA AGCAGTAGACG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
D3.3	GTATATCTCATTGCGGGACAT GAGACCGGAAG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
FRET-d-F	CCGCTCGAGGCCACCATGGA ACACAGCGGGATTCTG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
FRET-d-R	CCCAAGCTTAGATTTCTTGTT CCGGGG	Primers of fusion PCR to construct CD3 delta gamma chimeras.
FRET-g-F	CCGCTCGAGGCCACCATGGA GCAGAGGAAGGGTC	Primers of fusion PCR to construct CD3 delta gamma chimeras.
FRET-g-R	CCCAAGCTTCTTCTTCCTCAG TTGGTTTCC	Primers of fusion PCR to construct CD3 delta gamma chimeras.
miR30-Forward	CAGAAGGCTCGAGAAGGTAT ATTGCTGTTGACAGTGAGCG	Primers to amplify shRNAs from template 97 nucleotide DNA oligo and clone into LMP.
miR30-Reverse	CTAAAGTAGCCCCTTGAATTC CGAGGCAGTAGGCA	Primers to amplify shRNAs from template 97 nucleotide DNA oligo and clone into LMP.

Table 3.4: The list of primers used in this study.

3.1.9 DNA and Protein Molecular Weight Markers

DNA and protein molecular weight markers used in this project are listed in Appendix C.

3.1.10 DNA Sequencing

Sequencing service was commercially provided by McLab,CA,USA. (http://www.mclab.com/home.php)

3.1.11 Software and Computer Based Programs

The software and online programs used in this project are listed in Table 3.5:

Program	Website/Company	Use
VectorNTI 9.1.0	Invitrogen	Vector maps, restriction analysis, alignments.
SignalP3.0	http://www.cbs.dtu.dk/services/SignalP/	Predict location of signal peptide cleavage sites.
Sosui	http://bp.nuap.nagoya-u.ac.jp/sosui/	Secondary structure prediction of membrane proteins
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Blast sequencing results against human genome.
FinchTV 1.4.0	Geospiza Inc.	View and analyse the sequencing results.
FlowJo 7.5.5	http://www.flowjo.com/	View and analyse Flow Cytometry data
TMRPres2D	http://biophysics.biol.uoa.gr/TMRPres2D/	Visual representation of transmembrane proteins.

Table 3.5: Software and online bioinformatic programs.

3.2 METHODS

3.2.1 General Molecular Cloning Techniques

3.2.1.1 Bacterial Cell Culture

Bacterial culture growth: Bacterial cells were grown overnight (12-16h) at 37 °C shaking at 270 rpm in Luria Broth with or without selective antibiotic prior to any application. Bacterial cells either spreaded or streaked were grown on LB Agar Petri dishes overnight (12-16h) at 37 °C. For the glycerol stock preparation of bacterial cells, glycerol was added to the overnight grown bacteria cultures to a final concentration of 15%. Cells were frozen first in liquid nitrogen and then stored at -80°C.

Chemically competent bacterial cell preparation: *E. coli* DH5α competent cells were prepared starting from a single colony of previously streaked competent cells on solid growth media without selective agents. Single colony from the plate was grown overnight at 37°C, 270 rpm in 50 ml LB without any antibiotics. Next day 4 ml from the overnight grown culture was diluted within 400 ml LB and incubated at 37°C, 270 rpm until the OD_{590} reaches 0.375. The culture is then transferred into 50ml falcon tubes and incubated on ice for 10 min prior to centrifugation to pellet the cells in cold room at 3000g for 30min. The cell pellet is then resuspended in 10ml ice cold $CaCl_2$ solution and centrifuged in cold room at 1100g for 10 min. After resuspending the pellet again in 10ml ice cold $CaCl_2$ solution the resuspended cells are incubated on ice for 30 min. Following the final centrifuge in cold room at 1100 g for 10 min, the pellet is resuspended in 2ml ice cold $CaCl_2$ solution and dispensed into 150µl aliquots into prechilled eppendorf tubes. The prepared competent cells were frozen immediately in liquid nitrogen and then stored at -80°C. Each time the competent cells were prepared, the transformation efficiency was tested by transforming pUC19 plasmid.

Chemical transformation of bacterial cells: $CaCl_2$ treated chemically competent bacterial cells were taken out of -80°C and the DNA samples were added to each tube before completely thawing the cells on ice. The tubes were then stored on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and transferred back on the ice rapidly to chill for 60 seconds. 800 µl of autoclaved LB was added and the cultures were incubated for 45 minutes in a 37°C water bath to allow the bacteria recover and express the antibiotic resistance gene encoded by the plasmid. Transformed cells were spreaded on LB Agar plates with the appropriate antibiotics using 4mm sterile glass beads. The plates were then incubated overnight at 37°C.

Plasmid DNA isolation from bacterial cells: Plasmid DNA isolation was performed by the Mini- and Midi-Prep Kits from either Roche or QIAGEN. Single colony of *E. coli* from LB Agar plate or glycerol stock was grown overnight at 37°C, 270 rpm in liquid culture containing the selective antibiotics, prior to the plasmid isolation. If needed, a portion of overnight culture was used to make glycerol stock as explained previously. The concentration of the plasmid DNA isolated was determined quantitatively by UV Spectrophotometer. Several dilutions of plasmid DNA mini- or midi-prep were prepared and UV absorbance was measured at 260 nm by using quartz cuvettes.

3.2.1.2 Plasmid Construction

Polymerase Chain Reaction (PCR): Optimized PCR reaction and thermal cycle conditions are shown in Table 3.6. The thermal cycler conditions were as follows: initial denaturation at 95ºC for 4 minutes followed by 30 cycles of denaturation step (at 95ºC for 30 seconds), annealing step (at 56ºC for 60 seconds) and an extension step (at 72ºC for 120 seconds). These cycles were then followed by a final extesion step at 72ºC for 6 minutes.

For colony PCR: from bacterial cells: one colony was taken into 100µl distilled water and 3µl was taken to use as the template, from yeast cells: one colony was taken into 10µl 0.02N NaOH, swirled and lysed at 95°C for 5-10min. and used as the template.

PCR Reaction	Volume Used	Final Concentration
Template DNA	1-10 µl	2 pg/µl -4 ng/µl
10X *Taq* Polymerase Buffer with $MgCI_2$	5 µl	1X
dNTP Mix (10mM)	0.5 µl	0.2 mM
Primer forward (10µM)	2 µl	0.8 µM
Primer reverse (10µM)	2 µl	0.8 µM
Taq Polymerase (25u/µl)	0.5 µl	1.25 u/µl
ddH_2O	Up to 25 µl	
Total	25 µl	

Table 3.6: Optimized PCR conditions.

Restriction enzyme digestion: Digestion reactions mixtures were set by the addition of the DNA, the enzyme and the compatible buffer, and incubated at the optimum temperature of the enzyme used for 2- 2.5 hours. 1µg of DNA was digested for diagnostic purposes. 10 µg DNA or more was digested for gel extraction and cloning purposes.

Agarose gel electrophoresis and gel extraction: Agarose gels to observe the DNA according to their size were prepared in different concentrations ranging from 0.8% to 2.5% depending on the size of the fragments to be separated. In order to separate large fragments the percentage was low and vice versa for small fragments. Gel was prepared by dissolving determined amount of agarose powder in 0.5X TBE and heating 3-5 minutes in a microwave, after the gel cools to room temperature ethidium bromide was added to a final concentration of 0,001(v/v) and then the gel was poured in the gel apparatus. 0.5X TBE buffer was also used in the tank as running buffer. 6X loading dye was added into the samples, before loading. Gels were run at 100 Volts for 40-80 minutes, and the bands were observed under UV. For gel extraction of DNA samples, QIAGEN Gel Extraction Kit was used.

Ligation: The ligation reactions were performed by using T4 DNA Ligase (Fermentas), in 1:3, 1:5 or 1:10 vector to insert ratio, using 50-100ng vector. 5' overhangs of single digested vectors were dephosphorylated by calf intestinal alkaline phosphatase (CIAP) prior to the ligation. Ligation reaction was performed at 16^{o}C overnight. Then, the ligation mixtures were transformed into chemically competent DH5α bacteria.

3.2.2 The Membrane Yeast Two Hybrid System

3.2.2.1 Cloning of TCR Components into Bait and Prey Plasmids

PCR amplification of TCR components and cloning into bait/prey plasmids: Primers designed so that all TCR components would be cloned into the bait and prey plasmids by the help of the two directional SfiI recognition sites present in the forward and the reverse primers.(Table3.4) As the bait plasmid pBT3-SUC has the SUC region that targets the following peptide to the cell membrane the leader peptides of the TCR components was identified with SignalP3.0 program[89] and the forward primers were designed to exclude the leader peptides of the components. PCR was carried out using the plasmids containing the different chains as the templates. The PCR products obtained was digested with SfiI following gel extraction and cloned into the SfiI digested bait and/or prey plasmids. After cloning the correct plasmids were confirmed first by colony PCR and then by restriction enzyme digestion analysis.

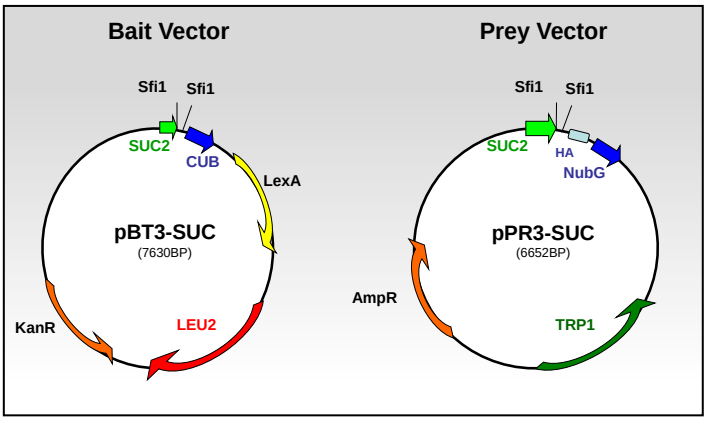

Figure 3.1: Maps of the bait (pBT3-SUC) and prey (pPR3-SUC) plasmids of the dual membrane system.

3.2.2.2 Transformation into Yeast

All the bait constructs are transformed into NMY51 strain with lithium acetate (LiOAc-PEG) transformation protocol. Wild type yeast grown on YPD plates were inoculated into 50 ml YPD for overnight growth at 29 ^{o}C, shaking and grown until OD_{546} is between 0.6 and 0.8 (if it is above 1.0, the culture is diluted to OD_{546} = 0.2 and re-grown to 0.6). Cells were pelleted at 2500g for 5 minutes at room temperature and pellet was resuspended in 2.5ml distilled water. PEG/LiOAc master mix was prepared freshly for each transformation and 300 µl of master mix was added on 1.5 µg of bait DNA to be transformed in an eppendorf. 100 µl of resuspended yeast was then added and the eppendorfs containing DNA to be transformed, yeast cells and master mix were incubated at 42 ^{o}C thermo mixer for 45 min. with slow shaking. The transformation reaction was then pelleted at 700g for 5 min at room temperature and the pellet was dissolved in 250 µl 0.9%NaCl . 50 µl from each transformation reaction were spread on SD selective plates and the plates were incubated at 29 °C for 2-3 days for the colonies to grow.

After selecting the yeast colonies that contain bait plasmid, prey plasmids are sequentially transformed into those NMY51 cells containing bait plasmids again with LiOAc-PEG protocol. Each transformation is coupled with the negative and positive control transformations. For large scale transformation that includes the bait plasmid and cDNA library plasmid 7µg of bait and prey plasmids were used.

3.2.2.3 Conformation and further analysis of primary colonies

The primary colonies grown on selective plates after library transformation was transferred into new selective plates and β-galactosidase assays were carried out via filter lift-off assays. An overlay mix containing 0.5% (w/v) agarose, 0.1mg/ml X-Gal stock solution (100mg/ml) in 1xPBS was prepared by heating in microwave and continuous stirring. 3MM Whatman papers cut to fit into 150mm petri dishes. Whatman papers were placed directly onto the agar plate containing the yeast colonies and incubated for 10

minutes. After the colonies sticked, the whatman paper was removed, incubated in liquid nitrogen for 5 min. and placed in the 150mm petri dish with colony side facing upwards. After letting the filters thaw for 5 min. overlay mixture was poured on such that the entire surface of the filter was covered. Allowing the agarose to solidify, the plates were incubated at room temperature until blue color developed. The intensity of blue color on the filter paper indicated the possible intensity of the interaction of the corresponding colony. All the β-galactosidase positive colonies were stocked in glycerol for further analysis.

3.2.2.4 Identification of Positive Colonies

The first strategy employed to identify the positive clones was doing colony PCR directly from the yeast colonies. For this purpose yeast cells were lysed by heating and the extract was used directly as the template for the PCR. The primers used for the PCR was the sequencing primers designed for the plasmid pDSL-Nx spanning the region of cDNA insert. (Table 3.4)

Plasmid recovery from yeast: The second strategy was the general strategy employed in literature to isolate DNA from yeast that has both bait and prey plasmids. Yeast colonies were grown overnight at 29°C in -leu-trp selective media. The cell pellet was resuspended in 250µl buffer P1(Qiagen) and transferred into eppendorf tube in which around 100µl of acid washed glass beads were added. Cells were lysed completely by vortexing the solution for 5 minutes. Qiagen miniprep protocol was employed for the rest of the isolation and in the last step DNA was eluded in 30 µl sterile water. 5 µl of miniprep yeast DNA was transformed into DH5α by standard chemical transformation procedure. The transformation product was spread on LB plates containing Ampicillin in order to select for the bacterial clones that were positively transformed with prey plasmid. Lastly plasmid isolation from DH5α was carried out and the isolated prey library plasmid was used directly for sequencing.

3.2.2.5 Reconfirmation of the interactions

The sequencing results were blast on human genomic transcripts using NCBI blast server to identify the clones. More than half of the clones were sequenced and the interesting ones are identified to be further confirmed. The prey plasmids identified to be further analyzed was transformed back into the yeast containing bait plasmid and secondary yeast two hybrid screens were carried out to reconfirm the interactions.

3.2.3 Interactions in Mammalian Cells

3.2.3.1 Cloning of CD3δ and selected cDNAs into mammalian expression plasmids

CD3δ coding region was amplified by PCR using designed FRET-d-F and FRET-d-R primers (Table 3.4) that amplifies all the coding region of CD3δ and introduces Xho1 and Hind3 restriction sites in the upstream and downstream of amplified region respectively. The digested PCR product was cloned into Xho1 and Hind3 digested mammalian expression plasmid pcDNA3.1Myc-His in frame with Myc tag. cDNA of interest was cut from the prey plasmid pDSL-Nx by the aid of SfiI restriction enzyme (that had one upstream and one downstream recognition sequence in the cDNA library plasmid) , it was then cloned into the mammalian expression plasmid pCMV-HA, in frame with the HA tag using the same SfiI sites present in the plasmid.

3.2.3.2 Transfection, cell lysis and immunoprecipitation

HEK293T cells were transfected by calcium phosphate procedure. Cells were split one to ten the day before transfection and on the day of transfection 10 μg of DNA to be transfected was mixed with 120μl of 1M$CaCl_2$ and the volume was completed to 500μl with distilled water in 15ml falcon tube. 500μl 2XHBS was added to the mixture drop by drop with bubbling and the resulting solution was vortexed immediately. The mixture was added on the cells after 10 min of incubation at room temperature in the hood. Three sets of transfections were carried out: one with the pcDNAMyc-CD3δ only, one with the

identified cDNA in pHAMex plasmid and the third one with both the pcDNAMyc-CD3δ and pHAMex-cDNA. Total amount of DNA transfected was 10μg in all of the reactions and all transformations had one positive control that is pcDNAGFP.

48 hours after transfection of HEK293T cells with the expression plasmids, cells were lysed in 1ml Triton-X Lysis buffer. The lysates were stored at -80^{0}C. For HA immunoprecipitations 50μl of anti-HA affinity matrix (Roche) is added in 450μl of lysate. For Myc immunoprecipitations 5ul anti-cMyc (Roche, 1μg/μl) plus 50μl proteinG sepharose 1:1 slurry was added in 450μl of lysates. Immunoprecipitations were carried out in cold room, rocking overnight. The next day beads were cleaned by precipitating and washing two times with PBS and after the final wash equal volume of SDS loading dye plus reducing agent was added. Samples were loaded on SDS gel after boiling for 5 minutes in water bath.

3.2.3.3 SDS Gel, transfer and western blot

All the SDS gels prepared in this study was 13% and after the samples were loaded the gel was run at constant 100V using BIORAD MiniProtean Tetra Cell and transferred using BIORAD Mini Trans-blot wet or BIORAD Trans-Blot semi-dry electrophoretic transfer cells. Membranes were blocked either overnight in cold room or for 1 hour at room temperature shaking in 10 ml PBST-milk. For the western blots anti-HA Peroxidase (Roche) or anti-Myc Peroxidase (Roche) was used. Antibody incubation was carried out at room temperature for 1 hour. Membranes were washed 3 times for 10 minutes in PBST after the antibody incubation and finally were visualized by the aid of SuperSignal West Dura Extended duration substrate.

3.2.4 Surface Markers of T Cell Lines

In order to visualize the surface markers, about 10^6 VL3.3M2 or 3B4.15 cells were precipitated, washed twice with PBS and then incubated with 1μg of the specific antibody in 100 μl PBS, on ice and in dark cold room, for 30 minutes. The cells were then washed

twice with PBS and directly analyzed by flow cytometry. The antibodies used for staining were all from BD biosciences unless indicated otherwise. The antibodies used in the study are listed in Table 3.7.

Antibody	Purpose (Stains)
H57.PE	TCRβ
2C11.FITC	Cd3δ/ε
GK1.5.PE	CD4
Ly-2.PE	CD8
CD69Bio with StreptavidinPE	CD69
CD81Bio with StreptavidinPE	CD81
CD5PE	CD5
CD69PE	CD69

Table 3.7: List of antibodies used for surface staining of T cell lines.

3.2.5 Stimulation of Double Positive and Single Positive T Cell Lines

3.2.5.1 Antibody Coating of Plates for Stimulation

24 well plates were coated with 0.025μg to 2.5μg of antiCD4 (GK1.5), anti-TCRβ (H57) or both in 1 ml of PBS per well. The plates were sealed with stretch film and incubated overnight at 4^0C. The next day, PBS is removed and 10^6 VL3.3M2 or 3B4.15 cells were added per well for stimulation. And the plate is incubated in CO_2 incubator until analysis.

3.2.5.2 Flow cytometric analysis of stimulation

24 hours after the stimulation, the stimulated cells were precipitated, washed and stained as explained in section 3.2.4 for the surface expression level of early activation marker CD69 or CD5. CD69FITC or CD69Bio coupled with Streptavidin PE and CD5PE were used for flow cytometric analysis.

3.2.6 Retroviral shRNA Mediated Expression Arrest

3.2.6.1 The pSM2 plasmid

To knock down the expression of CD3δ in T cell lines, retroviral plasmids coding shRNA towards CD3δ was purchased from Thermo Scientific Open Biosystems. (http://www.openbiosystems.com/RNAi/shRNAmirLibraries/pSM2Retroviral) Individual constructs were shipped as bacterial cultures of *E. coli* (DH10βpir116) in LB Broth with 8% glycerol and 25 μg/ml chloramphenicol and 25 μg/ml kanamycin and from that cultures glycerol stocks were prepared. Due to the tendency of all viral vectors to recombine we kept the incubation times as short as possible and avoided subculturing. Plasmids coding for the shRNAs towards EGFP (positive control) and CD3δ was isolated from the bacteria using Qiagen miniprep kit and analyzed by restriction enzyme analysis to ensure absence of recombination.

3.2.6.2 The LMP plasmid and cloning into LMP

Designing mir30 based shRNA template: MSCV-LTRmiR30-PIG (LMP) is a MSCV based retroviral vector that can be used to clone shRNA of interest and use for shRNA mediated gene silencing. In order to have a shRNA against the gene of interest, in our case CD81, the first step is designing the mir30-based shRNA template oligonucleotide. We first designed three different hairpin primers by selecting a sense sequence, of 22 nucleotide in length, from the coding sequence of CD81 gene. We then inserted the miR-30 loop sequence between the sense and the antisense sequences and finally added the appropriate flanking sequences of miR-30 miRNA to the 5' and the 3' end of the target. (Figure 3.2)

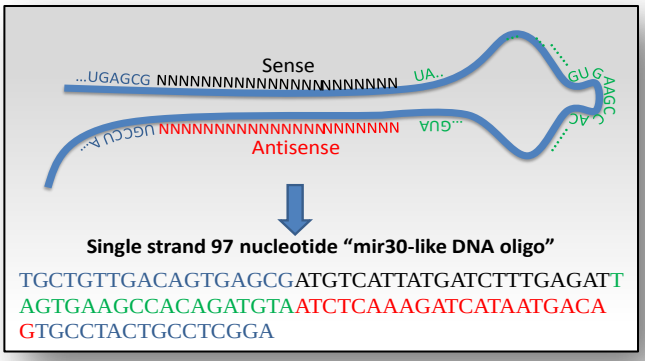

Figure 3.2: Design of oligonucleotides cloned into the LMP plasmid: A sense sequence of 22 nucleotide long (Black) is first selected from the gene of interest. miR30 loop sequence (green) is then inserted between the sense and antisense sequences. Finally, mir30 context (blue) is added to the 5' and 3' ends of the target. The formed 97 nucleotide long DNA oligonucleotide is used as the template to clone into LMP plasmid.

PCR amplification of mir30 based shRNA: Using the designed 97 nucleotide long oligonucleotides as the template PCR is carried out using mir30Forward and mir30 reverse primers. The PCR set up is given in Table 3.8. The thermal cycler conditions were as follows: initial denaturation at 94ºC for 1 minute followed by 30 cycles of denaturation step (at 94ºC for 30 seconds), annealing step (at 54ºC for 30 seconds) and an extension step (at 75ºC for 30 seconds). These cycles were then followed by a final extesion step at 75ºC for 10 minutes.

PCR Reaction	**Volume Used**
Template oligo(2nM)	10 µl
10X *Pfu* Polymerase Buffer with $MgCI_2$	10 µl
dNTP Mix (10mM)	2 µl
miR30 forward (10µM)	5 µl
miR30 reverse (10µM)	5 µl
DMSO	5 µl
Pfu Polymerase (25u/µl)	1 µl
ddH_2O	54 µl
Total	100 µl

Table 3.8: PCR amplification for shRNAmiR templates.

Cloning into LMP: The PCR product was then run on 2% agarose gel and the band corresponding to 135 bp. was extracted from the gel. Both the PCR product and the LMP plasmid purchased from Open Biosystems (Figure 3.3) were digested with EcoR1 and Xho1 and ligated. Correct ligation products were analyzed by restriction enzyme digestion.

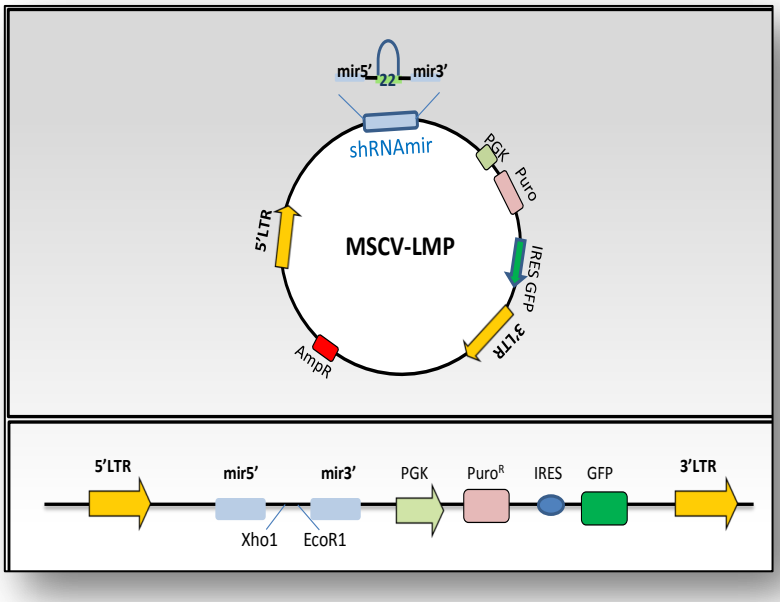

Figure 3.3: MSCV-LMP plasmid map with detailed shRNA insertion region at the bottom of the figure.

3.2.7 Retroviral Infection

3.2.7.1 Virus production using Phoenix cell line

Packaging cell line phoenix- Eco was transfected by Calcium phosphate transfection with 10µg of retroviral plasmid DNA and 2µg of packaging plasmid pCLEco. 48 hours after the transfection supernatant is collected and filtered through 0.45nm filters. Polybrene with final concentration of 6µg/ml was added to the filtered supernatant.

3.2.7.2 Infection of cell lines.

The cell lines to be infected in their mid to late log phases of growth were infected by spin infection procedure. NIH3T3 cell line is infected along with the desired cell lines as an internal control of infection efficiency. T cell lines to be infected were split one day before the infection in 24 well plates, NIH3T3 cells were also split one day before so that at time of infection they would be 60-80%confluent. The supernatant with polybrene from step 3.2.7.1 was used to infect the desired cell lines. For T cell lines in 24 well plate 1ml of supernatant was used per well, for the NIH3T3 cells 3 ml of viral supernatant was used. After addition of viral supernatant the plates were spinned at 32^{o}C and 1000g for 60 min. The plates were placed into the incubator and their mediums were changed after 2 hours. Secondary infection was also carried out, next day, following the same procedure. Infection efficiency was tested 48 hrs after the first infection.

3.2.8 Generation of Stable Cell Lines Expressing shRNAs

The cell line VL3.3M2 could not be infected by retroviral infection so they were transfected by electroporation with shRNA containing retroviral plasmids. BioRad GenepulserII was used for the electroporation procedures. VL3.3M2 cell line was split one day before transfection so that at the time of transfection they were in the log phase of growth. On the day of transfection 0.5-1 x 10^7 cells were washed once with serum free RPMI and resuspended in 500 μl serum free RPMI for transfection. Cells were transfected in 4mm electroporation cuvettes with 15 μg of DNA at different voltages as indicated in the results.

For the stable transfections, 48 hrs after the transfection cells were harvested and resuspended in RPMI with 1μg/ml puromycin and selected in 96well plates with limiting dilutions. For this selection procedure, one third of all the cells were used. Cells were resuspended in 1.6 ml of puromycin containing RPMI, and first 8 wells of 96 well plate were seeded with 200 μl of cell suspension/well. Cells in the first column were then diluted in 1:2 ratio with puromycin containing RPMI, serially through the following 10 columns.

Theoretically this seeding procedure with serial dilutions allows for 1 cell/well. Colonies became visible about 2-3 weeks after plating. The colonies that became visible were expanded in 24 well, 12 well and 6 well plates respectively. Surface CD81 expression levels along with GFP expression levels of the positive clones were assayed when the cells were at the 6 well plates. (Figure 3.4)

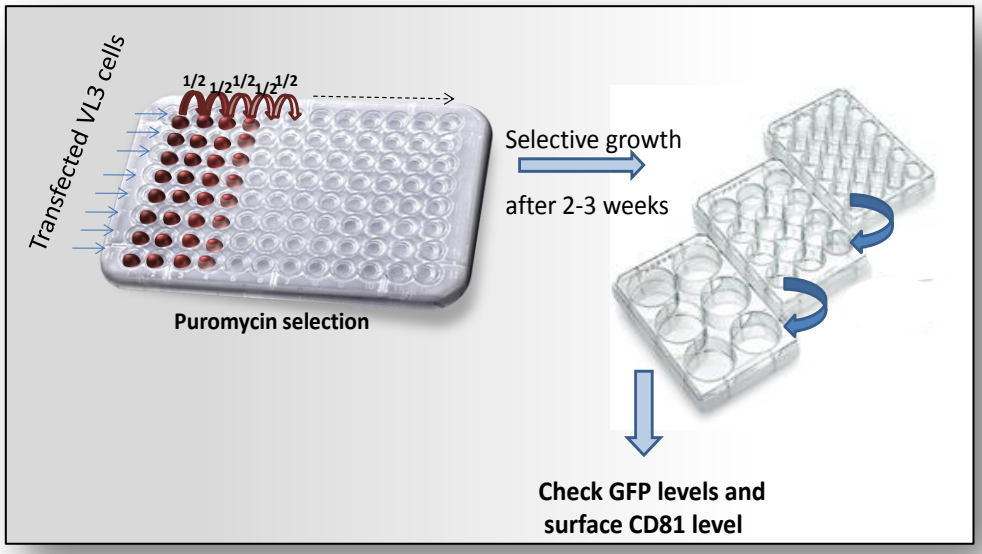

Figure 3.4: Flowchart describing the generation and selection of stable cell lines. VL3.3M2 cell lines were transfected with various plasmids by electroporation. 1:2 serial dilutions were performed in 96well plates under selection for puromycin. Colonies appearing in the lowest dilution were expanded under puromycin selection by transferring sequentially into 24, 12 and 6-well plates.

3.2.9 Activity of shRNAs

HEK293T cells were transfected with shRNA coding retroviral plasmids along with the expression plasmids of the target. The activity of shRNAs were assayed either with fluorescent cytometry (for the pSM2EGFP and LMPCD81) or with western blot (for pSM2CD3δ and LMPCD81) to see the decrease in the target gene expression.

4. RESULTS

4.1 THE DUAL MEMBRANE YEAST TWO HYBRID SYSTEM

4.1.1 Sub-Cloning of cDNAs Encoding TCR Subunits into Bait and Prey Plasmids

TCR components were amplified from the indicated plasmids with the designed primers (Figure 4.1). The PCR products were digested by the aid of the restriction sites introduced by PCR and cloned directly into the bait or prey plasmids. The correct ligation products were first analysed with colony PCR from the ligation colonies using the same primers used for amplification. (Figure 4.2) The colonies that gave positive result in colony PCR were used for DNA isolation and the isolated DNAs identity were confirmed by restriction enzyme analysis. (Figure 4.3)

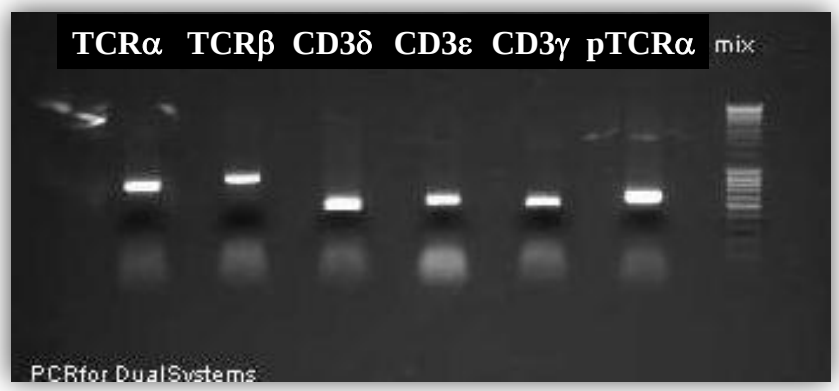

Figure 4.1: PCR amplification of TCR components. (TCRα: 890bp , TCRβ: 912bp ,CD3δ: 519bp, CD3γ:546 bp, CD3ε:620 bp, preTCRα: bp)

Figure 4.2: Successfully ligated clones identified by colony PCR. First row on the top shows different colonies from TCRα ligated bait plates. Presence of 890 bp band confirms the inserted TCRα. Second row shows TCRβ and CD3δ. 900bp band confirms correctly inserted TCRβ and 512 bp band confirms CD3δ respectively.

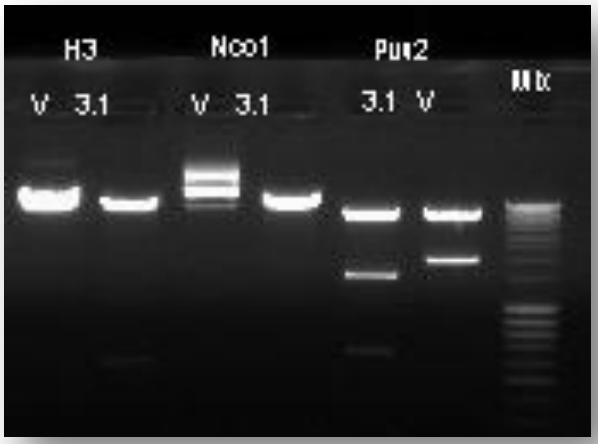

Figure 4.3: Confirmation of the pBT3SUC- CD3δ bait plasmid (colony3.1) H3:Hind3 has recognition sites in the upstream and downstream of SfiI sites used for cloning. Absence of any band in the vector alone digestion (V) but the presence of 530 bp band in colony 3.1 digestion shows an insertion in the SfiI site of the plasmid. Nco1: As there is no Nco1 site in the bait plasmid V shows undigested plasmid, whereas as the inserted CD3δ sequence introduces a single Nco1 site, plasmid from colony 3.1 is linearized with Nco1 digest. Pvu2: has two recognition sites in bait plasmid and CD3δ introduces two more, so the smaller 1935bp fragment in the vector alone digest becomes 1536,635 and 207 bp fragments in the colony 3.1 digest.

4.1.2 Plasmid Transformation into Yeast Cells- Positive and Negative Control Assays

Before transformation of the constructs into the NMY51 yeast strain, growth of NMY51 in selective and non-selective growth media was analyzed. After confirming that there was no visible growth neither on selective (-LTHA,-LTH) nor on non-selective (-LT) media, the pBT3-SUC- CD3δ construct is transformed into NMY51 strain. As there is Leu coding region in the bait plasmid, growth on –Leu plates confirmed the transformation. (Figure4.4)

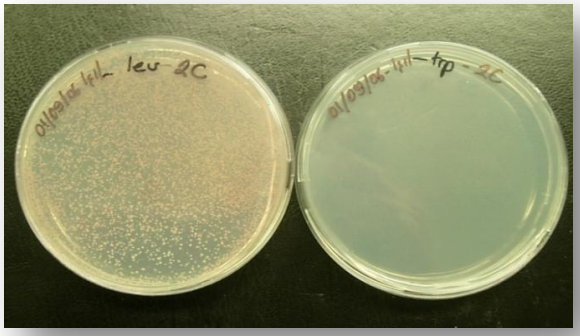

Figure 4.4: Growth of NMY51 yeast expressing bait proteins. Bait expressing NMY51 growth on –Leu plate (on the left) but not on –Trp plate(on the right).

Before going on with the library transformation positive and negative control assays were carried out. Positive control prey plasmid had Nub instead of NubG that would interact with the Cub region from the bait even in the absence of the protein interacting with the bait due to the strong affinity of Nub for Cub. Negative control prey had a yeast ER protein Alg5 fused to NubG which is unlikely to interact with our bait CD3δ. As NubG has no affinity towards Cub there were no colonies detected in this negative control assay. The results of the negative and positive assays were analyzed on the non-selective and selective plates. The non selective plates –Leu and –Trp indicated the presence of the bait and the prey plasmids respectively. The –Leu-Trp plate indicated the presence of both the bait and the prey plasmids in the yeast colonies and the selective plates –LTH and –LTHA indicated the activated reporter genes in the nucleus. (Figure 4.5 and Figure 4.6)

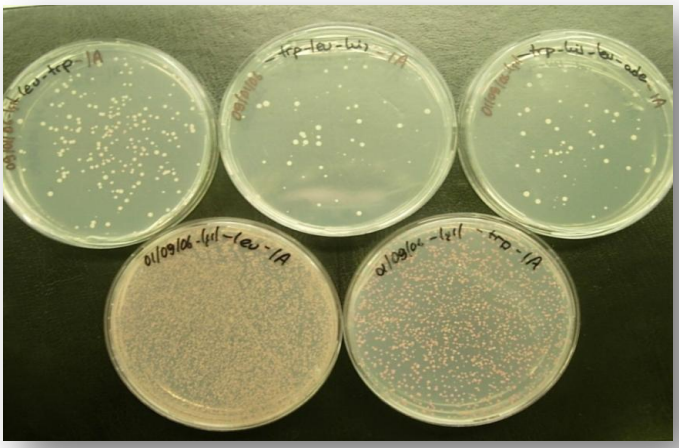

Figure 4.5: Positive control yeast two hybrid assay. On the top there are selective plates –Leu-Trp, -LTH and –LTHA respectively. On the bottom there are non selective plates –Leu and –Trp from left to right. The colonies that grow on –Leu plate are the ones containing bait plasmid. The colonies that grow on –Trp plate are the ones that contain prey plasmid. –Leu-Trp plate shows the yeast colonies that have both the bait and the prey plasmids. Growth on selective plates –LTH and –LTHA shows the presence of the interaction between the bait and the prey plasmids present.

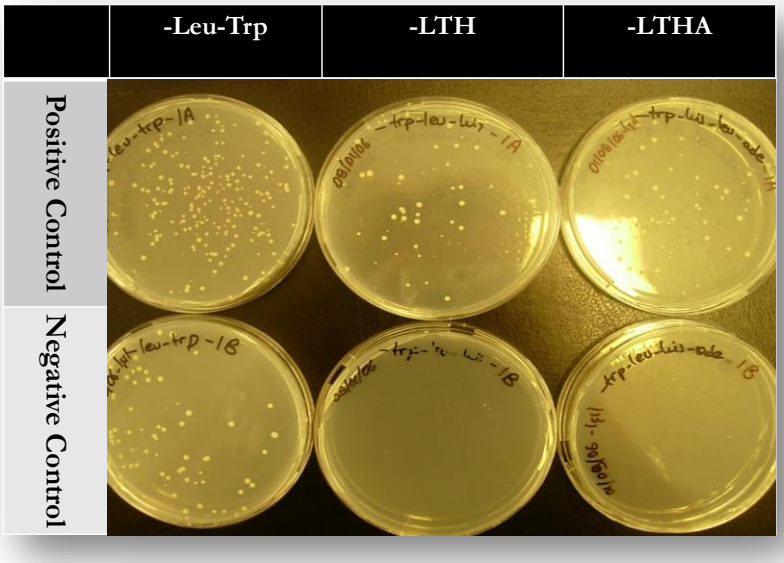

Figure 4.6: Positive and negative control yeast two hybrid assays. Both the assays have almost same number of colonies in the –Leu-Trp plates showing equal number of colonies that have both the bait and the prey plasmid. There are colonies on selective plates in positive control whereas none in negative control.

In order to eliminate the possibility that our bait is self activating empty prey plasmid was also transformed into bait containing NMY51. There were no growth on any of the selective plates making us conclude that our bait is not self activating meaning also that there is no background in the transformation plates.

4.1.3 Thymus cDNA Library Transformation and Yeast Two Hybrid Assay

After confirming that the system works properly, Jurkat Thymus cDNA library that was purchased from Dual Systems (#P02205, data sheet in Appendix D), was transformed into the NMY51 strain that contains pBT3-SUC- CD3δ as the bait. The plasmids encoding CD3δ as the bait (pBT3-SUC- CD3δ) and the Jurkat cDNA library as the prey (pDSL-Nx) plasmids and the protein products are shown schematically in Figure 4.7.

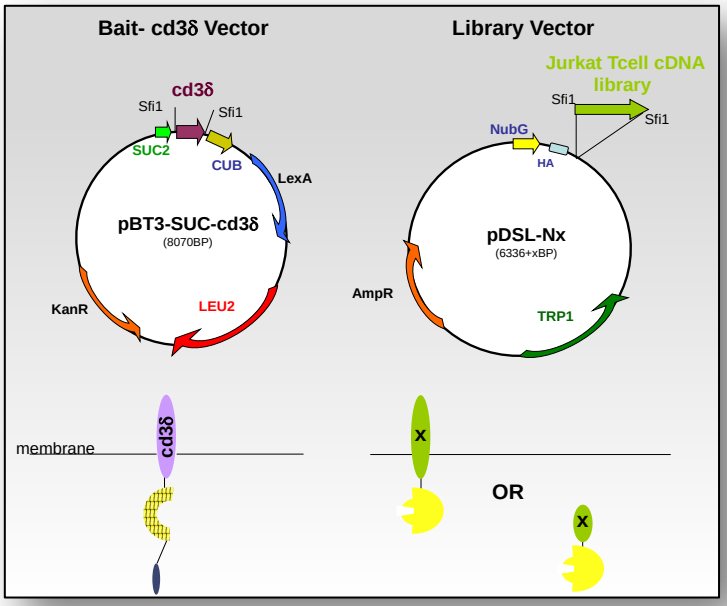

Figure 4.7: Schematic map of the bait and prey plasmids. Bait plasmid containing CD3δ cDNA and prey plasmid containing cDNA library and their possible protein products encoded by these plasmids: Bait plasmid expresses CD3δ coupled to Cub and LexA, the presence of upstream SUC2 coding region leads to membrane targeting of the expressed CD3δ in yeast cells, Leu4 selection marker makes it possible for yeast that is transformed with bait plasmid to grow in Leu4 lacking media, this plasmid can be selected with Kanamycin in bacterial media. pDSLNx prey plasmid expresses the cDNA fused to NubG, if the cDNA has a membrane localization signal it goes to the membrane or else it stays in the cytoplasm.

The colonies on the primary selective plates (542 colonies) were transferred on new selective plates (496 colonies) and β galactosidase assay was carried out for all the colonies to further eliminate the number of putative interacting colonies (426 colonies). An example of the colonies grown on the selective plate and β galactosidase assay result is given in Figure 4.8.

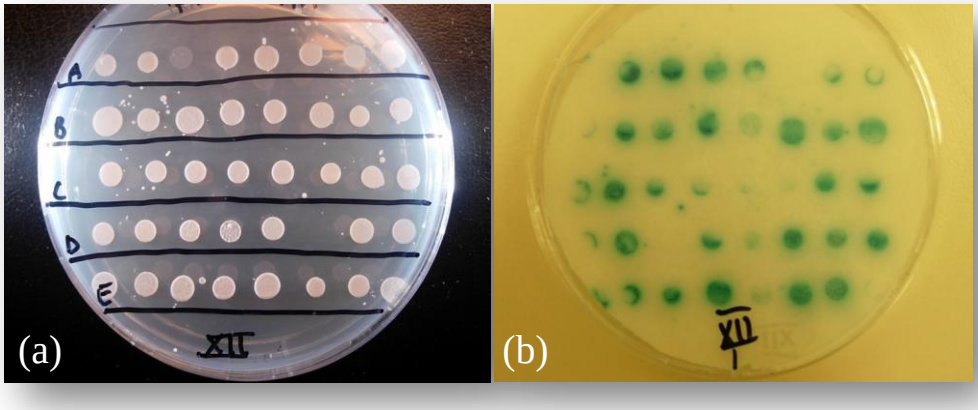

Figure 4.8: Growth of colonies on selective media(-LTHA)(a) and corresponding β-galactosidase filter lift-off assay(b) after library transformation. The positive colonies of the yeast two hybrid assay were taken into liquid cultures and 10 µl of each colony was transferred onto new selective plates(-LTHA), 3 days after the incubation growth was assayed in (a), the plate is then taken for β galactosidase filter lift off assay and the blue colonies were selected as β-galactosidase positive clones.

4.1.4 Identification of Positive Clones Encoding Proteins Interacting With CD3δ

To identify the positive clones the first strategy employed was direct colonyPCR (Figure 4.9) from the yeast colonies and then sequencing the PCR products. After sequencing the first 50 colonies in this manner, the rest of the colonies were identified by isolating the prey DNA from yeast and retransforming it to E.coli and isolating selectively the prey DNA from E.coli for further sequencing.

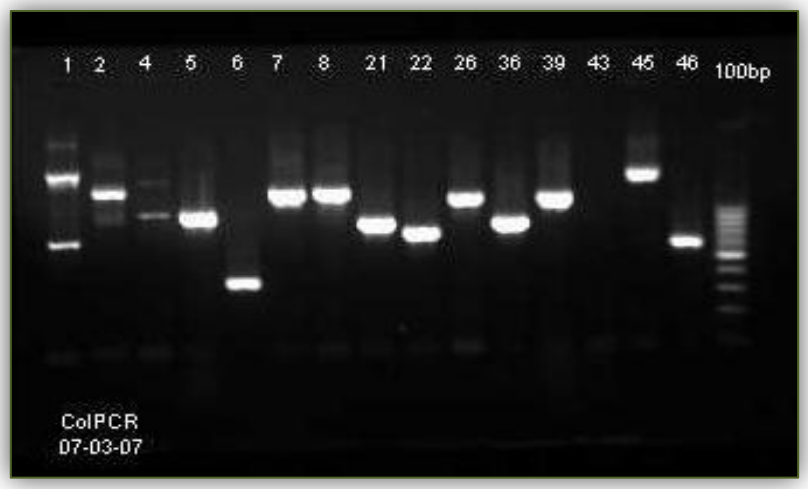

Figure 4.9: Direct colony PCR from yeast colonies using the forward and reverse sequencing primers designed for the library plasmid pDSL-Nx. Different colonies with the numbers on the gel, used as the template and different cDNAs in the colonies gave corresponding bands on the gel.

At the end of sequencing according to blast results of the different cDNAs, the interesting ones were selected. The chaperone proteins and previously identified protein partners of CD3δ were eliminated and the proteins either have a functional role in TCR assembly in the ER or TCR mediated stimulation were selected to be further analyzed. The proteins that have a predetermined role in B cell stimulation or BCR assembly were also included to the list to be further analyzed. (Table 4.1) These prey proteins were reconfirmed in yeast by the secondary yeast two hybrid screens. The plasmid DNA isolated from positive yeast clones were transformed into *E.coli* and grew on Amp containing LB to selectively isolate prey plasmids. The prey plasmid DNA isolated from bacterial cells was then transformed into yeast that already contains bait plasmid. Transformation products were streaked on selective media and growth was examined to confirm the interactions. (Figure 4.10)

CLONE	BLAST	
3A3a	NM_001040200.1	Homo sapiens claudin domain containing 1 (CLDND1), transcript variant 7, mRNA.
1G6/ 1I8a/ 3I3	NM_000873.2	Homo sapiens intercellular adhesion molecule 2 (ICAM2), mRNA**
1C8	NM_002208.3	Homo sapiens integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide) (ITGAE), mRNA.
1E2b/ (1E7-pcr-)	NM_021227	Homo sapiens DC2 protein (DC2), mRNA
1G4	NM_018845.1	Homo sapiens recombination activating gene 1 activating protein 1 (RAG1AP1), mRNA
3A2b	NM_004221.4	Homo sapiens interleukin 32 (IL32), transcript variant 2, mRNA.
3A5b	NM_025124.1	Homo sapiens transmembrane protein 134 (TMEM134), mRNA.
3A7b	NM_003329.2	Homo sapiens thioredoxin (TXN), mRNA*****
3B3b/ 3K8	NM_032927.2	Homo sapiens transmembrane protein 128 (TMEM128), mRNA
3C1	NM_005745.6	Homo sapiens B-cell receptor-associated protein 31 (BCAP31), mRNA***
3I1	NM_004356.3	Homo sapiens CD81 molecule (CD81), mRNA**
1G1	NM_198793.2	Homo sapiens CD47 molecule (CD47), transcript variant 2, mRNA**
1H4/ 3E1a	NM_144638.1	Homo sapiens transmembrane protein 42 (TMEM42), mRNA*
1J8A/ 1K1a/ 1K1b/ 3D5a	NM_014051.2	Homo sapiens transmembrane protein 14A (TMEM14A), mRNA*

Table 4.1: Table of different clones identified as positive interactors. First column shows the name given to the clone, second and third column shows the BLAST hit result of the cDNA with the NCBI accession numbers.

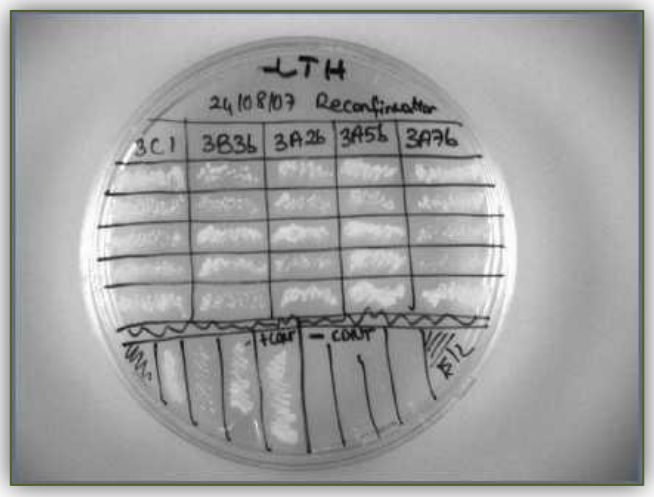

Figure 4.10: Reconfirmation of first 5 cDNAs identified in the yeast two hybrid screen: Following the second yeast two hybrid 5 different colonies(3C1,3B3b,3A2b,3A5b and 3A7b) grown on –Leu-Trp plates was streaked onto selective plate (-LTH), 5 different colonies from the positive and the negative control assays was also streaked on the selective plate. Growth of the cells that were transfected with the selected putative interactors and absence of growth in the negative control confirmed the interactions in yeast.

4.1.5 Binding Affinity between CD3δ and Other TCR Subunits

By using the membrane based yeast two hybrid system we also wanted to see the interactions between CD3δ and different subunits of TCR. We cloned the CD3δ, CD3ε, TCRα and TCRβ into the prey plasmid of dual systems, upstream of NubG so that the protein product will be the mentioned subunits bound to NubG in their C terminal. By transforming yeast containing CD3δ as the bait with those prey plasmids we performed two hybrid screens. The resulting number of colonies on the selective plates indicated the strength of the interaction between the subunits. After counting the number of colonies on the plates, we denoted the ones with too many colonies as TM and scaled the number of colonies from ++++ to + according to the relative numbers on plates. The results only showed the pair wise interactions that took place in the endoplasmic reticulum as the absence of other TCR subunits in yeast will result in these subunits retaining in the ER. (Table 4.2)

Plates / Prey	-Leu (Bait)	-Trp (Prey)	-LT (Bait +Prey)	-LTH (Interaction)	-LTHA (Interaction)
Positive Control	TM	TM	+++	+++	+++
Negative Control	TM	TM	++++	-	-
Empty Prey	TM	TM	++++	-	-
CD3d	TM	TM	++++	+	+
CD3e	TM	TM	++++	++	++
TCRa	TM	TM	++++	-	-
TCRb	TM	TM	++++	+	+
No Prey	TM	-	-	-	-

Table 4.2: Interaction between CD3δ and other TCR subunits, by using the membrane yeast two hybrid system. (TM= too many colonies, ++++ to + represents relative number of colonies on the plates + being around 50-70 colonies.)

4.2 IDENTIFICATION OF MOLECULAR INTERACTIONS IN MAMMALIAN TISSUE CULTURE CELLS

4.2.1 Cloning of CD3δ and Candidate cDNAs into Mammalian Expression Plasmids

The detected interactions should be confirmed in mammalian cells. For this purpose a strategy to transfer the bait (CD3δ) and identified cDNAs to mammalian expression plasmids that has tags for further co-immunoprecipitation experiments is devised as in Figure 4.11 and Figure 4.12.

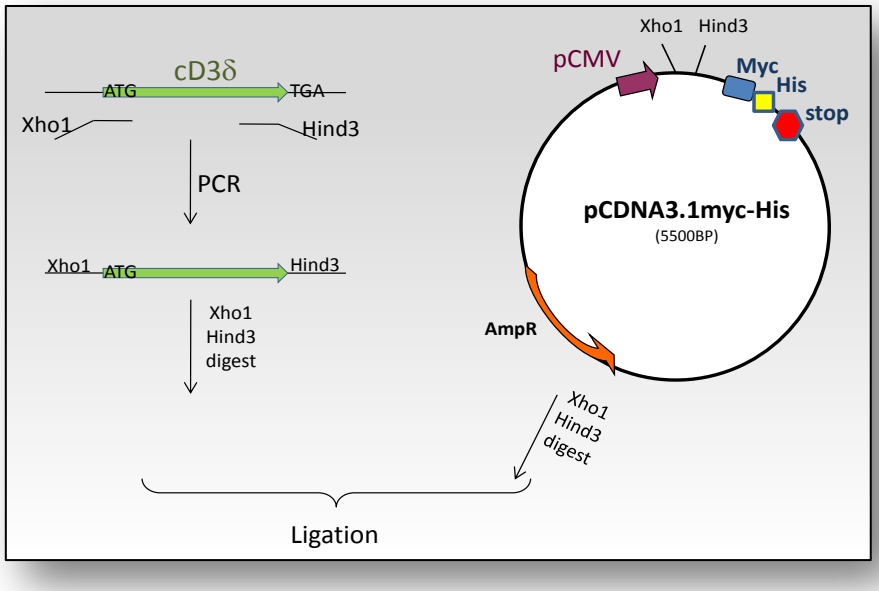

Figure 4.11: Strategy to transfer CD3δ cDNA to mammalian expression plasmid in frame with Myc epitope tag: First step is amplifying CD3δ using designed primers with Xho1 and Hind3 restriction sites, upstream and downstream respectively. PCR product that is digested with Xho1 and Hind3 is directly cloned into pCDNA3.1Myc-His plasmid in frame with the Myc epitope tag.

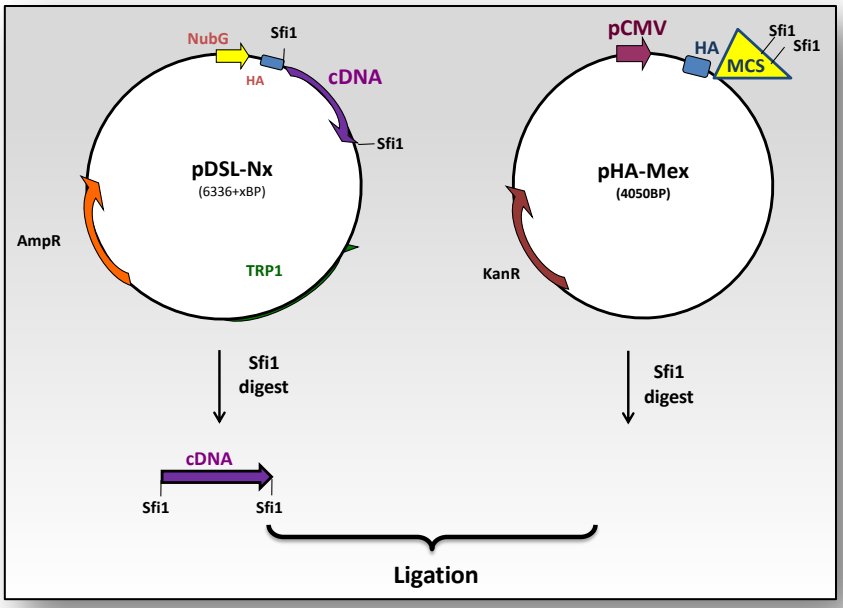

Figure 4.12: Strategy to transfer identified cDNAs into a mammalian expression plasmid in frame with an HA epitope tag.. cDNAs identified to be further analyzed is excised from library plasmid pDSL-Nx using the SfiI restriction sites, they are directly cloned into pHAMex plasmid in frame with the HA epitope tag using the SfiI restriction sites.

To clone CD3δ into pcDNA3.1MycHis plasmid, PCR was carried out with the designed CD3δ forward and reverse primers. The PCR product was digested with Xho1 and Hind3 and directionally cloned into pcDNA3.1MycHis plasmid. The colonies on the ligation plates were first analyzed with colony PCR using the same CD3δ forward and reverse primers. (Data not shown) The colonies with positive PCR bands were then used for plasmid isolation and the identity of the plasmids were analyzed by restriction enzyme digestion analysis. Xho1,Hind3 double digest confirmed the presence of inserted CD3δ and Xmn1 further confirmed the inserted CD3δ as it introduces a third recognition sequence to the plasmid.(Figure 4.13) The annotated plasmid map and diagnostic digests of pcDNA3.1Myc-CD3δ plasmid is in Appendix D.

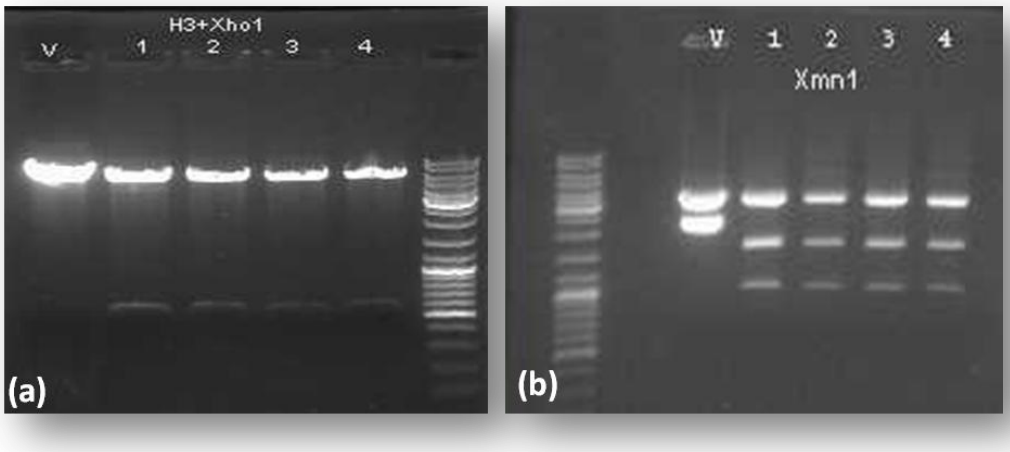

Figure 4.13: Confirmation of the identity of pcDNA3.1Myc-CD3δ plasmid: Hind3 and Xho1 digest(a) and Xmn1 digest(b) of four clones from ligation 1 to 4 and vector alone (pcDNA3.1MycHis) are given. In a, presence of 525bp fragment in Hind3 Xho1 double digest confirms the insertion of CD3δ into the plasmid. In b, pcDNA3.1MycHis plasmid having two recognition sequences for Xmn1 gives two bands of size 3400bp and 2420 bp ; presence of CD3δ sequence introduces a third Xmn1 recognition sequence and three bands of 3400,1500 and 1000 are seen in the digests.

According to the strategy in Figure 4.12 the cDNAs that we wanted to further analyze, were digested out from the library plasmid using SfiI restriction enzyme. (Figure 4.14) The cDNAs that were digested out from the library plasmid was gel extracted and cloned into pHAMex plasmid through the SfiI sites. The resulting mammalian expression plasmids of cDNAs were confirmed by restriction enzyme digestions. (Appendix D)

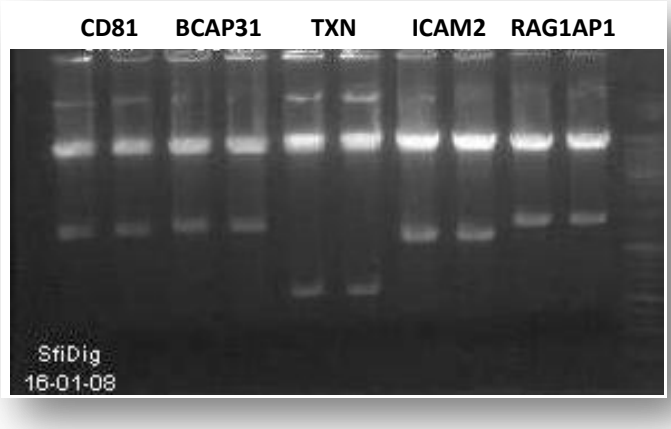

Figure 4.14: Purification of cDNAs from the pDSLNx library plasmid by SfiI restriction digestion. The upper bands are the pDSLNx library plasmid backbone and the lower bands correspond to the cDNAs with different sizes. Lower bands are extracted from the gel to clone into pHAMex plasmid.

4.2.2 Identification of CD81 as a CD3δ Interactor

cDNA sequence for each putative interactor to be further analyzed was constructed by aligning the forward and reverse sequencing results coming from different colonies of the same cDNA. The resulting cDNA sequence is compared with database cDNA sequence and the expected protein products were compared for their domain structure using secondary structure prediction program Sossui.[90] The predicted secondary structures of the proteins along with the database proteins were plotted by TMRPres2D.[91]

4.2.2.1 Secondary structure prediction of CD81

CD81 sequence in database corresponds to 231 amino acid peptide with four transmembrane regions one small and one large extracellular loop, that correlates with previous studies. The sequence we had from our cDNA, denoted as CD81 cDNA has 240 amino acids, that is different in first 36 amino acids from the database sequence. Overall secondary structure of the cDNA sequence that is predicted with Sosui is very similar to the one of database. When we blast the different aminoacid sequence to the human genome we found out that this 36 different aminoacid corresponds to the first intron of the CD81 gene. This made us propose that the contig we had in the library might be an alternative splice variant of CD81 that has the putative exon as the first exon.

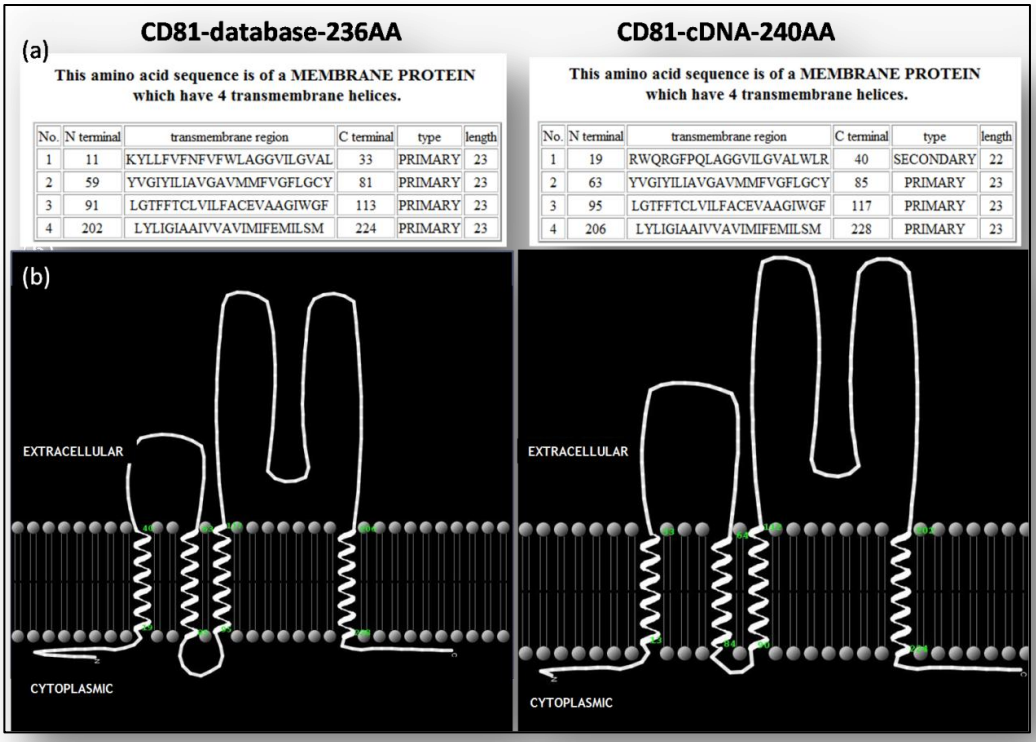

Figure 4.15: Secondary structure of CD81 protein: encoded from the database sequence (on the left) and cDNA sequence (on the right) Transmembrane regions are predicted with Sosui (a) and 2D structure is visualized by TMRPres2D. (b)

4.2.2.2 Identification of a Novel Exon in the CD81 Gene Locus

When we compare the cDNA sequences of CD81 from database and from the cDNA library, we have found an unidentified first exon in the cDNA of our library plasmid. To test whether this is an alternative splice variant of CD81 we designed primers in the upstream and downstream of both the known first and second exons and putative exon that we had. The schematical representation of genomic region of CD81 and the cDNAs of database and library CD81 cDNAs along with the primers designed to test the alternative splicing are shown in Figure 4.16.

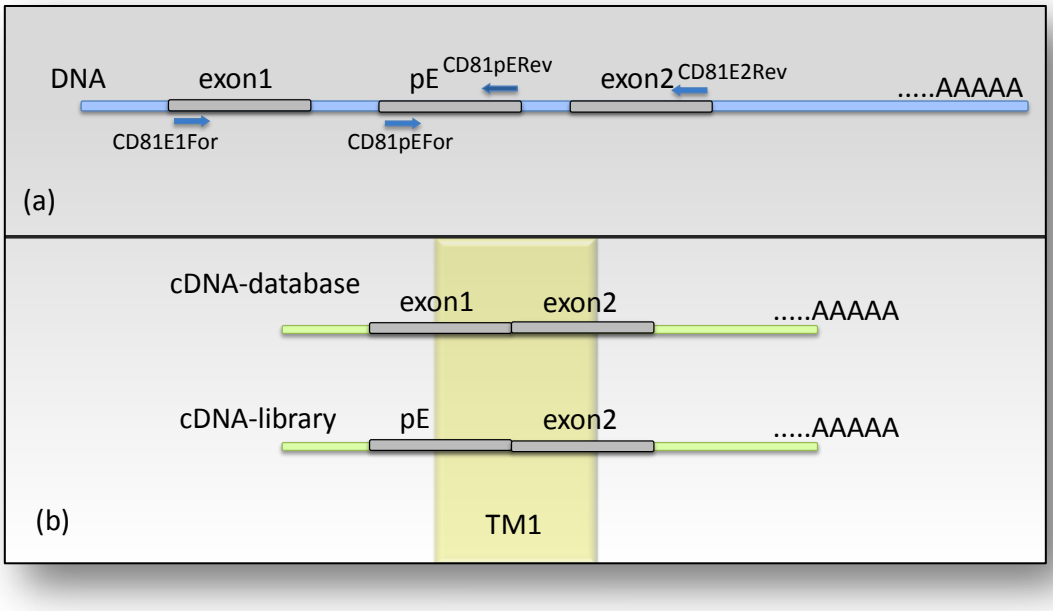

Figure 4.16: Genomic map of the CD81 gene locus, showing the locations of previously found first and second exons and the putative exon that we identified.(a) cDNAs of database sequence and library that we have showing the relative locations of exon1, exon2 and the putative exon. The exon1 exon2 junction in the database sequence corresponds to the first transmembrane region of the protein and the junction of putative exon and exon2 also corresponds to the first transmembrane region of the protein predicted by Sosui. (b).

This sequence of putative exon was found in human genome but not in mouse genome. RNA was isolated from different human cell lines. Using oligodT primer and reverse transcriptase, cDNA was produced from these cell lines and PCR with putative exon forward and exon 2 reverse was carried out to see if there will be a band. The cDNA plasmid that originally gave us this sequence was also included as a positive control template for the PCR. None of the cell lines tested gave this band from these primers indicating that this putative exon is not found in these cell lines. (Figure 4.17) Neither adding DMSO to the PCR reaction nor increasing the MgCl2 concentration changed the result. So, this putative exon that we found most probably comes from tumor specific splicing of the Jurkat cell line used at first place to prepare the cDNA library. But further structural studies revealed that the aminoacids coded from this putative exon is not responsible for the identified interaction of CD81 with CD3δ.

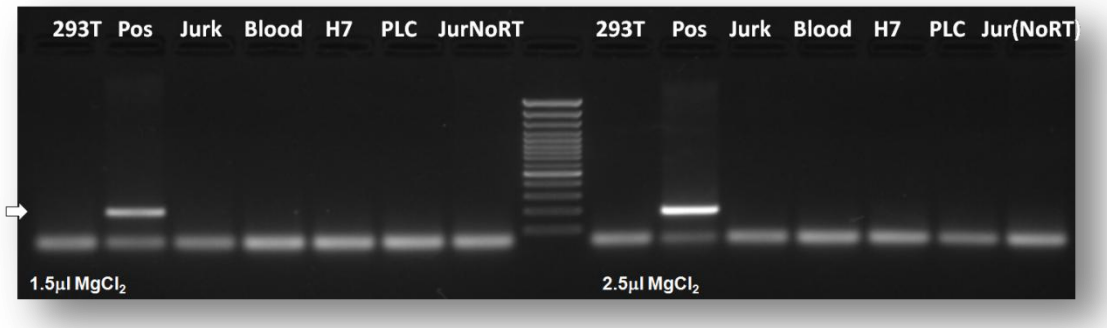

Figure 4.17: PCR amplification of alternatively spliced CD81 cDNAs (293T:HEK29T, Jurk:Jurkat, Blood, H7:HuH-7, PLC, JurNoRT: Jurkat RNA control) with putative exon forward and exon2 reverse primers. Expected band of 192bp is only observed in positive control (Pos) where the template was the plasmid containing CD81 Jurkat cDNA.

4.2.3 Identification of BCAP31 as a CD3δ Interactor

4.2.3.1 Secondary structure prediction of BCAP31

BCAP31 sequence in database corresponds to 246 amino acid sequence that is predicted to have three transmembrane domains that correlates with the previous studies. The cDNA sequence that we have in the library plasmid that is denoted as BCAP31 cDNA, corresponds to 285 aminoacid peptide and it contains the whole database sequence plus 39 aminoacids that is on the N terminal site and probably comes from the library plasmid. This peptide encoded by library cDNA has the same secondary structure as the database peptide with three transmembrane regions and the only difference is the N terminal extracellular portion of the peptide is 39 aminoacids longer. (Figure4.18)

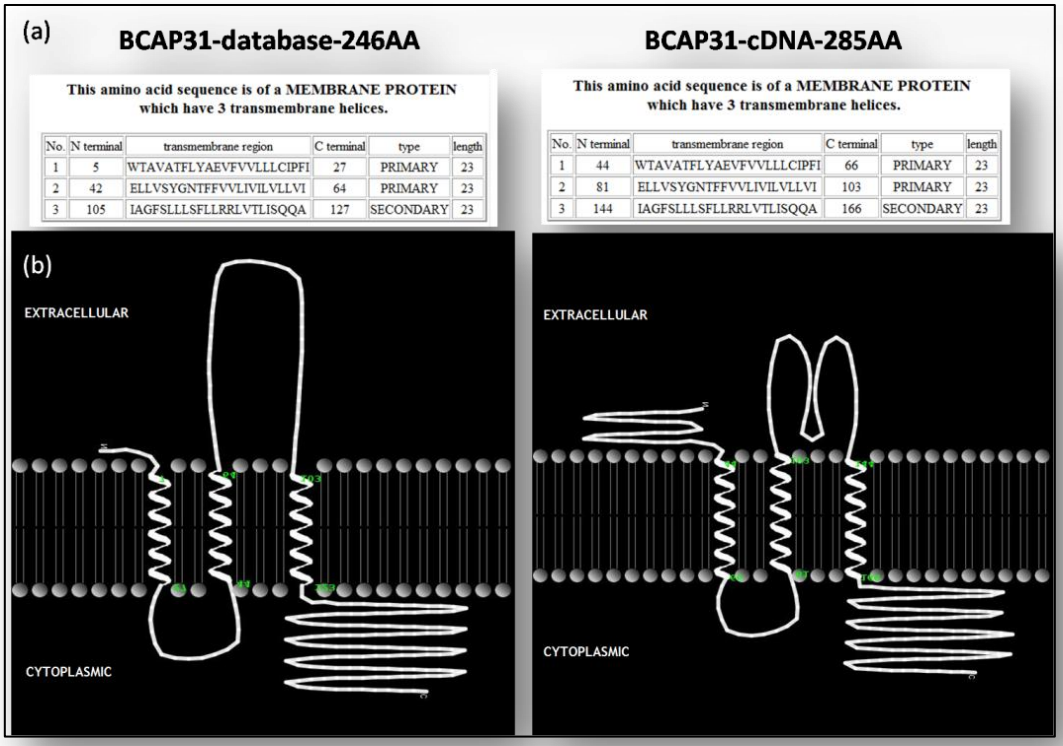

Figure 4.18: Secondary structure of BCAP31 protein encoded from the database sequence (on the left) and cDNA sequence(on the right). Transmembrane regions are predicted with Sosui (a) and 2D structure is visualized by TMRPres2D. (b)

4.2.4 Transfection, Cell Lysis and Immunoprecipitation

HEK293T cells with the known high transfection efficiency were used for the immunoprecipitation experiments. The protein products of the constructed mammalian expression plasmids and the logic of immunoprecipitation experiments are shown in Figure 4.19.

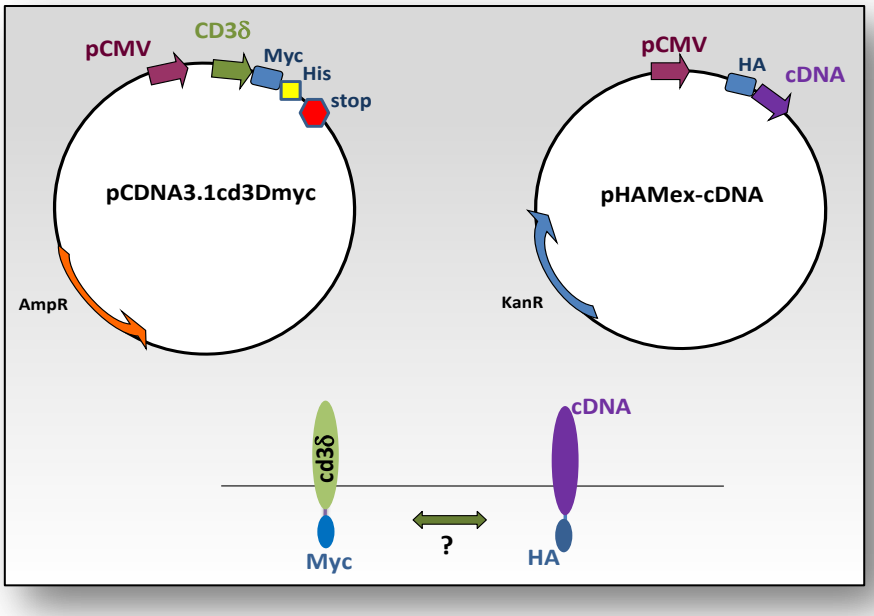

Figure 4.19: General strategy and logic of immunoprecipitation experiments: CD3δ is in mammalian expression plasmid in frame with the Myc epitope tag, so the expressed protein will be CD3δ-Myc, cDNAs cloned into pHAMex plasmid are also in frame with the HA epitope tag and the protein product will be HA-cDNA. So cotransfecting these two plasmids we assay whether there is any interaction between CD3δ and cDNA of interest.

4.2.4.1 Optimization of the Transfection Conditions of HEK293T Cells

pcDNAGFP plasmid (with GFP coding sequence in pcDNA plasmid) was used to transfect HEK293Tcells, 48 hours after the transfection cells were visualized under fluorescent microscope to see the expression of GFP that will indicate the transfection efficiency. Almost all the cells were visualized as green under the fluorescent microscope that corresponds to almost 90 % transfection efficiency. (Figure 4.20)

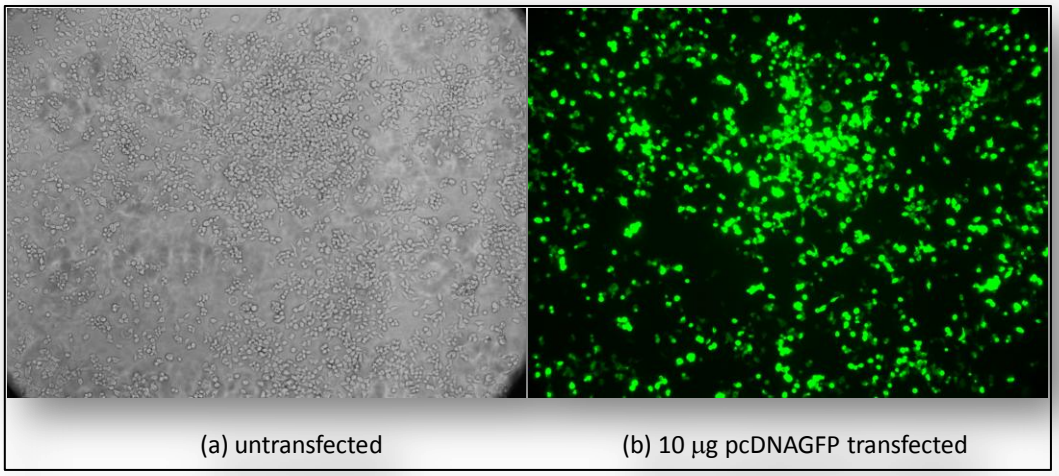

Figure 4.20: HEK293T cells untransfected (a) or transfected with pCDNAGFP (b) visualized under fluorescent microscope.

4.2.5 BCAP31 Interacts with CD3δ in HEK293T Cells

To confirm the identified interaction between BCAP31 and CD3δ we carried out immunoprecipitation experiments using the logic explained in Fig4.19. HEK 293T cells were transfected with pHA-BCAP31 or pCDNA3.1Myc-CD3δ or both and all the cells were lysed 48 hours after the transfection. Cell lysates were immunoprecipitated with anti-HA affinity matrix and the Immunoprecipitation products and lysates were loaded on SDS gel. The membrane after the transfer was blotted with anti-Myc Peroxidase and visualized using Peroxidase substrate on the film. Results clearly indicate that BCAP31 immunoprecipitates with the myc tagged CD3δ. Lysates blotted both with anti-Myc Peroxidase and anti-HA Peroxidase indicated that both the proteins were expressed from the plasmids. (Figure 4.21)

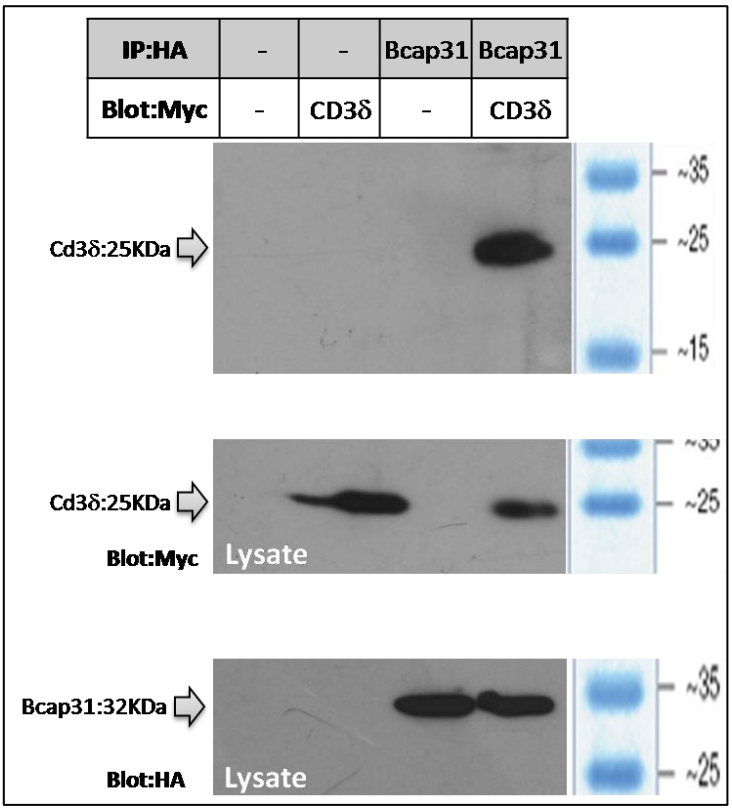

Figure 4.21: BCAP31 interacts with CD3δ. HEK293T cells were transfected with construct encoding BCAP31protein in the presence (lane 4) or absence of CD3δ (lanes 3). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-4) were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-myc antibodies. Anti-myc immunoblots show the presence of a 25kD band corresponding to the CD3δ protein co-immunoprecipitated with HA-BCAP31 protein. Middle row: anti-myc immunoblots of lysates demonstrates the expression of CD3δ specifically in cells transfected with this construct (lane 2-4). Bottom row: anti-HA immunoblots of lysates demonstrates the expression of BCAP31 in lanes 3-4.

4.2.6 CD81 Interacts with CD3δ in HEK293T Cells

To confirm the identified interaction between CD81 and CD3δ this time, we carried out immunoprecipitation experiments using the logic explained in Fig 4.19. HEK 293T cells were transfected with pHA-CD81 or pCDNA3.1Myc-CD3δ or both and all the cells were lysed 48 hours after the transfection. Cell lysates were immunoprecipitated with anti-Myc coupled to protein sepharose beads and the immunoprecipitation products and lysates

were loaded on SDS gel. The membrane after the transfer was blotted with anti-HA Peroxidase and visualized using Peroxidase substrate on the film. Results clearly indicate that CD81 immunoprecipitates with the myc tagged CD3δ. Lysates blotted both with anti--Myc Peroxidase and anti-HA Peroxidase indicated that both the proteins were expressed from the plasmids. (Figure 4.22)

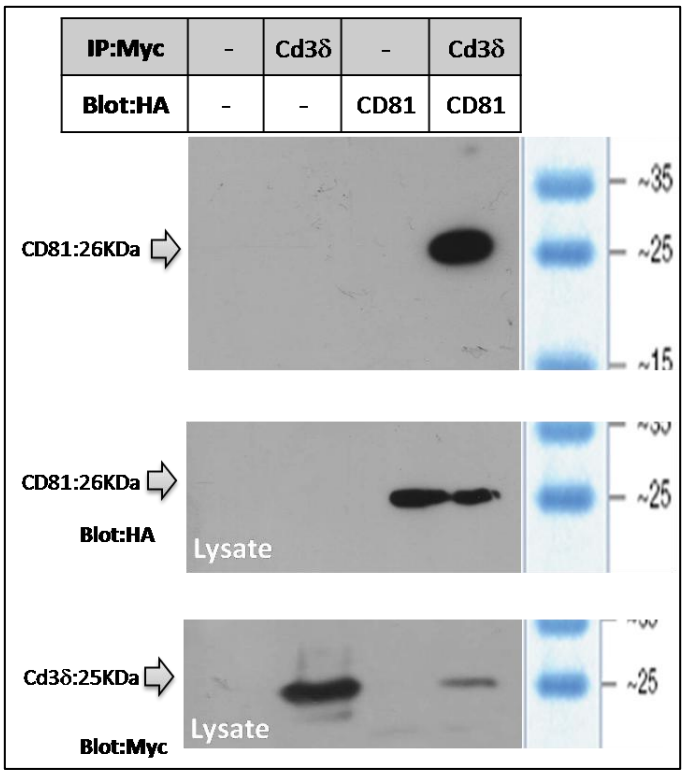

Figure 4.22: CD81 interacts with CD3δ. HEK293T cells were transfected with various construct encoding CD81protein in the presence (lane 4) or absence of CD3δ (lanes 3). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-4) were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblot shows the presence of a 26kD band corresponding to the CD81 protein co-immunoprecipitated with Myc- CD3δ protein. Middle row: anti-HA immunoblot of lysates demonstrates the expression of CD81 specifically in cells transfected with this construct (lane 3-4). Bottom row: anti-Myc immunoblot of lysates demonstrates the expression of CD3δ in lanes 2-4.

4.3 STRUCTURAL STUDIES

4.3.1 Constructs Encoding CD3γ/δ Chimeric Proteins

In order to identify the interaction domain of CD3δ with the identified cDNAs and also to see which parts of CD3δ are essential for its possible functions in TCR assembly, TCR stability and TCR stimulation, three different chimeras of CD3δ and CD3γ were constructed. The strategy was to construct chimeras with different extracellular, transmembrane or intracellular domains. The strategy that is based on fusion PCR with designed primers (Table3.4) is shown schematically in Figure 4.23.

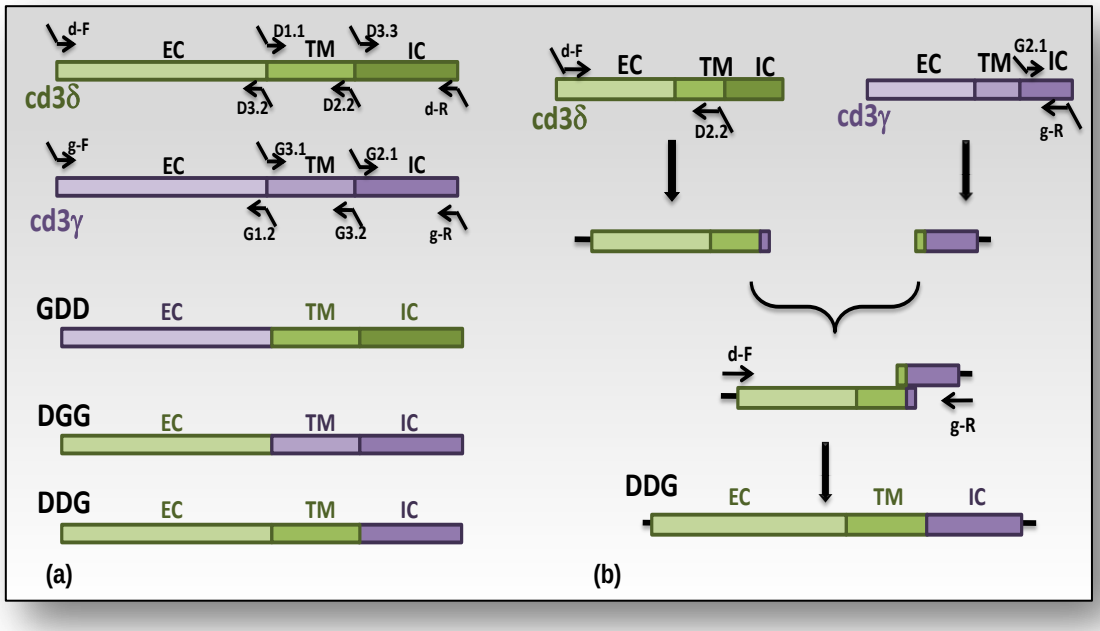

Figure 4.23: Fusion PCR strategy for the generation of the CD3γ/δ chimeras showing the primers designed (a) and two steps of PCR (b).

In order to construct CD3γ/δ chimeras the extracellular, transmembrane and intracellular regions of both CD3δ and CD3γ cDNA sequences were determined using VectorNTI program. The first step was the amplification of the regions to be fused (extracellular, transmembrane and intracellular) by the designed primers. The two PCR

products were then fused with 10 cycles of PCR without any primers and low annealing temperature, after the fusion the last step of PCR was carried out, using only the upstream and downstream primers with the Xho1 and Hind3 restriction sites. (Figure 4.24) By the aid of the Xho1 and Hind3 restriction sites in the forward and reverse primers, the amplified fusion PCR products were cloned into pcDNA3.1MycHis vector (same strategy as the cloning of CD3δ into the same plasmid.)

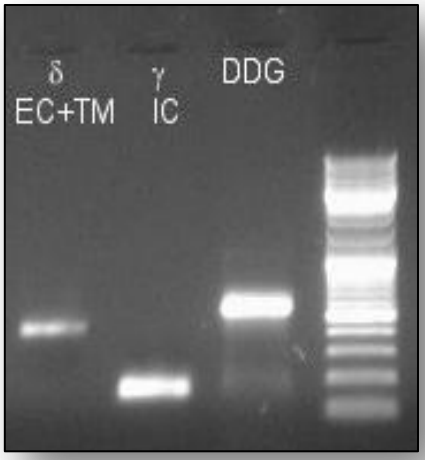

Figure 4.24: Example of fusion PCR for DDG chimera construction: Extracellular and transmembrane regions of CD3δ were amplified by designed primers (lane 1:δ EC+TM). Intracellular region of CD3γ was amplified with designed primers (lane 2: γ IC). These two PCR products were used in the fusion PCR to amplify the DDG chimera (lane 3: DDG)

4.3.2 Expression and Co-Immunoprecipitation of CD3γ/δ Chimeras with CD81 and BCAP31

The constructed CD3γ/δ chimeras in pcDNA3.1Myc plasmid along with the pHACD81 and pHABCAP31 were transfected to HEK293T cells, to see their expression and Immunoprecipitation. CD3γ could not be expressed from the pcDNA3.1MycCD3γ plasmid, although the plasmid is sequence verified. Interestingly chimera GDD that has the N terminal extracellular region coming from the CD3γ could neither be expressed. These two plasmids were sequenced both forward and reverse two times each and there seems nothing to be wrong with the sequence. Other plasmids that the expression was

successfully observed were pcDNA3.1MycDGG and pcDNA3.1MycDDG. Using these plasmids for transfecting the HEK293T cells with or without pHACD81 and pHABCAP31, lysates were subjected to anti-Myc immunoprecipitation. The expression levels of CD3δ, DGG and DDG were calculated to be equal and myc Immunoprecipitation samples when blotted with anti-HA, revealed that both CD81 and BCAP31 interact with DGG and DDG along with CD3δ. (Figure4.25) These data suggest that the interaction of both CD81 and BCAP31 is through the extracellular region of the CD3δ if these proteins do not interact with the CD3γ.

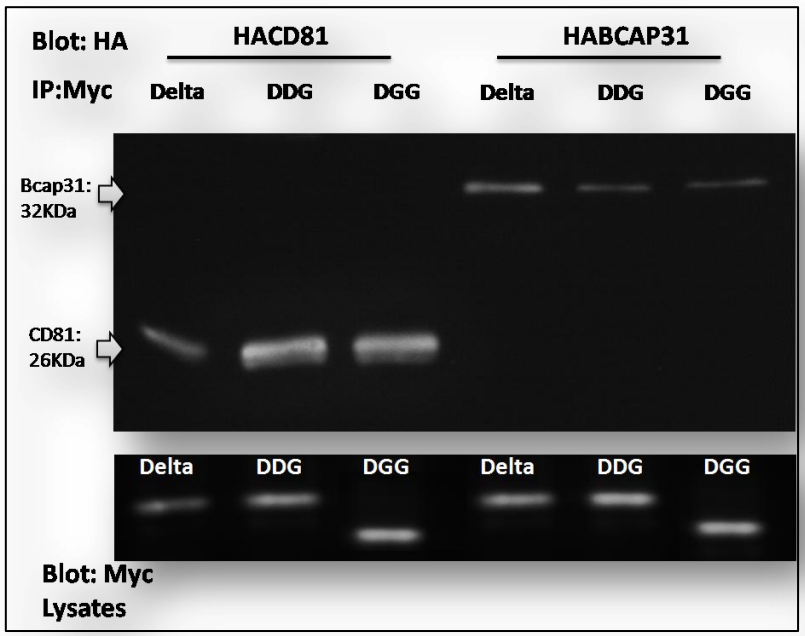

Figure 4.25: DGG and DDG chimeras interact with CD81 and BCAP31. HEK293T cells were transfected with constructs encoding CD81protein (lane1-3) or BCAP31 (lane 4-6) in the presence of CD3δ (lanes 1 and 4), DDG (lanes 2 and 5) or DGG (lanes 3 and 6). Top row: Lysates from transfected cells were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblot shows the presence of a 26kD band corresponding to the CD81 protein co-immunoprecipitated with Myc- CD3δ, DDG and DGG (lanes 1- 3) and 32kD band corresponding to the BCAP31 protein co-immunoprecipitated with Myc- CD3δ, DDG and DGG (lanes 4-6). Bottom row: anti-Myc immunoblot of lysates demonstrates the expression of CD3δ in lane1 and4, DDG in lane 2 and 5, and DGG in lane 3 and 6.

4.3.3 Constructs Encoding CD81/CD9 Chimeric Proteins

In order to structurally study the interaction of CD81 with CD3δ a panel of chimeric hCD81/hCD9 molecules were used. These chimeras in pBabeMN-IRESGFP plasmid was a gift from Shoshanna Levy from Stanford University.[44](Figure 4.26) The chimeras in these plasmids were amplified using the designed forward and reverse primers of CD81 and CD9. By the aid of the SfiI restriction sites in the forward and reverse primers, the amplified chimeras were directly cloned into pHAMex plasmid in the same manner as the cDNAs were cloned, in frame with HA tag.

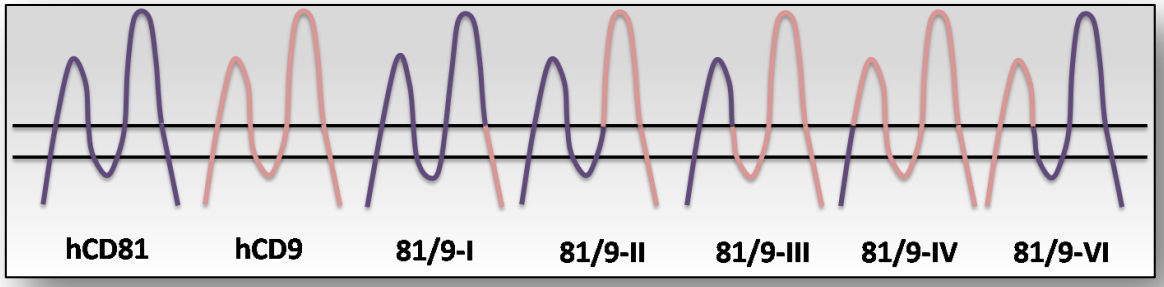

Figure 4.26: CD81/CD9 chimeric cDNAs express fusion tetraspanin proteins: CD81/CD9 chimeras with different regions coming from either hCD81 (purple) or hCD9 (pink) Both CD81 and Cd9 proteins are tetraspanin proteins that have 4 transmembrane domains, with their N and C termini facing the cytoplasm and a large extracellular loop between the 3rd and 4th transmembrane domains.

4.3.4 Cloning of CD81/9 Chimeric cDNAs into the pHAMex Plasmid

We amplified the indicated CD81/CD9 chimeric cDNAs by PCR using retroviral pBabeMN-IRESGFP plasmid acquired from Prof. Shoshanna Levy's laboratory at Stanford University as templates. Amplification of these cDNAs by designed CD81Forward, CD81Reverse, CD9Forward and CD9 reverse primers allowed the incorporation of SfiI restriction sites on either side of these chimeric cDNAs for cloning into the SfiI site of the pHAMex plasmid. (Figure 4.27) As seen in Appendix D, this plasmid has an N-terminal HA epitope tag that we used to tag these CD81/9 chimeric proteins. Amplified regions

were then extracted from agarose gel and ligated to pHAMex plasmid using SfiI restriction sites. Successful ligation products were first analyzed with SfiI restriction enzyme digestion to pop out the insert (Figure4.28) and then sent to sequencing.

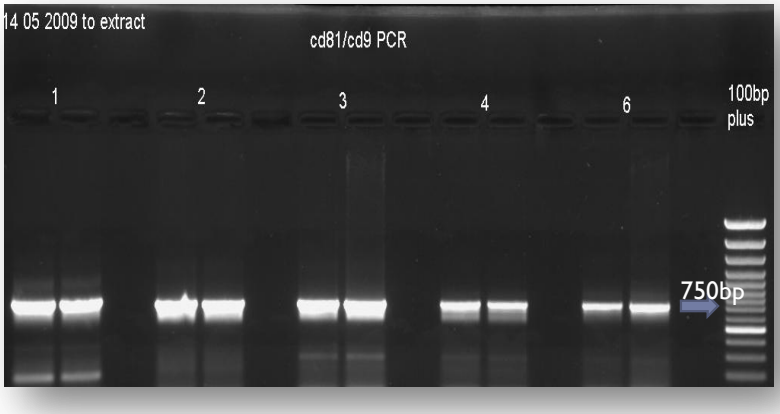

Figure 4.27: PCR amplification of CD81/CD9 chimeric cDNAs. Because the homologous CD81 and CD9 proteins are about the same size, all PCR products are approximately 750 bp long.

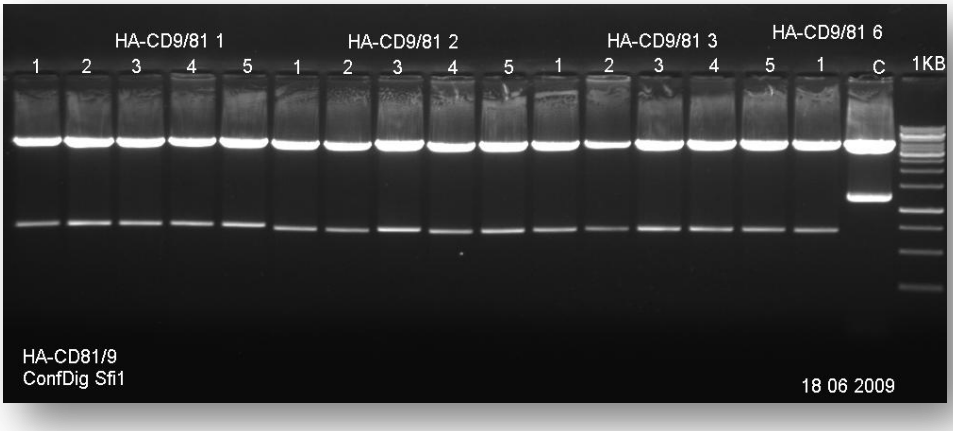

Figure 4.28: Identitiy of CD81/9 chimeric cDNAs inserted into the pHAMex plasmid. SfiI digestion of 5 plasmids purified from different colonies (1-5) for the CD9/CD81 chimeras I, II and III, and from one colony containing CD9/81 chimera VI show a band of 750 bp corresponding to the correct insert in the plasmid backbone. The lane marked C shows the restriction digestion of a plasmid pHACD81,used as a positive control giving 1305bp band. As all colonies contained the correct insertion, colony #1 from each set was used in further experiments.

4.3.5 All CD81/9 Chimeras Interact with CD3δ in HEK293T Cells

To identify the structural domains in CD81 that are responsible for interacting with the CD3δ protein, we expressed the CD81/CD9 chimeric proteins in mammalian tissue culture cells and performed immunoprecipitation experiments with co-expressed CD3δ proteins.

The CD81/9 chimeras I, II, III and VI that were successfully inserted into pHAMex plasmid were used for the immunoprecipitation experiments against Myc tagged CD3δ. For these co-immunoprecipitation experiments, the CD81/9 chimeras were N-terminally epitope tagged with the Ha epitope while the CD3δ protein was epitope tagged with the myc epitope. Immunoprecipitation with anti-myc antibodies followed by immunoblotting with anti-HA antibodies revealed that all chimeras interact with the CD3δ protein. (Figure 4.29). While control immunoprecipitations with no immunoprecipitating antibody only reveal the presence of background bands, immunoprecipitations with anti-myc antibodies reveal the presence of interacting Ha tagged CD81/9 chimeras in all immunoprecipitation reactions. As the large extracellular loop of the CD81 protein has previously been shown to interact with other proteins, we hypothesized that this domain was also responsible for binding the CD3δ protein. In previous experiments, the extracellular loop of the CD9 protein was shown not to interact with CD81 interaction partners. As we did not have a CD9 expression construct to demonstrate that CD9 in fact does not interact with CD3δ, we nonetheless wanted to use these CD81/9 chimeras to identify structural domains necessary for the CD81 CD3δ interaction. According to our hypothesis, we expected that all CD81/9 chimeras containing the CD81 large extracellular loop to interact with CD3δ. Thus, we expected CD81/9 I and VI to interact and II, II and IV not to interact with CD3δ. The co-immunoprecipitation experiment in Fig 4.29 shows that CD81/9 chimeras I, II, II and VI all interact with CD3δ. Note that we excluded CD81/9 chimera IV from this analysis because we could not demonstrate that this construct expressed a chimeric protein of the expected size. Consistent with our hypothesis that the extracellular loop of CD81 was responsible for CD3δ interaction, chimera I and VI interacted with CD3δ. However,

chimera II and III also interacted with the CD3δ protein, indicating that either a different region of CD81 is responsible for interaction or CD9 also interacts with CD3δ.

Although this result does not give us any idea of the interacting domain of the CD81 with CD3δ, the interaction of CD3δ with chimeras 1,2 and 3 confirms that the interaction we identified is not only true for the alternative variant of CD81 we had in the library plasmid. These chimeras, having the N terminal sequence same with the database sequence, also interact with CD3δ.

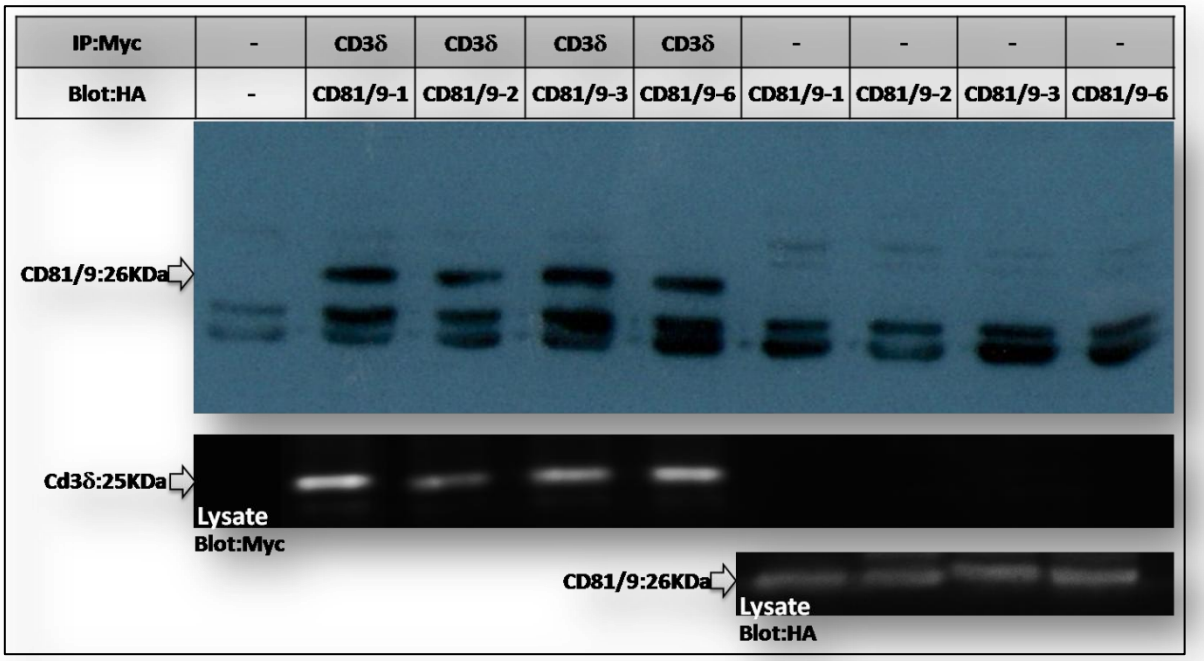

Figure 4.29: CD81/9 chimeric proteins interact with CD3δ. HEK293T cells were transfected with various constructs encoding CD81/9 chimeric proteins in the presence (lanes 2-5) or absence of CD3δ (lanes 6-9). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-9) were immunoprecipitated with anti-myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblots show the presence of a 26 kD band corresponding to the CD81/9 chimeric proteins co-immunoprecipitated with Myc-CD3δ proteins. Middle row: anti-myc immunoblots of lysates demonstrates the expression of CD3δ specifically in cells transfected with this construct (lane 2-5). Bottom row: anti-HA immunoblots of lysates demonstrates the expression of CD81/9 chimeras in lane 6-9.

4.3.6 Constructs Encoding Truncated BCAP31 Proteins

Different BCAP31 truncations were constructed with deletions from N terminal regions (BT1, BT2 and BT3) and the processed version of BCAP31 found in literature (BT5). The BCAP31 cDNA that we had in our library plasmid was longer than the one in database in N terminal. The first truncation denoted BT1 is the one that is found in database. In order to make the truncations, transmembrane regions of BCAP31 was determined and primers to amplify the truncated versions were designed as in figure 4.30. The schematical representation of formed truncations is shown in figure 4.31. It is worth to mention that the schematical representation is just to understand the length and regions of the truncations included, as some or all of them would be cytoplasmic proteins rather than membrane proteins without their membrane localization signals.

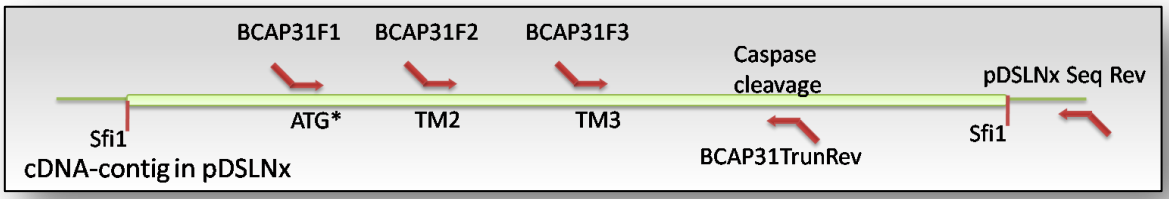

Figure 4.30: The location of oligonucleotides used to generate BCAP31 truncated cDNAs. *ATG is the start of the database BCAP31. BCAP31F1, BCAP31F2, BCAP31F3 with pDSLNx Seq Rev were used to construct BT1, BT2 and BT3 chimeras respectively. BT5 chimera is constructed using BCAP31F1 primer with BCAP31TrunRev primer. (BCAP31TrunRev primer corresponds to the caspase cleavage site of BCAP31) TM2 and TM3 denote the transmembrane regions of BCAP31.

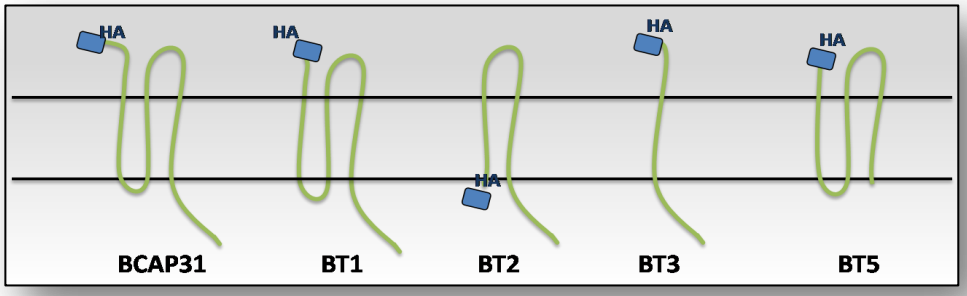

Figure 4.31: Schematical representation of BCAP31 truncations made. BCAP31 is the protein expressed from library cDNA, BT1 protein is identical to the database BCAP31 protein. BT2 is the first transmembrane lacking truncation of BCAP31, BT3 lacks both the first and the second transmembrane regions. BT5 is the caspase cleavage product of BCAP31 that is found in the literature.

BCAP31 truncations were all amplified from the cDNA of BCAP31 that we have in our library plasmid using the designed primers (Table 3.4) The PCR products were then digested with SfiI and loaded on gel to ligate with SfiI digested pHAMex plasmid (Figure 4.32)

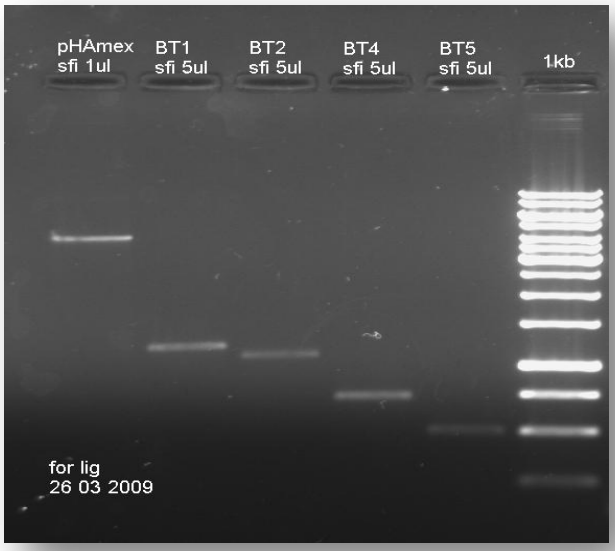

Figure 4.32: Amplification of BCAP31 truncated cDNAs by PCR. BCAP31 truncations 1, 2, 4 and 5 that were digested with SfiI restriction enzyme with sizes 1899bp, 1094bp, 725bp and 495bp respectively ready for ligation with SfiI digested pHAMex plasmid.

4.3.6.1 Expression of Truncated BCAP31 Proteins in Tissue Culture Cells

In order to confirm the expression from the constructed BCAP31 truncation plasmids, HEK293T cells transfected with the constructed BCAP31 truncation encoding plasmids. Cells were lysed and the lysates were blotted with anti-HAperoxidase. (Figure 4.33)

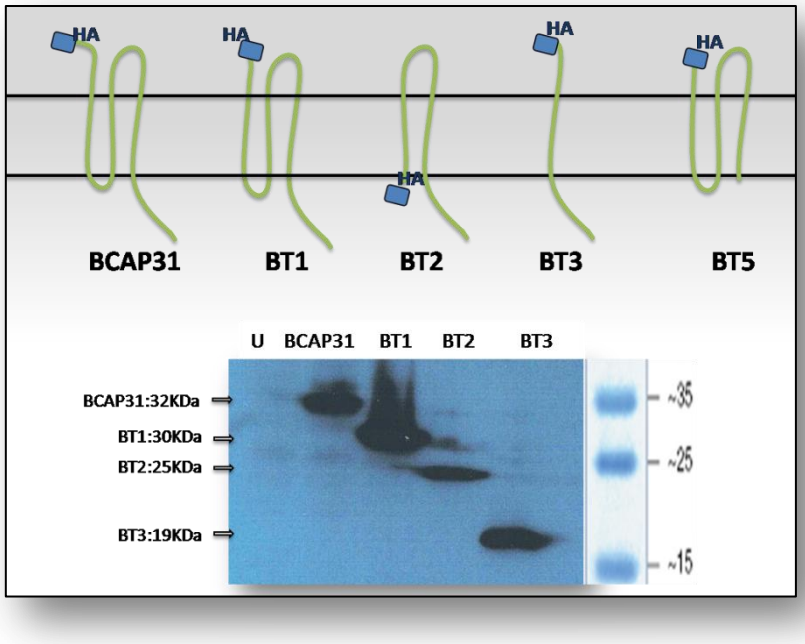

Figure 4.33: Detection of truncated BCAP31 mutant proteins. BCAP31 truncations BT1, BT2 and BT3 were used to transfect HEK293T cells and the lysates were blotted with anti-HA to see the expression of truncations. BCAP31 is used as a control and BT1, BT2 and BT3 all expressed the expected 30KDa, 25KDa and 19KDa proteins respectively.

4.3.7 BCAP31Truncations Interact with CD3δ in HEK293T Cells

BCAP31 Truncations prepared and confirmed for their expression were used for the immunoprecipitation experiments against myc tagged CD3δ. HEK293T cells were transfected with the pcDNA3.1Myc CD3δ plasmid along with the BCAP31 truncations cloned into pHAMex plasmid. The lysates were immunoprecipitated with anti-Myc agarose and blotted with anti-HA. The results indicate that CD3δ immunoprecipitates with all the BCAP31 truncations. (Figure 4.34, 4.35 and 4.36) When we look for the minimal interacting domain of these truncations, the third transmembrane domain of BCAP31 (the only transmembrane domain in BT3) seems to be the minimal interacting domain. When this third transmembrane region is further analyzed, two positively charged residues (117R and 118R) are proposed to have a role in a possible electrostatic interaction with the negative residue (111D) in CD3δ transmembrane region. (Figure 4.37) 111D residue in CD3δ was previously shown to play a role in the TCR assembly and interaction of CD3δ

with other TCR residues during the assembly process in the ER. As BCAP31 is an ER chaperone protein identified to bind proteins in the ER and function as a cargo for their export from ER and as TCR is assembled from the different subunits including CD3δ in the ER, we hypothesized that BCAP31 interacts with CD3δ in the ER through its third transmembrane domain and plays a role in recruiting CD3δ to the process of TCR assembly in the ER.

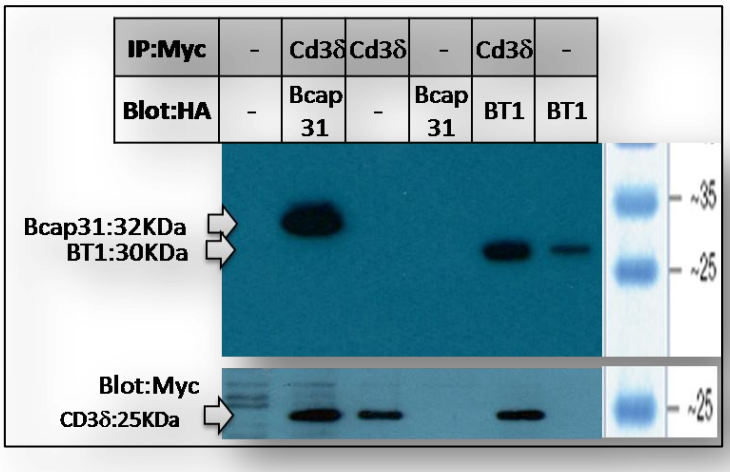

Figure 4.34: BCAP31 truncation BT1 interacts with CD3δ: HEK293T cells were transfected with constructs encoding BCAP31protein (lane2 and 4) and BT1protein (lane 5 and 6) in the presence (lanes 2, 3 and 5) or absence of CD3δ (lanes 4 and 6). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-6) were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblot shows the presence of a 32kD band corresponding to the BCAP31 protein and 30kD band corresponding to BT1 co-immunoprecipitated with Myc- CD3δ protein. There is also a band of 32kD in lane 6 that corresponds to BT1 bound to Myc beads. Bottom row: anti-Myc immunoblot of lysates demonstrates the expression of CD3δ specifically in lanes 2, 3 and 5.

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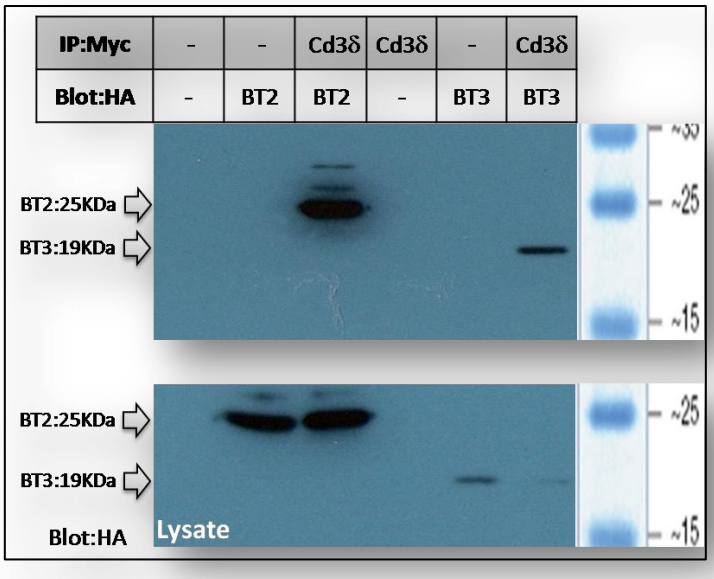


Figure 4.35: BCAP31 truncations BT2 and BT3 interact with CD3δ: HEK293T cells were transfected with constructs encoding BT2protein (lane2 and 3) and BT3 protein (lane 5 and 6) in the presence (lanes 3 and 6) or absence of CD3δ (lanes 2 and 5). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-6) were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblot shows the presence of a 25kD band corresponding to the BT2 protein and 19kD band corresponding to BT3 protein co-immunoprecipitated with Myc- CD3δ protein. Bottom row: anti-HA immunoblot of lysates demonstrates the expression of BT2 of 25Kd (lanes 2 and 3) and BT3 of 19kD (lanes 5 and 6).

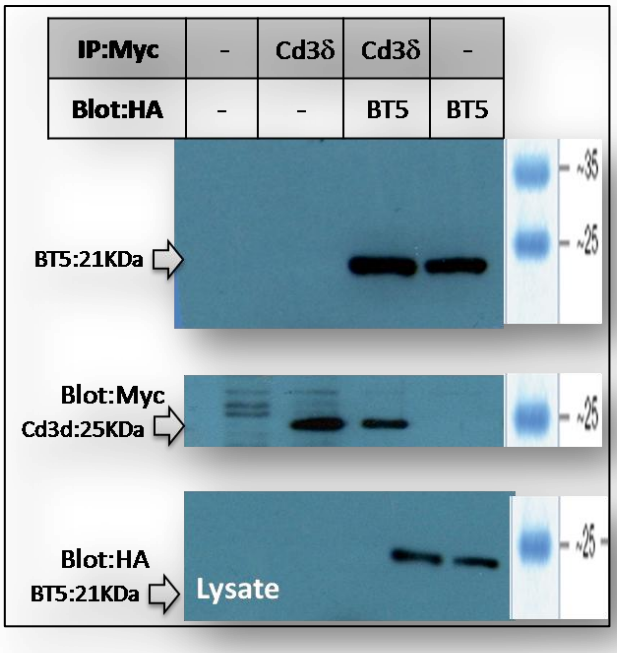


Figure 4.36 :BCAP31truncation BT5 interacts with CD3δ: HEK293T cells were transfected with construct encoding BT5 protein (lane4 and 5) in the presence (lane 4) or absence of CD3δ (lane 5). Top row: Lysates from untransfected HEK293T cells (lane 1) or transfected cells (lanes 2-4) were immunoprecipitated with anti-Myc antibodies and immunoblotted with anti-HA antibodies. Anti-HA immunoblot shows the presence of a 21kD band corresponding to the BT5 protein co-immunoprecipitated with Myc- CD3δ protein.(lane 3) and BT5 protein that is bound to the anti-Myc beads(lane 4) Middle row: anti-Myc immunoblot of lysates demonstrates the expression of CD3δ specifically in lanes 2 and 3. Bottom row: anti-HA immunoblot of lysates demonstrates the expression of BT5 in lanes 4 and 5.

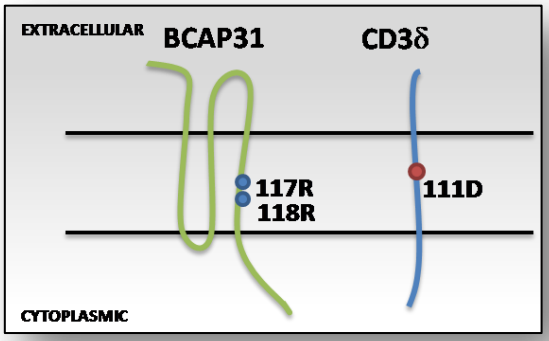


Figure 4.37: The structure of the transmembrane domains of BCAP31 and CD3δ showing the electrostatic residues (negatives: red, positives: blue).Two positively charged residues (117R and 118R) in third transmembrane domain of BCAP31 might play a role in a possible electrostatic interaction with the negative residue (111D) in CD3δ transmembrane region.

4.3.8 The Sub-cellular Localization of BCAP31 Proteins

As the BCAP31 sequence that we used for the study was 39 aminoacids longer than the database BCAP31 we wanted to see whether it is localized in the ER as the database BCAP31 as it was predicted by looking at the sequence in figure4.18. To test this we used pHABCAP31, pHABT1 (that is the database BCAP31 cloned in HA plasmid) and pHABT3 (that is identified as the minimal interacting truncation of BCAP31 with only the third transmembrane region) plasmids to transfect HeLA cells along with an ER marker plasmid ERYFP. 48 hours after the transfection cells were stained with anti-HA rhodamine antibody to visualize the HA tagged proteins. The results indicate that both BCAP31 and BT1 co localize with the ER marker and therefore localized in the ER. The BT3 truncation, lacking the membrane localization signal does not localize specifically to ER as it does not co localize with the expressed ERYFP marker. As the microscope we use to visualize the localization slides is not a confocal microscope we cannot conclude whether the BT3 expression we see is in the cytoplasm or nucleus of the cells. (Figure 4.38)

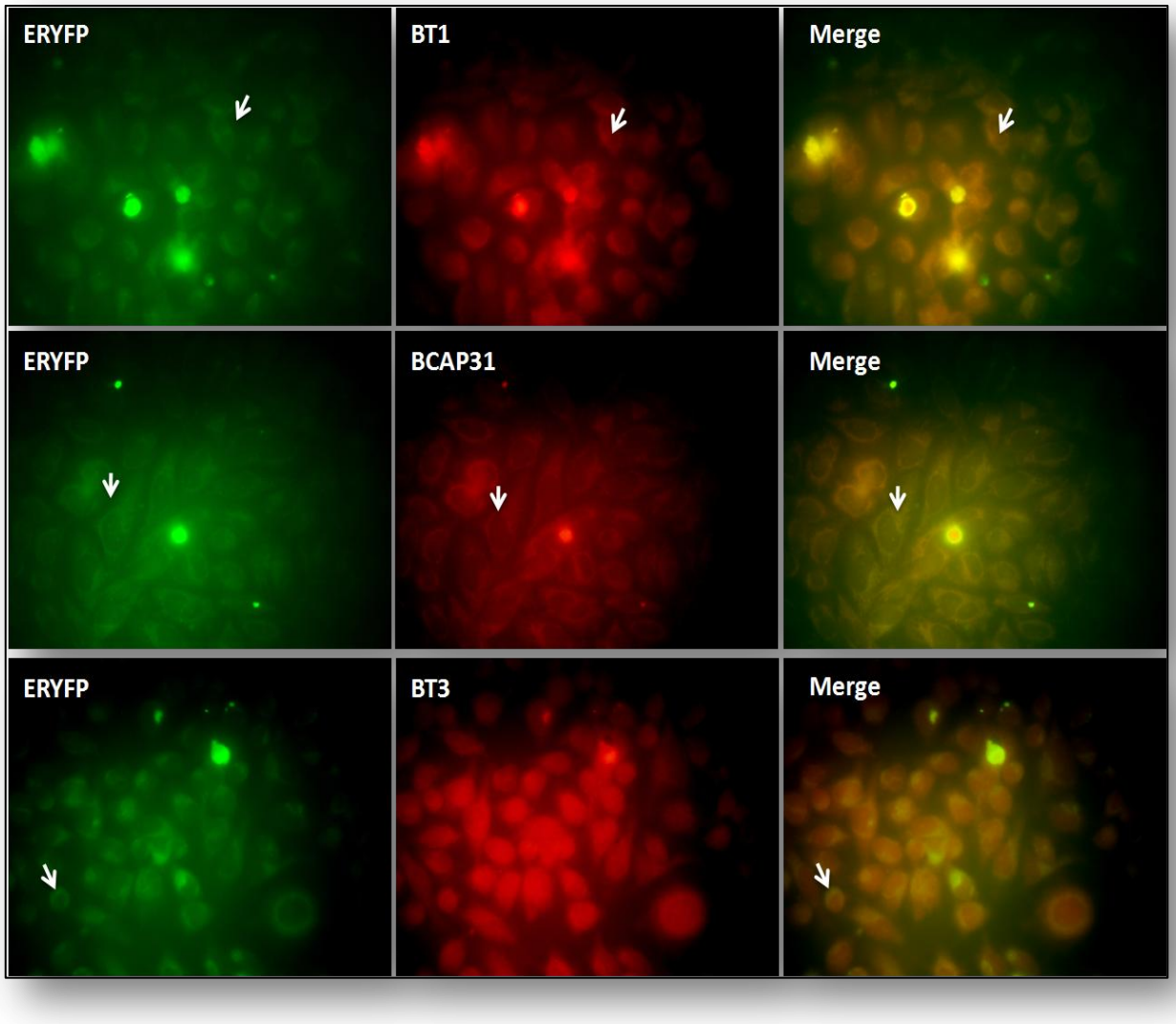


Figure 4.38: Subcellular localization of BCAP31 truncated mutant proteins. Localization of BT1 (first row), BCAP31 (second row) and BT3 (third row) along with the ER marker. HeLa cells expressing ERYFP (first column) and indicated HA tagged BCAP31 truncations were stained with anti HA rhodamine (second column). In the third column where two images are merged, BT1 and BCAP31 are shown to co localize with the ER marker, whereas BT3 is expressed in the cytoplasm. The arrows indicate the ER of the cells that is shown with green due to YFP expression. The bright spots in the figure are artifacts of staining.

4.4 shRNA MEDIATED EXPRESSION ARREST

As we aimed to test the functional relevance of the interaction between CD3δ and CD81 in murine T cell lines, we decided to knock down the expression of CD81 in these cells and observe its effect on TCR signaling. We employed the expression arrest strategy that depends on the expression of shRNAs from retroviral plasmids as shown in figure1.10. The first step was the production of the virus that contains the pSM2 plasmid towards EGFP that will be used as the positive control of the shRNA mediated expression arrest system. The EGFP shRNA coding pSM2 retroviral plasmid is purchased from Open Biosystems. In the pSM2 plasmid, the shRNA cassette is placed between the 5' long terminal repeat (5'LTR) and the drug selection markers. The design of the hairpin cassette incorporates sequences of the human miRNA, mir-30. By adding the mir-30 loop and 125nt of mir-30 flanking sequence on either side of the hairpin results in more than 10 fold greater Drosha and DICER processing of the expressed shRNA resulting in a greater potency for expressed hairpins.[81] The retrovirus was produced in phoenix packaging cells by co-transfection with pSM2-shRNA plasmids and pCL-ECO packaging plasmid. The supernatant of transfected phoenix cells was used for infecting the cell line of interest. The overall strategy of retroviral infection is given in figure 4.39.

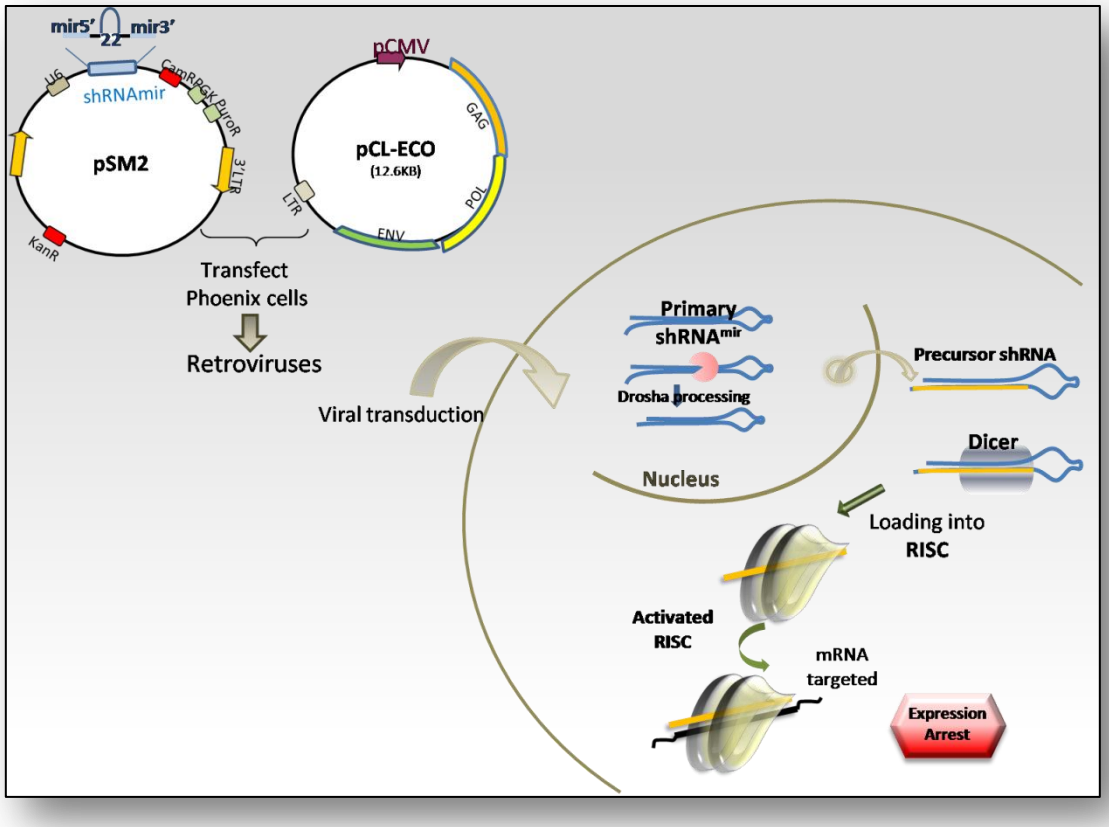


Figure 4.39: Flowchart describing the production of retrovirus coding for shRNAs, retroviral transfer of shRNA and shRNA mediated expression arrest. Retroviral plasmids (pSM2 or LMP) that encodes the shRNA of interest with the flanking mir30 hairpin sequences was used to transfect phoenix packaging cell lines in the presence of pCLEco retroviral packaging plasmid. Retroviruses with the desired shRNA constructs are produced from the phoenix cell lines and supernatant of this cell line containing the retroviral particles is used to infect the cell line of interest. After the successful infection of the target cell line, the primary shRNA produced in the nucleus of the cell is first processed by Drosha and then exported to the cytoplasm as a precursor shRNA. In the cytoplasm with the activity of Dicer this precursor shRNA is loaded into the RISC complex and following activation of RISC the expression arrest of the target mRNA takes place.

4.4.1 EGFP shRNA Efficiently Knocks Down EGFP Expression.

HEK293T cells were transfected with constructs expressing EGFP (pIRES2EGFP) in the absence or presence of constructs that express shRNA against EGFP (pSM2EGFP) or shRNA against CD3δ (pSM2CD3δ). Mean GFP expression of untransfected or transfected

cells was measured in flow cytometry. There is increased GFP due to expression of EGFP from pIRES2EGFP plasmid compared to untransfected cells. When there is pSM2EGFP plasmid that expresses shRNA against EGFP, the GFP amount decreases indicating the efficient knocking down of EGFP by shRNA. (Figure 4.40)

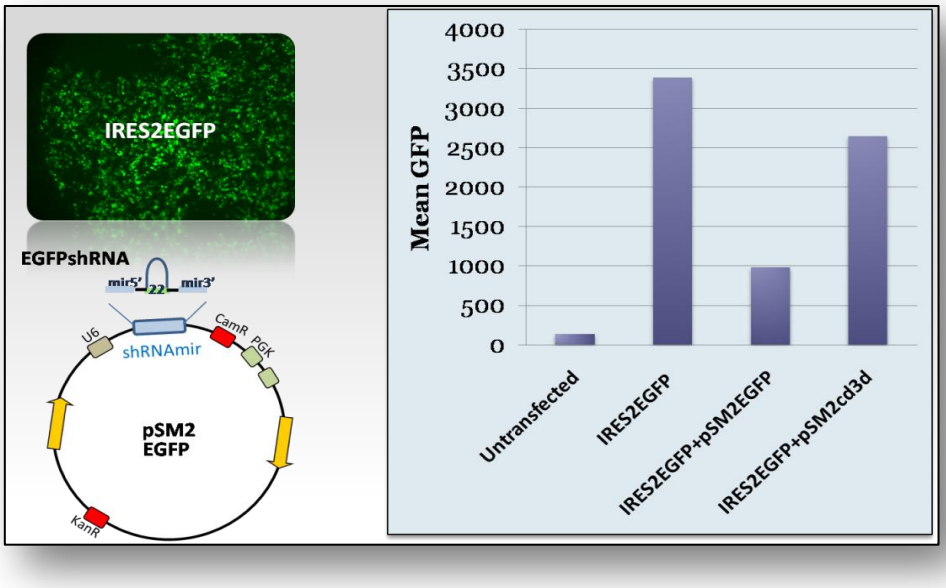


Figure 4.40: Positive control assay for pSM2 plasmids coding shRNA. HEK293T cells expressing IRES2EGFP in the absence or presence of psM2EGFP (plasmid coding shRNA towards EGFP) or pSM2CD3δ (plasmid coding shRNA towards CD3δ) were analyzed in flow cytometry. Mean GFP expression of cells visualized by flow cytometry was plotted and efficiency of EGFPshRNA in knocking down the expression of EGFP was shown indicated by the decrease of mean GFP of the cells that express both EGFP and EGFPshRNA, when compared to EGFP alone or EGFP plus CD3δshRNA expressing cells.

4.4.2 CD3δ shRNAs (K and L) Efficiently Knocks Down CD3δ Expression in HEK293T Cells

In order to check the efficiency of shRNAs against CD3δ in pSM2 plasmid, lysates of the HEK293T cells transfected with pcDNAMyc-CD3δ plus pSM2 plasmids, was checked for the presence of Myc tagged CD3δ by anti-myc blot. When pSM2- CD3δ (K) and pSM2- CD3δ (L), but not pSM2EGFP, were co expressed with CD3δ Myc, the expression of CD3δ decreases. (Figure 4.41)

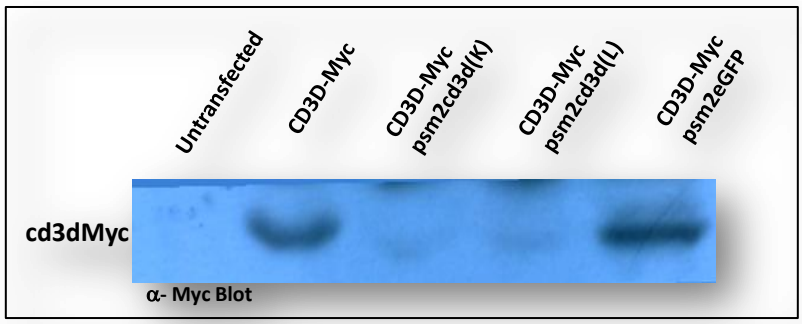


Figure 4.41: Western blot showing CD3δ Myc expression with or without the shRNA expression. First lane there is the lysate from the untransfected HEK293T cells, lane 2: pcDNA3.1MycCD3δ expresses CD3δ. Lanes 3 and 4: pSM2CD3δ -K and pSM2CD3δ -L two different shRNA coding plasmids cotransfected with pcDNA3.1MycCD3δ, there is decrease in CD3δ expression. Lane 5: pSM2EGFP shRNA coding plasmids cotransfected with pcDNA3.1MycCD3δ, there is no decrease in CD3δ expression as the shRNA encoded is not specific to CD3δ.

4.4.3 Cloning of Oligonucleotides Encoding CD81shRNA into the LMP Plasmid

In order to knock down the expression of CD81 in murine T cell lines, we first designed the shRNAs to clone and express in LMP retroviral plasmids. First we designed 97 nucleotide long DNA oligonucleotides to be used as the templates. The designed three different template oligonucleotides are shown in table 4.3. Having these oligonucleotides as the template, PCR was carried out using mir30Forward and mir30Reverse primers that introduce EcoR1 and Xho1 restriction enzyme recognition sequences to the PCR product to aid in cloning of the product into LMP plasmid. (Figure 4.42) PCR products were extracted from the agarose gel and following digestion with EcoR1 and Xho1 restriction enzymes, cloned into LMP using the same restriction sites. The miniprep DNA of positive colonies after ligation was diagnosed for the correct insertion of shRNA using Xho1 and Hind3 restriction sites. The expected fragment sizes and the colonies diagnosed are shown in figure 4.43.

	5’miR-30 Sense Loop Antisense 3’miR-30
1	5’TGCTGTTGACAGTGAGCGATGTCATTATGATCTTTGAGATTAGTGAAGCCACAGA TGTAATCTCAAAGATCATAATGACAGTGCCTACTGCCTCGGA 3’
2	5’TGCTGTTGACAGTGAGCGCGCAGCCATTGTGGTAGCTGTCTAGTGAAGCCACAGA TGTAGACAGCTACCACAATGGCTGCATGCCTACTGCCTCGGA 3’
3	5’TGCTGTTGACAGTGAGCGCGCTGTCATTATGATCTTTGAGTAGTGAAGCCACAGA TGTACTCAAAGATCATAATGACAGCTTGCCTACTGCCTCGGA 3’

Table 4.3: Designed template oligonucleotides for CD81 shRNA amplification: A sense sequence of 22 nucleotide long (Black) is first selected from the CD81 gene. miR30 loop sequence (green) is then inserted between the sense and antisense sequences. Finally, mir30 context (blue) is added to the 5’ and 3’ ends of the target.

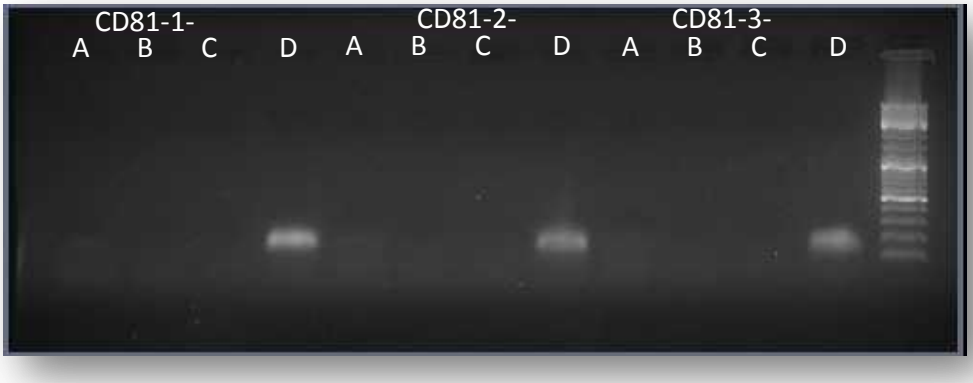


Figure 4.42: PCR of CD81 shRNAs from three different templates (1, 2 and 3) A, B and C are negative control of PCRs with no template, no forward primer and no reverse primer respectively. D is the PCR that has both the template and primers that shows the successful amplification of shRNAs with size 137bp.

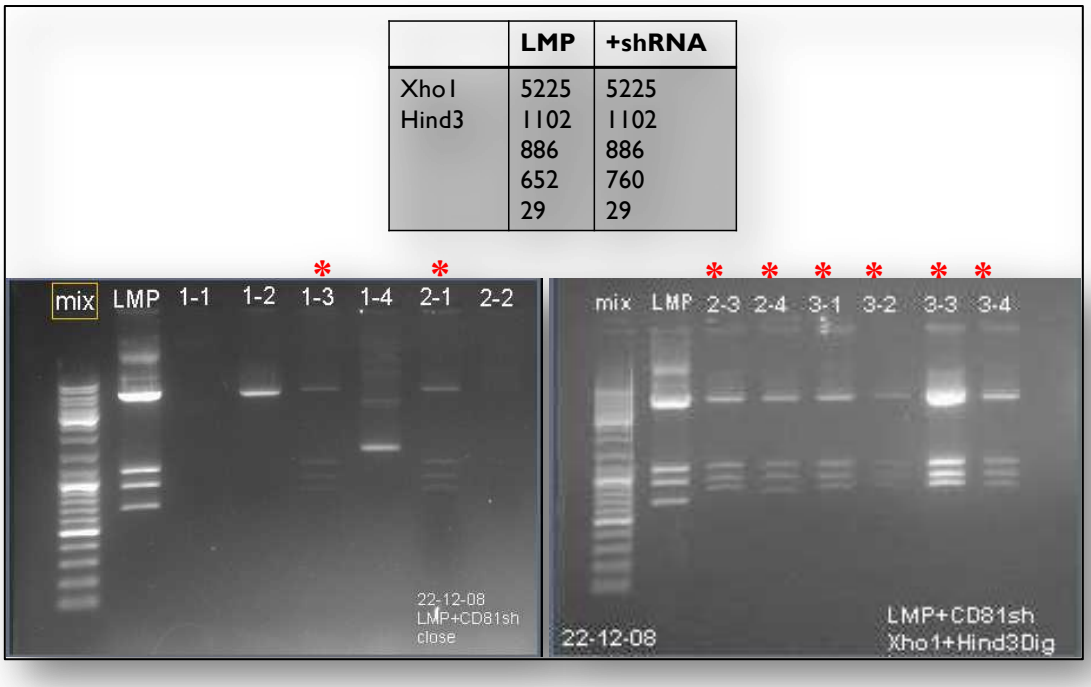


Figure 4.43: Confirmation of the identity of LMP-CD81shRNA plasmids. Xho1 and Hind3 double digest was used for diagnosis of correct insertion of CD81 shRNAs. Xho1 and Hind3 together has 5 recognition sites in empty LMP plasmid and introduction of shRNA in between two of this sites results in a 760 bp band instead of 652 bp band. Four colonies from CD81shRNA1 (1-1 to 1-4), 4 colonies from CD81shRNA2 (2-1 to 2-4) and four colonies from CD81shRNA3 (3-1 to 3-4) were diagnosed along with empty LMP in two different agarose gels. The colonies with the asterix above seemed positive when compared to LMP alone digestion as seen in the expected band lengths above the figure.

4.4.4 The LmpCD81shRNA Clone 2.3 But Not 3.3 Decreases The Expression of CD81 Protein Levels in HEK293T Cells.

Different LMPCD81 plasmids that were diagnosed to be correct were assayed for their shRNA efficiency. Clone 2.3 denoted as LMPCD81-2 and clone 3.3 denoted as LMPCD81-3 were used to transfect HEK293T cells along with pHACD81 to observe the possible decrease in the CD81 band intensity in anti-HA western blot. Anti-calnexin was also used in the same experiment to show equal protein amount in different lanes. The results indicate that lysates that were transfected with LMPCD81-2 but not LMPCD81-3 or empty LMP plasmid shows decreased CD81 expression. (Figure 4.44) When LMPCD81-2

and LMPCD81-3 were sequenced there found to be mutation in the shRNA coding region of the LMPCD81-3 but not LMPCD81-2 and we concluded that this was the reason for the difference in their efficiencies to knock down CD81 protein expression. For the further experiments LMPCD81-2 was used.

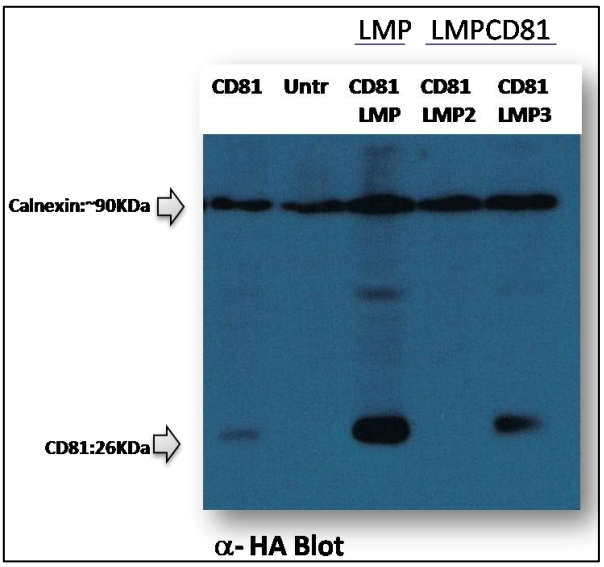


Figure 4.44: CD3δ shRNAs knock down CD3δ protein expression. Western blot showing CD3δ Myc expression with or without the shRNA expression. Lysates from HEK293T cells expressing CD81 alone(lane1), untransfected(lane 2), expressing CD81 along with empty LMP(lane 3) and CD81 along with LMPCD81 clone 2.3-LMP2- or LMPCD81 clone 3.3 –LMP3- (lanes 4 and 5 respectively) were blotted with anti-HA to see the expression of HA tagged CD81 of size 26KDa. Equal loading was confirmed by blotting the same membrane with anti-calnexin that gives around 90KDa band corresponding to calnexin. CD81 expression is downregulated by the expression of LMP2 but not LMP3.

Having confirmed the efficiency of LMPCD81 clone 2.3 to decrease protein expression level of CD81 in total cell lysates, we wanted to further see if it decreases surface CD81 levels so that we can diagnose the further clones that express efficient shRNA only by looking at the surface CD81 levels. We infected NIH3T3 cells with around 90 percent efficiency with LMPCD81 clone 2.3, empty LMP or irrelevant pSM2EGFP retroviral plasmids and checked the surface CD81 expression by surface labeling the cells with antiCD81.Bio coupled with streptavidinPE antibodies. The results indicated that surface CD81 level of NIH3T3 cells that are infected with LMPCD81 clone 2.3, but not the other retroviral plasmids, decreases 70%. (Figure 4.45)

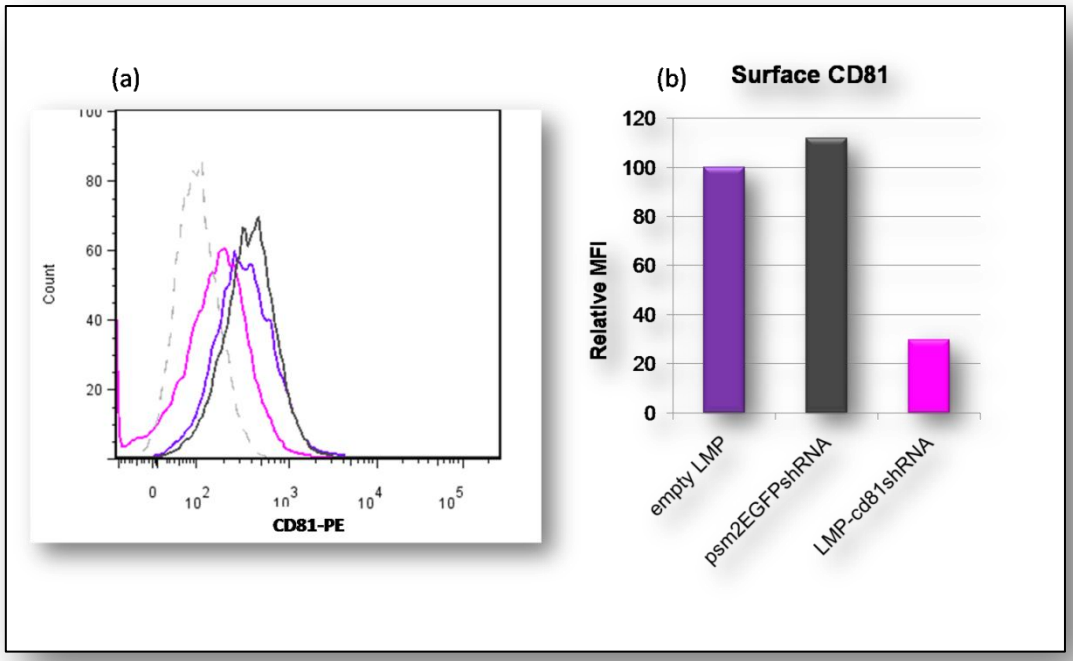


Figure 4.45: EGFP shRNA knocks down EGFP protein expression. NIH3T3 cells infected with retrovirus coding shRNA against EGFP (pSM2EGFP) shown in gray, empty LMP (shown in purple) or LMPCD81 (shRNA against CD81) shown in pink were stained with antiCD81biotin and streptavidinPE. Histogram shows the CD81PE level of the cells(a) and mean fluorescence intensity optimized with respect to empty LMP (set to 100) is plotted on a bar graph.(b)

4.4.5 Identification of Surface Markers for Murine T Cell Lines

VL3.3M2 and 3B4.15 were the two cell lines used in the studies and before going on with the stimulation experiments we wanted to check the surface markers of these two cell lines and validate the antibodies that we are going to use for the further staining procedures. Surface TCR and CD3 amounts were tested with H57 and 2C11 antibodies respectively. The presence of coreceptors CD4 and CD8 were also tested. As seen in figure 4.46 and 4.47 VL3.3M2 cells are double positive cells with both CD4 and CD8 on their surface as coreceptors, 3B4.15 cells are single positive cells with only CD4 as the coreceptor on their surface.

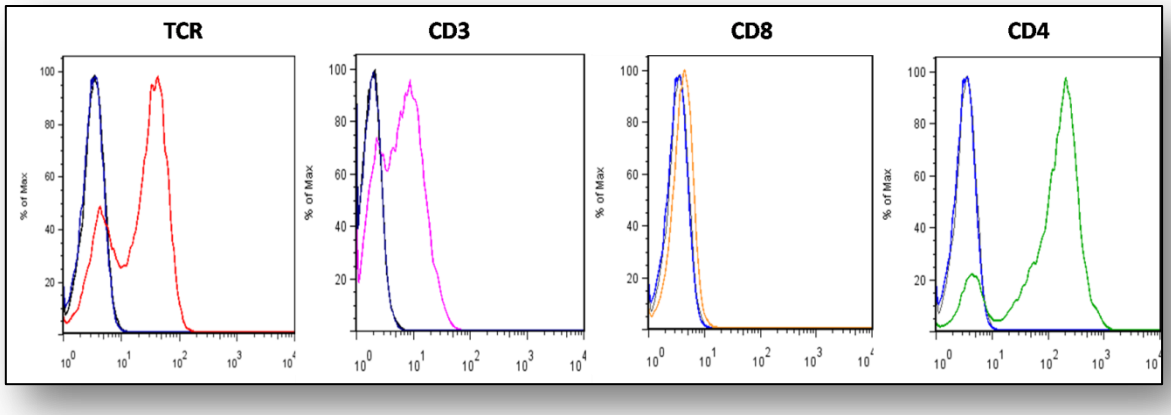


Figure 4.46: Surface markers of 3B4.15 cells. In the first histogram, surface TCR expression is shown (red histogram) relative to an isotype control antibody staining (blue histogram). In the second histogram surface CD3 expression is shown (pink histogram) relative to an isotype control antibody staining (blue histogram) .In the third histogram surface CD8 expression is shown (orange histogram) relative to an isotype control antibody staining (blue histogram) In the last histogram surface CD4 expression is shown (green histogram) relative to an isotype control antibody staining (blue histogram) The 3B4.15 cells are TCR and CD3 positive T cell lines that have only the CD4 as coreceptors on their surface as they are CD4 single positive murine T cell lines.

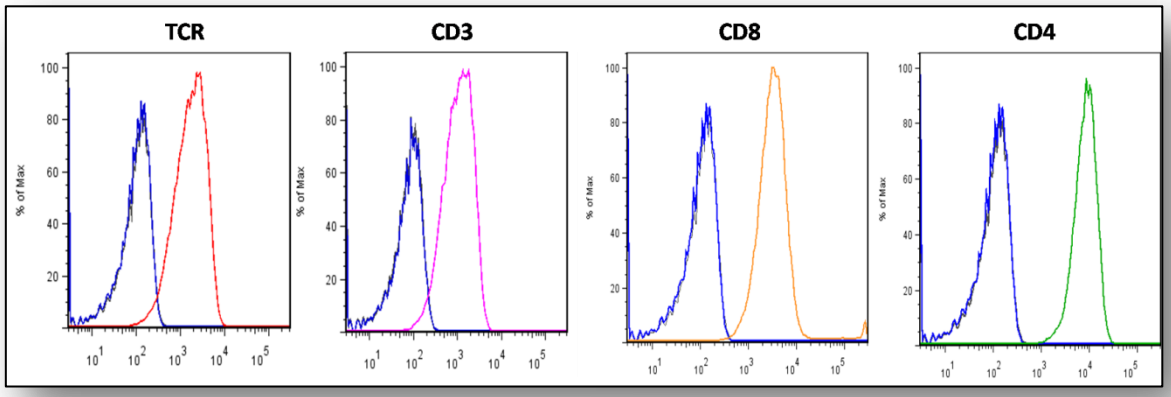


Figure 4.47: Surface markers of VL3.3M2 cells. In the first histogram surface TCR expression is shown (red histogram) relative to an isotype control antibody staining (blue histogram). In the second histogram surface CD3 expression is shown (pink histogram) relative to an isotype control antibody staining (blue histogram). In the third histogram surface CD8 expression is shown (orange histogram) relative to an isotype control antibody staining (blue histogram). In the last histogram surface CD4expression is shown (green histogram) relative to an isotype control antibody staining (blue histogram). The VL3.3M2 cells are TCR and CD3 positive T cell lines that have both the CD8 and CD4 as coreceptors on their surface as they are double positive T cell lines.

4.4.6 Introduction of LmpCD81 Plasmid into VL3.3M2 Cells

As the VL3.3M2 cells could not be infected by retroviral infection, the cells were electroporated with retroviral plasmids coding shRNA against CD81 (LMPCD81) or empty retroviral plasmid (LMP). Two different conditions were used for the electroporation of VL3.3M2 cells and the cells from both of these electroporations were used for further analysis. Efficiency of transfection by electroporation was visualized by the expression of GFP from the LMP backbone by flow cytometry. (Figure 4.48) The electroporated cells were then selected for LMP expression using puromycin. There were two set of cells that were selected, one set was the original electroporation products (that is pool of cells that express LMPCD81 or LMP) the second set of cells were the ones that are single cell clones which are selected starting from single cells by preparing limiting dilution of starting electroporated cells as explained in figure 3.4. The pool of cells that were splitted in puromycin containing medium for 4 weeks were assayed for their GFP expression levels after two months to see whether the selection was successful. GFP positive population of both of the electroporations with different voltages increased almost to 100% showing successful selection of cells expressing LMP or LMPCD81 plasmids with GFP coding region. (Figure 4.49)

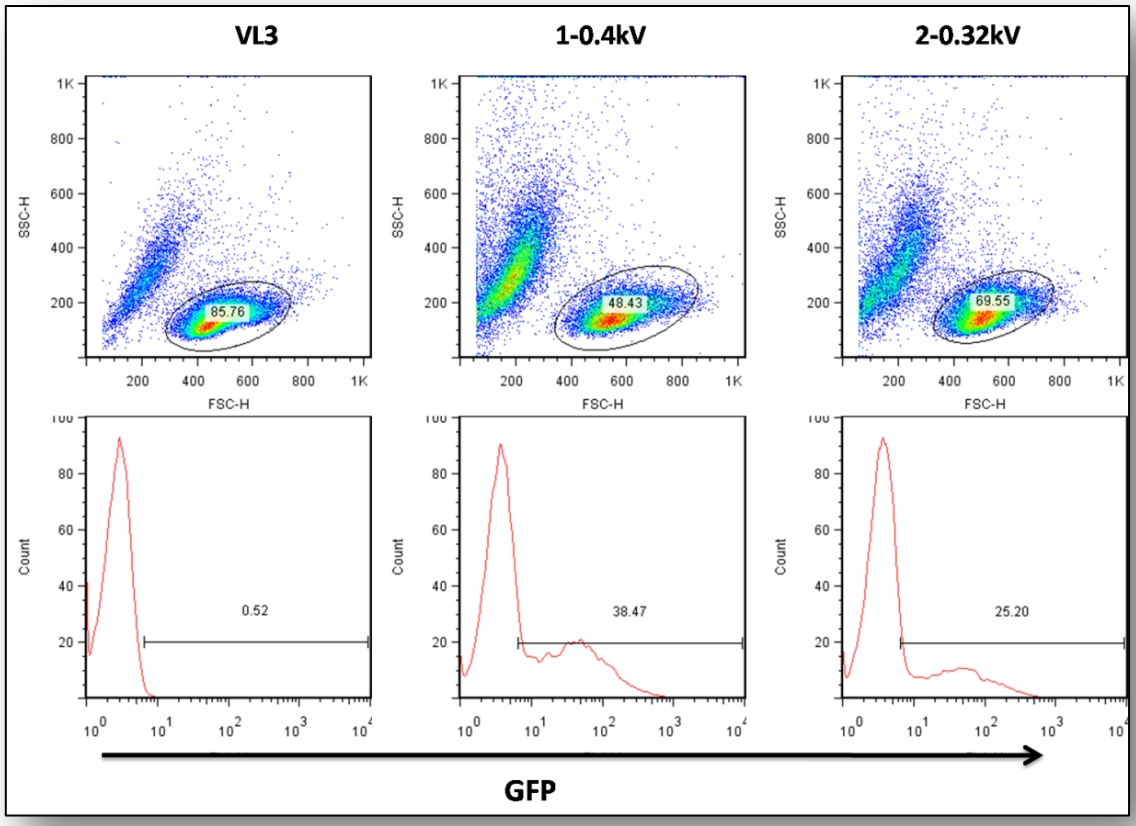


Figure 4.48: Expression of GFP reflects the transfection efficiency of VL3.3M2 cell lines transfected with LMP or LMPCD81shRNA plasmids. Upper row shows the FSC vs. SSC of untransfected, 0.4kV electroporation and 0.32kV electroporation from left to right. Lower row shows the corresponding GFP histograms of alive cells and GFP positive population is gated with the percentages shown on above the gates. When the cells were electroporated at 0.4kV, almost 50% of the cells were alive and the GFP positive cells were 38.47% of the alive cells. When the cells were electroporated at 0.32kV this time almost 70% of the cells were alive, but only 25% of alive cells were GFP positive. Both cell populations of different electroporation conditions were used for selection.

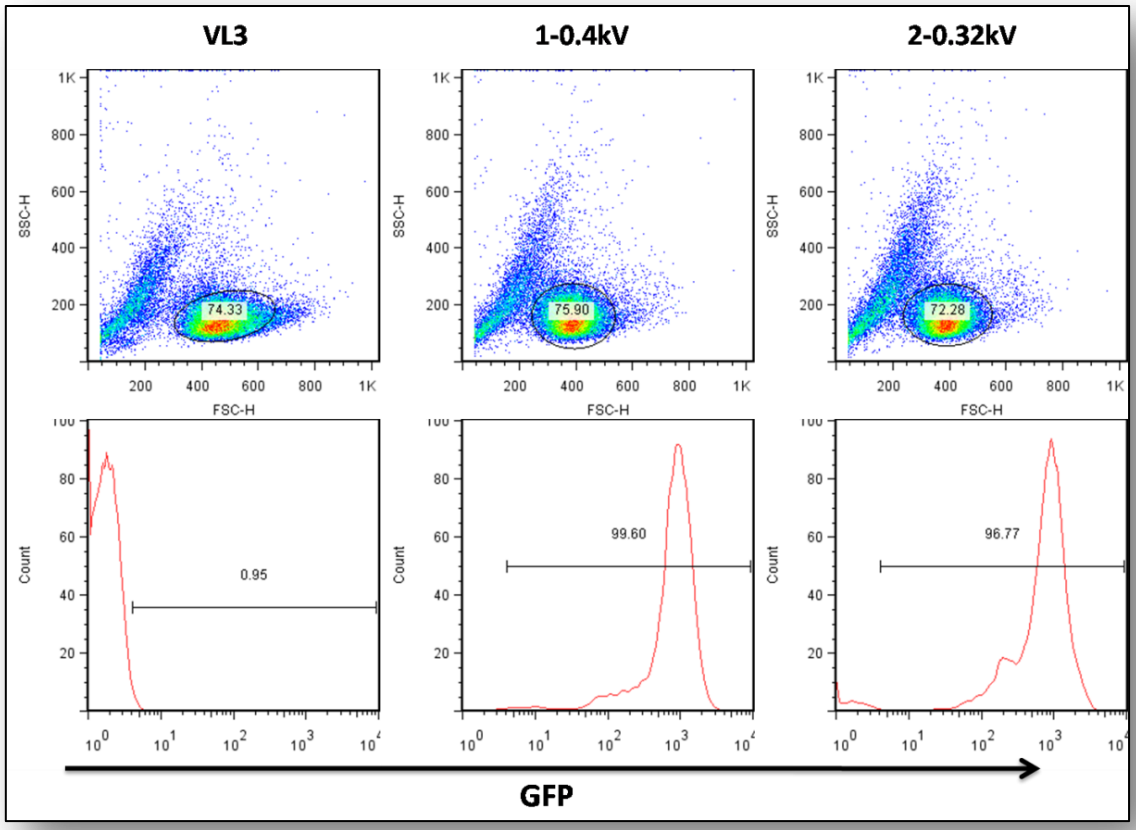


Figure 4.49: Selection of transfected VL3.3M2 cell lines results in stably transfected cells. Upper row shows the FSC vs. SSC of untransfected VL3.3M2, 0.4kV electroporation and 0.32kV electroporation products after selection from left to right. Lower row shows the corresponding FL-1 histograms of alive cells and GFP positive population is gated with the percentages shown on above the gates. As seen from the FSC vs. SSC dot plots, in both of the cell populations that were selected, the alive cells were 75% as the untransfected VL3.3M2 cells. The GFP expressing percentage of cells was 99.6% in 0.4kV electroporated cells and 96.77% in 0.32kV electroporated cells. Both populations of cells were successfully selected for GFP expression that is encoded by the LMP plasmid used for transfecting the cells.

4.4.7 Surface CD81 Expression Levels in Stable Cell Lines Expressing CD81shRNA

After selecting the cell lines that express CD81 shRNA stably, the efficiency of shRNAs to knock down the expression of surface CD81 was tested. The pool of electroporated cells and also the selected stable clones were stained with antiCD81Bio coupled with streptavidinPE to see the surface CD81 levels on the cells. The results of pool of the electroporated cells is shown in figure 4.50. The surface CD81 level of untransfected

VL3.3M2, empty LMP expressing VL3.3M2 and LMPCD81 expressing VL3.3M2 was analyzed via flow cytometry by looking at the mean PE fluorescence of GFP positive cells. The surface CD81 level of LMPCD81 expressing pool of cells was downregulated by 30%. (Figure 4.50)

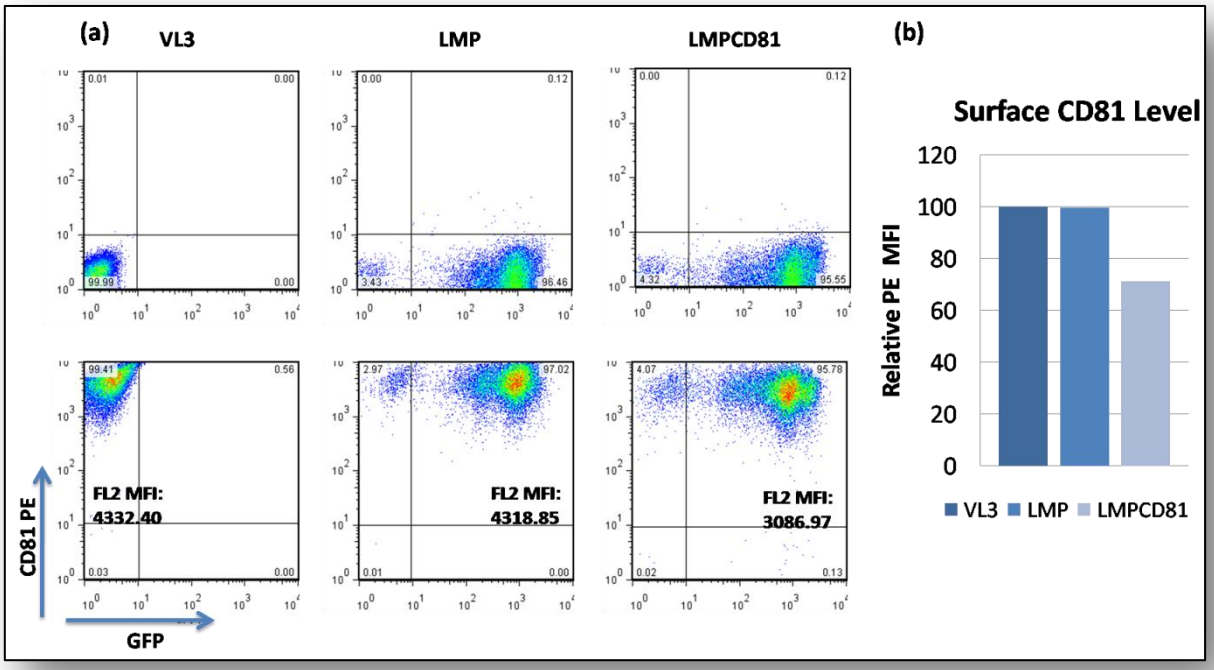


Figure 4.50: Surface CD81 expression on stably transfected VL3.3M2 cells. VL3.3M2 cells that are untransfected (left) and stably LMP expressing VL3.3M2 cells (middle) and stably LMPCD81 expressing VL3.3M2 cells (right) were analyzed by flow cytometry for the surface CD81 expression levels. Dot plots of GFP versus PE are given in (a) for the unstained cells (upper row) and for CD81 biotin plus streptavidinPE stained cells (lower row). MFI (Mean fluorescence Intensity of PE of each cell population (that represents surface CD81) was plotted in a bar graph. Relative surface CD81 level of cells with respect to untransfected VL3.3M2 cells (set to 100) was plotted on a bar graph, which shows 30% decrease in surface CD81 level of LMPCD81 expressing VL3.3M2 cells. (b)

On the other hand, stable single cell clones were also tested for their surface CD81 levels in the same manner. The results of three different experiments are shown in figure 4.49. The average mean GFP of selected clones was plotted in six different clones. The selected six clones were empty LMP transfected negative control clones 1b5, 1e4 and LMPCD81 transfected 3a4, 4a10, 3b3 and 3b4 clones. The surface CD81 expression levels were shown with respect to highest CD81 expressing 1b5 clone. In clone 4a10, expression

of surface CD81 was downregulated 35%, whereas in clone 3a4 expression was downregulated almost 60 %.(Figure 4.51)These two positive clones along with 1b5 and 1e4 negative control clones were used for the further analysis.

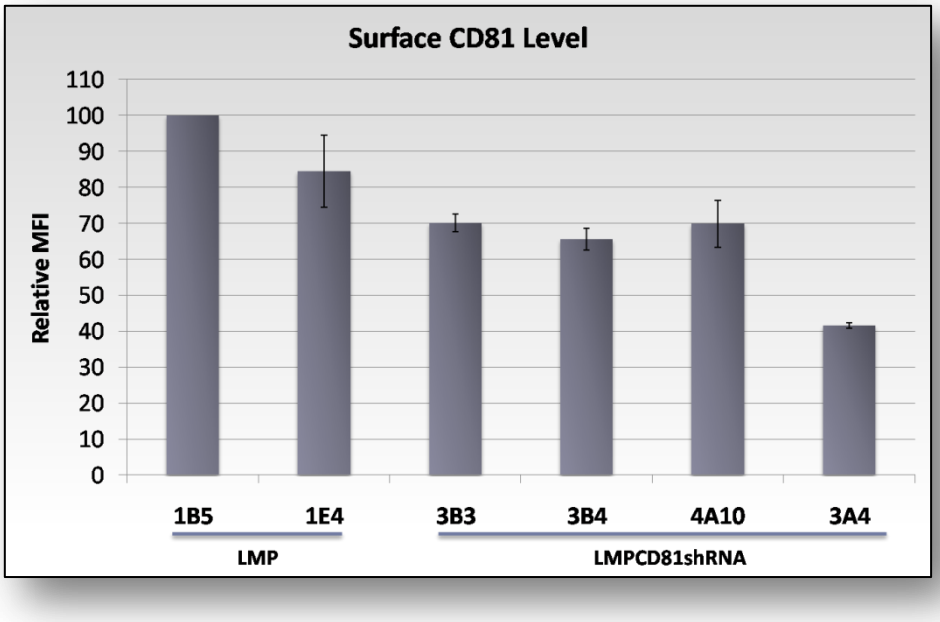


Figure 4.51: Surface CD81 expression on single cell clones of stably transfected VL3.3M2 cell lines (3b3, 3b4, 4a10 and 3a4) along with empty LMP stable cell lines 1e4 and 1b5. Surface CD81 levels are shown as percentage of negative control 1b5 clone. The surface CD81 levels are shown as relative mean fluorescence intensities, with higher CD81 expressing cell line 1B5 amount set to 100.

The stable cell lines that showed decreased surface CD81 (Figure 4.51) were this time assayed for the amount of surface TCRβ expression. Surface TCRβ amounts of the negative control cell lines (1e4 and 1b5) and CD81 shRNA expressing cell lines (3a4 and 4a10) were same with the surface TCRβ amount of untransfected VL3.3M2 cells. (Figure 4.52) We decided to use these clones for the further functional studies as the only difference among them would be the difference of the surface CD81 levels. As there was a small fluctuation on the surface TCRβ amount of 3b4 and 3b3 clones, they were not used for the further functional studies.

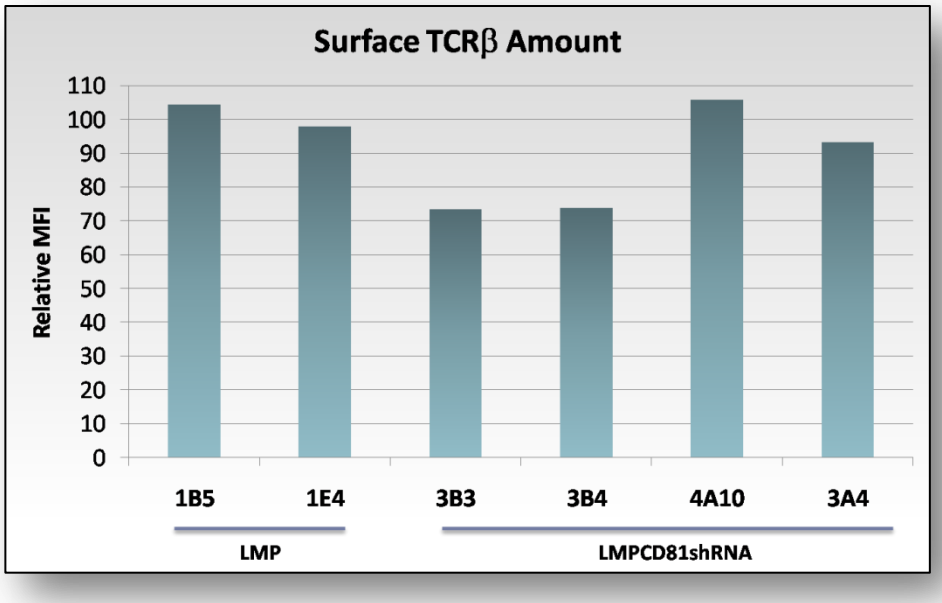


Figure 4.52: Surface TCRβ expression on single cell clones of stably transfected VL3.3M2 cell lines (3a4,4a10,3b4 and 3b3 are the cell lines that stably express shRNA against CD81; 1e4 and 1b5 are negative control cell lines that stably express empty LMP with no shRNA). Relative MFI of the clones were plotted with respect to negative control 1b5 (set to 100).

4.5 STIMULATION OF T CELL LINES BY ANTIBODY TREATMENT

To assess the efficiency of TCR signaling in the absence of CD81, we incubated the 3B4.15 and VL3.3M2 T cell lines on plates coated with immobilized antibodies against TCRβ and CD4 were used to stimulate. Stimulation was quantified by measuring the amount of surface CD69 expression. CD69 is the earliest inducible cell surface glycoprotein acquired during lymphoid activation and used as a stimulation marker both in-vivo and in-vitro experiments. [60, 92]

4.5.1 Stimulation and Co-Stimulation of 3B4.15 T Hybridoma Cell Lines

In order to see the effect of surface CD81 level in TCR signaling we first needed to optimize the stimulation conditions for murine T cell lines. We wanted to observe not only the TCR mediated stimulation but also the costimulation effect of CD4 on TCR

stimulation. As shown in fig. 4.46, 3B4.15 cells are positive for both the TCR and the CD4 co-receptor. Costimulation with antibodies against these two surface markers is thought to sequester the Lck tyrosine kinase which phosphorylates tyrosine amino acids in the ITAM motifs in the cytoplasmic tails of TCR subunits. There are two pools of Lck in T lymphocytes, a membrane associated pool that resides in rafts and a CD4 tail associated pool, pre-bound to the cytoplasmic tail of the CD4 co-receptor. It is thought that anti-TCRβ antibody crosslinking activates TCR signaling by activating the former pool of Lck and anti-TCRβ plus CD4 co-crosslinking activates the latter pool of coreceptor associated Lck. We found that in high concentration of antibodies used for stimulation, surface CD69 level of 3B4.15 cell line increased both with TCRβ alone and TCRβ plus CD4 stimulation. However, the effect of TCRβ/CD4 costimulation is only observed when the antibody concentrations used for stimulation were lowered to 0.1μg/ml and even 0.05μg/ml. (Figure 4.53)

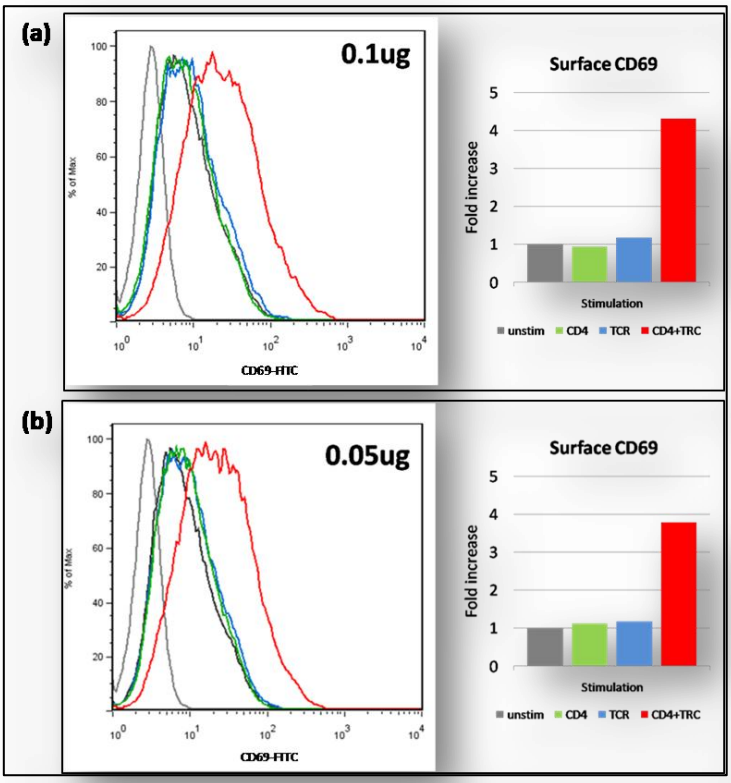


Figure 4.53: Co-stimulation of 3B4.15 cells using 0.1µg (a) or 0.05µg (b) of antibody for stimulation. The histograms show the FITC intensity that represents the labeled surface CD69 with CD69FITC antibody. The graphs next to histograms represent the fold increase in surface CD69 levels that is normalized to unstimulated surface CD69 level.

4.5.2 Stimulation and Co-Stimulation of VL3.3M2 Cells

VL3.3M2 cells were also stimulated by coating the plates with different amounts of TCRβ and CD4 antibodies. Costimulation was also observed in stimulations that had low amounts of coating antibodies as in 3B4 cells. Using 0.5ug of antibody to coat the plates and stimulate the cells, surface CD69 levels of TCRβ alone stimulated cells increased around 2.5fold and costimulation increased the surface CD69 level by almost 4 folds. (Figure 4.54). This amount of antibody and also lower and higher amounts of antibodies were used to stimulate the CD81shRNA expressing cell lines.

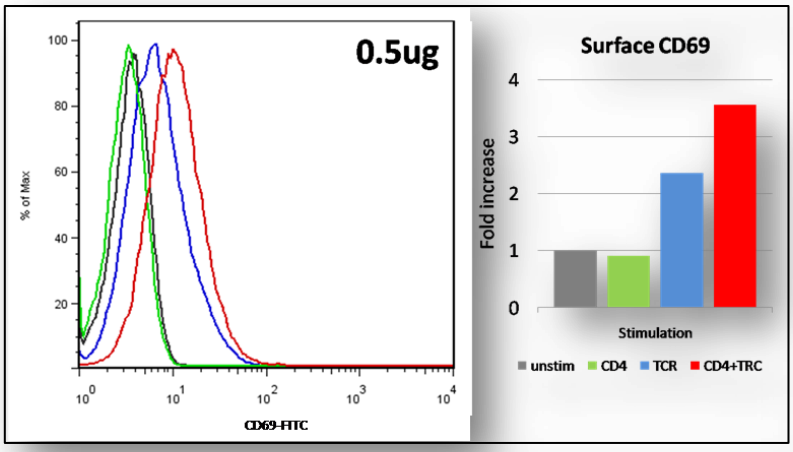


Figure 4.54: Co-stimulation of VL3.3M2 cells using 0.5µg of antibody for stimulation. The histogram shows the FITC that is labeled surface CD69 with CD69FITC antibody. The graph next to the histogram represents the fold increase in surface CD69 levels that is normalized to unstimulated surface CD69 level.

As the expression of retroviral plasmid LMP could alter the stimulation of T cells, one of the negative control cell lines 1b5 was first stimulated with 0.25µg and 0.1µg of antibodies. Using 0.1µg of antibody for stimulation, costimulation effect of CD4 was observed for 1b5 cell line, and this amount of antibody was used for the start point of further stimulation experiments (Figure 4.55).

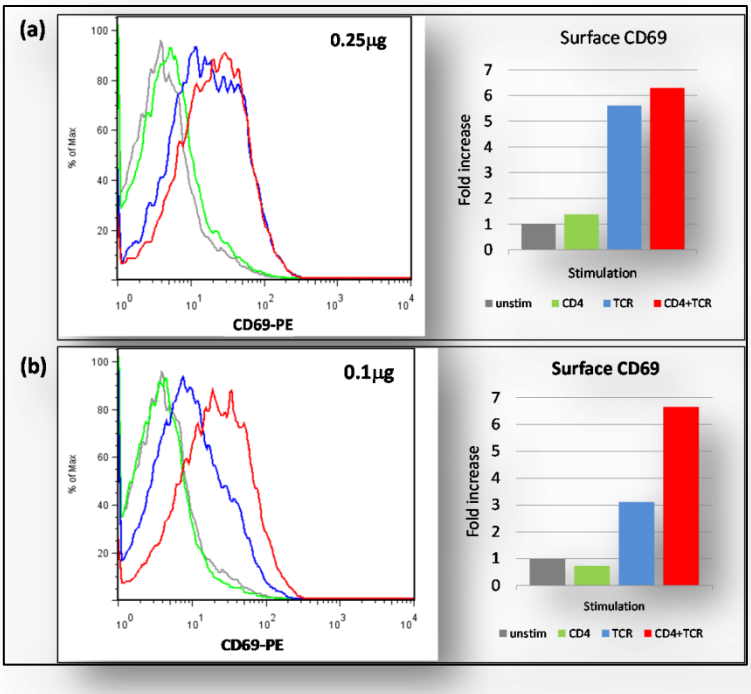


Figure 4.55: Co-stimulation of VL3.3M2-1B5 cells using 0.25µg (a) or 0.1µg (b) of antibody for stimulation. The histograms show the mean FL1 intensity that is labeled surface CD69. The graphs next to histograms represent the fold increase in surface CD69 levels that is normalized to unstimulated surface CD69 level.

4.6 STIMULATION OF CD81shRNA EXPRESSING STABLE CELL LINES

4.6.1 Stimulation of LMP or LMPCD81 expressing VL3.3M2 Cells

In order to see the effect of CD81 on T cell receptor mediated stimulation of T cells, after optimization of stimulation conditions of VL3.3M2 cells, the stable electroporated pool of VL3.3M2 cells and single clone stable cell lines that express either CD81shRNA along with GFP (from LMPCD81 plasmids) of GFP alone (from empty LMP) were stimulated with TCR and CD4 antibodies. When pool of VL3.3M2 cells that stably express LMP or LMPCD81 were stimulated with 0.1µg of antibody, the results of three different experiments indicated that TCR mediated stimulation of CD81 low cells increases when compared to untransfected VL3.3M2 or empty LMP expressing stable VL3.3M2 cells. (Figure 4.56) This result makes us hypothesize that CD81 presence on the surface of

T cells regulates the TCR mediated signaling. Interaction of CD81 on the cell surface with the TCR through CD3δ chain might involve in a primary control step of TCR mediated signaling. Normally the idea behind the stimulation of T cells leading to increased expression of surface CD69 level is that TCR antibody coated to the tissue culture plate leads to crosslinking of TCR chains on the surface of T cells and leads to concentrating the TCR and initiate downstream signaling events. Although we do not know the exact step of stimulation that is altered leading to an increased level of CD69 expression, it is evident that binding of CD81 to TCR complex decreases its translocation to the lipid rafts and therefore when surface CD81 level is lowered, the TCR mediated stimulation increases.

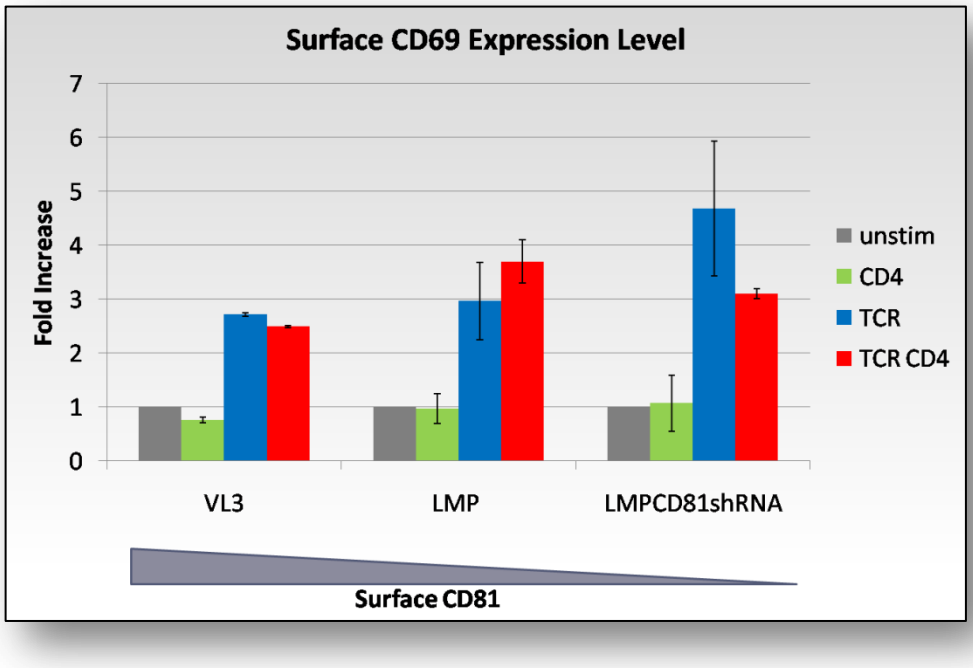


Figure 4.56: Surface CD69 expression on stimulated VL3.3M2 cells. CD69 levels of unstimulated (grey), CD4 stimulated (green), TCRβ stimulated (blue) or TCRβ plus CD4 stimulated (red) VL3.3M2 cells, LMP expressing VL3.3M2 cells and LMPCD81 expressing VL3.3M2 cells. Results are given as fold CD69 optimized to unstimulated CD69 levels of different cells. Mean of three different experiments are given with error bars corresponding to standard deviations. As seen from the blue bars, TCR mediated stimulation increases by decreasing amounts of CD81.

4.6.2 Stimulation of Single Clone Stable VL3.3M2 Cells Expressing LMP or LMPCD81

As we found an increase in TCR mediated stimulation with decreasing amounts of CD81, and the pool of cells we used showed up to 30% of decrease in surface CD81 levels, we wanted to test single cell clones that express CD81shRNAs for the stimulation experiments. The single cell clones that has the highest decrease in surface CD81 level was 60%. For the single cell clones of stable cell lines, experiments with different amounts of antibodies (0.025μg.0.1μg and 1μg) were carried out. The histograms that show the overlay of CD69PE intensities of TCRβ, CD4 or TCRβ CD4 stimulated cells clearly demonstrated that at 0.025μg antibody concentration used for stimulation, there is almost no stimulation of the cells and stimulation increases by increasing amount of antibodies used for stimulation. The results of three representative clones with different surface CD81 expression levels indicate that the effect of CD4 costimulation increases by decreasing amount of CD81 levels. (Figure 4.57) When the trend of this increase in CD69 levels was observed for three representative clone of cells with different surface CD81 levels (CD81 high,CD81 intermediate and CD81 low) there seems to be an increase in TCRβ and CD4 costimulation of VL3.3M2 cells by decreasing amount of surface CD81.(Figure 4.58). In the insight of previous studies that show the interaction of CD81 with CD4, we hypothesized that CD81 interacting with TCR through CD3δ chain might involve in regulation of CD4 proximity to TCR and thus might alter the CD4 mediated costimulation of T cells. Observing an increase in the CD4 mediated costimulation of T cells by decreasing amounts of surface CD81 suggests that CD81 indeed functions in regulating the CD4 mediated costimulation of T cells.

This trend of difference in surface level expression is not only true for surface CD69 level, as we also checked surface CD5 expression, another T cell stimulation marker, after stimulation in two different experiments. Although the surface CD5 expression is always higher than the surface CD69 expression, the trend in the increase of expression for all the clones was same. Surface CD5 levels were increased with decreasing amounts of surface CD81 level following TCRβ-CD4 costimulation. (Figure 4.59)

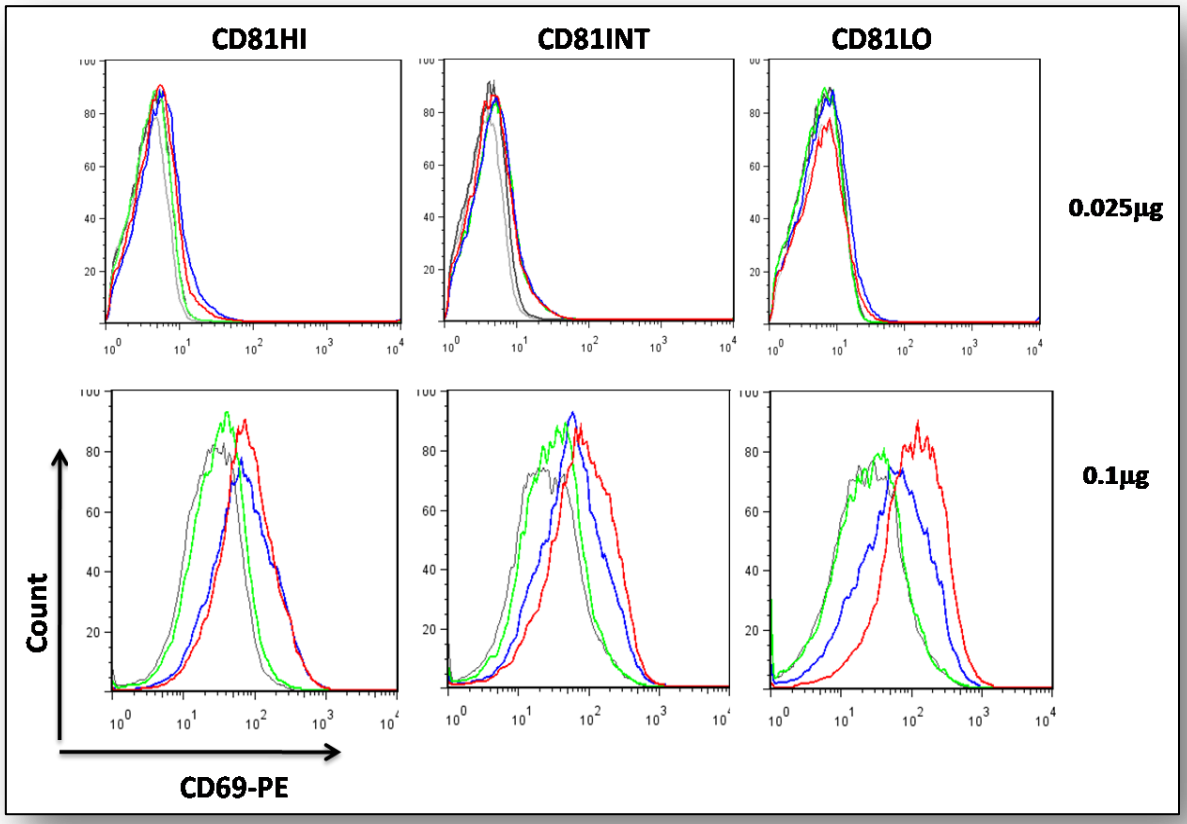


Figure 4.57: Stimulation of VL3.3M2 cell clones with different surface CD81 levels: PE histograms of three representative cell lines with high, intermediate and low surface CD81 levels (CD81HI,CD81INT and CD81LO), that were stimulated with 0.025µg of (Upper row) or 0.1µg of TCR (Blue) ,CD4(green) or TCR plus CD4(red) antibodies. The grey lines in each histogram represent the surface CD69 level of unstimulated cells.

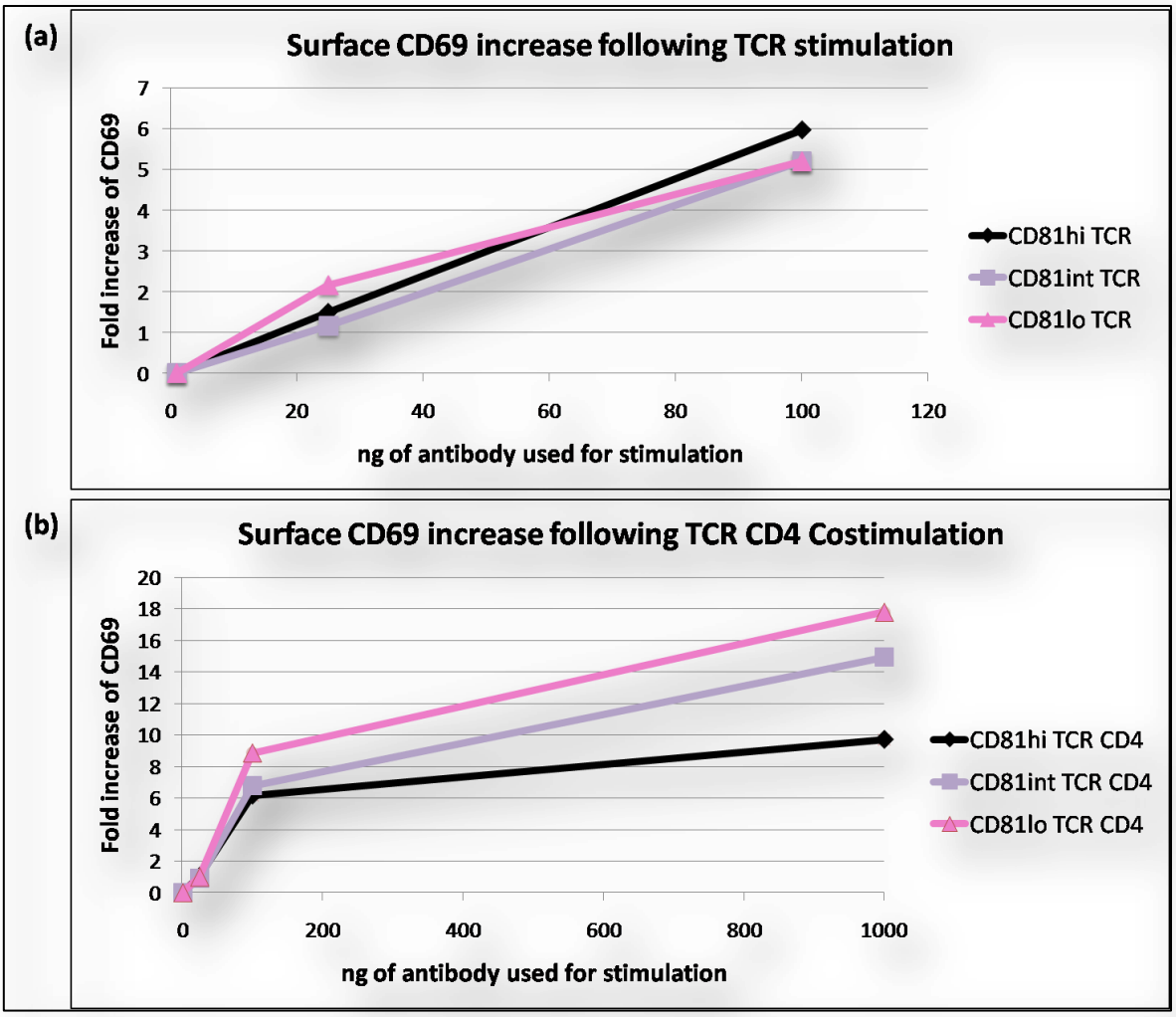


Figure 4.58: Surface CD69 Levels of three representative VL3.3M2 cell lines that express different levels of surface CD81 (denoted as CD81lo: low level expression-pink line-, CD81int: intermediate level expression-purple line- and CD81hi:high level of expression-black line-) Fold increase of surface CD69 levels are plotted for three different cell lines following their stimulation with plate bound 25ng, 100ng and 1000ng of antibody. Following TCR alone stimulation there is no difference in between three cell lines.(a) Regardless of the amount of surface CD81 expression, stimulation by increasing TCR+CD4 antibodies results in increasing CD69 expression. In all cases TCR+CD4 costimulation results in more CD69 upregulation compared to TCR stimulation alone. The responsiveness of these three cell lines to TCR+CD4 costimulation increases with decreasing surface CD81 levels.(b)

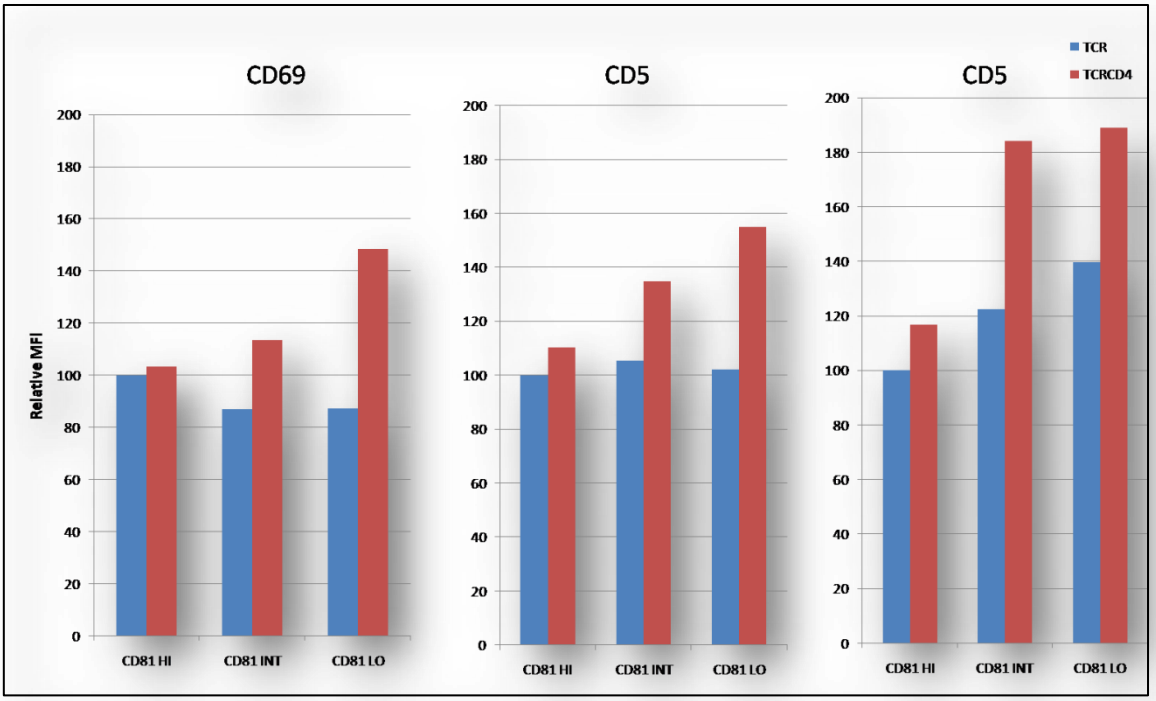


Figure 4.59: Relative Mean fluorescence intensity (MFI) of CD69 surface expression on stimulated VL3 clones. The first graph shows the surface CD69 expression level and the other two graphs show surface CD5 expression level following stimulation with either 0.1µg of TCRβ alone (blue) or with 0.1µg TCRβ and 0.1µg CD4. The relative MFI was calculated as we set mean PE of CD81HI cells stimulated with TCRβ alone to 100 and calculated the others relative to this.

5. DISCUSSION

The regulation of immune responses is the major medical goal of research in immunology. This goal is two-fold: suppression of immune responses during autoimmune disease and the stimulation during infectious disease or cancer. The best way to do this is to understand and modify the T cell development in the thymus. The thymus generates a functional T cell repertoire while mature T cells that migrate to secondary lymphoid organs from the thymus results in proliferation, cytotoxic killing, cytokine secretion and induction of programmed cell death. All of these T cell activation events are initiated by the T cell receptor complex (TCR) on the cell surface of T lymphocytes. TCR is composed of ligand binding TCRα and TCRβ subunits along with signaling subunits CD3δ, CD3ε, CD3γ and TCRζ.[34] The T cell immune response is initiated upon engagement of TCR and coreceptors (CD4 or CD8) by cognate antigen presented on MHC molecules by antigen presenting cells. This engagement that is highly regulated with a diverse set of proteins on the cell surface induces the activation of biochemical signaling pathways that direct the outcome of the immune response.

In this study we focused on the CD3δ subunit of the TCR to identify putative interaction partners that might have a role in TCR signaling or assembly. The reason to focus on CD3δ subunit is that CD3δ knockout mice do not have any peripheral T lymphocytes.[36] In fact, these mice have arrested development at the positive selection stage of T lymphocytes in the thymus. This phenotype indicates that the CD3δ subunit is required either for signaling by the TCR, or for the expression of this receptor on the surface of developing thymocytes. Interestingly, the CD3δ subunit is unique among CD3 subunits because its inactivation does not prevent the DN to DP transition in the thymus. It is known that this transition is signalled by a receptor called preTCR which is composed of preTCRα, TCRβ and CD3 subunits. The major difference between TCR signaling and

preTCR signaling is the requirement of either CD4 or CD8 co-receptors for TCRs. Because the preTCR signals in DN thymocytes that do not express these co-receptors, it does not require help from these molecules, CD3δ may be a critical subunit for co-receptor dependent signaling of T cell receptors.

In the first part of the project, we used a modified yeast two hybrid system to identify putative interaction partners of the CD3δ subunit of TCR. The membrane based yeast two hybrid system that we used in the study is a novel technology that aids in identification of interaction partners of membrane proteins. The classical yeast two hybrid system, commonly used to identify novel protein interaction partners is limited to the analysis of nuclear protein-protein interactions. The membrane yeast two hybrid system overcomes this drawback as it uses split ubiquitin technology where membrane anchored bait and prey proteins interact to cause reporter gene activation as a result of the translocation of a transcription factor from the yeast cell plasma membrane to the nucleus. As explained in detail in the Section 1.9 of this thesis, we employed this novel technology to identify interacting partners of yeast membrane localized CD3δ. We first cloned a CD3δ coding cDNA into the bait plasmid of the system fused to SUC2 reading frame that encodes a yeast membrane localization signal for the downstream protein. One crucial step in designing the primers to amplify and clone CD3δ subunit into the bait plasmid was to exclude the membrane signaling region of CD3δ and clone the CD3δ in frame with the SUC2 reading frame so that the protein product would be targeted successfully to the yeast membrane.

After testing the correct functioning of the system by the aid of positive and negative control assays, a Jurkat thymic cDNA library was used as the prey to screen for the interaction partners of CD3δ. Jurkat is a human leukaemic T cell line that was developed in 1977[93] and since then, has been widely used as an in vitro T cell stimulation and signaling system. Screening resulted in approximately 500 colonies and the identity of cDNAs were determined by isolating DNA from yeast cells, retransforming into bacterial cells, isolating cDNA plasmid from the bacterial cell followed by DNA sequencing. The interaction between proteins encoded by cDNA clones with a potential role in TCR signaling or

assembly and CD3δ was confirmed with secondary yeast and mammalian interaction experiments. Two proteins from the screen that show a putative interaction with CD3δ, were selected for further analysis in mammalian tissue culture cell lines.

One drawback of this membrane based yeast two hybrid system was the subcellular localization of the mammalian proteins expressed in yeast cells. CD3δ was targeted to yeast cell membrane using a SUC2 yeast membrane localization signal. However, we could not test whether the encoded CD3δ is localized to the yeast plasma membrane, therefore we could not conclude whether the identified interactions took place on the cell membrane or in the intracellular membrane compartments such as ER. One other drawback of this system was that the CD3δ subunit was expressed by itself in yeast cells, without the other TCR subunits. It is known that when individual TCR subunits cannot assemble into full TCR complexes, they are degraded in the ER in mammalian cells.[4] Even if some CD3δ proteins escaped from the yeast ER to the plasma membrane, their protein interaction partners may be different than the interaction partners of TCR subunits assembled into a full TCR complex. This obstacle could be overcome by expressing the full TCR complex on the surface of yeast cells. A strategy to express all TCR subunits in yeast cells could be to use P2A sequences that allow the expression of poly-cistronic mRNAs in eukaryotic cells, placed in between cDNAs of individual TCR/CD3 subunits in yeast expression plasmids. P2A sequences, stands for picornaviral 2A, are utilized to construct polycistronic plasmids as the P2A-like sequences mediate a cotranslational cleavage event resulting in the release of each individual protein product.[94, 95] A proposed yeast expression plasmid containing individual TCR subunit cDNAs separated by p2a sequences is shown in fig.5.1. We predict that if CD3δ cDNAs fused to Cub are co-expressed with such a yeast TCR expression plasmid in yeast cells, Cub tagged CD3δ may assemble into a full TCR in the yeast ER and this complex may efficiently transport to the yeast plasma membrane. Repeating the membrane two hybrid screen with our Jurkat prey cDNA library expressed in these yeast cells may result in the identification of proteins that interact with CD3δ that is expressed in yeast cells in the context of the full TCR complex.

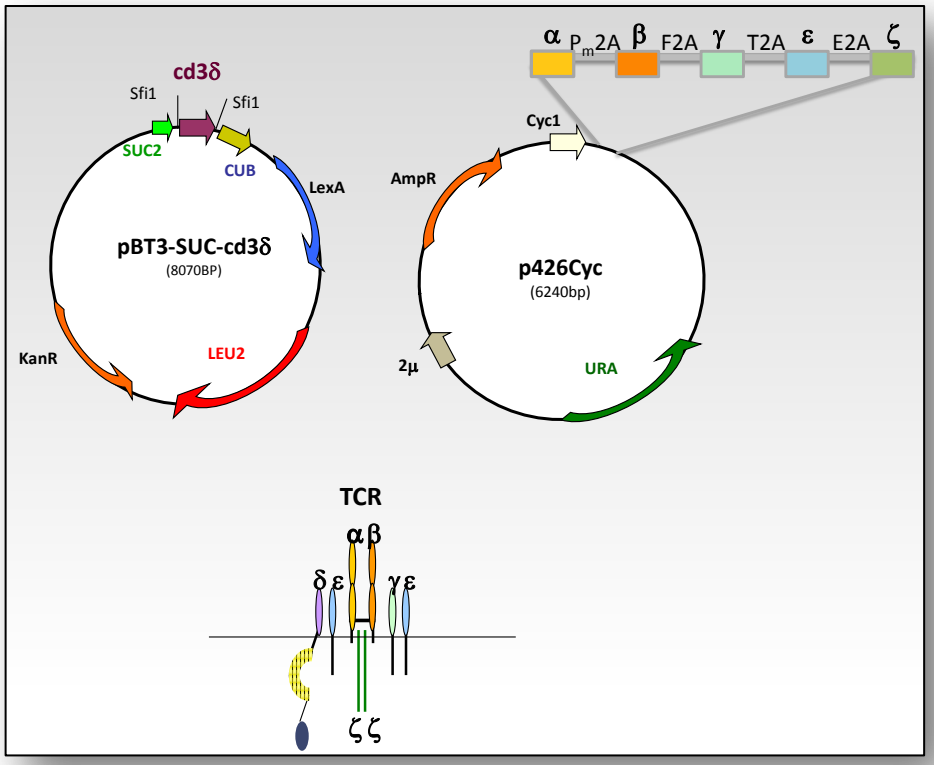


Figure 5.1: Strategy to fully assamble the TCR complex on the yeast cell plasma membrane. Yeast expression plasmid containing cDNAs for TCR subunits, separated by p2A like sequences is shown (on the right). When this plasmid is coexpressed with the bait plasmid encoding CD3δ (on the left) Cub tagged CD3δ may assemble into a full TCR in the yeast ER and this complex may efficiently transport to the yeast plasma membrane.

Two proteins CD81 and BCAP31, interacting with CD3δ in the yeast cells, were tested for their interaction to CD3δ in mammalian tissue culture cell lines. For this purpose the first step was analyzing the sequence of cDNA in the original library plasmid for the protein product that it encodes. The sequencing results of cDNA clones coming from forward direction sequencing and reverse direction sequencing were aligned to come up with the sequence of the cDNA used. In case of BCAP31, the sequencing result indicated that the protein product is 38 aminoacids longer than the one in the database. This 38 amino acid was at the very N terminal end of the protein and after the 39th residue the full length amino acid sequence was conserved. As the cDNA sequence that codes for this 38 amino acid sequence is not found in the genomic region of the BCAP31 we concluded that this part is from the cDNA library plasmid that was introduced in the process of preparing

the cDNA library. We wanted to see whether this extra portion will change the proposed 2D structure of BCAP31 by using web based Sosui transmembrane prediction program. Comparing the predictions for database sequence and our amino acid sequence, there seemed to be no difference in any of the three transmembrane regions, only that N terminal extracellular portion of the protein encoded was longer than the database protein.

In case of CD81, the protein sequence encoded by the cDNA that we have was different than the one found in database in the N terminal portion. When we blasted this different cDNA sequence on the human genome we found that this region corresponds to CD81 genomic region, that is proposed to be the first intron. As the difference in the sequence is only from the first exon, we thought this sequence might be a novel exon for CD81, that is found in an alternative splice variant found in Jurkat cell line. In order to test this hypothesis, we designed forward and reverse primers against this putative exon and previously identified first and second exons. Using this designed primers we tested for amplification from cDNA templates of different human tissue culture cell lines along with human blood cDNA, but could not amplify any of the cell line cDNAs tested. As the positive control for this PCR that includes cDNA library plasmid as the template gave the expected band, we concluded that there was nothing wrong with the designed primers. As the cDNAs used in this assay was confirmed for quality for different PCR assays, there was also nothing wrong with the cDNAs prepared. So, the conclusion could be that this exon we identified could be from an alternative splice variant from the Jurkat cell line that was used in the first place to prepare the cDNA library. Jurkat cells might have undergone tumor specific splicing and that might be the reason for us not to observe this variant in any of the cell lines tested. Nevertheless, future interaction assays of CD81chimeras that did not include this alternative exon of our cDNA confirmed that the interaction of CD81 with CD3δ we identified does not depend on the part encoded from this alternative portion of cDNA.

After analyzing the proteins encoded from the cDNAs, we cloned this cDNAs into mammalian expression plasmids in frame with an N terminal HA epitope tag, the same frame in the cDNA library plasmid was used to clone the cDNAs into the mammalian

expression plasmids. Coimmunoprecipitation experiments to see the interaction of identified proteins with CD3δ in mammalian tissue culture cells were carried out. All the immunoprecipitations were carried out in HEK293T human embryonic kidney cells, as these cells are widely used cells for expression analysis having a high rate of transfection efficiency and accompanied high level of expression of transfected constructs. For this immunoprecipitation experiments CD3δ coding cDNA, this time with its own membrane signaling peptide coding region is cloned into mammalian expression plasmid in frame with a C terminal myc epitope tag. Carrying out immunoprecipitation experiments in transfected HEK293T cell lines, we confirmed that both BCAP31 and CD81 interact with CD3δ in mammalian tissue culture cell lines. We do not know whether CD3δ transfected HEK293T cells express CD3δ in their surface or not, so the interactions we identified in mammalian tissue culture cells might either be on the plasma membrane or on the ER membrane. For BCAP31 it is not a problem as the BCAP31 itself is localized in the ER in mammalian cells. For CD81, even if the interaction that we identified herein takes place in the ER, it might still let us confirm the fact that the partnership between CD81 and its associated proteins is initiated early during biosynthesis in the ER/pre-Golgi compartments. This was previously shown for the partnership between CD81 and endo-H-sensitive pCD19 glycoform.[44] Our findings here, supports the proposition that tetraspanin web assembly is initiated in the ER, where the tetraspanin-partner building blocks are first formed, and then the web is further assembled with other tetraspanins and their partners during intracellular processing in the Golgi.[41]

For the further analysis of the identified interactions, first set of experiments designed were structural experiments to identify interacting domains of CD3δ, BCAP31 and CD81. We constructed cDNAs that code for chimeric CD3δ and CD3γ proteins. For this experiment we identified the extracellular, transmembrane and intracellular regions of both CD3δ and CD3γ and designed forward and reverse primers with overlapping sequences to amplify and fuse different portions of cDNAs forming chimeric protein products. The reason to use CD3γ as the chimeric partner of CD3δ was that they resemble each other both in domain and 2D structure. In this part of the project where we designed three different chimeras denoted as DDG, DGG and GDD, indicating which cDNA that

each domain , extracellular, transmembrane and intracellular, comes from. We cloned all three of these chimeras and full length CD3γ cDNA into the mammalian expression plasmids with fused C terminal Myc epitope tag and sequence verified the constructed plasmids. Although the sequencing results indicated no single point or frame shift mutation in the coding sequences in any of the plasmids, we could not express full length CD3γ and GDD chimera in HEK293T cell lines. We still wanted to go on with the other chimeric constructs that encode DDG and DGG and see the interaction of these chimeras with CD81 and BCAP31. The results showed that both the chimeras interacted with both CD81 and BCAP31 indicating either the interacting domain of CD3δ with this protein is in the cytoplasmic domain or more probably CD3γ that we could not express also interacts with the identified proteins.

In order to find the minimal interacting domain of BCAP31 with CD3δ, this time we constructed several N terminal deletion truncated versions of BCAP31. We designed different forward primers to each of the three transmembrane regions of BCAP31 and one reverse primer to amplify this deletion truncated BCAP31s, also one primer set to amplify the caspase cleaved form of the BCAP31 found in the literature was designed. PCR products amplified from the cDNA template were cloned into mammalian expression plasmid in frame with an N terminal HA epitope tag. It is worth to note that as the cDNA that we had for the BCAP31 was longer in 5 terminus than the database sequence, the first deletion construct denoted as BT1 is the same protein as database BCAP31. Having successfully expressed these truncated BCAP31s in HEK293T cells, we carried out immunoprecipitation experiments to find the minimal interacting domain of BCAP31 with CD3δ. In course of this immunoprecipitations we realized that BT1 that corresponds to database BCAP31 protein and BT5 that corresponds to caspase cleavage product of BCAP31 found in literature, bind non specifically to the Myc beads used for immunoprecipitation. Although the immunoprecipitation of CD3δ and this two protein products is clearly demonstrated, presence of this non specific binding might lead to the question whether the real interaction occurs or not. In order to resolve this misleading data, as a future study we could try the reverse reciprocal immunoprecipitation of the lysates with HA beads and blot the membranes with immunoprecipitated lysates with anti-myc

immunoblotting to see if the interaction is present independent of the proteins interacting with the myc beads. Nevertheless, the results indicated that BT3 with only the third transmembrane region interacts with CD3δ. Analyzing this third transmembrane region of BCAP31, there are two charged residues found. Previous studies on TCR assembly showed that charged residues found in the transmembrane regions of TCR subunits is what mediates the stepwise assembly of TCR complex.[4] CD3δ subunit of the TCR also has a charged residue in its transmembrane region and this made us propose that the charged residues in the transmembrane regions are important in interaction of BCAP31 and CD3δ.

This same truncated BCAP31 protein expressing constructs were used to transfect HeLa cells in the presence of ERYFP marker to test the subcellular localization of encoded proteins. Full length BCAP31, BT1 (corresponding to database sequence) and BT3 (minimal interacting deletion truncation) were expressed with an HA epitope tag in the mammalian cell lines from this constructs. We used antiHA rhodamine to stain and see the HA fused proteins and check colocalization of this proteins with the ERYFP markers on the merged pictures. Our cDNA encoded BCAP31 colocalizes with the ERYFP marker as the database protein sequence (BT1). The BT3 construct having only the third transmembrane region did not colocalize with the ER marker, and this is due to the fact that it lacks the membrane localization signal therefore is not targeted to ER. Further localization of the BCAP31 in the presence of CD3δ might also be tested as a future study to confirm that the interaction between CD3δ and BCAP31 happens in the ER.

The last set of structural study was the one involving CD81/CD9 chimeric constructs. CD9 is also a tetraspanin molecule that is a close partner of CD81 in the tetraspanin web. Their domains and 2D structures are very similar and this CD81/CD9 chimeric molecules were used in previous studies.[41] Chimeric constructs in retroviral plasmids were a gift from Prof. Shoshana Levy from Stanford University, and we used these plasmids as templates to amplify the cDNAs coding for different chimeras. Amplified cDNAs were cloned into mammalian expression plasmids in frame with N terminal HA epitope tag. Immunoprecipitation experiments with CD3δ revealed that all the chimeric constructs interact with CD3δ, and could not identify the interacting domain of

CD81. The reason could be that CD9 also interacts with CD3δ, that we could not test as we did not have CD9 coding cDNA. As a future study CD9 interaction with CD3δ can be tested that could lead into a better conclusion about these immunoprecipitations. However, the interaction of CD3δ with this chimeras made us confirm that the interaction between CD81 and CD3δ does not depend on the alternative N terminal portion of the encoded protein from our cDNA.

The last part of the project focuses on TCR signaling and any possible effect of CD81 on T cell stimulation. CD81 is a tetraspanin molecule that functions in the tetraspanin web which might allow immune cells to attain such specific and highly regulated responses to a constantly changing environment. Tetraspanins function as specific membrane docks that cluster their associated membrane proteins with intracellular membrane-proximal signaling proteins. CD81 has been demonstrated to act as a costimulatory molecule in human and mouse B and T cells[41], suggesting that coengagement of CD81 facilitates the reorganization of the membrane, thereby reducing the threshold of cell activation. This costimulatory function of CD81 has also been demonstrated by the engagement of a natural ligand, the hepatitis C virus envelope protein[96] to CD81 lacking the immunoreceptor tyrosine-phosphorylated activation motifs, thus the possible mechanism of signaling by the CD81 molecule remains to be defined. In the light of this information we wanted to find out whether CD81 surface expression level has any effect on TCR mediated stimulation of VL3.3M2 double positive T cell lines. We used shRNA mediated expression arrest to decrease the CD81 expression level in VL3.3M2 T cell lines. We started by designing the shRNA templates, amplified and cloned the shRNAs into LMP retroviral plasmid. LMP contains human mir30 based hairpin sequences that aids in activation of shRNAs in the target cells. We confirmed the identity of the constructed shRNA plasmids and efficiency of shRNAs to decrease both surface CD81 and CD81 protein expression levels.

The step of introducing retroviral plasmids into T cell tissue culture cell lines was the most difficult part of the project. We first optimized retroviral infection using Phoenix cells as a packaging system and infecting NIH3T3 cells. As a result of successfully

optimizing the amount of viral supernatant to be used to infect the cells and infection conditions, we could infect the NIH3T3 cell line with almost 95% efficiency, visualized by the expression of GFP from the LMP retroviral plasmid that we used. However, when we tried to infect the T cell lines, neither CD4 single positive 3B4.15 T lymphomas nor VL3.3M2 double positive T cell lines could be infected with the same viral supernatant. We tried to optimize the infection procedure further by using spin infection and changing amount of polybrene used, unfortunately we could not obtain any infection in any of the cell lines. We thought this might be due to low level of retroviral receptor expression on these cells so further studies in our lab to express the transgenic retroviral receptor gene in these cell lines is being carried out. As we could not infect the T cells of interest we decided to introduce the retroviral plasmids into these cell lines by electroporation. We optimized the electroporation conditions and visualized the efficiency of electroporation by the aid of GFP coding region of the LMP retroviral plasmid that we have. Among the two T cell lines, only the double positive VL3.3M2 T cell lines could be transfected with high efficiency by electroporation and therefore we used these cells to study the TCR stimulation.

In the effort of optimizing stimulation conditions so that we would be able to see the effect of not only TCRβ mediated stimulation but also the CD4-TCRβ mediated costimulation of cells, we tried different amounts of antibodies to stimulate the cells. When we lower the amount of antibodies used for stimulation, we could see the costimulation effect of CD4 in 3B4.15 T cell lines. Although the extent of costimulation was lower in VL3.3M2 cells, we could also optimize the antibody concentration for stimulation of these shRNA transfected cells. We selected for stable cell lines that express either empty LMP construct (that does not have any shRNA coding region , only the GFP) or LMPCD81shRNA constructs (that express shRNA against CD81). For selection of stable cell lines we devised two different strategies, first we used pool of cells that were electroporated with LMPCD81shRNA and second we did limiting dilutions to select for single clones that express LMPCD81shRNAs. The reason to include both set of cells in the stimulation experiments is that both sets have their own advantage and disadvantage. The disadvantage of using pool of cells is that the cumulative surface CD81 level is

downregulated only to 30% so the conclusion from this set of cells could not reveal the overall effect of CD81. On the other hand the single cell stable clones that showed higher reduction on the surface CD81 expression levels to an extend of 70% might mislead us if the insertion of retroviral plasmid for the ancestor single cell, abolished other coding regions than CD81. So, we used both of this sets of cells for the stimulation experiments.

The results of stimulation experiments showed that decreasing amount of surface CD81 expression level results in an increase in the expression of surface CD69, that is the earliest activation marker of T cells. Further analysis of single cell clones revealed that decreasing CD81 expression level on T cells increases CD4 TCR costimulation of the cells, again indicated by an increased expression of surface CD69 levels. In order to confirm that CD69 was not the only marker that showed increased expression following stimulation. Two more sets of experiments were done in which this time surface expression of CD5, another T cell activation marker, was assayed. The same pattern of increase in expression is observed in CD5. Overall these results made us conclude that CD81 absence on the cell surface leads to increased TCR signaling, therefore the CD81 interacting with CD3δ subunit of the TCR controls the TCR mediated signaling.

Previous data showed that CD81 interacts with CD4 coreceptor on the membrane of T cells, although the functional relevance of this interaction is not revealed ,it was shown that CD4 that is bound to Lck can no longer interact with CD81. Keeping these data in mind and looking at our results, the role of CD81 on T cell surface in TCR stimulation can be hypothesized as: in unstimulated resting T cells, Lck is bound to CD4 and CD81 is bound to TCR through CD3δ. Following stimulation, as TCR and CD4 translocate to lipid rafts in the immune synapse, CD81 dissociates from TCR, binding to CD4 that is now free from Lck as Lck is phosphorylating ITAMs of TCR signaling subunits and initializing the downstream TCR signaling. In cells that lack CD81 on their surface, TCR is not sequestered from CD4 bound Lck and stimulation of T cells is therefore higher.

Further studies to correlate the CD4 CD81 interaction with CD3δ CD81 interaction might be carried out to better understand the role of CD81 especially in CD4 mediated

costimulation of T cells. Also one better strategy to be employed when studying TCR signaling in more detail, would be to check the initial events like Lck phosphorylation or TCR signaling subunit phosphorylation rather that checking the final CD69 or CD5 activation marker expression on the cell surface.

6. CONCLUSION

The aim of this project was to understand the role of the CD3δ subunit of the TCR. The basic approach was to identify novel proteins that interact with the CD3δ subunit of the TCR complex and study the structural and functional importance of these interactions in T cell lines in tissue culture. The first part of the project was identification of the putative interactors using a novel modified yeast two hybrid system. The novel membrane based yeast two hybrid system that we used in this study relies on the idea of using split ubiquitin technology to assay for the interactions of membrane localized proteins. We cloned CD3δ coding sequence in the bait yeast expression plasmid of the Dual Membrane Systems and screened CD3δ against Jurkat Thymic cDNA library. The results of the screen gave us around 500 colonies and by using different strategies as direct colony PCR from yeast cells or DNA isolation from yeast cells; we sequenced DNA from most of these positive colonies. As the system uses three reporter genes and includes several positive and negative controls, we focused on putative proteins that might have a role in TCR mediated signaling, assembly of TCR in the ER or previously shown to have a role in B cell receptor mediated signaling. From the list of putative interactors, 16 of them were further confirmed by secondary yeast two hybrid screens.

Two proteins after this confirmation were selected for further analysis. The first protein selected was the transmembrane protein CD81. CD81 was previously shown to express on the cell surface of lymphocytes and has been suggested to have diverse roles in lymphocyte development. CD81 is believed to be essential for B cell development and regulating activation of B cells as it associates with CD19 and CD21 forming a signal transduction complex. Role of CD81 in T cells is not precisely known, except the presence of data that shows altered peripheral T cell proliferation in the absence of CD81 and more

importantly promoted powerful proliferative response of T cells following CD3 and CD81 crosslinking. Although the literature indicates that physical proximity of CD81 and CD3 on the same cell surface is required, there is no physical data that shows interaction of CD81 and CD3. Only physical interaction of CD81 reported in T cells is CD4-CD81 interaction and as this interaction is abolished when CD4 that is engaged with Lck could not associate with CD81.[51] All of these data suggested a role of CD81 in TCR mediated signaling and stimulation of T cells, and therefore performed structure-function analysis to better understand the significance of the identified interaction of CD81 and CD3δ.

In order to see if CD81 has a role in TCR mediated T cell stimulation, we used shRNA mediated expression arrest to decrease CD81 expression on T cell lines and study the effect on stimulation. We constructed shRNA coding retroviral plasmids. After testing the efficiency of shRNAs to decrease the expression of CD81 protein and surface CD81 expression, we used these constructed retroviral plasmids to electroporate VL3.3M2 double positive T cell lines. We produced two sets of stable cell lines that express shRNA against CD81.The first set of cells, which were pools of cells selected for LMPCD81 or LMP expression that showed around 30% decrease in surface CD81 levels. Stimulation results of this pool of cells showed an increased TCR mediated stimulation by decreasing amount of CD81 on the cell surface. These data suggest a role of CD81 on TCR stimulation as its absence upregulates the TCR mediated stimulation. The second set of cells was single cell cloned stable cell lines that express shRNA against CD81. Three different representative single cell clones that downregulate surface CD81 levels by 60% and 35% or no downregulation, were used for stimulation experiments. The results indicate not only an increase in TCR mediated stimulation of cell lines but also an increase in CD4-TCR costimulation of cells by decreasing amounts of surface CD81. This result confirmed our hypothesis that CD81 interacting with CD3δ and previously with CD4, has a functional role of inhibiting CD4 TCR costimulation of T cell lines.

The second protein we found that interacts with CD3δ was BCAP31. BCAP31 was initially identified as a B cell Associated Protein 31 (BAP31) and is a component of the ER quality control compartment. Its association with proteins in the ER mediates its function

as a cargo protein between ER and the cisGolgi, especially for the membrane associated proteins. It was previously shown to modulate the surface level of expression and surface stability of MHCI and IgD molecules that are both crucial fot the immune system.

We wanted to identify the localization of BCAP31 protein that we identified to interact with CD3δ and also the domain of BCAP31 that is responsible for the CD3δ interaction. For both of these purposes we constructed deletion truncated versions of BCAP31, lacking first or both first and second transmembrane regions. Successfully expressing these constructed truncations of BCAP31 in mammalian cell lines we carried out immunoprecipitation experiments to identify the minimal interacting domain of BCAP31. BT3 truncation that has only the third transmembrane domain, interacted with CD3δ and made us conclude that the third transmembrane region is the minimal interacting domain. Further analysis of this third transmembrane domain revealed the presence of two charged residues, that might play a role in a possible electrostatic interaction between BCAP31 and CD3δ as CD3δ also has a charged residue in its transmembrane domain. This charged residue of CD3δ was previously shown to play a role in electrostatic interactions between TCR subunits leading to a stepwise assembly of the TCR complex in the ER.[4] On the other hand, to identify the localization of BCAP31 protein that interacts with CD3δ, we transfected these constructs expressing truncated versions of BCAP31 along with fluorescent ER marker. Following immunostaining of expressed full length and truncated BCAP31s we identified that BCAP31 protein that we identified to interact with CD3δ colocalizes with the ER marker to ER of the transfected Hela cells, like the database BCAP31 protein. Among the constructed truncations,BT3 lacking the ER localization signal did not colocalize with the ER marker and showed expression in the cytoplasm or nucleus of the transfected HeLa cells.

As a result in this current study, we identified two proteins, CD81 and BCAP31 that interact with the CD3δ subunit of the T cell receptor complex. CD81 protein interaction showed a functional relevance as it alters the TCR signaling in T cell lines. BCAP31 and CD3δ interactions takes place in the ER and are mediated by the third transmembrane

domain of BCAP31 protein. This interaction of BCAP31 with the CD3δ subunit of TCR likely has a role in the assembly of the TCR complex in the ER.

APPENDIX

APPENDIX A: Chemicals Used In The Study

Chemicals & Media Components	Supplier Company
Acetic Acid	Merck, Germany
Acid Washed Glass Beads	Sigma, Germany
Acrylamide/Bis-acrylamide	Sigma, Germany
Agarose	peQLab, Germany
Anti-HA Affinity Matrix	Roche, Germany
Anti-HA Peroxidase	Roche, Germany
Anti c-Myc Antibody	Roche, Germany
Anti-c-Myc Agarose Affinity Gel Antibody	Sigma, Germany
Anti-Myc Peroxidase	Roche, Germany
Ammonium Persulfate	Sigma, Germany
Ammonium Sulfate	Sigma, Germany
Ampicillin Sodium Salt	CellGro, USA
Bacto Agar	BD, USA
Bacto Yeast Extract	BD, USA
Bacto Tryptone	BD, USA
Boric Acid	Molekula, UK
Bradford Reagent	Sigma, Germany
Bromophenol Blue	Sigma, Germany
CSM-Ade-His-Leu-Trp	Q-Biogene, USA
CSM-His-Leu-Trp-Ura	Q-Biogene, USA
DMSO	Sigma, Germany
D-Glucose	Sigma, Germany
D-Galactose	Sigma, Germany
DMEM with L-Glutamine	PAN, Germany
LB Agar	BD, USA
LB Broth	BD, USA

Chemicals & Media Components	Supplier Company
Distilled water	Milipore, France
DNA Gel Loading Solution, 5X	Quality Biological, Inc, USA
DPBS	CellGro, USA
EDTA	Applichem, Germany
Ethanol	Riedel-de Haen, Germany
Ethidium Bromide	Sigma, Germany
Fetal Bovine Serum (FBS)	Biological Industries, Israel
Glycerol Anhydrous	Applichem, Germany
Glycine	Applichem, Germany
HBSS	CellGro, USA
HEPES	Applichem, Germany
Hydrochloric Acid	Merck, Germany
Isopropanol	Riedel-de Haén, Germany
Kanamycin Sulfate	Gibco, USA
L-(+)-Arabinose	Merck, Germany
L-(-)-Fucose	Sigma, Germany
L-Glutamine	Hyclone, USA
Liquid nitrogen	Karbogaz, Turkey
Lithium Acetate Dihydrate	Sigma, Germany
Magnesium Chloride	Promega, USA
2-Mercaptoethanol	Sigma, Germany
Methanol	Riedel-de Haen, Germany
Monoclonal Anti-HA−Agarose Antibody	Sigma, Germany
PEG-4000	AppliChem, Germany
Penicillin-Streptomycin	Sigma, Germany
Phenol-Chloroform-Isoamylalcohol	Amersco, USA
PIPES	Sigma, Germany
Potassium Acetate	Merck, Germany
Potassium Chloride	Fluka, Germany
Potassium Hydroxide	Merck, Germany
Protease Tablets (EDTA-free)	Roche, Germany
ProtG Sepharose	Amersco, USA

Chemicals & Media Components	Supplier Company
RNase A	Roche, Germany
RPMI 1640 with L-Glutamine	PAN, Germany
SDS Protein Gel Solution, 2X	Quality Biological, Inc, USA
SDS Pure	Applichem, Germany
Skim Milk Powder	Fluka, Germany
Sodium Azide	Amresco, USA
Sodium Chloride	Applichem, Germany
TEMED	Applichem, Germany
Deoxyribonucleic acid Sodium Salt Type III from Salmon Testes	Sigma, Germany
Terrific Broth	BD, USA
Tris Buffer Grade	Amresco, USA
Tris Hydrochloride	Amresco, USA
Triton X100	Promega, USA
Tween20	Sigma, Germany
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific, USA
YNB with Ammonium Sulfate	Q-Biogene, USA
YNB without Ammonium Sulfate and without Aminoacids	Q-Biogene, USA
YPD Agar	Sigma, Germany
YPD Broth	BD, USA

APPENDIX B: Equipment Used In The Study

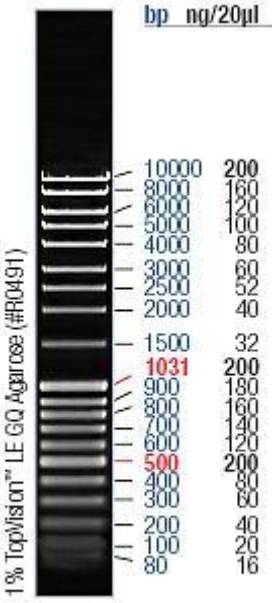
Equipment	Company
Autoclave	Hirayama,Hiclave HV-110,Japan
Balance	Sartorius, BP221S, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Cell Counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany
	Hitachi, Sorvall RC5C Plus, USA
CO_2 Incubator	Binder,Germany
Deepfreeze	-80^{0}C, Forma,Thermo ElectronCorp.,USA
	-20^{0}C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis	Biogen Inc., USA
Apparatus	Biorad Inc., USA
Elecroporation Cuvettes	Eppendorf, Germany
Electroporator	BTX-ECM630, Division of Genetronics, Inc, USA
Filter Membranes	Millipore,USA
Flow Cytometer	BDFACSCanto,USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater	Thermomixer Comfort,Eppendorf,Germany
Hematocytometer	Hausser Scientific,Blue Bell Pa.,USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany
	Memmert, Modell 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton,3000RS,USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France
	Eppendorf, Germany
Microscope	Olympus CK40,Japan
	Olympus CH20,Japan
	Olympus IX70,Japan

Equipment	Company
Microwave Oven	Bosch,Turkey
pH meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	Bosch,Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan
	Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Vortex	Velp Scientifica,Italy

APPENDIX C: DNA and Protein Molecular Weight Markers

MassRuler™ DNA Ladder Mix

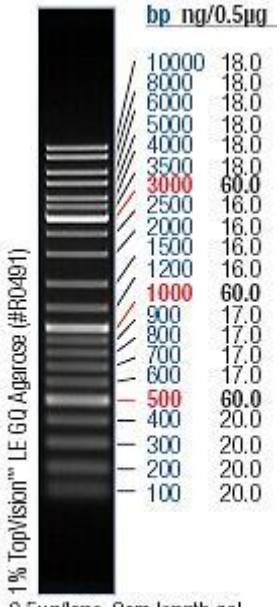
Fermentas, Germany



10µl/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

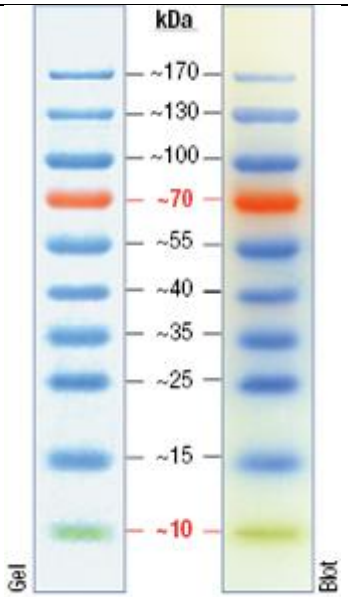
GeneRuler™ DNA Ladder Mix

Fermentas, Germany



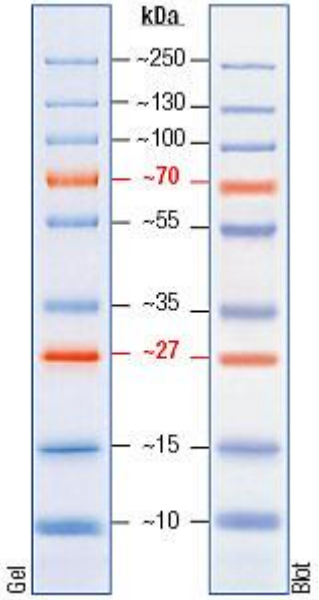
0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

PageRuler™ Prestained Protein Ladder Fermentas, Germany



4-20% Tris-glycine SDS-PAGE

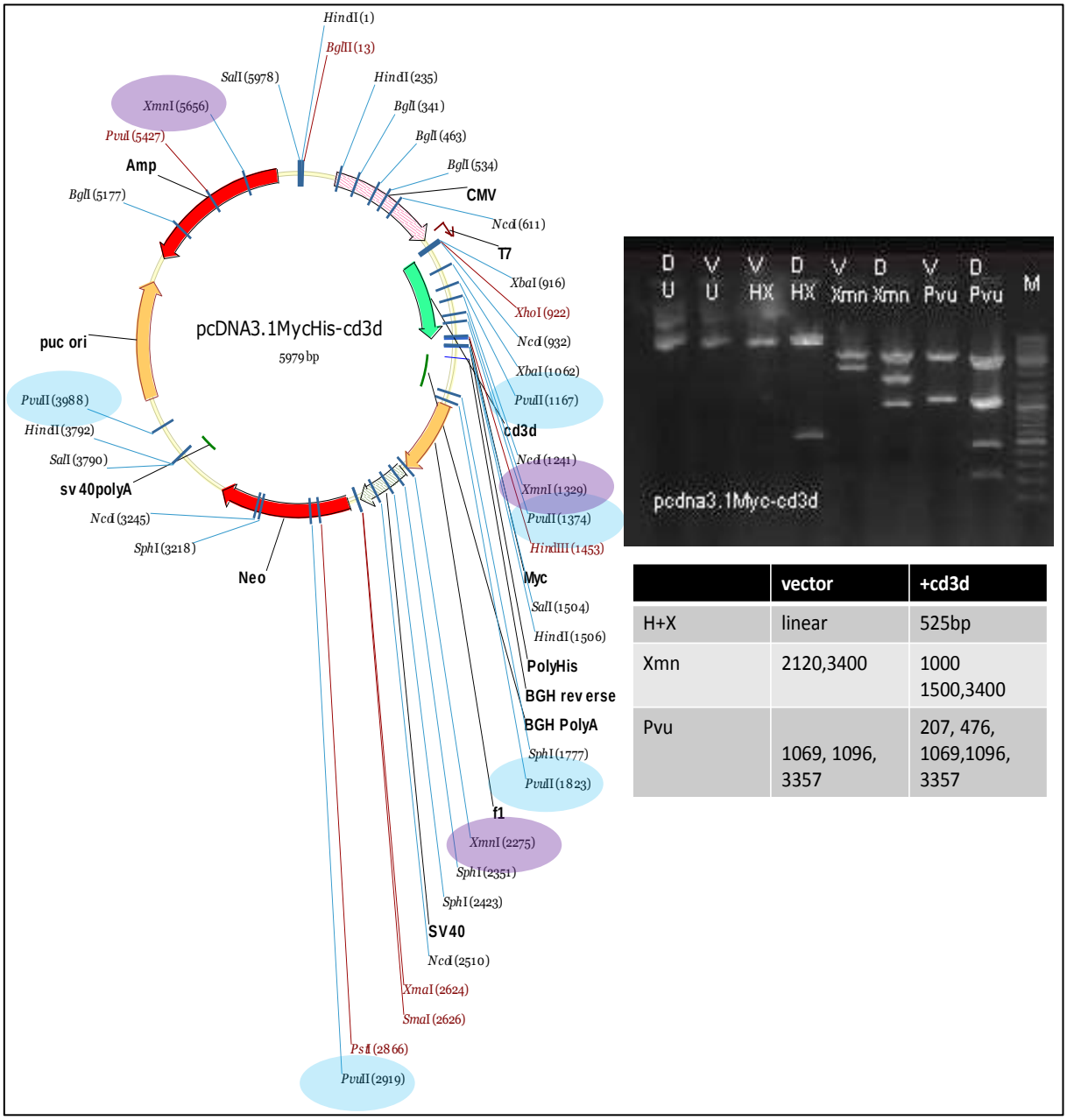
PageRuler™ Plus Prestained Protein Ladder Fermentas, Germany



8-16% Tris-glycine SDS-PAGE

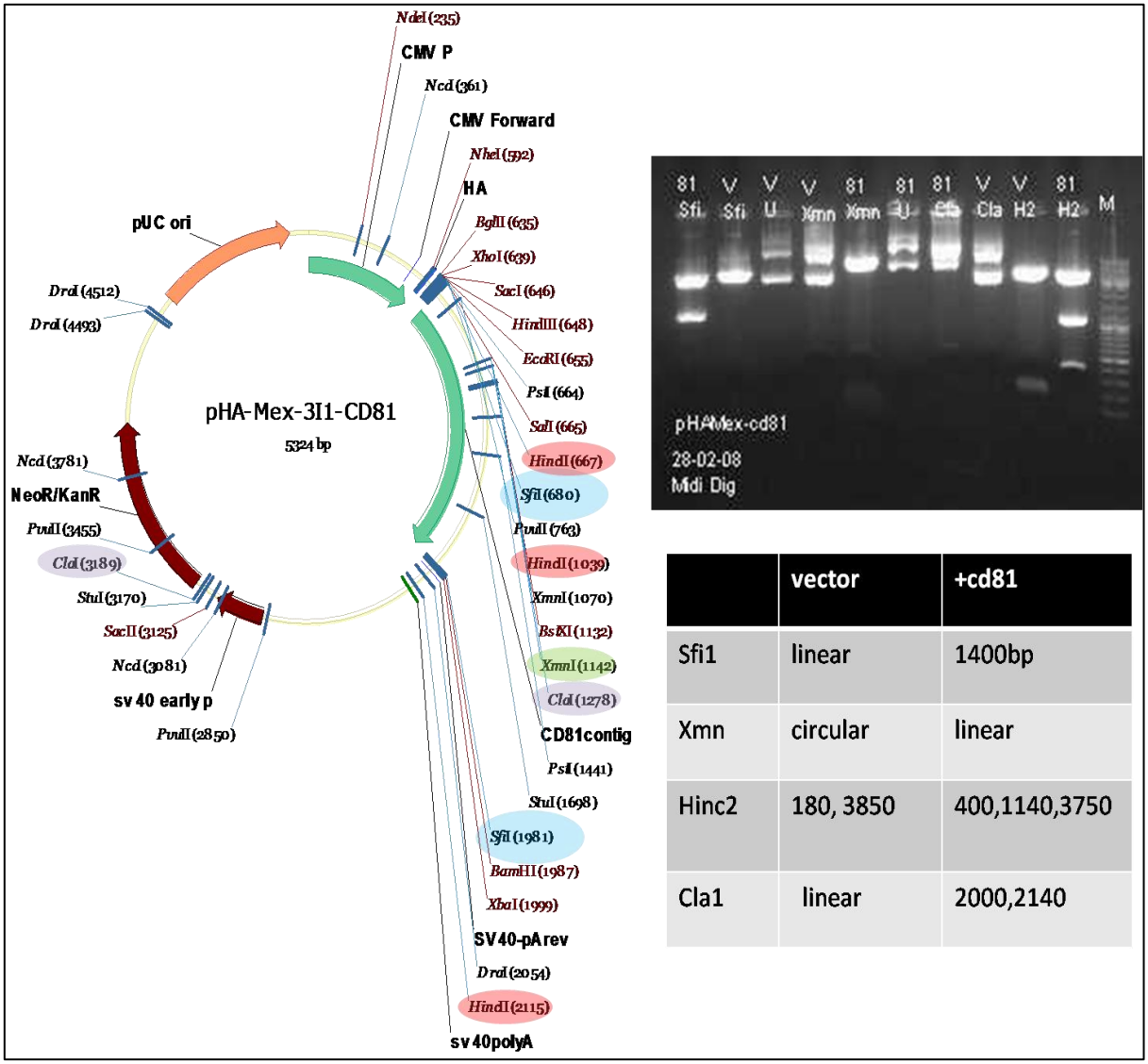
APPENDIX D: PLASMIDS CONSTRUCTED AND USED IN THE STUDY

pCDNA3.1Myc- CD3δ



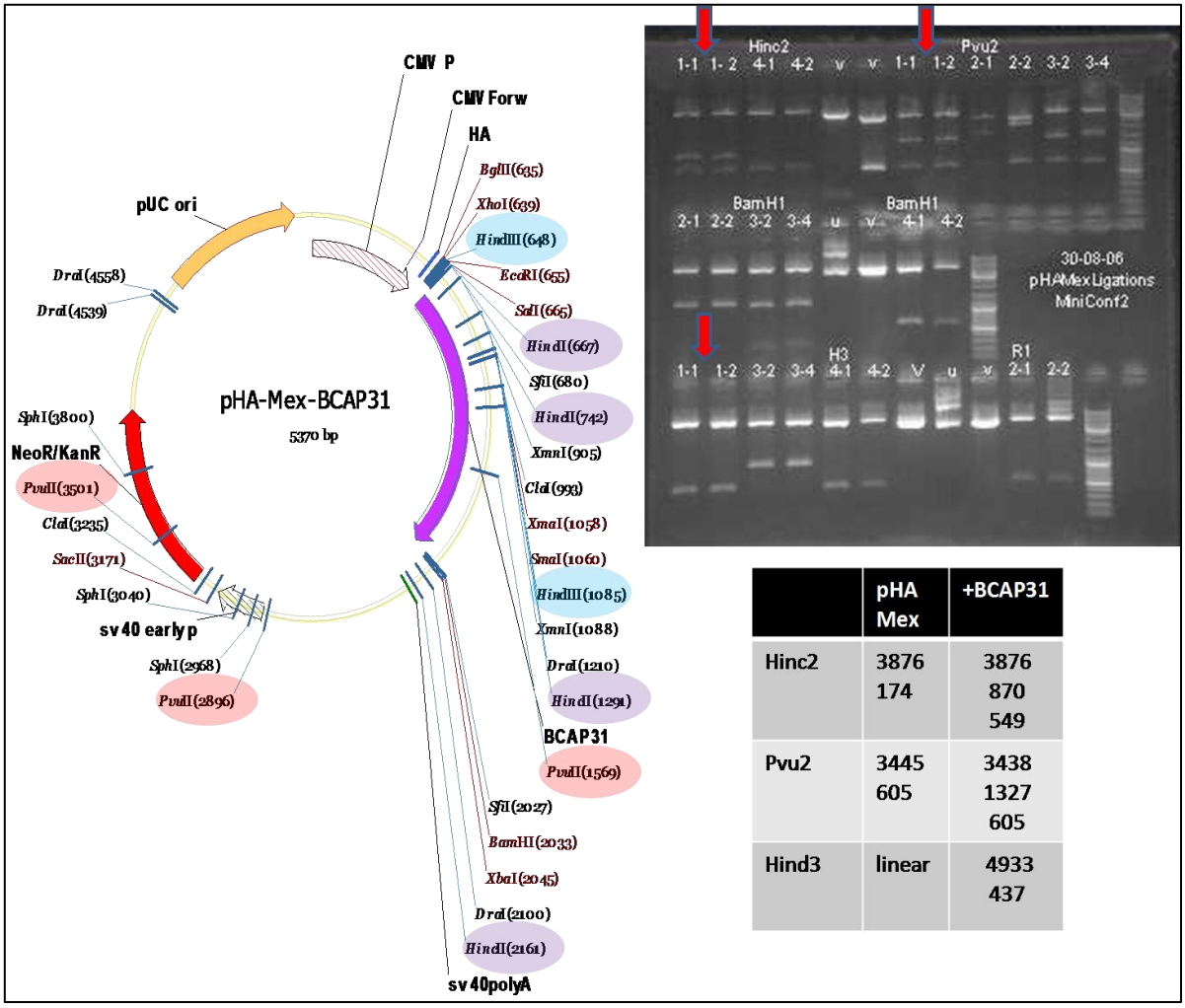
	vector	+cd3d
H+X	linear	525bp
Xmn	2120,3400	1000 1500,3400
Pvu	1069, 1096, 3357	207, 476, 1069,1096, 3357

pHAMEx-CD81



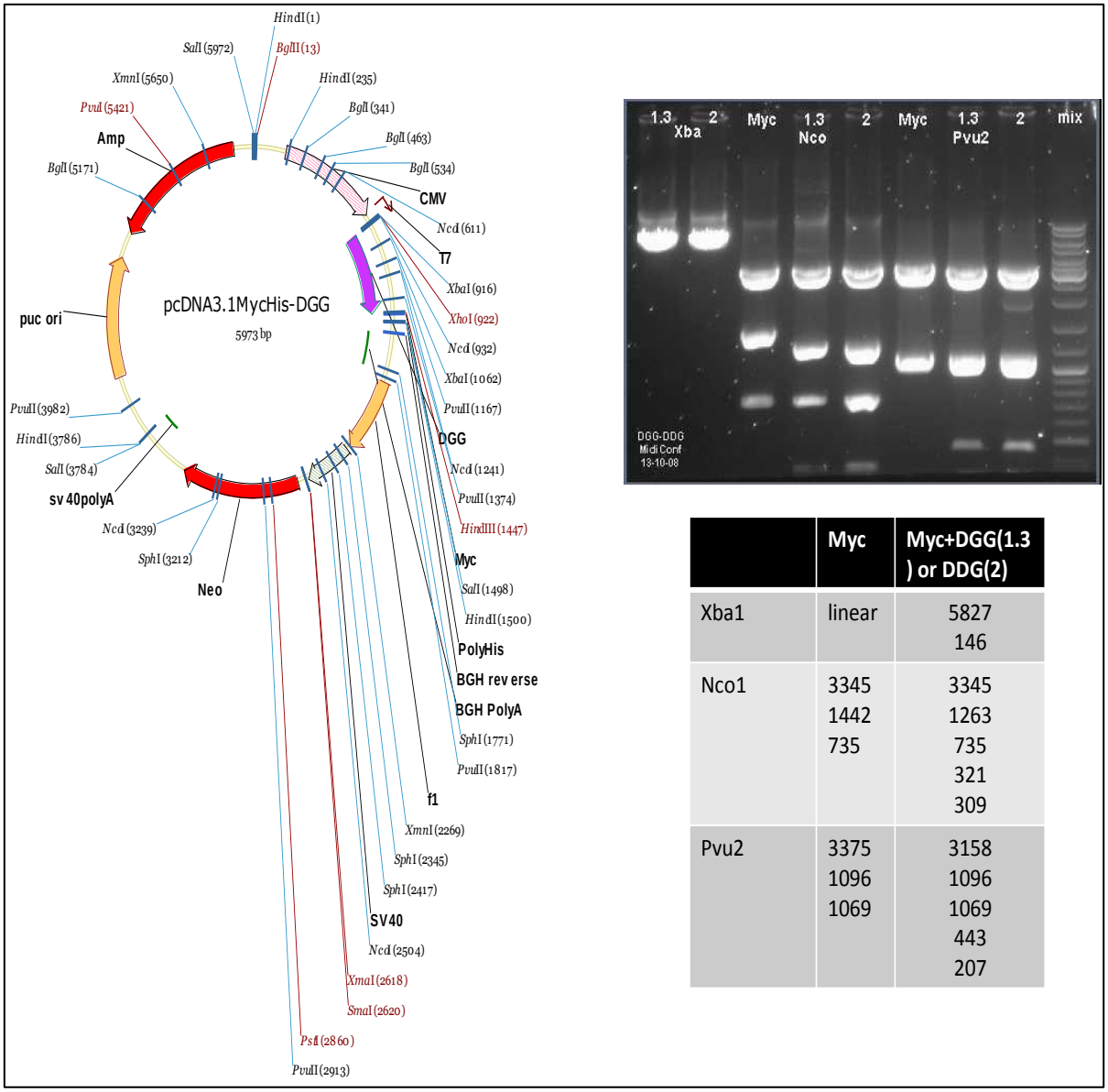
	vector	+cd81
Sfi1	linear	1400bp
Xmn	circular	linear
Hinc2	180, 3850	400,1140,3750
Cla1	linear	2000,2140

pHAMEX-BCAP31



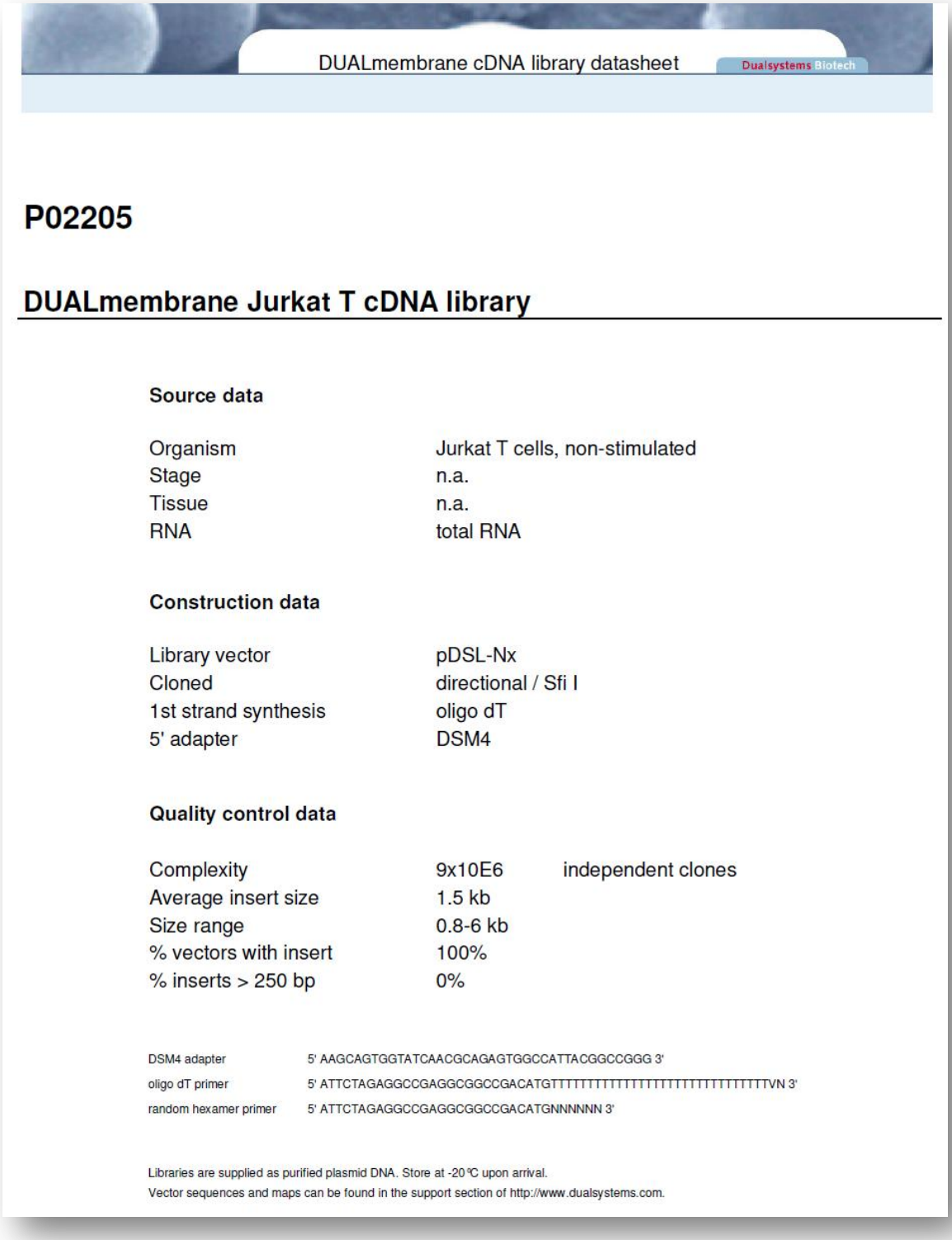
	pHA Mex	+BCAP31
Hinc2	3876 174	3876 870 549
Pvu2	3445 605	3438 1327 605
Hind3	linear	4933 437

pcDNAMyc-DGG and pcDNAMyc-DDG



	Myc	Myc+DGG(1.3) or DDG(2)
Xba1	linear	5827 146
Nco1	3345 1442 735	3345 1263 735 321 309
Pvu2	3375 1096 1069	3158 1096 1069 443 207

cDNA LIBRARY



DUALmembrane cDNA library datasheet

Dualsystems Biotech

P02205

DUALmembrane Jurkat T cDNA library

Source data

Organism	Jurkat T cells, non-stimulated
Stage	n.a.
Tissue	n.a.
RNA	total RNA

Construction data

Library vector	pDSL-Nx
Cloned	directional / Sfi I
1st strand synthesis	oligo dT
5' adapter	DSM4

Quality control data

Complexity	9x10E6	independent clones
Average insert size	1.5 kb	
Size range	0.8-6 kb	
% vectors with insert	100%	
% inserts > 250 bp	0%	

DSM4 adapter	5' AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG 3'
oligo dT primer	5' ATTCTAGAGGCCGAGGCGGCCGACATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN 3'
random hexamer primer	5' ATTCTAGAGGCCGAGGCGGCCGACATGNNNNNN 3'

Libraries are supplied as purified plasmid DNA. Store at -20°C upon arrival.
Vector sequences and maps can be found in the support section of http://www.dualsystems.com.

APPENDIX E: PROTEIN SEQUENCES

- CD3δ Encoded from pcDNA3.1MycCD3δ

MEHSGILASLILIAVLPQGSPFKVQVTEYEDKVFVTCNTSVMHLDGTVEGWFAKNKTLNLGKGVLDPR
GIYLCNGTEQLAKVVSSVQVHYRMCQNCVELDSGTMAGVIFIDLIATLLLALGVYCFAGHETGRPSGA
AEVQALLKNEQLYQPLRDREDTQYSRLGGNWPRNKKSKLGPEQKLISEEDLNSAVDHHHHHH

- CD81 Contig Sequence Encoded from pHACD81:

MYPYDVPDYAPRSRAQASNSAVDPAITAGGISEGASGGWSILGGGSRWQRGFPQLAGGVILGVAL
WLRHDPQTTNLLYLELGDKPAPNTFYVGIYILIAVGAVMMFVGFLGCYGAIQESQCLLGTFFTCLVILFA
CEVAAGIWGFVNKDQIAKDVKQFYDQALQQAVVDDDANNAKAVVKTFHETLDCCGSSTLTALTTSV
LKNNLCPSGSNIISNLFKEDCHQKIDDLFSGKLYLIGIAAIVVAVIMIFEMILSMVLCCGIRNSSVY

- BCAP31 Contig Sequence Encoded from pHABCAP31:

MYPYDVPDYAPRSRAQASNSAVDPAITAGGGRGCCGRVRLLRRGLHVDCGKLGNKLTSSCGKPSSNR
MSLQWTAVATFLYAEVFVVLLLCIPFISPKRWQKIFKSRLVELLVSYGNTFFVVLIVILVLLVIDAVREIRK
YDDVTEKVNLQNNPGAMEHFHMKLFRAQRNLYIAGFSLLLSFLLRRLVTLISQQATLLASNEAFKKQA
ESASEAAKKYMEENDQLKKGAAVDGGKLDVGNAEVKLEEENRSLKADLQKLKDELASTKQKLEKAEN
QVLAMRKQSEGLTKEYDRLLEEHAKLQAAVDGPMDKKEE

- BT1 Encoded from pHABT1:

MYPYDVPDYAPRSRAQASNSAVDPAITASLQWTAVATFLYAEVFVVLLLCIPFISPKRWQKIFKSRLVE
LLVSYGNTFFVVLIVILVLLVIDAVREIRKYDDVTEKVNLQNNPGAMEHFHMKLFRAQRNLYIAGFSLLL
SFLLRRLVTLISQQATLLASNEAFKKQAESASEAAKKYMEENDQLKKGAAVDGGKLDVGNAEVKLEEE
NRSLKADLQKLKDELASTKQKLEKAENQVLAMRKQSEGLTKEYDRLLEEHAKLQAAVDGPMDKKEE

- BT2 Encoded from pHABT2:

MYPYDVPDYAPRSRAQASNSAVDPAITAELLVSYGNTFFVVLIVILVLLVIDAVREIRKYDDVTEKVNLQ
NNPGAMEHFHMKLFRAQRNLYIAGFSLLLSFLLRRLVTLISQQATLLASNEAFKKQAESASEAAKKYME
ENDQLKKGAAVDGGKLDVGNAEVKLEEENRSLKADLQKLKDELASTKQKLEKAENQVLAMRKQSEG
LTKEYDRLLEEHAKLQAAVDGPMDKKEE

- BT3 Encoded from pHABT3:

MYPYDVPDYAPRSRAQASNSAVDPAITAIAGFSLLLSFLLRRLVTLISQQATLLASNEAFKKQAESASEA
AKKYMEENDQLKKGAAVDGGKLDVGNAEVKLEEENRSLKADLQKLKDELASTKQKLEKAENQVLAM
RKQSEGLTKEYDRLLEEHAKLQAAVDGPMDKKEE

- BT5 Encoded from pHABT5:

MYPYDVPDYAPRSRAQASNSAVDPAITAMSLQWTAVATFLYAEVFVVLLLCIPFISPKRWQKIFKSRLV
ELLVSYGNTFFVVLIVILVLLVIDAVREIRKYDDVTEKVNLQNNPGAMEHFHMKLFRAQRNLYIAGFSLL
LSFLLRRLVTLISQQATLLASNEAFKKQAESASEAAKKYMEENDQLKKGAAVD

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