IDENTIFICATION OF THE INTERACTING PROTEIN PARTNERS OF THE ThPOK TRANSCRIPTION FACTOR

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ABSTRACT

Key words: T cell, T cell Development, ThPOK, Yeast Two Hybrid Screening, Immunoprecipitation.

T lymphocytes are an essential part of the adaptive immune system. In the course of T lymphocyte development, one crucial step is the decision to commit to one of two lineages: CD4 and CD8 single positive (SP) T cells. The cellular and molecular mechanisms underlying T cell lineage commitment has long been the subject of intense debate.

ThPOK, T-helper-inducing POZ/Krüppel like factor, is a zinc finger transcription factor containing both a Krüppel-like zinc finger domain and a BTP/POZ domain, which has been linked to homodimerization and recruitment of other proteins. During the development of thymocytes, the ThPOK protein mediates the differentiation of MHC-II restricted thymocytes into the CD4 SP lineage, using its N-terminal BTB/POZ domain. We screened a human thymic cDNA library against the BTB/POZ domain of the ThPOK protein to identify its interacting protein partners by using a conventional yeast two hybrid system. We identified putative interacting proteins by performing DNA sequencing and bioinformatics analysis on relevant prey protein encoding plasmids. We re-confirmed these interactions in yeast cells by secondary yeast two hybrid screens. We confirmed the biological relevance of the identified interactions by performing co-immunoprecipitation experiments in transfected mammalian tissue culture cells. We demonstrated that seven proteins, named POMP, MEF2B, TCF7, ZNF384, DPP7, HINT2 and PARP12 can interact with the BTB/POZ domain of the ThPOK protein.

TRANSKRİPSİYON FAKTÖRÜ ThPOK ILE ETKİLEŞİMİ OLAN PROTEİNLERİN BELİRLENMESİ

ÖZET

Anahtar kelimeler: T hücresi, T hücre gelişimi, ThPOK, Maya ikili hibrit taraması, Immün çökeltme.

T hücreleri kazanılmış bağışıklık sisteminin önemli bir parçasıdır. T lenfositlerin gelişiminde önemli aşamalardan biri T hücrelerinin CD4 veya CD8 tekli pozitif hücreler olarak kökenleşmesidir. Bu kökenleşmenin altında yatan hücresel ve moleküler mekanizmaların belirlenmesi uzun süredir önemli bir araştırma konusu olmuştur.

ThPOK (Yardımcı T hücrelerini uyaran POZ/Krüppel-benzeri faktör), homodimerizasyon ve diğer proteinler ile olan etkileşimde rol oynayan BTP/POZ ve Krüppel-benzeri zinc finger bölgelerine sahiptir. Timositlerin gelişimi sırasında ThPOK proteini N-terminal BTB/POZ bölgesi aracılığıyla, MHC-II kısıtlı timositlerin CD4 tekli pozitif hücrelere dönüşmesinde aracı olmaktadır. Bu çalışmada geleneksel "maya ikili hibrit" sistemini kullanarak, insan timus cDNA kütüphanesini ThPOK proteininin BTB/POZ bölgesine karşı taradık ve bu protein ile etkileşim içindeki proteinleri belirlemeyi amaçladık. DNA sekanslanması ve BLAST analizi sonucunda, etkileşim gösteren proteinleri belirledik. Belirlenen etkileşimleri ikincil "maya ikili hibrit" taraması aracılığıyla yeniden doğruladık. Belirlenen proteinlerin aktarıldığı memeli hücrelerinde immün çökeltme deneyleri yaparak, varolan etkileşimleri biyolojik olarak ilişkilendirdik. Çalışma sonucunda bu proteinlerden POMP, MEF2B, TCF7, ZNF384, DPP7, HINT2 ve PARP12 olmak uzere yedi tanesinin, ThPOK proteinin BTB/POZ bölgesiyle etkileşime girdiğini gösterdik.

To my family

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
δ	Delta
γ	Gamma
AD	Activation Domain
ADE	Adenine
APC	Antigen Presenting Cell
Amp	Ampicillin
bp	Basepair
BAF	Brg- or hBrm-Associated Factor
BAZF	Bcl6-Associated Zinc Finger Protein
Bcl6	B Cell Lymphoma 6 Protein
BD	Binding Domain
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BLASTP	Protein BLAST
BTB	Broad Complex/Tramtrack/Bric-a-Brac
BTF3	Human Basic Transcription Factor 3
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

DPP7	Dipeptidyl peptidase 7 Protein
EDTA	Ethylenediaminetetraaceticacid
Erk	Extracellular Signal Related Kinases
E8	CD8 Enhancer Element
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GATA-3	Transcription Factor GATA Binding Protein-3
GFP	Green Fluorescent Protein
HCFC1	Human Host Cell Factor 1 Protein
HDAC	Histone Deacetylases
HEK	Human Embriyonic Kidney
HINT2	Histidine Triad Nucleotide Binding Protein 2
HIS	Histidine
HMG	High Mobility Group
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-7R	Interleukin-7 Receptor
IP	Immunoprecipitation
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
Kan	Kanamycin
LB	Luria Broth
Lck	Lymphocyte-Specific Protein-Tyrosine Kinase
Lef	Lymphoid Enhancer Factor
LEU	Leucine
MAZR	Myc-Associated Zinc-Finger Protein Related Factor
MCS	Multiple Cloning Site
MEF2B	Myocyte Enhancer Factor 2B
MHC	Major Histocompatibility Complex
NCBI	National Center for Biotechnology Information
Neo	Neomycin
NK	Natural Killer Cells
OD	Optimal Density

PARP-12	Poly(ADP-ribose) polymerase family member 12 Protein
PEG	Polyethylenglycol
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
POMP	Proteasome Maturation Protein
POZ	Poxvirus and Zinc-Finger
rpm	Revolution per minute
RNA	Ribonucleic Acid
Runx	Runt-Related Gene Family
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
ThPOK	T Helper Inducing POZ-Kruppel Factor
Th	T Helper Cell
TOX	Thymus High Mobility Group Box Protein
TRP	Tryptophan
URA	Uracil
USCS	University of California at Santa Cruz
YPD	Yeast Extract-Peptone-Dextrose
ZF	Zinc-Finger
Zfp	Zinc-Finger Protein

1 INTRODUCTION

1.1 T CELL DEVELOPMENT

Bone marrow derived hematopoietic stem cells can give rise to all blood cells including lymphocytes. B and natural killer lymphocyte lineages originate and develop within the bone marrow. On the other hand, lymphocyte precursors formed in the bone marrow migrate to the thymus which is the unique microenvironment for T cell development.[1-3] In the thymus, early progenitor cells pass through a series of developmental stages. Specialized thymic regions direct T cell maturation at each major stage. Thymocyte populations at distinct stages of maturation differ in the expression of cell-surface molecules.[4] The proteins commonly used to track different stages of T lymphocyte development are CD3-T cell receptor (TCR) complex and the co-receptors CD4 or CD8 are the three characteristic cell-surface markers of mature T lymphocytes.

Thymocyte maturation can be subdivided into three stages. In the first developmental stage, progenitor cells lack all the mature T cell markers and they are called Double Negative (DN) thymocytes. These DN cells begin to express the adhesion molecule CD44 and then the α-chain of CD25. In the DN stage, expression of the surrogate preTCRalpha molecule along with the CD3 molecules are followed by the productive rearrangement of β-chain.[5, 6] The surrogate preTCRalpha along with the newly arranged TCR-β assembles into a multi-subunit receptor complex named the preTCR, which triggers the proliferation of thymocytes and the expression of the CD4 and CD8 co-receptors. Thymocytes in this stage of development are called Double Positive (DP), $CD4^+$ $CD8^+$, and the rearrangement of TCR-α-chains initiates at this stage.

Due to the random genetic rearrangements, there is a possibility that many rearranged TCRs do not recognize the antigen presenting self- MHC (Major Histocompatibility Complex) molecules or are potentially auto reactive. T cells carrying these rearrangements would be useless or even deleterious. Thus, developing thymocytes are both positively and negatively selected according to the specificity and affinity of the TCR that they express on their surface. DP thymocytes expressing low-levels of TCRαβ recognize cortical epithelial cells expressing MHC plus self peptides with adequate affinity and they are positively selected by receiving a survival signal.

Negative selection of the DP cells occurs when positively selected DP cells are unable to bind bone marrow derived APC (Antigen Presenting Cells) expressing MHC plus self peptides at least with a low affinity and die through programmed cell death.[7, 8] Alternatively, those DP thymocytes that receive very strong self peptide-MHC signals also undergo negative selection. The DP cells that survive positive and negative selection continue on to the third stage of T cell development. Either the CD4 or CD8 co-receptor is downregulated and lineage committed $CD4^+$ and $CD8^+$ SP (Single Positive) T cells are generated, whereby there is a high level of TCR expression on their cell surface at this stage. Finally, these mature thymocytes undergo a limited number of cellular divisions in the thymic medulla and migrate to peripheral lymphoid organs.[9, 10] (Figure 1.1)

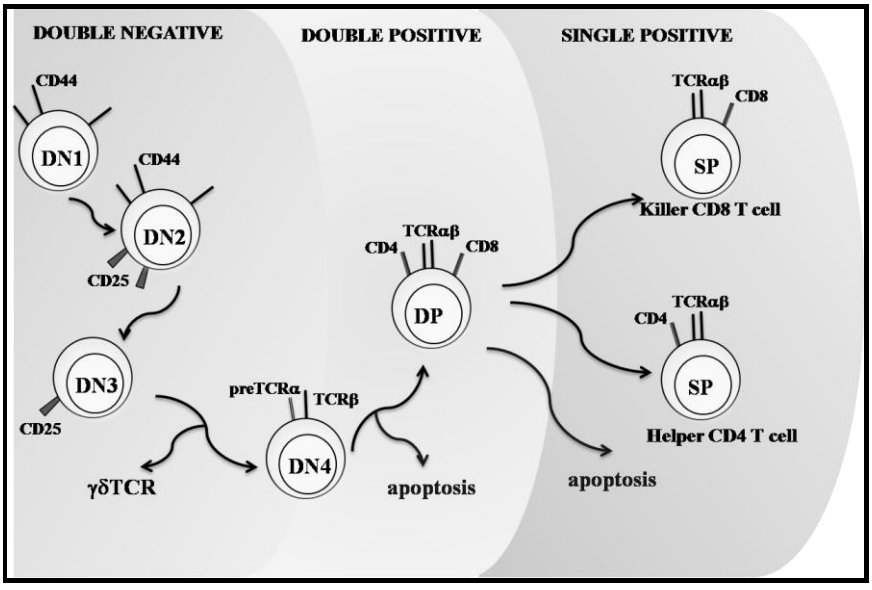


Figure 1.1: The developmental stages of T lymphocytes.
DN: Double Negative, DP: Double Positive, SP: Single Positive

There is also a small population of T lymphocytes that express the γδ-TCR on their surface instead of αβ-TCR. When T cell precursors enter the thymus, they can differentiate into this alternative lineage of T cells expressing γδ-TCR which undergo productive rearrangement of both the γ and δ TCR gene segments. The details of the positive and negative selection processes controlling the development of this minor population of T cells are still unrevealed. [6, 11, 12]

1.2 THYMOCYTE LINEAGE COMMITMENT

Maturation of CD4 SP and CD8 SP T cells eventually requires matching of the MHC specificity of the TCR with that of the co-receptor. Thymocytes that express MHC-II specific TCR differentiate into CD4 SP T cells, whereas thymocytes that express MHC-I specific TCR differentiate into CD8 SP T cells. Many models have been proposed to elucidate CD4 versus CD8 lineage commitment. Several transcription factors controlling this choice have been identified.

The classical view of CD4 versus CD8 lineage choice is that the transcriptional shutdown of one or the other co-receptor gene upon TCR signaling of DP T cells. According to this model, this irreversible termination of CD4 or CD8 gene expression occurs selectively as a result of the same TCR signals during positive selection. It has long been a matter of debate how a signal generated by the same TCR can result in the generation of two different outcomes (CD4 or CD8 downregulation).

1.2.1 THE INSTRUCTIVE MODEL

According to the instructive model of lineage commitment, interaction of TCR on the surface of DP thymocytes with either MHC-I or MHC-II instructs the cell to differentiate into the CD4 or CD8 lineage. Binding of TCR on DP thymocytes to MHC-I results in downregulation of CD4 to produce CD8 SP thymocytes, whereas binding of

TCR to MHC-II results in the downregulation of CD8 to produce CD4 SP thymocytes.[13] (Figure 1.2)

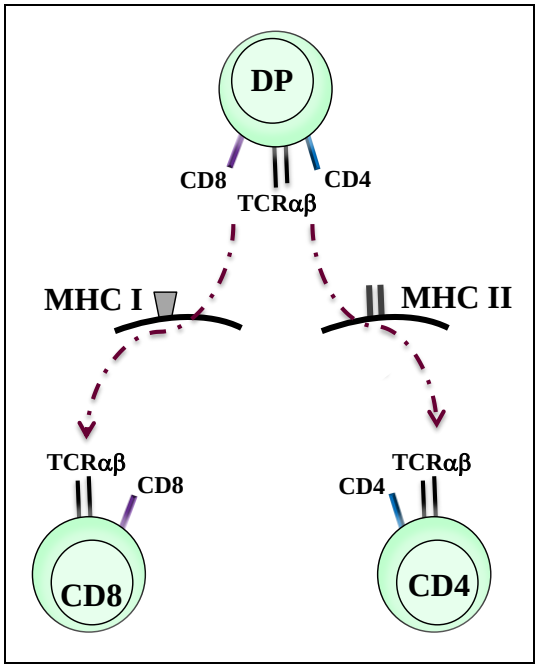


Figure 1.2: The instructive model of thymocyte lineage decision.

1.2.2 THE STOCHASTIC / SELECTIVE MODEL

The stochastic/selective model proposes that DP thymocytes randomly downregulate their CD4 and CD8 co-receptor gene expression independent of their TCR specificity during positive selection. The problem with such a model is that the MHC-I or MHC-II specificity of the TCR is generated by random somatic rearrangements of the TCRα and TCRβ gene loci, and some DP thymocytes will have MHC-I specific TCR's but would have randomly downregulated CD8 expression, and vice versa. Therefore, this model also proposes a rescue step in which only thymocytes which have matched TCR-MHC specificity and co-receptor expression are rescued. (Figure 1.3)

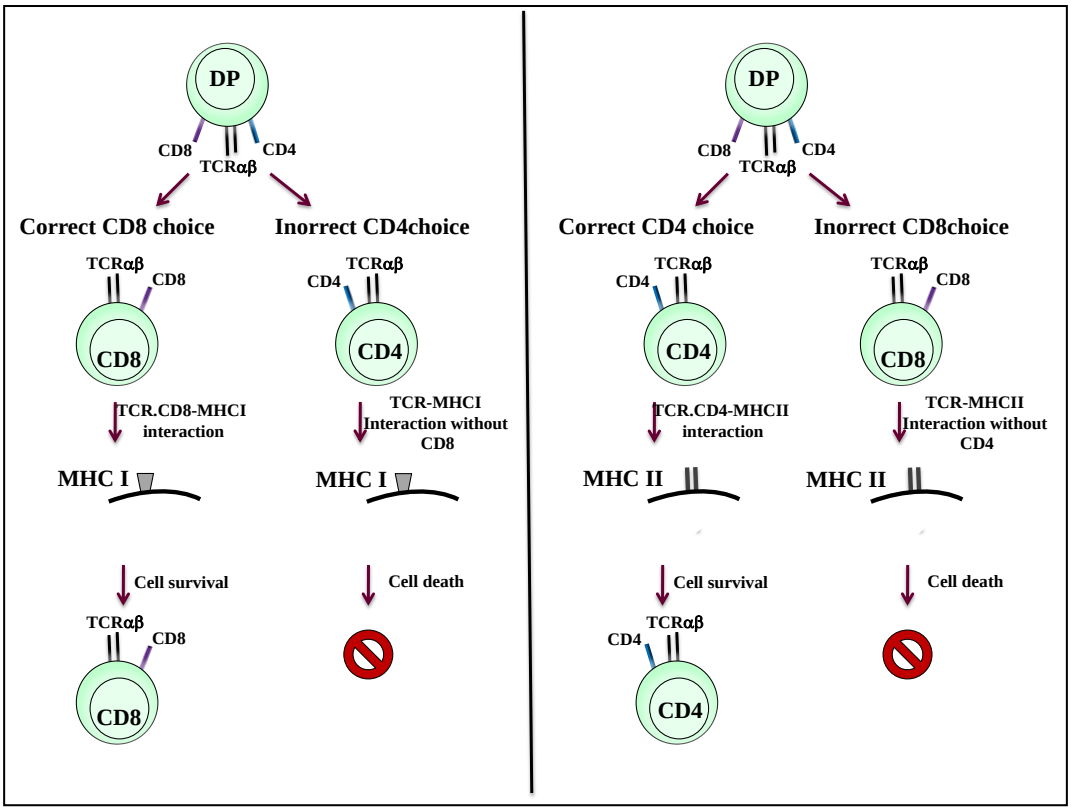


Figure 1.3: The stochastic/selective model of thymocyte lineage decision.

The discovery of transitional cells found in MHC-I or MHC-II deficient mice supported the stochastic/selective model as these cells express wrong co-receptors.[14] It was later found that DP thymocytes become CD4 or CD8 SP thymocytes by following a transitional route. (Figure 1.4) Transitional thymocytes are post-double positive cells that have received TCR signals and have downregulated the expression of either or both of the co-receptor gene loci, resulting in the downregulation of the surface expression of CD4 and/or CD8 protein.

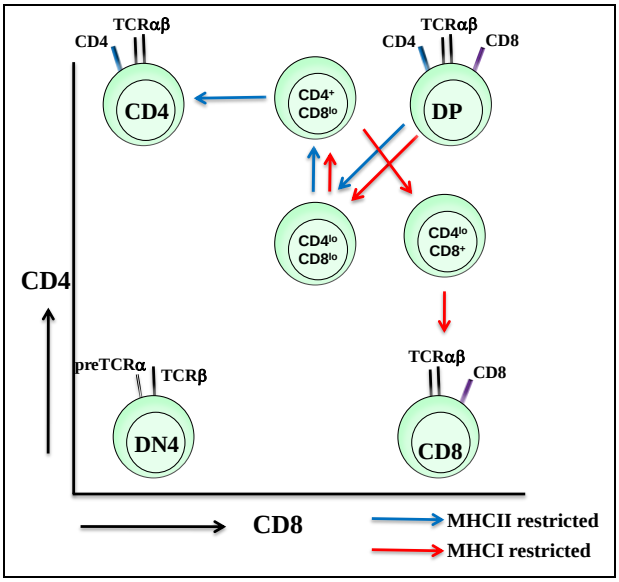


Figure 1.4: Thymocyte development and lineage decision according to the stochastic/selective model.

Both MHC-I and MHC-II restricted DP thymocytes become $CD4^{low}CD8^{low}$ cells, and then yield $CD4^{high}CD8^{low}$ cells. At this transitional stage where CD4 expression is more than CD8 expression, CD4 lineage committed $CD4^{high}CD8^{low}$ cells loose expression of CD8 and become CD4 SP thymocytes, whereas $CD4^{high}CD8^{low}$ cells that are committed to CD8 lineage first increase expression of CD8, followed by a loss in CD4 expression and become CD8 SP thymocytes.[14-16] However, these transitional thymocytes with mismatching TCR and co-receptors do not die and need TCR mediated rescue signals contradicting the assumptions of the stochastic/selective model.[17, 18]

1.2.3 THE STRENGTH OF SIGNAL MODEL

In the instructive model, DP thymocytes were thought to be instructed by qualitatively distinct signals transduced from different MHC specific TCR/co-receptor engagements to downregulate mismatched co-receptors. However, CD8 co-engagement was not necessarily required for CD8 lineage commitment, because MHC-II restricted DP thymocytes could give rise to CD8 SP thymocytes.[19] This model proposes that CD4 and CD8 lineage commitment results from quantitatively different signals of co-

engaged TCR and co-receptor molecules, whereas weak signals favor CD8 lineage and stronger signals favor CD4 lineage choice. (Figure 1.4)

A matter of fact, Lck, Lymphocyte-specific protein-tyrosine kinase, associates with CD4 more strongly than with CD8, a consequence of which is that the Lck-dependent signaling pathways is more highly activated in MHC-II restricted thymocytes than in MHC-I restricted ones.[20, 21] Thus, increasing Lck activity in DP thymocytes results in promoting CD4 lineage differentiation, whereas reducing Lck activity results in promoting CD8 lineage differentiation.[22-24] Also, increasing activity of other intracellular kinases (Tec-family kinases and extracellular signal related-kinases (Erk)) in DP thymocytes favored CD4 lineage choice, whereas decreasing kinase activity favored CD8 lineage choice. (Figure 1.5)

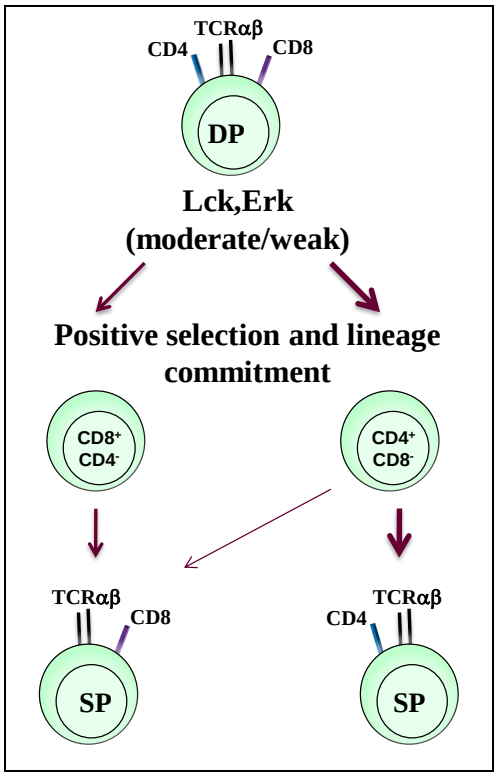


Figure 1.5: The strength of signal model of thymocyte lineage decision.
The intensity of signal is represented by the thickness of arrow.

Experimentally changing the number of tyrosine-based activation motifs (ITAMs) within each TCR signaling complex was presented as a test for strength of

signal instructive model. TCR signaling intensity and the number of the SP thymocytes generated decreased with the reduction in the number of ITAMs in TCR complex. However, this change in ITAM numbers, and following change in TCR signaling did not affect the CD4 versus CD8 lineage commitment.[25] This observation contradicted with the strength of signal model.

1.2.4 THE DURATION OF SIGNAL MODEL

$CD4^{high}CD8^{low}$ thymocytes are the cells where most or all the CD4 versus CD8 lineage commitment occurs.[15, 16, 26] The decreased expression of CD8 on the $CD4^{high}CD8^{low}$ thymocytes results in short duration and weak intensity of MHC-I specific TCR signals, because these thymocytes loose the CD8 component of the TCR co-receptor signal. On the other hand these thymocytes can continue to receive long duration and strong intensity of MHC-II specific TCR signals, because they do not loose the CD4 component of the TCR co-receptor signal.[26] According to the duration and intensity of the MHC specific TCR signals, the duration of signal model proposes that DP thymocytes which are $CD4^{high}CD8^{low}$, shutdown one of the co-receptor gene expression, and differentiate into other co-receptor SP thymocytes. The MHC-I specific TCR signals with short duration and weak intensity favors CD8 lineage commitment; the MHC-I specific TCR signals with long duration and weak intensity promotes CD4 lineage commitment by turning off the expression of CD4 and CD8 co-receptors respectively. On the other hand, MHC-II specific TCR signals with short duration and weak intensity also favor CD8 lineage commitment. [27, 28] (Figure 1.6)

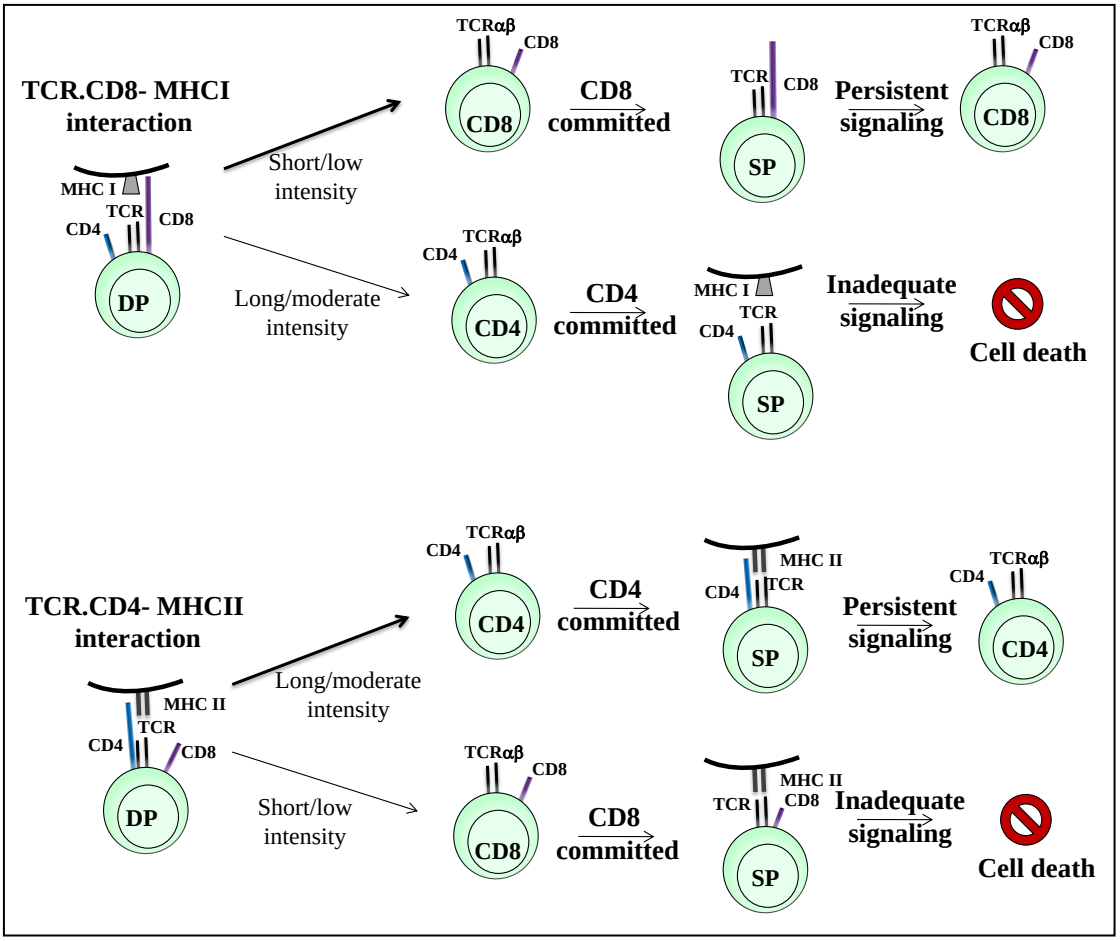


Figure 1.6: The duration of signal model of thymocyte lineage decision.

1.2.5 THE KINETIC SIGNALING MODEL

Unlike the classical CD4 versus CD8 lineage commitment models, kinetic signaling model proposes that positive selection and lineage commitment occurs sequentially. CD8 gene transcription is terminated during positive selection in TCR signaled DP thymocytes regardless of the MHC specificity of TCR and these TCR signaled DP thymocytes become transcriptionally $CD4^{high}CD8^{-}$ transitional cells which are still lineage uncommitted. The CD4 versus CD8 lineage commitment occurs at this transcriptionally $CD4^{high}CD8^{-}$ intermediate stage according to the properties of the TCR signal. Most transcriptionally $CD4^{high}CD8^{-}$ intermediate cells appear $CD4^{high}CD8^{low}$ phenotypically.

According to the kinetic signaling model, uncommitted $CD4^{high}CD8^{low}$ intermediate cells favor CD4 lineage differentiation and become CD4 SP thymocytes when TCR mediated positive selection signals continue despite terminated CD8 gene transcription. If positive selection signaling by TCR stops in the absence of CD8 gene expression, uncommitted $CD4^{high}CD8^{low}$ cells commit to the CD8 SP lineage. They terminate transcription of CD4 gene and re-express CD8 molecule on their cell surface to become CD8 SP thymocytes.

The kinetic signaling model also proposes that duration of TCR signaling controls signaling by the IL-7 and other γ-chain cytokine receptors. TCR signal duration is directly proportional to cytokine signaling which serves as sensor of TCR signal duration. Termination of CD4 gene transcription and re-initiation of CD8 gene expression in $CD4^{high}CD8^{low}$ cells give rise to CD8 SP thymocytes and this co-receptor reversal is favored by IL-7 and other γ-chain cytokines signaling.[29] On the other hand, continuous TCR signaling disrupts IL-7 signaling and promotes CD4 lineage differentiation. At the stage of $CD4^{high}CD8^{low}$ cells, when TCR signaling is disrupted, Il-7 receptor signals cause the termination of CD4 gene expression and initiation of CD8 gene expression.

The regulation of CD4 and CD8 gene expression underlines the kinetic signaling model of thymocyte lineage commitment. Activity of a silencer element restricts the tissue specificity of CD4 gene expression and enhancer elements define the tissue and stage specificity of CD8 gene expression. CD8α gene expression is controlled by five enhancer elements that have been identified to date. The activities of these enhancer elements show stage specificity during thymic development.[30, 31] The activity of the enhancer element that controls CD8 gene expression in DP thymocytes, $E8_{III}$, is disrupted by TCR signals during positive selection and consequently CD8 expression is terminated.[18] Also it has been experimentally shown that IL-7 regulates CD8 gene expression. IL-7 signaling targets the enhancer element, $E8_I$, which is only active in CD8 lineage thymocytes and up regulates the CD8α gene expression regardless of MHC specificity of the TCR. The transcriptional regulation of the CD8 gene follows the kinetic signaling model. An expectation of the kinetic signaling model is that CD4

lineage choice should be driven by TCR signaling and independent of IL-7, whereas CD8 lineage choice should be controlled by cytokine signaling.[32]

1.3 KEY MOLECULES AFFECTING CD4 AND CD8 DEVELOPMENT

1.3.1 High Mobility Group (HMG) Box Proteins

High Mobility Group (HMG) Box proteins include transcription factors and share DNA-binding motifs. They bind DNA in a sequence specific or sequence independent manner. These proteins can regulate gene expression by changing local chromatin structure and forming regulatory protein complexes with other nuclear proteins. T-cell factor (TCF), Lymphoid enhancer factor (Lef) and TOX are HMG box proteins highly expressed in thymic tissue. The expression of TOX has been found to be up-regulated during the positive selection of thymocytes.[33-35] TOX deficiency in mice results in the preferential positive selection of CD8 SP cells and a lack of CD4 SP thymocytes. It is thought that the absence of $CD4^{high}CD8^{low}$ intermediate cells in these mice plays a role in this defect. Transgenic re-expression of TOX in these knockout mice results in the rescue of the CD4 SP lineage. [36]

1.3.2 GATA-3

GATA-3 is one of the key factors involved in thymocyte lineage fate. Trans-acting T-cell-specific transcription factor GATA-3 is a zinc finger protein that binds to the enhancer of TCR alpha and delta genes. GATA-3 is expressed during the all stages of thymocyte development and its expression is up regulated during the transition from DP to CD4 SP cells. On the other hand, GATA-3 expression decreases during the differentiation to the CD8 SP lineage. Inhibition or deletion of GATA-3 results in the loss of CD4 SP thymocytes, but this deficiency does not have any affect on CD8 lineage development. Interestingly, in GATA-3 deficient mice, DP thymocytes with MHC-II specific TCR are not directed to CD8 lineage choice and over-expression of

GATA-3 does not promote MHC-I specific DP thymocytes to CD4 development. These experiments indicate that GATA-3 is required for CD4 development, that it is not a lineage determining transcription factor. [37-40]

1.3.3 Runx Family of Transcription Factors

The Runx family of transcription factors shares a highly conserved DNA binding domain. The Runx family consist of three proteins; Runx1, Runx2 and Runx3.[41, 42] Runx1 and Runx3 are expressed in DN and CD8 SP thymocytes, respectively. Of these, two Runx proteins bind to the CD4 silencer element and down regulate the expression of CD4 in DN and CD8 SP thymocytes. Runx3 can also bind to CD8 enhancer, $E8_I$, and contribute to the CD8 expression. The inactivation of Runx1 in DN thymocytes results in de-repression of CD4. [43-46] Runx3 expression was first detected in $CD4^{high}CD8^{low}$ cells at low levels. In Runx3 deficiency, CD4 expression is de-repressed and the number of CD8 SP thymocytes was reduced. Over expression of Runx3 in transgenic mice down regulates the CD4 expression and favors CD8 lineage differentiation.[46, 47] It is thought that at $CD4^{high}CD8^{low}$ thymocyte stage, Runx3 terminates CD4 expression by binding to the CD4 silencer element, and re-initiates CD8 expression by binding to CD8 enhancer, $E8_I$.[43-45]

1.3.4 T Helper Inducing POZ-Krüppel Factor (ThPOK) and other BTB-ZF Proteins

In HD (Helper-deficient) naturally occurring mutant mice, CD4 SP thymocytes could not be generated because MHC-II restricted DP thymocytes differentiated into CD8 lineage T cells.[17] A spontaneous recessive mutation was identified in HD mice and the mutated locus was identified as the zinc finger transcription factor ThPOK (for 'T helper-inducing POZ-Krüppel factor'; also called cKrox or Zfp67; encoded by the Zbtb7b gene). The point mutation was in the second zinc finger domain of the ThPOK protein. Transgenic expression of ThPOK in HD background resulted in the

differentiation of positively selected DP thymocytes into the CD4 lineage, regardless of their TCR-MHC specificity.[48, 49] (Figure 1.7)

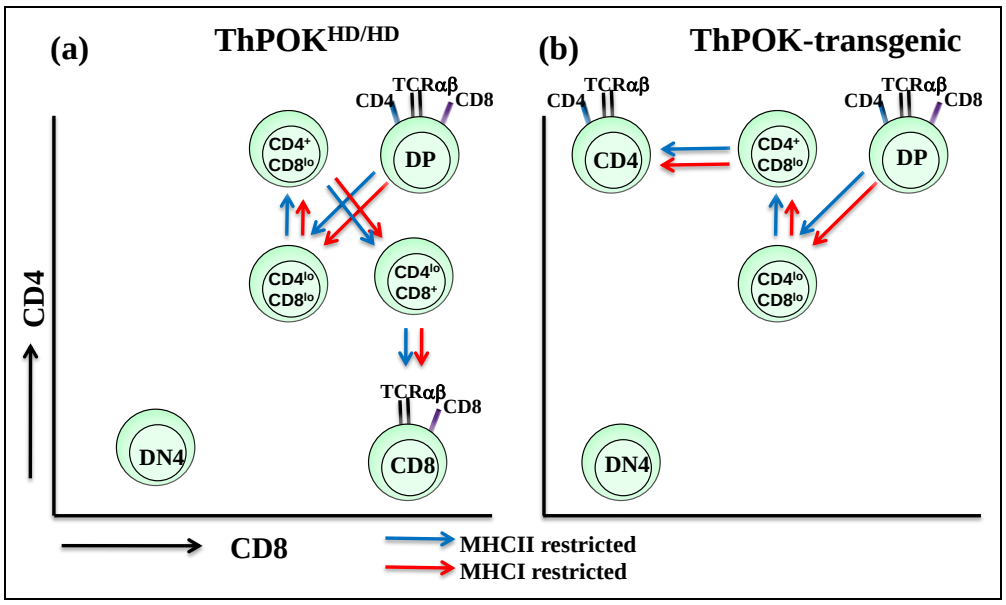


Figure 1.7: The effect of ThPOK protein on thymocte lineage decision.
In ThPOK$^{HD/HD}$ mice, all positively selected thymocytes differentiate into the CD8 lineage (a), whereas when ThPOK is transgenically expressed, all positively selected cells differentiate into the CD4 lineage (b).

Although widely expressed in other tissues, ThPOK shows a tightly regulated and highly lineage- and stage-specific expression pattern during thymic development. ThPOK is first expressed in CD4^{high}CD8^{low} thymocytes and then in CD4 SP, but not in CD8 SP thymocytes. [50]

ThPOK proteins antagonize the Runx dependent down regulation of CD4 and ThPOK gene expression by binding the CD4 and ThPOK silencer elements, and in so doing, promotes CD4 lineage commitment.[43] ThPOK expressed in retrovirally transduced CD8 SP thymocytes caused decreased CD8 expression, and induced CD4 lineage characteristics to these terminally differentiated cells. ThPOK can repress the activity of $E8_I$ CD8 enhancer, activity and CD8 gene expression.[51] These observations are consistent with the notion that ThPOK is necessary for the generation of the CD4 lineage, but not the sole master regulator controlling this event.[52-54] When GATA-3 is deficient in DP thymocytes, CD4 SP thymocytes do not develop. One reason for this may be that GATA3 binds in the proximal regulatory element of Zbtb7b gene encoding

ThPOK and that in GATA3 deficient mice; $CD4^{high}CD8^{low}$ thymocytes cannot initiate ThPOK expression.

Transgenic expression of ThPOK in GATA-3 deficient DP thymocytes did not result in generation of CD4 SP thymocytes, but there was a loss in CD8 SP thymocytes. Therefore, it can be concluded that GATA-3 is necessary for the expression of ThPOK and promotes its function in the generation of CD4 lineage lymphocytes, but that ThPOK has a GATA-3 independent function in downregulating CD8 expression.[38, 52, 54] (Figure 1.8)

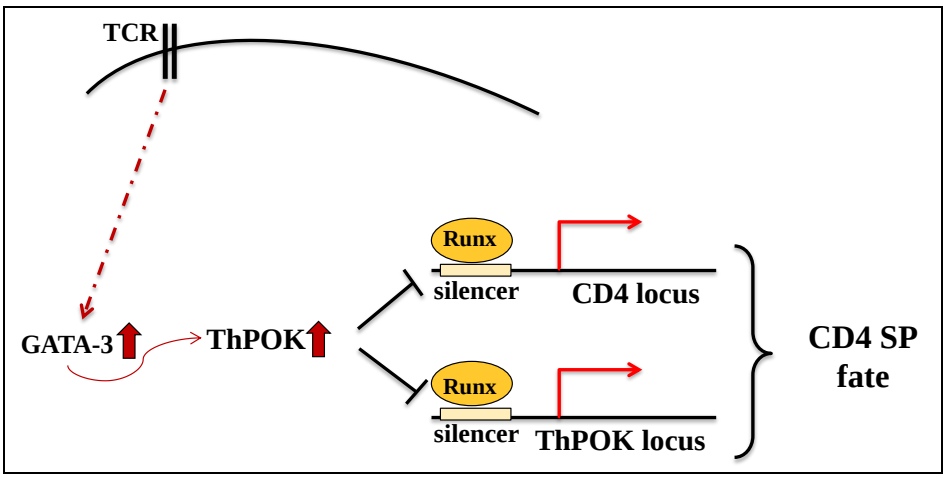


Figure 1.8: The effect of GATA-3 on the expression of CD4 and ThPOK.

The C_2H_2-type zinc finger protein ThPOK contains both a C-terminal Krüppel-like zinc finger domain for DNA binding and a N-terminal BTB (for 'broad complex/tramtrack/bric-a-brac' ; also called POZ) domain involved in protein homodimerization and interaction with other transcription factors.[55, 56] (Figure 1.9)

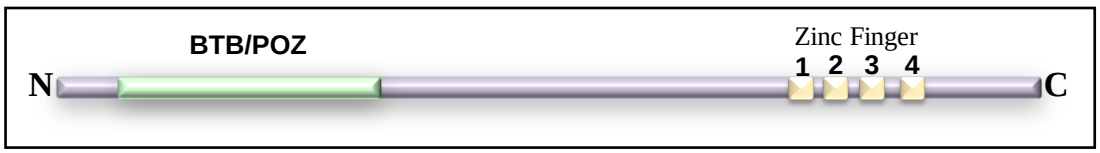


Figure 1.9: The schematic representation of the ThPOK protein showing its domains.

The BTB-POZ domain is a protein-protein interaction motif that is involved in different cellular processes such protein targeting for ubiquitination, cytoskeleton dynamics, ion channel assembly and gating, and transcriptional regulation.[57] The BTB-POZ domain is required for ThPOK function during thymocyte lineage commitment. Transgenic expression of ThPOK in ThPOK deficient mice disrupted CD8 SP thymocyte development and promoted CD4 SP thymocyte differentiation. Expression of a ThPOK variant lacking the BTB-POZ domain did not have a phenotypic effect, indicating the necessity of the BTB-POZ domain and the importance of protein-protein interactions in the function of the ThPOK protein.[58] (Figure 1.10)

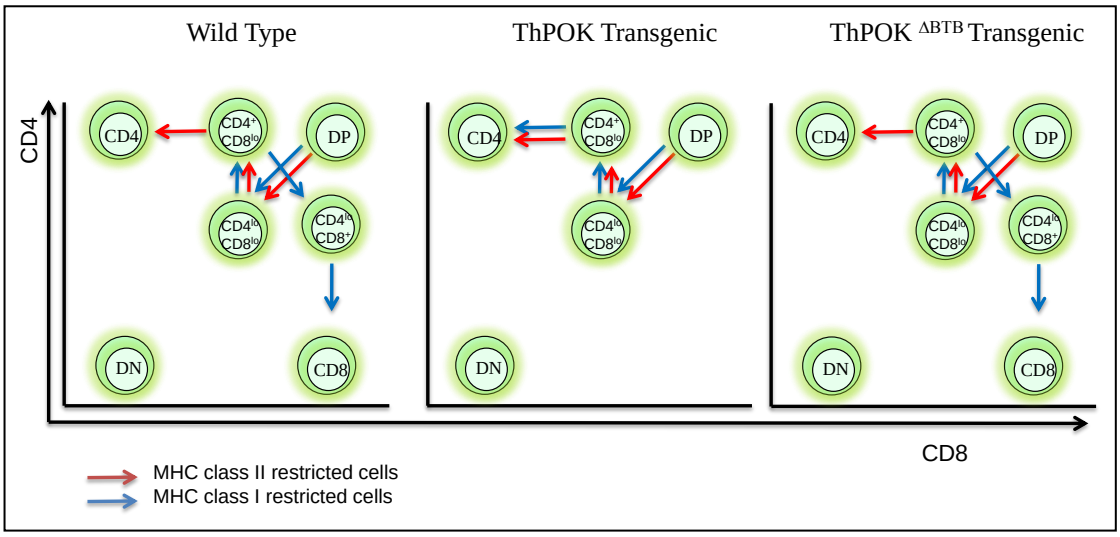


Figure 1.10: Flow cytometric analysis of wild type, ThPOK transgenic, ThPOK $^{\Delta BTB}$ transgenic thymocytes.

The BTB-POZ domain directs protein homo and heterodimerization of BTB-zinc finger (BTB-ZF) proteins, and changes their DNA-binding properties. The BTB-POZ domain of ThPOK may be involved in recruiting other protein complexes to ThPOK target enhancers and silencers or may affect the DNA binding specificity of this transcription factor.[59]

BTB-ZF proteins have been implicated in transcriptional repression and activation. However, the domains responsible for repression and activation have not been identified in BTB-ZF proteins.[60-62] Up to now, five BTB-ZF proteins have been found to have a role in T cell development and function. (Table 1.1)

Murine protein names	Gene Name	Human Homologues
MAZR	*Zfp278*	ZN278_HUMAN
Th-POK	*Zbtb7b*	ZBTB7B
Bcl6	*Bcl6*	BCL6
BAZF	*Bcl6b*	ZNF62
PLZP	*Zbtb32*	ZBTB32

Table 1.1: The BTB-ZF proteins; their gene names and human homologues that have a role in T cell development and/or function.

Only the protein protein interaction domains of these T cell BTB-ZF proteins are highly conserved, indicating that their mechanism of transcriptional repression and activation may be similar. On the other hand, the number and sequence of amino acids within C-terminal zinc finger domains of these BTB-ZF proteins are not similar. Also, their expression patterns differ during T cell development.[63] (Figure 1.11, Figure 1.12, Figure 1.13)

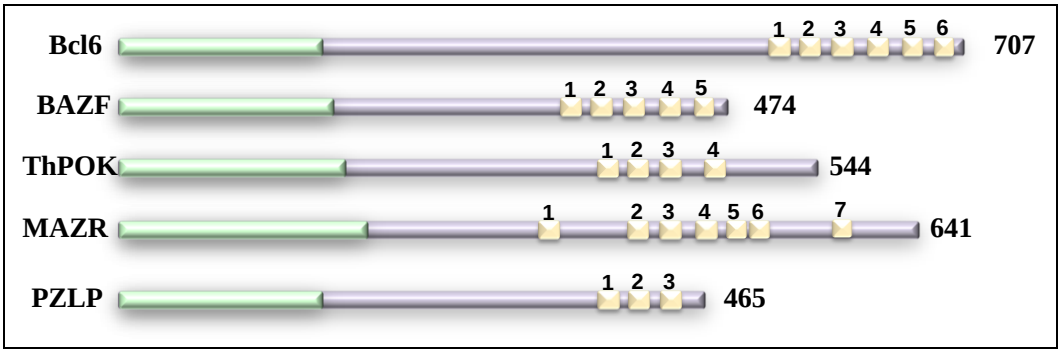


Figure 1.11: The BTB-ZF proteins.

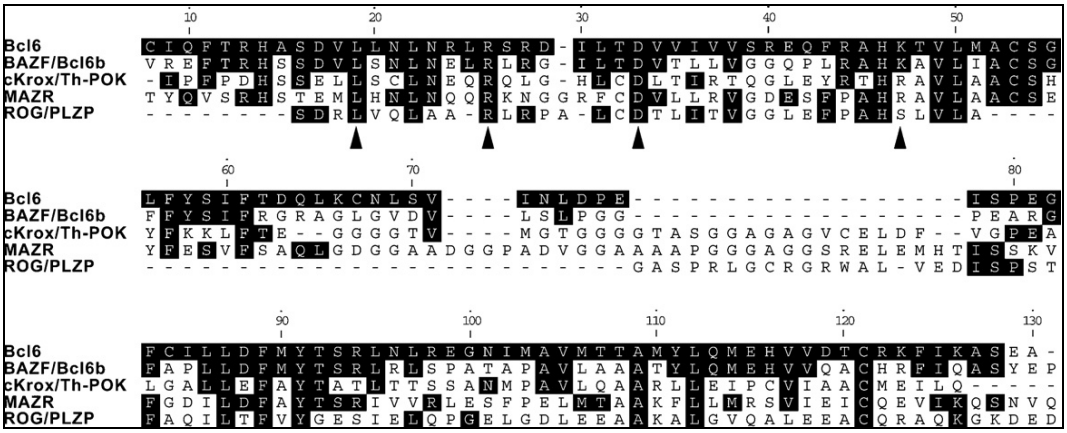


Figure 1.12: Sequence alignment of the BTB domains of BTB-ZF proteins known to play a role in thymocyte development and T cell function.
The arrows indicate the conserved amino acid residues required for the interaction with corepressors and dimerization

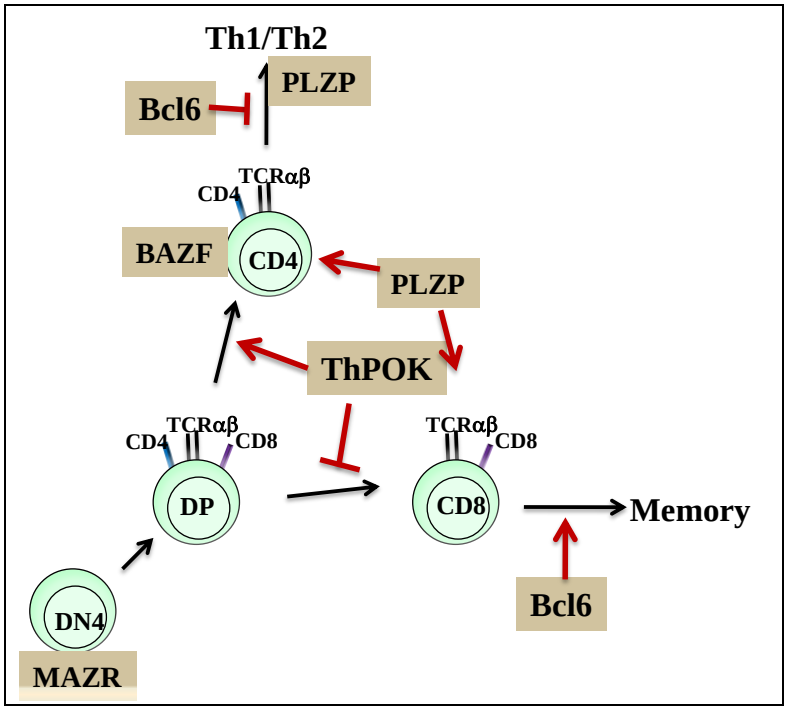


Figure 1.13: The expression pattern and the function of different BTB-ZF proteins during T lymphocyte development.

It was recently shown that the BTB-ZF protein MAZR, encoded by the Zfp268 gene, negatively regulates CD8 expression. The role of MAZR was first identified as a binding protein partner of CD8 enhancer element, $E8_{II}$. In developing DN thymocytes, MAZR interacts with several CD8 cis-regulatory elements and nuclear repressor complexes via its BTB-POZ domain. Nuclear co-repressors are part of multi-subunit

complexes consisting of the BAF complex, methyl- DNA binding proteins and histone deacetylases (HDACs). At the DP thymocyte stage, MAZR is thought to be involved in keeping local chromatin at the CD8 gene loci transcriptionally terminated. The BTB-POZ domain of MAZR may function to recruit repressor protein complexes to the CD8 enhancer. Upon progression to the DP thymocyte stage, MAZR expression is downregulated and CD8 gene transcription starts. Forced expression of MAZR in DP thymocytes disrupts activation of CD8 expression. [59, 63-66]

The BTB-ZF protein ROG/PLZP, encoded by the Zbtb32 gene, is expressed in the thymus, spleen, lymph nodes and naive T cells at low levels. Upon stimulation with anti-CD3, its expression increases in CD4 and CD8 T cells. ROG/PLZP protein was first identified as a repressor of GATA-3 protein which is involved in T cell development and CD4 lineage differentiation. Also, ROG/PLZP deficiency results in increased T cell proliferation and induced cytokine production. It involves in the control of T cell proliferation and regulating cytokine expression.[67-69] ROG/PLZP binds GATA-3 via its C-terminal zinc finger domain, inhibiting the DNA binding of GATA-3. Also, ROG/PLZP leads to histone hypo-acetylation at IL4 gene loci and termination of IL-4 production in T-cytotoxic 2 cells via BTB-POZ domain dependent recruitment of HDAC molecules.[70]

The BTB-ZF protein Bcl-6 was first found in B lymphocytes. It involves in germinal center formation, memory B cell generation, and was shown to be involved in repressing B cell differentiation to plasma cells. Bcl-6 deficiency in mice leads to an increase in T helper cell 2 (Th2) cytokine responses and Th2 type inflammation. [61, 71] Bcl-6 also plays a role in the development of memory CD8 T cells after T cell activation.[72] These results indicate that Bcl-6 may be downstream of TCR signals. Another BTB-ZF protein BAZF is highly homologous to Bcl-6 in its C-terminal zinc finger domain as well as its BTB domain. These homologous proteins can heterodimerize and bind to DNA binding sites in the STAT6 gene.[73] The BTB domain of BAZF protein can recruit nuclear co-repressors through its interaction with the Bcl-6 protein. However, BAZF can function as a transcriptional repressor, independently of Bcl-6 as well.[74, 75] BAZF is thought to be important for naive T cell activation and controlling secondary response of memory CD8 T cells.[76, 77]

2 AIM OF THE STUDY

In this project, we aimed to identify putative proteins that interact with the BTB domain of the zinc finger transcription factor, ThPOK. ThPOK mediates the differentiation of MHC-II restricted thymocytes into the CD4 SP lineage, using its N-terminal BTB domain. Because it is a protein-protein interaction motif, the BTB domain is thought to mediate the function of the ThPOK protein by recruiting other proteins.

The cellular and molecular mechanisms underlying T cell lineage commitment has long been the subject of intense debate. Identification of interacting putative proteins with ThPOK through its BTB domain could reveal the unknowns in the function of ThPOK during T cell lineage differentiation.

We used the classical yeast two hybrid assay, which is one of the most powerful methods for identification of interaction partners of nuclear proteins, in this study. We screened a human thymic cDNA library against the BTB domain of the ThPOK protein to identify any potential interactors. We further performed co-immunoprecipitation experiments in mammalian cells to study the interesting putative interactors. Because of ThPOK's critical importance in lineage commitment, elucidating the interaction pattern of these proteins with the ThPOK BTB domain will enhance our understanding of thymocyte lineage differentiation.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All the chemicals used in this project are listed in Table 3.1.

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
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
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 Amino acids	Q-Biogene, USA
YPD Agar	Sigma, Germany
YPD Broth	BD, USA

Table 3.1: The list of the chemicals used in this study.

3.1.2 Equipment

All the equipment used in this project are listed in Table 3.2.

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	-80^0C, Forma,Thermo ElectronCorp.,USA
	-20^0C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA
	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
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

Table 3.2: The list of the equipment used in this study.

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.[78]

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.

Calcium Chloride ($CaCl_2$) Solution: 60mM $CaCl_2$ (diluted from 2M stock), 15% Glycerol ,10mM PIPES (pH=7) and the solution prepared was autoclaved at 121 °C for 15 min and stored at 4 °C.

Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g of KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml distilled H_2O. pH was adjusted to 7.4 by drop wise addition of concentrated HCl and the buffer was completed to 1 L with distilled H_2O.

2X HEPES-buffered saline: 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.

TX100 Wash Buffer: For 50ml buffer, 0.5 ml 20% TX100, 2.5 ml 1 M Tris (pH7.4), 1.5 ml 5 M NaCl, 0.2 ml, 0.5 M EDTA was dissolved in 45.3 ml water.

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 that ıncludes 5XLoadıng Dye and 20X Reducing agent 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.

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

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

3.1.4 Growth Media

Bacterial Growth Media:

Luria Broth from BD (Beckton, Dickinson and Company) 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 autoclaving .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.

Yeast Growth Media:

Yeast extract-Peptone-Dextrose (YPD) Broth from BD was used for liquid culture of wild type yeast strain AH109. 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. Synthetic Complete (SC) Drop-out Medium was used for liquid culture of plasmid transformed yeast. CSM-Ade-His-Leu-Trp and CSM-His-Leu-Trp-Ura from Q-Biogene were used

to prepare liquid and solid SC Drop-out medium. For liquid SC Drop Out Medium, 6.7g Yeast Nitrogen Base without amino acids and ammonium sulfate from Difco, 0.6g CSM-His-Leu-Trp-Ura from Q-Biogene, and 20g D-glucose from Sigma were dissolved in 1L distilled water and autoclaved at 121^{o}C for 15 min. For selection, Histidine at a final concentration of 10 g/L, Tryptophan at 10 g/L, Adenine at 2 g/L and Leucine at 2 g/L were added to the medium prior to use. For solid SC Drop-out Medium, 20g Bacteriological Agar from Biolab, 6.7g Yeast Nitrogen Base withouto amino acids and ammonium sulfate from Difco, 0.6g CSM-His-Leu-Trp-Ade from Q-Biogene, and 20g D-glucose were dissolved in 1L distilled water and autoclaved at 121^{o}C for 15 min. 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.

Mammalian Cell Growth Media:

Adherent cell lines (HEK 293T) were grown in filter-sterilized DMEM that was supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-Glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin. The cells 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, QIAGEN,Germany
QIAGEN Plasmid Maxi Kit, QIAGEN, Germany
Qiaprep Spin Miniprep Kit, QIAGEN,Germany
Qiaquick Gel Extraction Kit, QIAGEN,Germany
ROCHE Plasmid Miniprep Kit, ROCHE, Germany
ROCHE Plasmid Midi Kit, ROCHE, Germany
ROCHE PCR Purification Kit, ROCHE,Germany
MATCHMAKER GAL4 Two-Hybrid System 3, Clontech, USA

3.1.6 Enzymes

Enzymes and their corresponding 10X reaction buffers used in this project are listed in Table 3.3.

Enzyme	Buffer	Company
BamHI	BamHI	Fermentas
BglII	Orange	Fermentas
CIAP (Alkaline Phospahase)	CIAP	Fermentas
EcoRI	EcoRI	Fermentas
EcoRV	Red	Fermentas
HindIII	Red	Fermentas
Nco1	Yellow	Fermentas
Sal1	Orange	Fermentas
SacI	SacI	Fermentas
SfiI	Green	Fermentas
Taq Polymerase	Taq	Fermentas
Pfu Polymerase	Pfu	Fermentas
T4 DNA Ligase	T4 DNA Ligase	Fermentas
Xba1	Yellow	Fermentas
Xho1	Red	Fermentas

Table 3.3: The list of restriction enzymes and their corresponding buffers used in this study.

3.1.7 Cell Types

S. cerevisiae wild type AH109 strain (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1*) was used for Yeast Two Hybrid experiments. Yeast strain AH109 is designed for detecting protein interactions during a

two-hybrid screen. AH109 contains distinct ADE2, HIS3, lacZ, and MEL1 reporter constructs that are only expressed in the presence of GAL4-based protein interactions. Strain AH109 virtually eliminates false positive protein interactions that arise during a typical GAL4-based two-hybrid screen. *E. coli* DH-5α (*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_K^- m_K^+), λ–) competent cells were used for bacterial transformation of plasmids. For the mammalian confirmation experiments, a variant of Human Embryonic Kidney cell line, HEK293T was used for transfection.

3.1.8 Vectors and Primers

Vectors and primers used in this project are listed in the Table 3.4 and Table 3.5. The vector maps are given in Appendix A.

Plasmid	Company
pACT2 AD	Clontech
pCDNA 3.1 Hygro	Invitrogen
pCMV-HA	Clontech
pCMV-MYC	Clontech
pGBKT7 BD	Clontech
pCDNAGFP	Lab Construction

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

Primer name	Sequence
cKroxM 21-158 FWD	5'CCGGAATTCCTGAGCTGCCTGAACGAGCAGC3'
cKroxM 21-158 REV	5'CCGGAATTCTCGCTCACAGTCATCCTCATCG3'
pACT2 AD 3’	5'AGATGGTGCACGATGCACAG 3'
pACT2 AD 5’	5'TACCACTACAATGGATG3'

Table 3.5: The list of primers and their sequences 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 B.

3.1.10 DNA Sequencing

DNA Sequencing service was commercially provided by McLab,CA,USA.

3.1.11 Software and Computer Based Programs

The software and online programs used in this project are listed in alphabetical order:

UCSC Genome Browser	http://genome.ucsc.edu/cgi-bin/hgGateway
Protein Calculator v3.3	http://www.scripps.edu/~cdputnam/protcalc.html
NCBI BLAST Server	http:/ncbi.nlm.nih.gov/blast.cgi
FinchTV	http://www.mclab.com

3.2 METHODS

3.2.1 Vector Construction

Polymerase Chain Reaction (PCR):

Optimized PCR reaction and thermal cycle conditions are shown in Table 3.6.

PCR Reactıon	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: The optimized PCR reaction conditions.

The PCR 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 7 minutes.

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 of DNA

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 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. Then the $CaCl_2$ solution was used for the successive centrifuge steps and isolation of competent cells. The cells prepared were frozen immediately in liquid nitrogen and then stored at -80°C. The competency of the prepared cells were tested by transforming 10/100 pg of pUC19 plasmid.

Transformation of Chemically Competent DH5α 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 either by the alkaline lysis protocol[78] or by the Mini- and Midi-Prep Kits from Roche and 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.

3.2.3 Yeast Cell Culture

Yeast Culture Growth

Yeast cells were grown for 20-24 hours at room temperature on a rotator in YPD Broth or SC Drop-out liquid medium. Yeast cells either streaked with a toothpick or spreaded with 4mm glass beads, were grown on YPD or SC Drop-Out Agar Petri dishes for 2-4 days at 29 °C. For the glycerol stock preparation of yeast cells, glycerol was added onto overnight grown cultures to a final concentration of 25%.

Plasmid DNA Isolation from Yeast Cells

A single colony of *S. cerevisiae* AH109 from YPD or SC Drop-out agar plate was grown at room temperature on a rotator in 2-5 ml of liquid growth medium. Roche and Qiagen mini-prep kits were used to isolate yeast plasmid DNA. Sterile acid washed glass beads were used during re-suspension of yeast pellets prior to lysis step.

Transformation into Yeast Cells

A single AH109 yeast cell on YPD agar plates was inoculated using a sterile toothpick with 50 ml of YPD broth and grown for 20- 24 hours at 29^{o}C and 250 rpm. When the OD_{600} of the culture reached between 0.4 and 0.6, it was transferred to 50 ml falcon tubes and centrifuged at 4^{o}C at 2500g. The pellet was dissolved within 2.5 ml of distilled water. PEG/LiOAc was prepared from 50% PEG, 1M LiOAc and ssDNA. 300 ul of this solution was added onto 1.5 μg of the plasmid in an 1.5 ml eppendorf tube

and after a brief vortexing, 100 µl of re-suspended yeast cells were added onto the DNA/PEG/LiOAc/ssDNA suspension. After a 45 min of incubation at 42^{o}C with slow shaking, the culture was pelleted at room temperature. 0.9% of NaCl was used to dissolve the pellet. Yeast cells were then spreaded onto agar plates with selective amino acids by using glass beads, and incubated for 2-4 days at 29^{o}C. Library transformation was performed according to the Matchmaker Gal4 Two-Hybrid System 3 & Libraries User Manual.

Yeast Colony PCR

Yeast colonies were swirled in 10 µl 0.02N NaOH and boiled at 95^{0}C for 8-10 minutes. Optimized PCR reaction and thermal cycle conditions used for yeast colony PCR are shown in Table 3.4. The primers, pACT2 AD 5' and pACT2 AD 3', were used for the colony PCR of yeast cells. The Colony PCR conditions were as follows: initial denaturation at 95ºC for 2 minutes followed by 30 cycles of denaturation step (at 95ºC for 30 seconds), annealing step (at 55ºC 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.

Yeast Cell Lysis

Yeast cells grown on selective solid medium were inoculated into various selective liquid culture media and grown at room temperature for 2 days. The yeast culture was then centrifuged at 4ºC for 5 minutes at 13200 rpm. After discarding the supernatant, yeast pellets were dissolved in 100ml TX100 lysis buffer and stored at -80ºC. 20ml of yeast cells were incubated at 99ºC for 5 minutes and centrifuged at 13200rpm at room temperature before performing SDS-PAGE.

The Yeast Two Hybrid Method

The conventional yeast interaction trap is a powerful method for in vivo analysis of protein-protein interactions. More than 50% of known proteins have been identified by the Yeast Two Hybrid method. The yeast, *S. cerevisiae*, is the model organism for the interaction trap of eukaryotic proteins, because of being an eukaryotic organism

which has a neutral internal pH and reduced state of glutathione, and also enabling formation of tertiary protein complexes. The principle of this method is based on the activation of downstream reporter genes by binding of a eukaryotic transcription factor onto upstream activation sequences. The transcription factor is split into two separate fragments: a DNA Binding Domain (BD) and an Activation Domain (AD). The system needs two separate plasmids which are constructed to express the protein of interest fused to the BD fragment of the transcription factor and another protein fused to the AD fragment of the transcription factor. The protein fused to the BD fragment is referred to as the 'bait protein', and it is generally a known protein for identification of its interacting partners. The protein fused to the AD fragment of the transcription factor is referred to as the 'prey protein', and it can be either a single known protein or a library of proteins derived from a cDNA pool. When these bait and prey plasmids are introduced into auxotrophic mutant yeast strains which cannot grow when certain nutrients are absent in the growth medium, the interaction of bait and prey proteins brings the AD and BD fragments of the transcription factor together to mediate the activation of transcription of the reporter genes and enable yeast cells to survive on the selective growth medium lacking essential nutrients. Examples of reporter genes used in this system are the *HIS3* gene required for histidine (His) synthesis, the *LEU2* gene required for leucine (Leu) synthesis, the *URA3* gene required for uracil (Ura) synthesis, the *ADE2* gene required for adenine (Ade) synthesis and the LacZ gene producing β-galactosidase for selection of the cells in which interaction occurs.[79-87] (Figure 3.1)

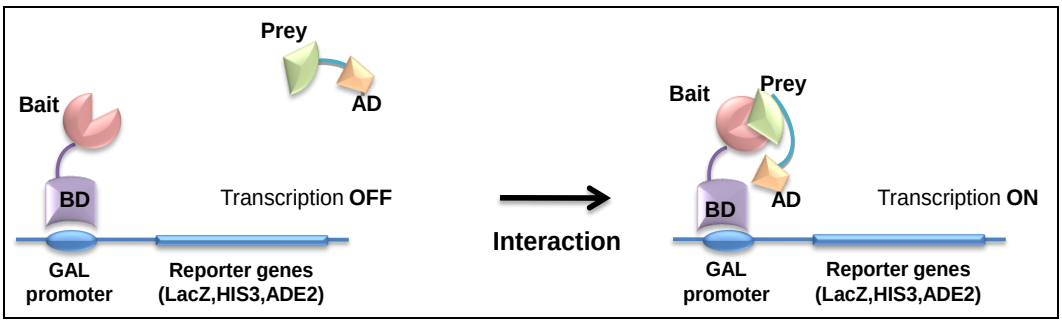


Figure 3.1: The Matchmaker Gal4 Two-Hybrid System.

The classical yeast two hybrid system screens for the interaction between over-expressed bait and prey proteins in the yeast nucleus. The over-expression of fused proteins may lead to many false positive colonies growing on the selective media for the interaction trap. Also, the post-translational modifications in yeast might affect the

interaction of the fused proteins and lead to false negatives that can not grow on the selective media.[87]

The Matchmaker Gal4 Two-Hybrid System 3 from Clontech was used in this project. pGBKT7 BD was used as a bait plasmid which expresses the protein of interest fused to the GAL4 DNA-binding domain, whereas pACT2 AD was used as a prey plasmid which expresses different cDNA'S from a Human Thymic cDNA library fused to the GAL4 Activation domain. The BTB domain of ThPOK (The plasmid bearing *M. musculus* Zbtb7b gene was provided from Remy Bosselut, NCI, NIH, USA) was PCR amplified and cloned into the pGBKT7 BD bait plasmid in frame with GAL4 DNA-binding domain. The bait and prey plasmids used in this project contain the auxotrophic genes for TRP and LEU synthesis, respectively. The wild type auxotrophic yeast strain AH109 was used as model organism which cannot synthesize ADE, HIS, LEU, TRP and URA.[79] The strategy used to analyze and verify putative positive interactions is described in the Figure 3.2.

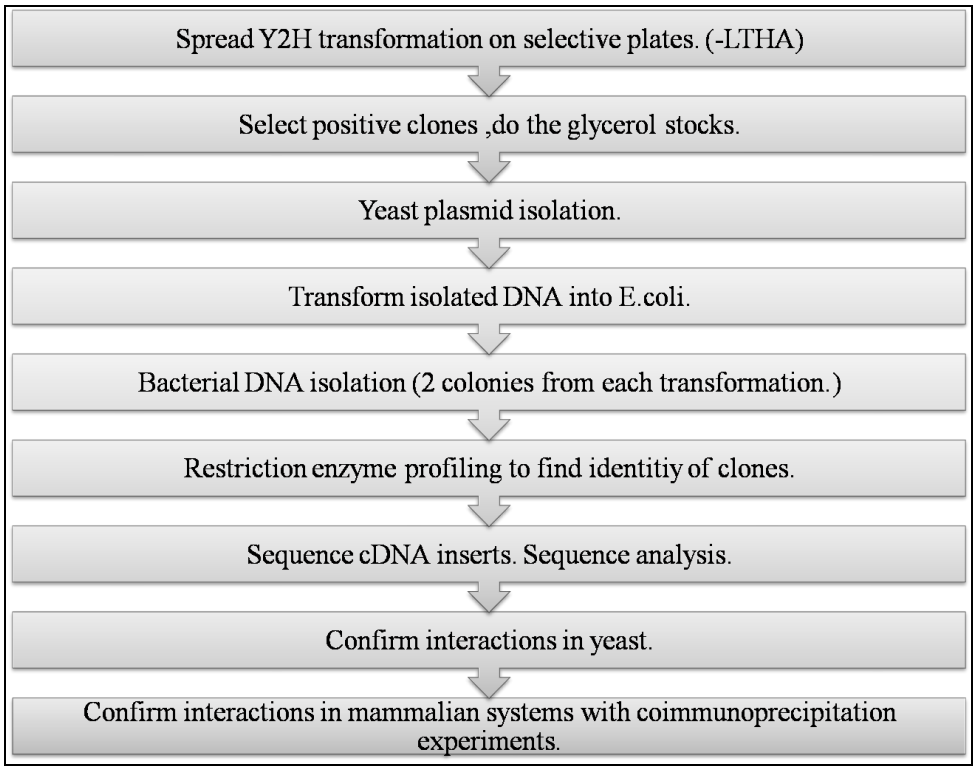


Figure 3.2: The strategy used to analyze putative positive interactions.

3.2.4 Mammalian Cell Culture

Mammalian Culture Growth

Adherent cell lines (HEK293T) were grown in a humidified atmosphere of 5% CO_2 at 37 °C in filter-sterilized DMEM supplemented with heat-inactivated 10% FBS (Fetal Bovine Serum), 2mM L-Glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin. Cell cultures were split in every 2-3 days by 1/10 dilutions. For the preparation of frozen stock of HEK293T cells, cells at mid to late growth phase were resuspended in freezing medium (10%DMSO in FBS) and stored at -80°C for 48 hours. For longer storage, the cells were maintained in liquid nitrogen tank. After thawing, the cells were immediately washed with growth medium to get rid of DMSO.

Transfection of HEK 293T cells

Transfection of HEK293T cells with plasmid DNAs was achieved by formation of a calcium phosphate/DNA co-precipitate. HEK293T cells were split to 30% confluency in either 60mm or 100mm tissue culture dishes. Split cells in tissue culture dishes were incubated for 16-24 hours at 37°C and 5% CO2 and the medium was changed 1 hr before transfection. On the transfection day, Calcium phosphate/DNA co-precipitate was prepared as: 120µl of 1 M $CaCl_2$, total 10µg of plasmid DNA and 380 µl of ddH_2O were combined with 500µl of 2X HBS and the mixture was incubated at room temperature for 10-15 min. Then, the mixture was added drop by drop on cells in tissue culture dishes. Transfected cells were incubated at 37°C and 5% CO2 the incubator for 48 hrs. Transfection efficiency of HEK293T cells was determined by transfecting with pCDNA-GFP plasmid, and examining the percentage of cells expressing GFP under the fluorescent microscope or in the flow cytometer machine.

Cell Lysis

48 hours after transfection, HEK293T cells were de-attached from 6-well plates by pipetting and centrifuged at 1000 rpm, for 5 minutes at room temperature. Cells were washed once with 1X PBS and were lysed in 50µl of TX100 Lysis buffer; cells

from 100 mm dishes were lysed in 1 ml of TX100 Lysis buffer. After incubation on ice for 30 minutes, lysates were centrifuged at 4°C for 10 minutes. Supernatants were stored at -80°C until use.

3.2.5 Protein Gel Electrophoresis

3.2.5.1 Western Blotting and Immunoprecipitation

For western blotting, separating and stacking gels were prepared according to the protocol described in the Materials and Methods section. 25µl of protein samples were mixed with 5X Loading Dye and boiled at 99°C for 5 minutes. Pre-stained Protein Marker from Fermentas was used as a molecular weight marker. Gel running was performed at constant 100V at room temperature, whereas transfer of proteins to the PVDF membrane from MILIPORE was carried out at 4°C for 60 minutes at a constant voltage of 100V using a BIORAD Transfer apparatus. Ponceau staining was performed on membranes to confirm the transfer of proteins to the membrane. Blocking was performed either at room temperature for 60 minutes or overnight at 4°C in 10mL blocking solution described in materials and methods. Anti-c-Myc Peroxidase and anti-HA Peroxidase from Roche were used to blot proteins of interest on the membranes after blocking. Membranes were incubated with 10µl of anti-c-Myc Peroxidase in 4mL blocking solution (1:400) in 50ml falcon tubes at room temperature for 1 hour on a rotator. For HA-blotting, membranes were incubated with 10µl of anti-HA Peroxidase in 4 ml blocking solution (1:400) at 4°C overnight in 50ml falcon tubes on a rotator. The membranes blotted with relevant antibodies were washed three times for 5 minutes with PBST. Detection of the labeled proteins was done in the dark room after treating membranes with SuperSignal West Pico Chemiluminescent Substrate (1:1) from Thermo-Scientific.

Immnuprecipitation from HEK293T cell lysates co-transfected with bait and prey plasmids was performed using anti-c-Myc Agarose Affinity Gel Antibody, Monoclonal Anti-HA−Agarose Antibody from Sigma and also Anti-HA Affinity Matrix from Roche. HEK293T cells transfected in 100mm tissue culture dishes were lysed in 1ml of

fresh TX 100 Lysis Buffer. After washing 50µl of affinity matrix containing the relevant antibodies five times with sterile PBS (without azide), 450µl of lysates was added on the beads and incubated in eppendorf tubes while gently rotating at 4°C overnight. Protein bound beads were washed again with 1X sterile PBS (without azide) for five times. 25µl of 2X Protein Loading dye was added to beads and boiled at 99°C for 5 minutes before loading to SDS gels and western blotting with the relevant antibodies.

4 RESULTS

4.1 CLONING OF THE ThPOK BTB DOMAIN INTO THE pGBKT7 BD BAIT PLASMID

The N-terminal BTB domain between the 21st and 158th amino acids of the mouse ThPOK was amplified by PCR using cKroxM 21-158 Reverse and cKroxM 21-158 Forward primers. The length of the amplified BTB domain was 432bp and EcoRI restriction sites were inserted at either end of the PCR product for cloning purposes. The amplified BTB domain was inserted into the pGBKT7 BD plasmid through the EcoRI site. (Figure 4.1) The plasmid was confirmed with restriction enzyme digestion and the sizes of the expected bands are shown in the table. (Figure 4.2) This strategy and the cloning were performed by Halil İbrahim Aksoylar.

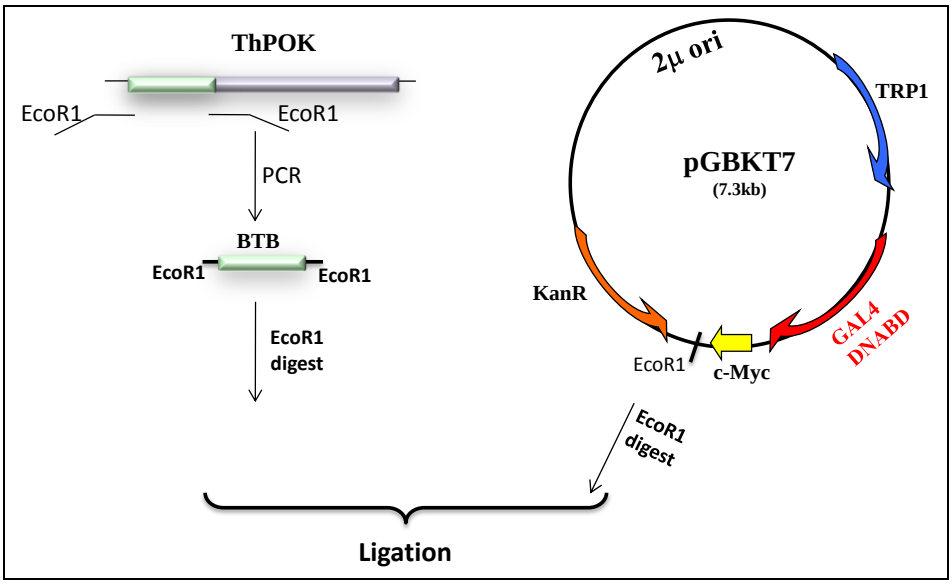
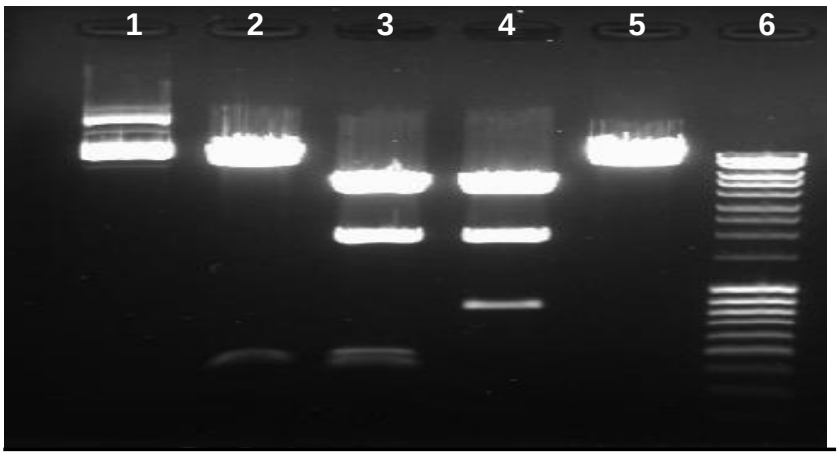


Figure 4.1: The cloning strategy of the ThPOK BTB domain into the pGBKT7 BD bait plasmid.

The pGBKT7 BD plasmid contains a 2 micron sequence necessary for plasmid replication in yeast cells, a Trp1 selectible gene (in blue), a Kanamycin resistance gene for selection in bacterial cells (in orange), and a fusion cDNA encoding the GAL4 BD, a c-Myc epitope.



	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Plasmid	pGBKT7 BD-BTB	pGBKT7 BD-BTB	pGBKT7 BD-BTB	pGBKT7 BD-BTB	pGBKT7 BD-BTB	DNA Ladder Mix
Restriction enzyme	Undigested	EcoRI	BamHI + PvuII	PvuII	BamHI	
Band size	7724	432	4853, 1993, 479, 398	4854, 1993, 877	7724	

Figure 4.2: Confirmation digestion of the pGBKT7 BD-BTB plasmid.
The expected restriction fragment sizes are indicated for each restriction digest below the gel in base pairs.

4.2 YEAST TWO HYBRID SCREEN WITH DIFFERENT STRINGENCY CONDITIONS

The growth of wild type yeast strain AH109 on different stringency conditions was tested by transforming positive control bait and prey plasmids that were previously identified to express interacting proteins. The human thymic cDNA library plasmid, pACT2 AD, purchased from Clontech was amplified by transforming it into chemically competent DH5α cells. The cDNA library was isolated by Qiagen DNA maxi-prep kit. Yeast cells were transformed with the bait plasmid expressing the cloned BTB domain of ThPOK. The clones that grew on -Trp selective plates (containing bait plasmid) were

grown again for the secondary transformation with the chimeric cDNA-AD expressing prey plasmid, and were grown on either -Trp -Leu selective plates or -Leu -Trp -His –Ade (-L-T-H-A) selective plates to either calculate transformation efficiency or to select for plasmids encoding interacting proteins.At the end of the large scale yeast two hybrid assay screen, 552 colonies grew on the -L-T-H-A selective plates. These 552 colonies were stored for the further analysis. This part of the project was conducted by Ibrahim Aksoylar in Dr. Erman's laboratory, and resulted in the generation of 552 individually frozen glycerol stocks.

4.3 ISOLATION OF POSITIVE CLONES

The first strategy employed to identify clones encoding interacting proteins was to perform colony PCR on yeast colonies grown under selective conditions. For this purpose, yeast cells were first lysed with heating and the extract was used directly as the template for PCR reactions. Primers annealing on either side of the pACT-AD cDNA library plasmid MCS were used to amplify cDNA's from individual colonies.

The second strategy employed to identify interacting clones was to isolate plasmid DNA from yeast colonies grown on -L-T-H-A selective plates containing both bait and prey plasmids. Isolated plasmid DNAs from yeast cells were low in quantity and quality, so they was re-transformed into *E.coli* DH5α competent cells and plasmid isolation from bacteria was carried out by selecting only for the ampicillin resistant prey plasmids. Two colonies from each transformation plate were selected for the bacterial plasmid isolation. Restriction enzyme profiling of the isolated plasmid DNA was performed and clones containing unique inserts were identified by DNA sequencing. (Figure 4.3) Isolated bacterial plasmids were digested with BglII and HindIII restriction enzymes to compare the identities between two colonies of transformed yeast clones. If the restriction enzyme profile of two colonies of the same clone were similar, only one of the colonies was analyzed further. For example, the clone number 542, restriction digestion analyses of colonies 542A and 542B gave the same restriction digestion profile. On the other hand, analysis of colonies 525A and

525B from the clone number 525 indicated that these colonies contained different inserts. (Figure 4.4)

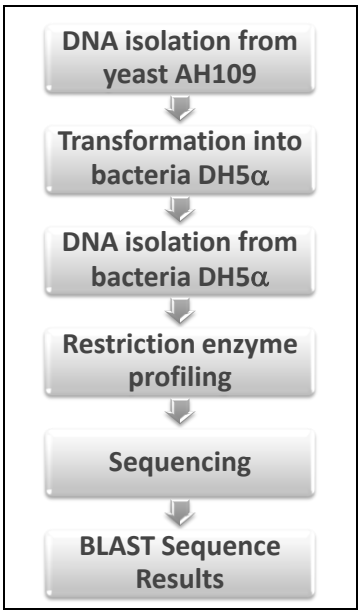


Figure 4.3: The strategy for sequence analysis of the identified clones.

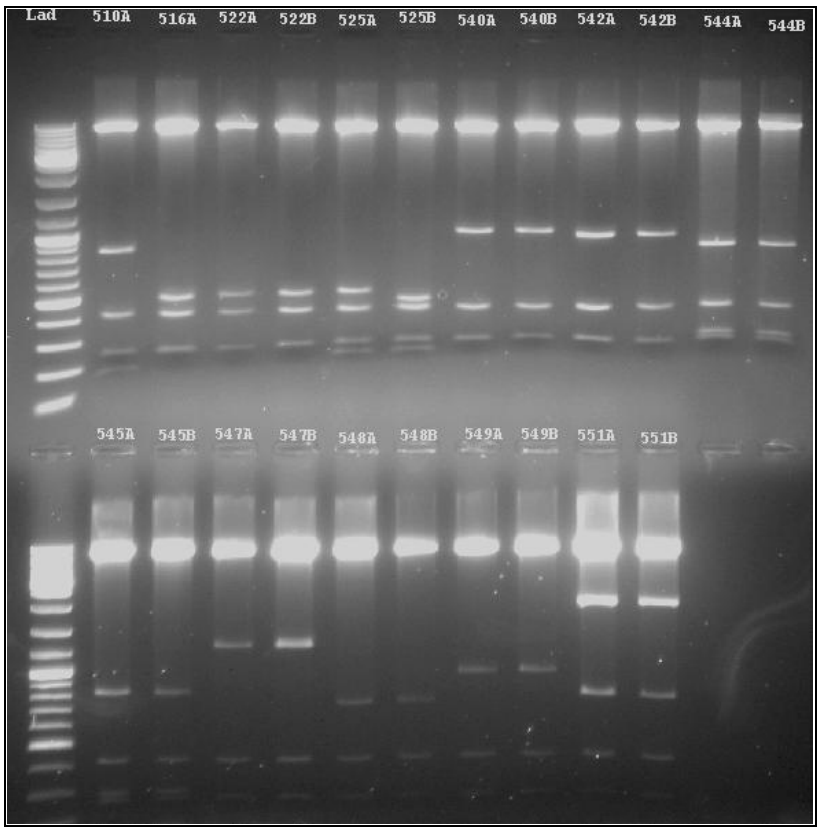


Figure 4.4: Restriction enzyme profiling of isolated plasmid DNAs from different clones with BglII and HindIII double digestion.

4.4 SEQUENCE ANALYSIS OF PUTATIVE INTERACTIONS

Ten microliters of bacterial plasmid DNA mini-preps having a concentration of at least 50 ng/μl was were sequenced at MCLAB Company, a biotechnology company in San Francisco, CA, USA. Sequences were analyzed with the FinchTV, DNA sequencing chromatogram trace viewer program provided by MCLAB. Protein sequences of the identified prey clones are shown in Appendix C. Sequencing results were analyzed by the BLAST program, comparing them to human genomic transcripts using the NCBI BLAST server (BLASTN).[88] Up to date, half of the 552 clones were sequenced and clones of interest were determined for further analysis. Reading frames of the cDNA sequences in pACT2 AD prey plasmids were identified by using the Vector NTI 9.0 program. Protein sequences of the identified prey clones were aligned to the human protein database by using the NCBI BLAST server (BLASTP) to confirm that the identified sequence corresponds to an expressed protein. The BLASTN and BLASTP results of different prey clones are shown in Table 4.1 and Table 4.2 respectively.

Clone	**BLASTN**	
9	NM_001037637.1	Homo sapiens basic transcription factor 3 (BTF3)
19	NM_015932	Homo sapiens proteasome maturation protein (POMP)
20	NM_005919	Homo sapiens myocyte enhancer factor 2B (MEF2B)
66	NW_001838952	Homo sapiens transcription factor 7 (TCF7)
74	NM_001039920	Homo sapiens zinc finger protein 384 (ZNF384)
88	NM_013379.2	Homo sapiens dipeptidyl-peptidase 7 (DPP7)
475	NM_005334	Homo sapiens host cell factor C1 (HCFC1)
481	NM_032593	Homo sapiens histidine triad nucleotide binding protein 2 (HINT2)
528	NT_007914	Homo sapiens poly (ADP-ribose) polymerase family, member 12 (PARP12)

Table 4.1: BLASTN results of the identified prey clones.

Clone	BLASTP	
9	**NP_001032726.1**	Homo sapiens basic transcription factor 3 (BTF3)
19	**NP_057016.1**	Homo sapiens proteasome maturation protein (POMP)
20	**NP_001139257.1**	Homo sapiens myocyte enhancer factor 2B (MEF2B)
88	**NP_037511.2**	Homo sapiens dipeptidyl-peptidase 7 (DPP7)
481	**NP_115982.1**	Homo sapiens histidine triad nucleotide binding protein 2 (HINT2)
528	**NP_073587.1**	Homo sapiens poly (ADP-ribose) polymerase family, member 12 (PARP12)

Table 4.2: BLASTP results of the identified prey clones.

Clone	Molecular Functions	
9	**BTF3**	Regulation of transcription, DNA dependent RNA Polymerase II transcription factor activity
19	**POMP**	Immune response Protein folding Protein binding Signal transducer activity Nucleic acid binding
20	**MEF2B**	Sequence specific DNA binding Transcription factor activity
88	**DPP7**	Proteolysis Protein binding Serine-type endopeptidase activity Aminopeptidase activity
481	**HINT2**	Hydrolase activity Nucleotide binding
528	**PARP12**	Zinc ion binding Nucleic acid binding Metal ion binding RNA binding NAD+ ADP- ribosyltransferase activity

Table 4.3: Reported molecular functions of the identified prey clones.[89-94]

4.5 CONFIRMATION OF PUTATIVE INTERACTIONS IN YEAST CELLS

To ensure that bait-prey interactions between the ThPOK BTB domain and the identified prey proteins were solely responsible for yeast cell growth in our interaction trap screen, we re-transformed bait containing yeast cells with prey plasmid DNA isolated from E.coli colonies previously transformed with prey plasmids extracted from selected yeast colonies. The bait construct, pGBKT7 BD-BTB, was transformed into the AH109 yeast strain by the LiOAc-PEG transformation protocol. After selecting yeast colonies that contain the bait plasmid, the prey plasmids selected for further analysis were sequentially transformed back into the yeast cells expressing the bait protein again by the LiOAc-PEG transformation protocol. Yeast cells sequentially transformed with the pGBKT7 BD-BTB bait plasmid and different pACT2 AD prey plasmids were streaked on SD (–LT) and SD (–LTHA) selective plates. Yeast cells that contain both the bait plasmid and the prey plasmid, grew on SD (–LT) plate. The presence of an interaction between the BTB bait protein and the prey protein activated His and Ade synthetic genes, and enabled yeast cells grow on SD (–LTHA) plate. Yeast cells bearing the pGBKT7 BD-BTB plasmid and the pACT2 AD empty prey plasmid did not grow on SD (-LTHA) selective plate. This observation showed that the BTB domain as a bait protein is not auto-reactive and the transcription of reporter genes is not activated without an interacting prey protein. (Figure 4.5)

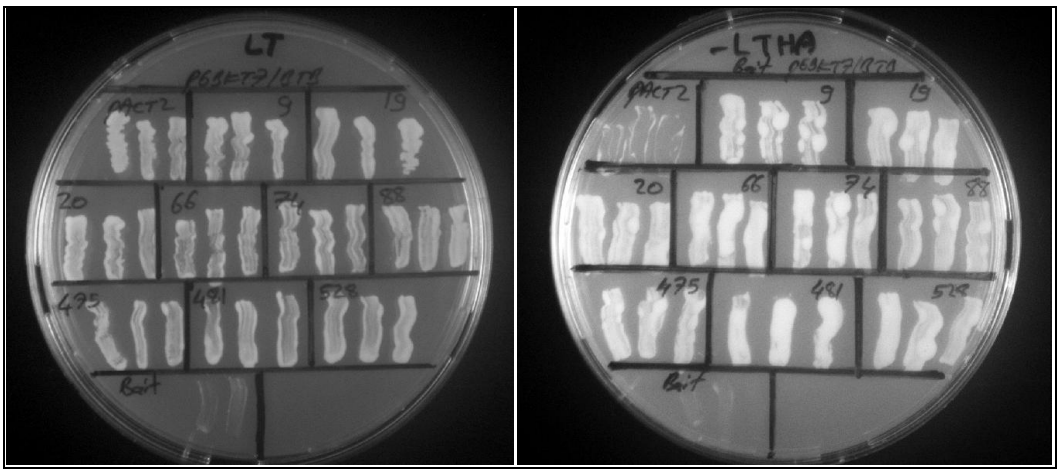


Figure 4.5: Confirmation of putative interactions in yeast cells containing the pGBKT7 BD-BTB plasmid.

Left: SD (-LT) plate, Right: SD (-LTHA) plate, Three colonies for each transformation was streaked onto each plate. All yeast contained the pGBKT7 BD-BTB bait plasmid, in addition, colonies were transformed with the following prey plasmids: clone numbers from left to right: pACT2, 9, 19; 20, 66, 74, 88; 475, 481, 528; pGBKT7 BD-BTB

In order to test the auto-reactivity of the prey proteins, the identified prey plasmids were also sequentially transformed into the yeast cells already transformed with the pGBKT7 BD empty bait plasmid using the LiOAc-PEG transformation protocol. Yeast cells sequentially transformed with the pGBKT7 BD empty bait plasmid and different pACT2 AD prey plasmids, were streaked on SD (–LT) and SD (–LTHA) selective plates. Yeast cells that contain both the bait plasmid and the prey plasmid, grew on SD (–LT) plate. Yeast cells bearing the pGBKT7 BD empty bait plasmid and pACT2 AD prey plasmid did not grow on SD (-LTHA) selective plate. This observation showed that the identified prey proteins are not auto-reactive and that the transcription of His and Ade reporter genes is not activated without the presence of an interacting ThPOK BTB domain. (Figure 4.6)

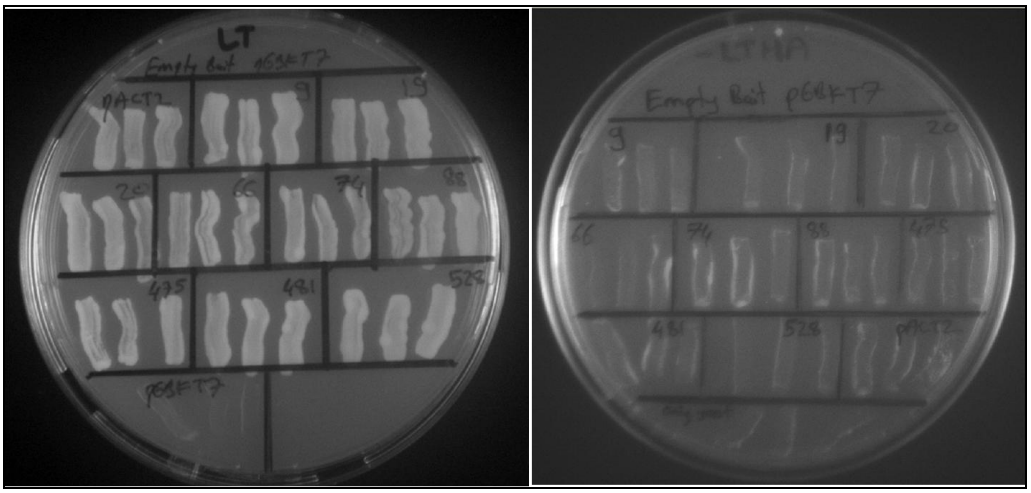


Figure 4.6: Confirmation of putative interactions in yeast cells containing the pGBKT7 BD empty bait plasmid.
Left: SD (-LT) plate, Right: SD (-LTHA) plate, Three colonies for each transformation was streaked onto each plate. All yeast contained the empty pGBKT7 BD bait plasmid, in addition, colonies were transformed with the following prey plasmids: clone numbers from left to right: pACT2, 9, 19; 20, 66, 74, 88; 475, 481, 528; empty bait plasmid

One of the other transcription factors containing a BTB/POZ domain, the *M. musculus* MAZR protein, was used to test the specificity of the identified interactions between the BTB/POZ domain of the ThPOK protein and the 9 different human thymic prey proteins that were selected for further analysis. The plasmid expressing the MAZR protein was kindly provided by Wilfried Elmeier (Institute of Immunology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Austria). The coding region of MAZR was cloned into the pCMV-Myc plasmid using EcoRI restriction sites in frame with the Myc tag. After this sub-cloning step, the MAZR coding region was excised with SfiI and SalI restriction sites and cloned into the pGBKT7 BD plasmid in frame with the GAL4 DNA Binding Domain. (Figure 4.7) The pGBKT7 BD-MAZR plasmid was confirmed using different restriction enzyme digestions. The expected band lengths are shown. (Figure 4.8)

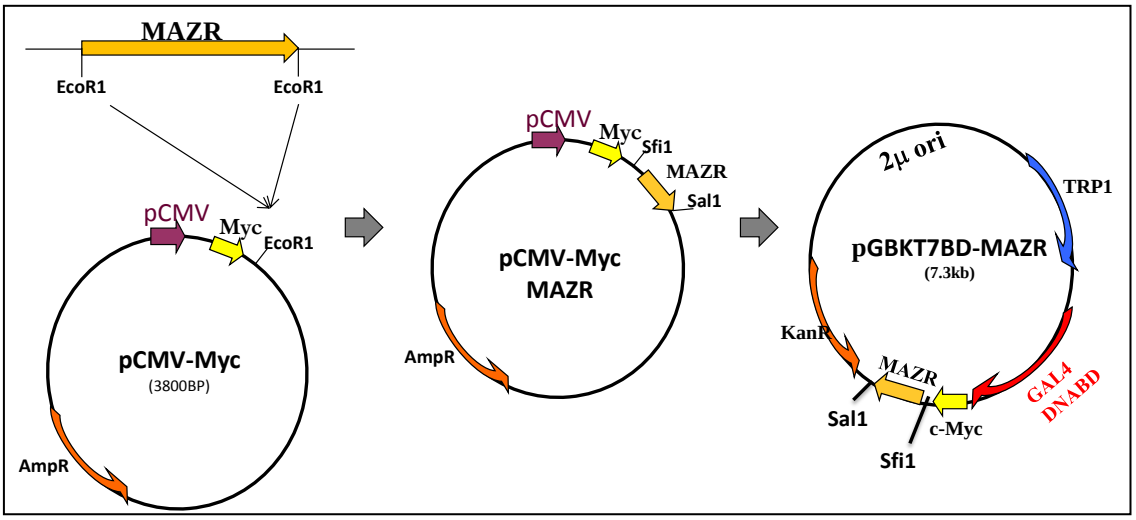
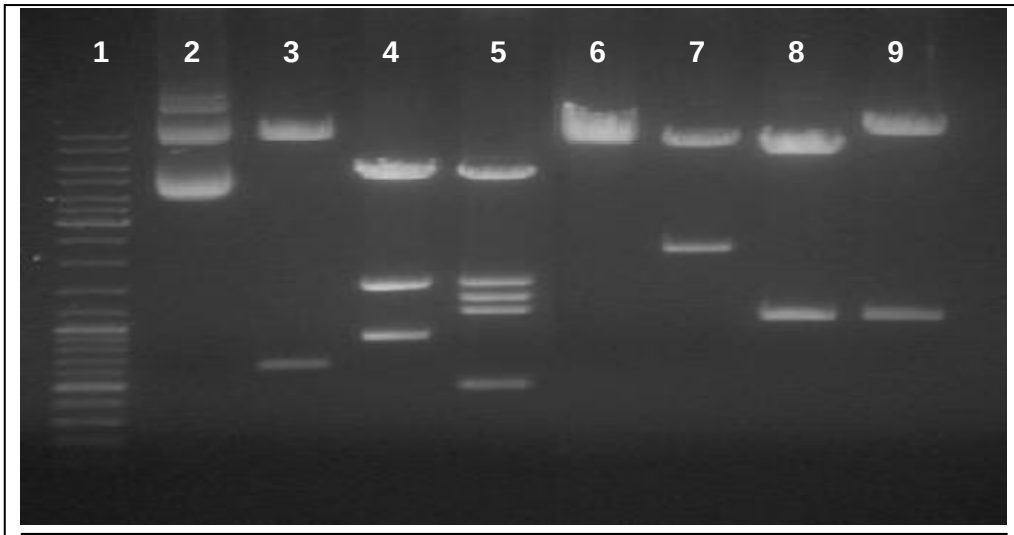


Figure 4.7: The cloning strategy of the pGBKT7 BD-MAZR plasmid.
The pGBKT7 BD-MAZR plasmid contains a 2 micron sequence necessary for plasmid replication in yeast cells, a Trp1 selectible gene (in blue), a Kanamycin resistance gene for selection in bacterial cells (in orange), and a fusion cDNA encoding the GAL4 BD, a c-Myc epitope and the coding sequence of the MAZR protein. The pCMV-Myc plasmid contains a CMV promoter for expression of proteins in mammalian cells (in purple), an Ampicillin resistance gene for selection in bacterial cells and a c-Myc epitope. The pCMV-Myc/MAZR eukaryotic expression plasmid was used as an intermediate to generate the pGBKT7 BD-MAZR yeast bait protein expression plasmid. In future studies we plan to use this intermediate plasmid in co-immunoprecipitation experiments in HEK 293T cell lines.



	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
Plasmid	DNA Ladder Mix	pGBKT7 BD	pGBKT7 BD-MAZR	pGBKT7 BD	pGBKT7 BD-MAZR	pGBKT7 BD	pGBKT7 BD-MAZR	pGBKT7 BD	pGBKT7 BD-MAZR
Rest. enzyme		BglII	BglII	HindIII	HindIII	SalI + SfiI	SalI + SfiI	XbaI	XbaI
Band size		No site	8635, 624, 12	4938, 1498, 868	4938, 1498, 1271, 1112, 452	7282, 22	7282, 1989	6316, 988	8283, 988

Figure 4.8: Confirmation digestion of the pGBKT7 BD-MAZR plasmid.
The expected restriction fragment sizes are indicated for each restriction digest below the gel in base pairs.

In order to test the specificity of the bait-prey protein interactions, isolated prey plasmids were transformed into the yeast cells containing MAZR protein expression plasmid, using the LiOAc-PEG transformation protocol. Yeast cells were sequentially transformed with the pGBKT7 BD-MAZR bait plasmid and different pACT2 AD prey plasmids were streaked on SD (–LT) and SD (–LTHA) selective plates, using the LiOAc-PEG transformation protocol. Yeast cells that contain both the bait plasmid and the prey plasmid, grew on SD (–LT) plates. The presence of an interaction between the MAZR bait protein and different prey proteins activated expression of the essential nutrients, His and Ade, and consequently enabled yeast cells grow on SD (–LTHA) plates. As expected, yeast cells containing the pGBKT7 BD-MAZR plasmid and the pACT2 AD empty prey plasmid did not grow on SD (-LTHA) selective plates. This observation showed that the MAZR protein expressed as a bait in AH109 cells is not auto-reactive and that the transcription of reporter genes is not activated without an interacting prey protein. (Figure 4.9)

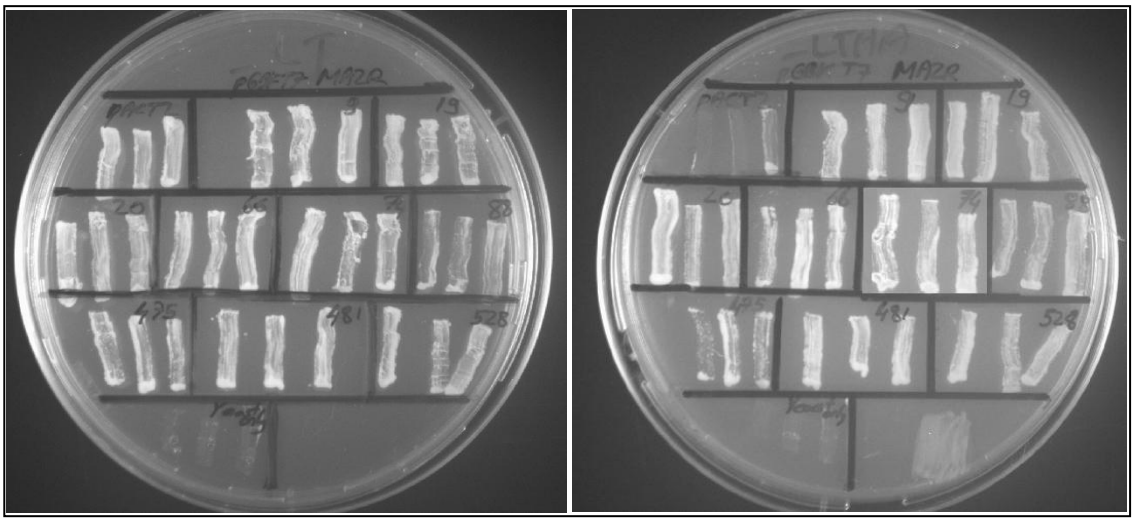
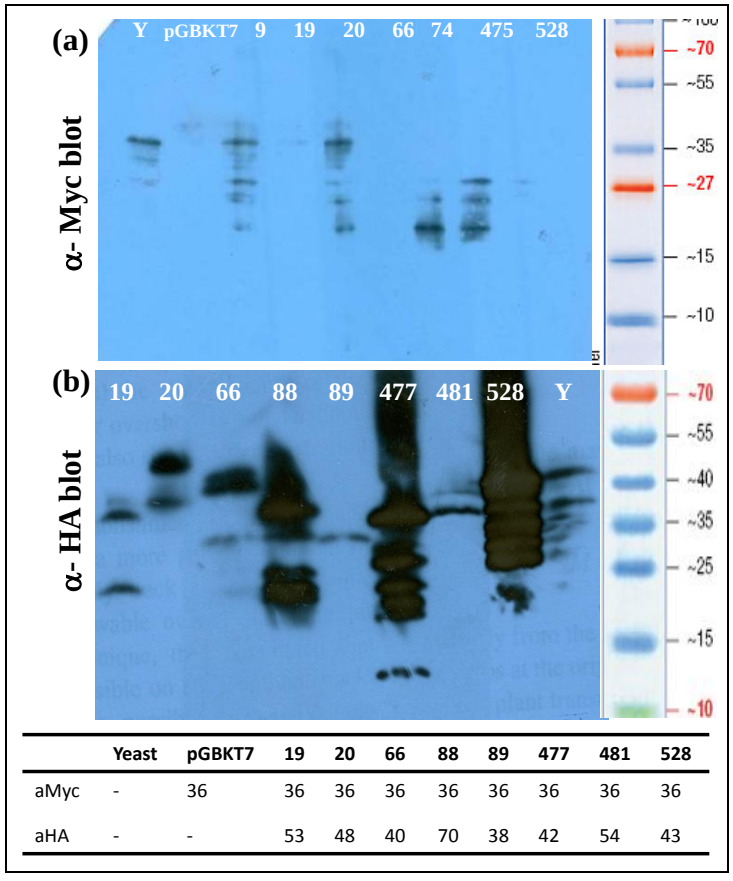


Figure 4.9: Confirmation of putative interactions in yeast cells containing the pGBKT7 BD-MAZR plasmid.
Left: SD (-LT) plate, Right: SD (-LTHA) plate, Three colonies for each transformation was streaked onto each plate. All yeast contained the pGBKT7 BD-MAZR bait plasmid, in addition, colonies were transformed with the following prey plasmids: clone numbers from left to right: pACT2, 9, 19; 20, 66, 74, 88; 475, 481, 528; pGBKT7 BD-MAZR

4.6 CONFIRMATION OF BAIT AND PREY PROTEIN EXPRESSION IN YEAST CELLS

To demonstrate that bait and prey proteins could be expressed in AH109 yeast cells, we performed western blotting experiments. Yeast cells expressing both bait and prey proteins were lysed, run on 13% denaturing acrylamide gels, transferred onto PVDF membranes and blotted with peroxidase coupled anti-c-Myc antibodies to detect the Myc epitope tagged GAL4 BD-BTB protein and peroxidase coupled anti-HA antibodies to detect the HA epitope tagged GAL4 AD-prey proteins. Wild type AH109 strain yeast cells and yeast cells transformed with both pACT2 AD and pGBKT7 BD empty plasmids were used as controls. In the α-Myc blotted membranes, the expected 36KDa band that corresponds to GAL4 BD fused BTB domain with Myc tag could not be observed. However, in the lanes of containing clones 9, 20, 66 and 74, a 20kDa band was observed that may correspond to the Myc tagged BTB protein. (Figure 4.10a)

In the α-HA blotted membranes, clones 20, 66 and 528 gave the expected bands of 48kDa, 40kDa and 43kDa, respectively that correspond to GAL4 AD fused prey proteins with the HA tag. We observed the bands of 25kDa, 42kDa and 14kDa that may correspond to only HA tagged prey proteins, in blots containing lysates from yeast cells expressing clones 19, 88 and 477, respectively. The other clones gave unexpectedly smaller bands that were either nonspecific bands or degraded versions of the GAL4 AD fused prey proteins with the HA tag. (Figure 4.10b) We conclude that these experiments demonstrate the expression of some of the (19, 20, 66, 88, 477, 528) prey clones in AH109 yeast cells, but that further experiments need to be conducted to definitively demonstrate that all clones of interest can be expressed in these cells. The inability to detect robust protein expression in yeast cells likely stems from the known problems of conducting western blots using yeast lysates which express small amounts of heterologous proteins and contain large amounts of proteases that degrade proteins.



	Yeast	pGBKT7	19	20	66	88	89	477	481	528
aMyc	-	36	36	36	36	36	36	36	36	36
aHA	-	-	53	48	40	70	38	42	54	43

Figure 4.10: Yeast western blots of different clones.
Y stands for untransformed yeast lysates. Detection of the Myc tagged bait protein (a) and detection of HA tagged prey proteins (b). The expected protein sizes are indicated for each blot below the gel in kiloDaltons.

4.7 RE-CONFIRMATION OF PUTATIVE INTERACTIONS IN MAMMALIAN SYSTEMS

4.7.1 Cloning of bait and prey coding regions into mammalian expression plasmids

Next, we wanted to re-confirm the interactions we identified in yeast cells in mammalian cells by overexpressing bait and prey proteins without the GAL4 BD and AD fusions in HEK 293T cell lines. To do this, we first transferred the bait and various prey cDNAs from yeast expression plasmids into mammalian expression plasmids. The pCDNDA3.1 Hygro from Invitrogen, pCMV-HA and pCMV-Myc plasmids from Clontech were used to express bait and prey proteins in mammalian systems. A fragment encoding the BTB domain of the ThPOK protein was excised from the pGBKT7 BD-BTB plasmid using SfiI and SalI retriction enzymes, and was cloned into SfiI and SalI restriction enzyme sites of the pCMV-Myc plasmid, in frame with the sequence encoding Myc epitope of the pCMV-Myc plasmid. (Figure 4.11)

Three different strategies were used to clone the coding regions of prey proteins into mammalian expression plasmids. As the human thymic cDNA library was cloned into the pACT2 AD prey plasmid through EcoRI and XhoI restriction sites, the first strategy was to use EcoRI and XhoI restriction sites to transfer the prey coding regions into the pCMV-HA plasmid in frame with the HA-tag. For the prey sequences including internal EcoRI restriction sites, BamHI and XhoI restriction enzymes were used to transfer the relevant sequences into the pCMV-HA plasmid using the compatible BglII and XhoI restriction sites in frame with the sequences encoding HA-tag. As a third strategy, the prey cDNA sequences having more than one XhoI site were excised out of the pACT2 AD plasmid along with sequences encoding the HA tag using BglII restriction sites and cloned into the pCDNA3.1 Hygro plasmid using the compatible BamHI restriction site. (Figure 4.12) Prey cDNAs, 9, 66, 74, 88, 475, 481 and 528 were cloned into the pCMV-HA plasmid and the prey cDNAs 19 and 20 were cloned into the pCDNA3.1-Hygro plasmid.

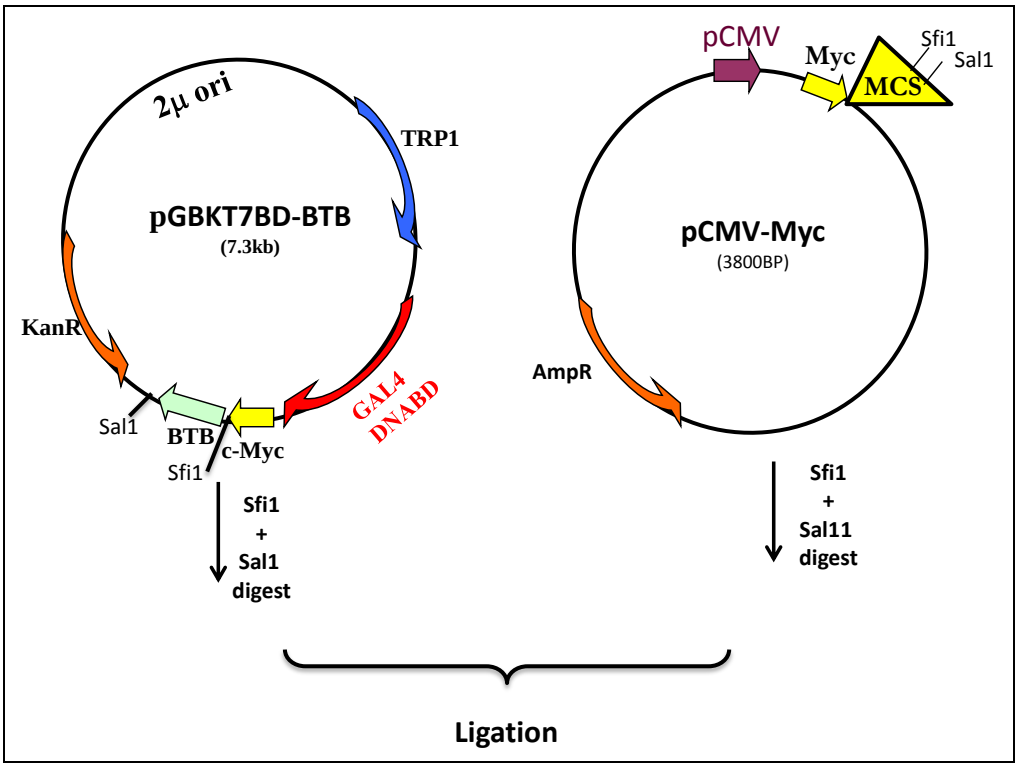


Figure 4.11: The cloning strategy employed to transfer the coding region of the BTB domain into the pCMV-Myc plasmid.

The pGBKT7 BD-BTB plasmid contains a 2 micron sequence necessary for plasmid replication in yeast cells, a Trp1 selectible gene (in blue), a Kanamycin resistance gene for selection in bacterial cells (in orange), and a fusion cDNA encoding the GAL4 BD, a c-Myc epitope and the ThPOK BTB domain. The pCMV-Myc plasmid contains a CMV promoter for expression of proteins in mammalian cells (in purple), an Ampicillin resistance gene for selection in bacterial cells and a c-Myc epitope.

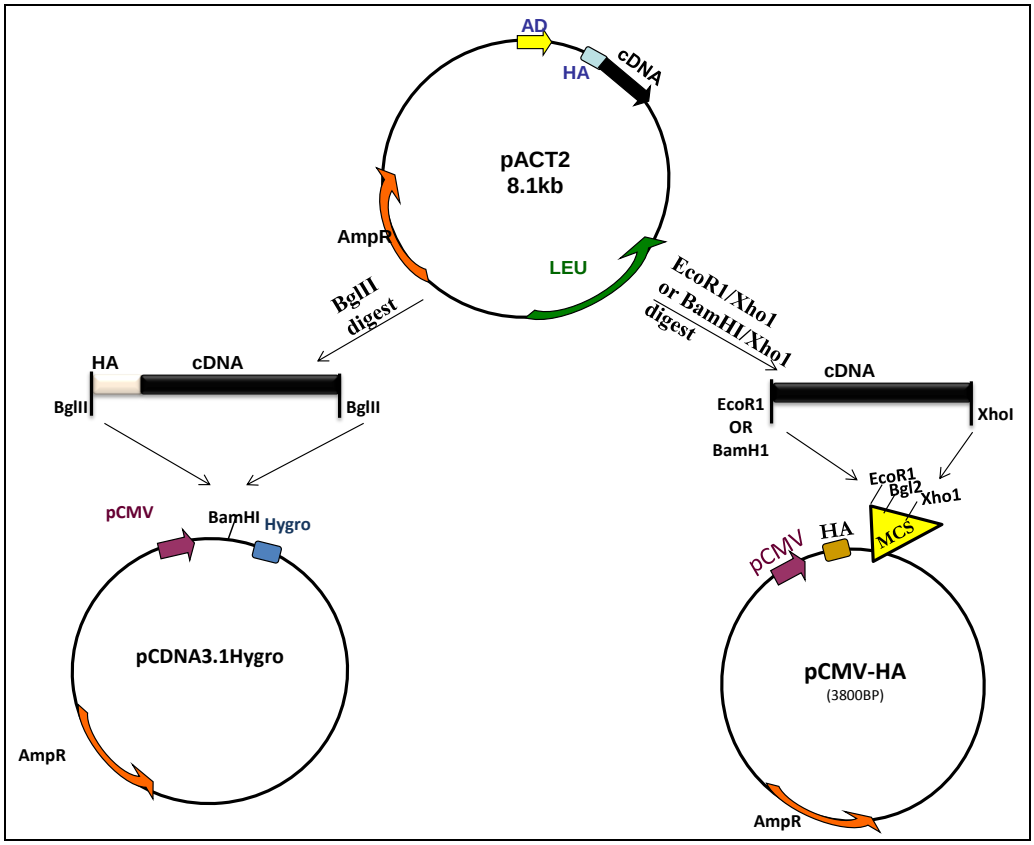
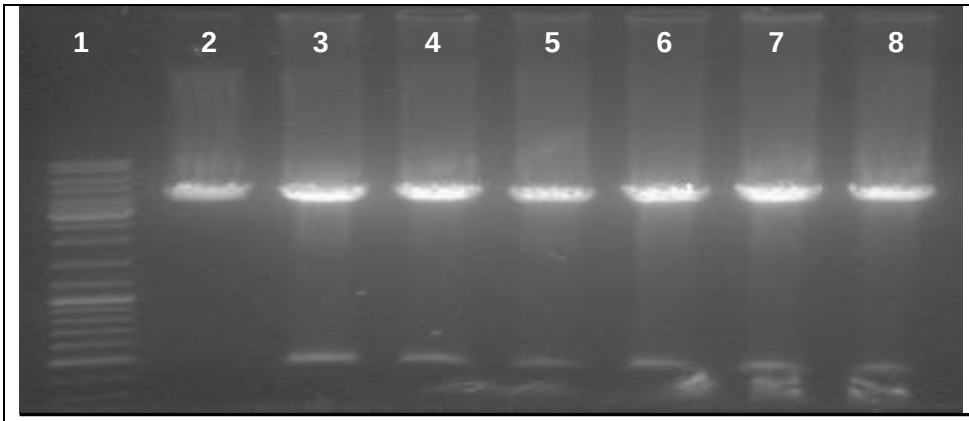


Figure 4.12: The cloning strategy employed to transfer the coding regions of prey proteins into different mammalian expression plasmids.

The pACT2 AD plasmid contains a 2 micron sequence necessary for plasmid replication in yeast cells, a Leu selectible gene (in green), an Ampicillin resistance gene for selection in bacterial cells (in orange), and a fusion cDNA encoding the GAL4 AD, an HA epitope and the prey cDNA. The pCMV-HA plasmid contains a CMV promoter for expression of proteins in mammalian cells (in purple), an Ampicillin resistance gene for selection in bacterial cells and an HA epitope.pCDNA3.1 Hygro plasmid contains a CMV promoter for expression of proteins in mammalian cells (in purple), an Ampicillin resistance gene for selection in bacterial cells and a Hygromycin resistance gene for selection in mammalian cells.

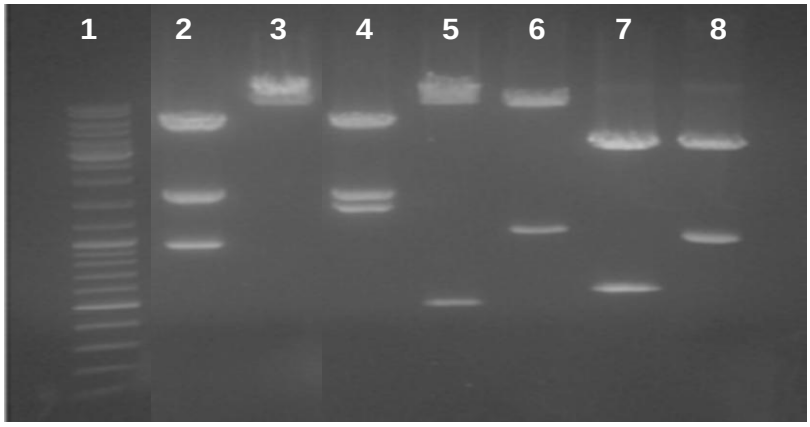
The constructed plasmids were confirmed by different restriction enzyme digestions. The BTB domain cloned into the pCMV-Myc plasmid was confirmed by digesting it with the EcoRI and BglII restriction enzymes. (Figure 4.13) Further confirmation of the plasmid pCMV-Myc BTB along the pGBKT7 BD-BTB plasmid was performed with different restriction enzymes and the expected bands are shown. (Figure 4.14) The prey cDNA clones that are cloned into different mammalian expression plasmids were confirmed with different restriction enzyme digestions. The

expected restriction fragment sizes are indicated for each restriction digest below the gel in base pairs. (Figure 4.15, Figure 4.16 and Figure 4.17)



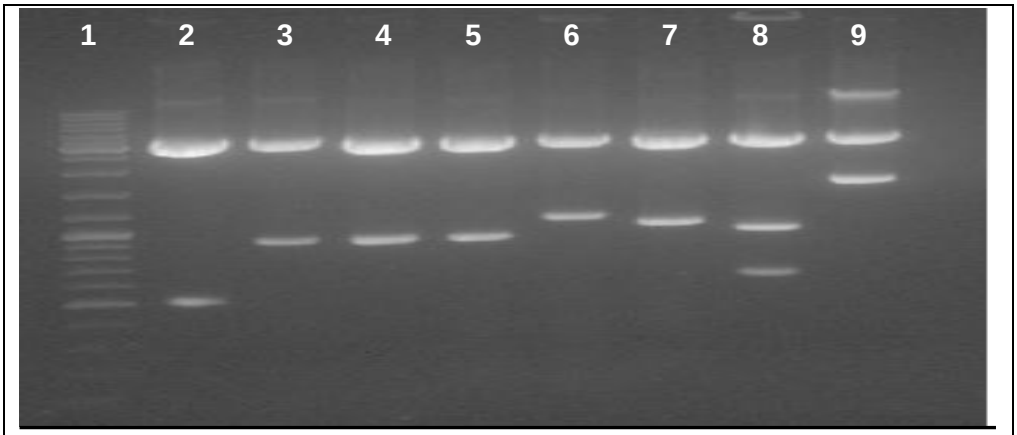
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Plasmid	DNA Ladder Mix	pCMV-Myc	pCMV-Myc BTB 1	pCMV-Myc BTB 2	pCMV-Myc BTB 3	pCMV-Myc BTB 4	pCMV-Myc BTB 5	pCMV-Myc BTB 6
Rest. enzyme		EcoRI + BglII	EcoRI + BglII	EcoRI + BglII	EcoRI + BglII	EcoRI + BglII	EcoRI + BglII	EcoRI + BglII
Band size		3776, 15	3775, 420, 24	3775, 420, 24	3775, 420, 24	3775, 420, 24	3775, 420, 24	3775, 420, 24

Figure 4.13: Confirmation digestion of the pCMV-Myc BTB plasmid



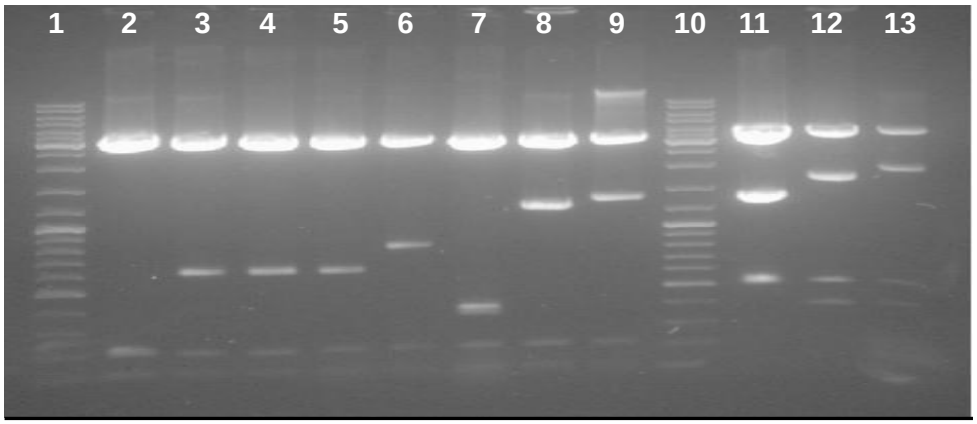
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Plasmid	DNA Ladder Mix	pGBKT7 BD	pGBKT7 BD	pGBKT7 BD-BTB	pGBKT7 BD-BTB	pGBKT7 BD-BTB	pCMV-Myc	pCMV-Myc BTB
Rest. enzyme		HindIII	EcoRI	HindIII	EcoRI	XbaI	XbaI	XbaI
Band size		4938, 1498, 868	7304	4938, 1498, 1238	7304, 432	6736, 988	3282, 509	3282, 937

Figure 4.14: Confirmation digestion of the pGBKT7 BD-BTB and pCMV-Myc BTB plasmids.



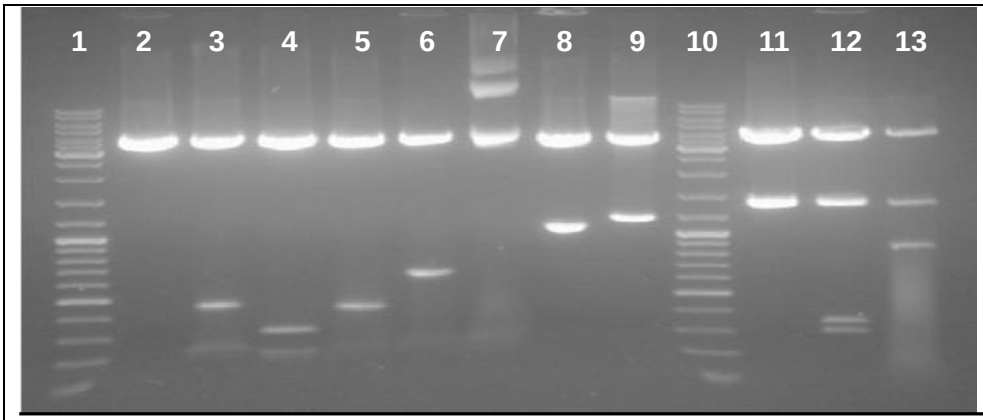
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
Plasmid	DNA Ladder Mix	pCMV-HA	pCMV-HA 9	pCMV-HA 66	pCMV-HA 74	pCMV-HA 475	pCMV-HA 481	pCMV-HA 528	pCMV-HA 88
Rest. enzyme		XbaI	XbaI	XbaI	XbaI	XbaI	XbaI	XbaI	XbaI
Band size		3282, 500	3282, 929	3282, 940	3282, 896	3282, 1100	3282, 1130	3282, 956, 749	3282, 1641

Figure 4.15: Confirmation digestion of the mammalian plasmids expressing different prey proteins with the XbaI restriction ezyme.



	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13
Plasmid	DNA Ladder Mix	pCMV-HA	pCMV-HA 9	pCMV-HA 66	pCMV-HA 74	pCMV-HA 475	pCMV-HA 481	pCMV-HA 528	pCMV-HA 88	DNA Ladder Mix	pCDNA3.1 Hygro	pCDNA3.1 Hygro-19	pCDNA3.1 Hygro-20
Rest. En.		BamHI	BamHI	BamHI	BamHI	BamHI	BamHI	BamHI	BamHI		NcoI	NcoI	NcoI
Band size		3294, 212, 198, 78	3294, 641, 198, 78	3294, 652, 198, 78	3294, 608, 198, 78	3294, 812, 198, 78	3294, 450, 392, 198, 78	3294, 1417, 198, 78	3294, 1353, 198, 78		3740, 1352, 509	3740, 1684, 509, 379	3740, 1899, 509, 379

Figure 4.16: Confirmation digestion of the mammalian plasmids expressing different prey proteins with BamHI and NcoI restriction enzymes.



	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13
Plasmid	DNA Ladder Mix	pCMV-HA	pCMV-HA 9	pCMV-HA 66	pCMV-HA 74	pCMV-HA 475	pCMV-HA 481	pCMV-HA 528	pCMV-HA 88	DNA Ladder Mix	pCDNA 3.1 Hygro	pCDNA 3.1 Hygro-19	pCDNA 3.1 Hygro-20
Rest. En.		EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI	EcoRI + XhoI		EcoRI	EcoRI	EcoRI
Band size		3761, 21	3761, 450	3761, 294, 125	3761, 417	3761, 600	3761, 651	3761, 1205	3761, 1162		4191, 1410	4191, 1410, 351, 302	4191, 1410, 868

Figure 4.17: Confirmation digestion of the mammalian plasmids expressing different prey proteins with EcoRI, XhoI and NcoI restriction enzymes.

4.7.2 Co-Expression of bait and prey proteins in mammalian cells

All mammalian bait and prey expression plasmids were transfected into HEK 293T cells by the Calcium phosphate protocol. The pCDNAGFP plasmid was also transfected in order to check for the transfection efficiency of HEK293T cells. (Figure 4.18) These transfected cells were analyzed under the fluorescent microscope using a either a visible light or GFP excitation/emission filter indicate that the transfection efficiency was more than 80%.

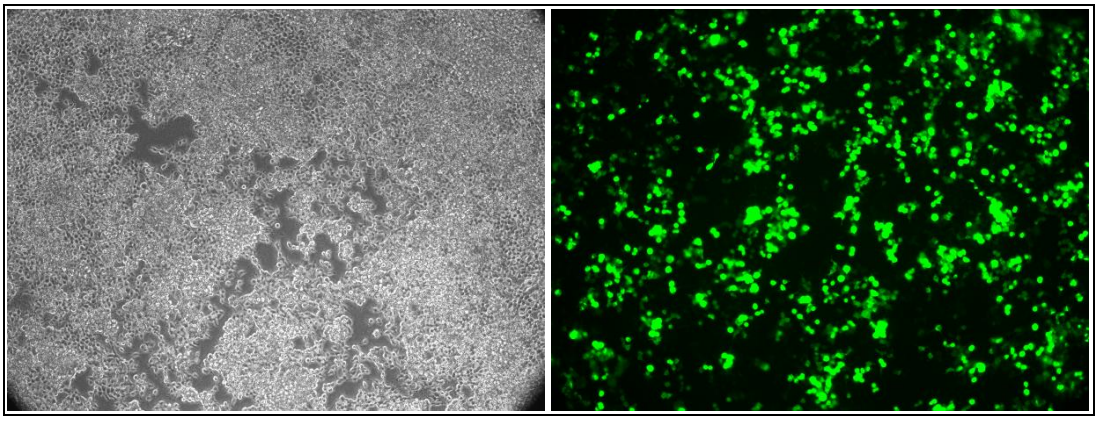
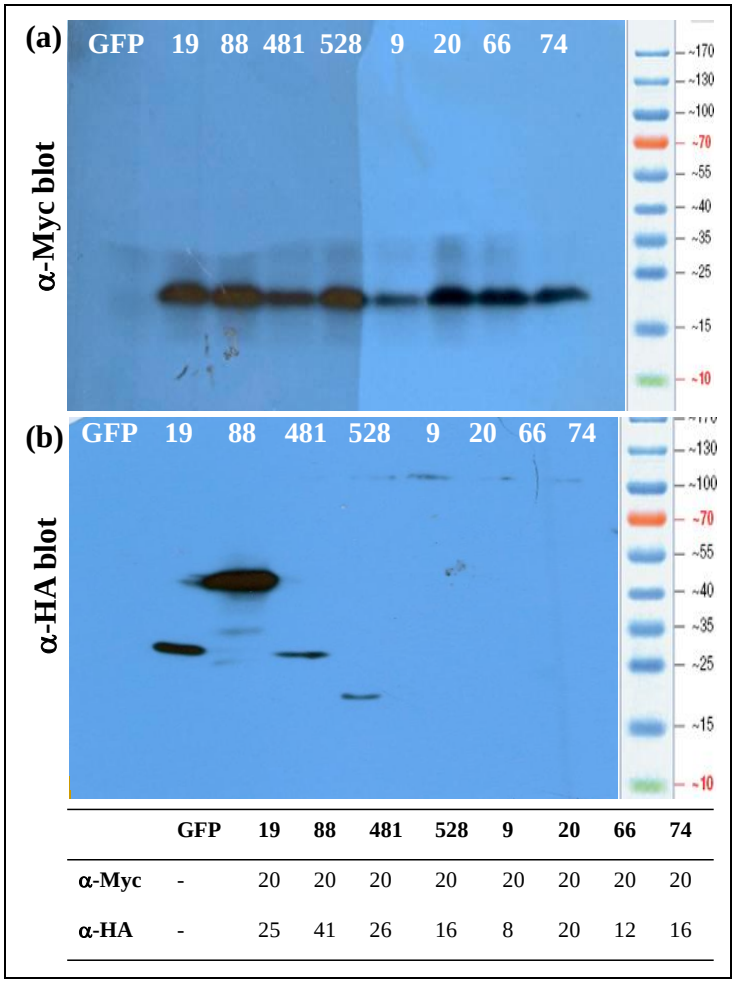


Figure 4.18: HEK 293T cells transfected with the pCDNAGFP plasmid examined under the fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

In order to detect proteins encoded by the transfected bait and prey plasmids, we performed western blot analysis using antibodies against epitope tags present in these plasmids. Equal amounts of lysates from transfections were examined. Anti-c-Myc Peroxidase was used to detect the expression of Myc-tagged bait protein (BTB/POZ domain of the ThPOK protein), whereas anti-HA Peroxidase was used to blot the expression of HA-tagged prey proteins from the same lysates. HEK 293T cells were co-transfected with the pCMV-Myc BTB plasmid and different mammalian prey plasmids. The lysates from each transfection were loaded on two different SDS gels and blotted with either anti HA or -Myc. In the anti Myc blot, an expected band of 20kDa that corresponds to the Myc tagged BTB protein is observed in all lysates of cells transfected with the pCMV-Myc BTB plasmid. In the anti HA blot, clones 19, 88, 481 and 528 gave the expected bands of 25kDa, 41kDa, 26kDa and 16kDa, respectively. For the other clones, expected bands could not be detected. (Figure 4.19)



	GFP	19	88	481	528	9	20	66	74
α-Myc	-	20	20	20	20	20	20	20	20
α-HA	-	25	41	26	16	8	20	12	16

Figure 4.19: Expression of the Myc tagged bait protein and HA tagged prey proteins in HEK 293T cells
Detection of the Myc tagged BTB protein (a) and detection of HA tagged prey proteins (b). The expected protein sizes are indicated for each blot below the gel in kiloDaltons.

4.7.3 Immunoprecipitation of the bait and putative interacting prey proteins

For each immunoprecipitation experiment, the transfection efficiency of HEK293T cells was identified by co-transfecting the pCDNA-GFP plasmid. (Figure 4.20, 4.22, 4.24, 4.26 and 4.28) After co-transfection of mammalian bait and prey plasmids into HEK293T cells in 100mm tissue culture dishes, lysates were immunoprecipitated with either immobilized anti-c-Myc beads to precipitate the Myc tagged BTB protein or immobilized anti-HA beads to precipitate HA-tagged prey proteins. In order to confirm the putative interaction between the bait and prey proteins in mammalian cells, anti-c-Myc immunoprecipitated (IP) lysates were blotted with anti-

HA Peroxidase and then with the anti-c-Myc Peroxidase for confirmation, whereas anti-HA immunoprecipitated lysates were blotted with anti-c-Myc Peroxidase and then with the anti-HA Peroxidase for confirmation. HA tagged putative interactors 19, 481 and 528 were first immunoprecipitated with anti-HA beads and blotted with the anti Myc to detect the Myc tagged BTB protein in the presence of an interaction.

In all the western blots shown in this section, the first four lanes contain lysates of transfected HEK 293T cells to demonstrate that bait and prey proteins indeed are overexpressed in tissue culture experiments and the last four lanes contain anti-HA antibody immonuprecipitated lysates of the same transfections.

Immunoprecipitation of the clone 19 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 19 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein. The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged prey proteins. (Figure 4.21a)To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. The detection of a 25kDa band in lane 7 and lane 8 indicates that in the anti-Myc blot, there was HA tagged prey protein 19 in these anti-HA immunoprecipitates. This result demonstrates that anti-HA immunoprecipitation specifically brings down Myc epitope tagged BTB proteins only in lysates that contain both proteins. (Figure 4.21b)

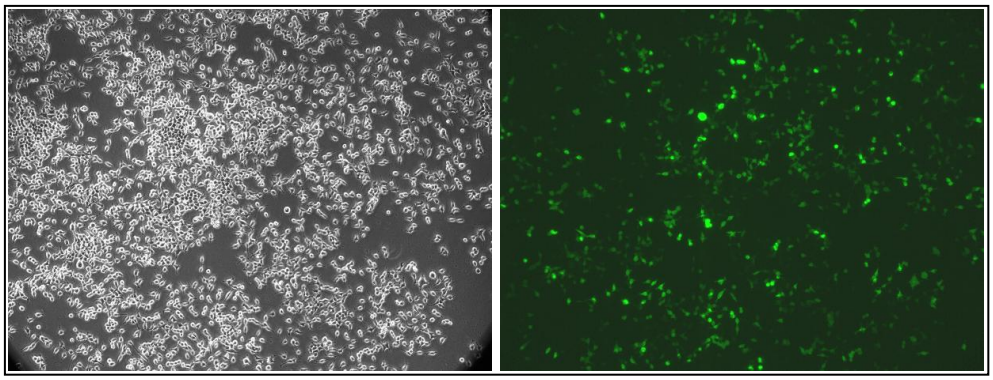


Figure 4.20: Clone 19 IP experiment: HEK 293T cells transfected with the pCDNAGFP plasmid were examined under the fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

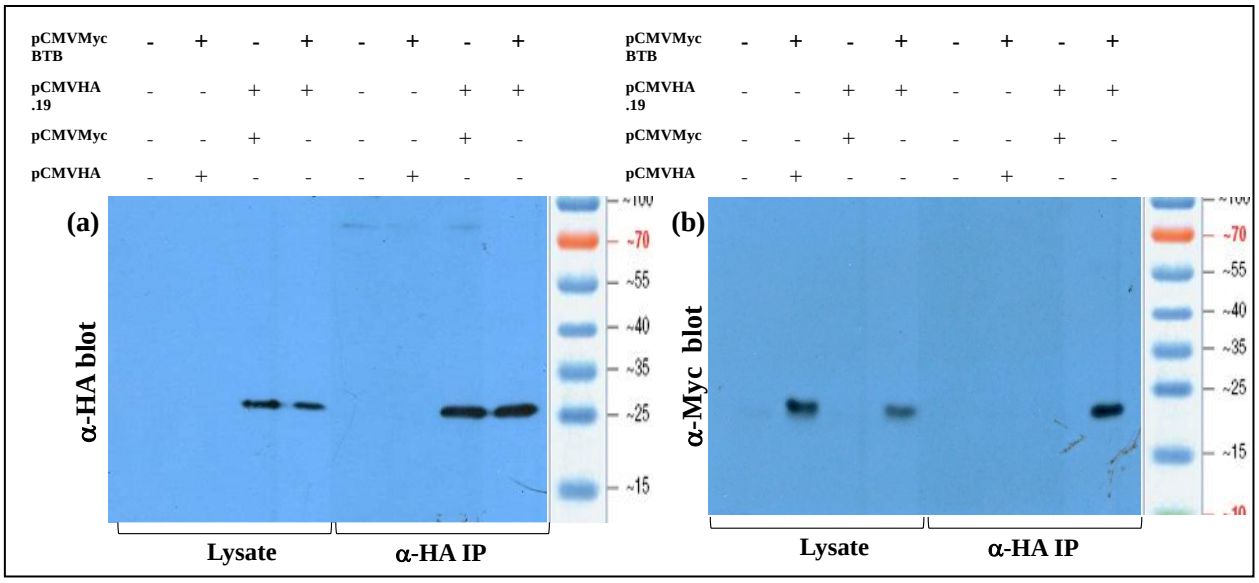


Figure 4.21: Interaction of the clone 19 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP followed by α-HA (a) and α-Myc (b) blots. In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 481 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 481 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged prey proteins. (Figure 4.23a) To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. The detection of a 26kDa band in lane 7 and lane 8 indicates that in the anti-Myc blot, there was HA tagged prey protein 481 in these anti-HA immunoprecipitates. This result demonstrates that anti-HA immunoprecipitation specifically brings down Myc epitope tagged BTB proteins only in lysates that contain both proteins. (Figure 4.23b)

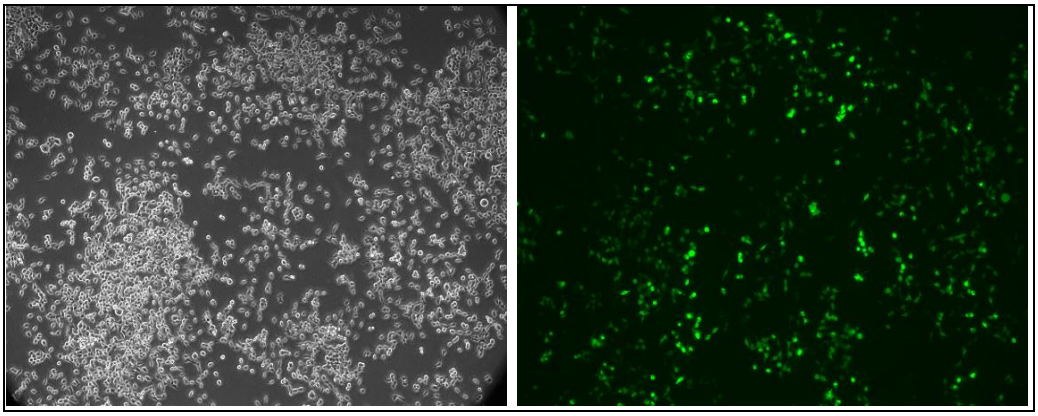


Figure 4.22: Clone 481 IP experiment: HEK 293T cells transfected with the pCDNAGFP plasmid were examined under fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

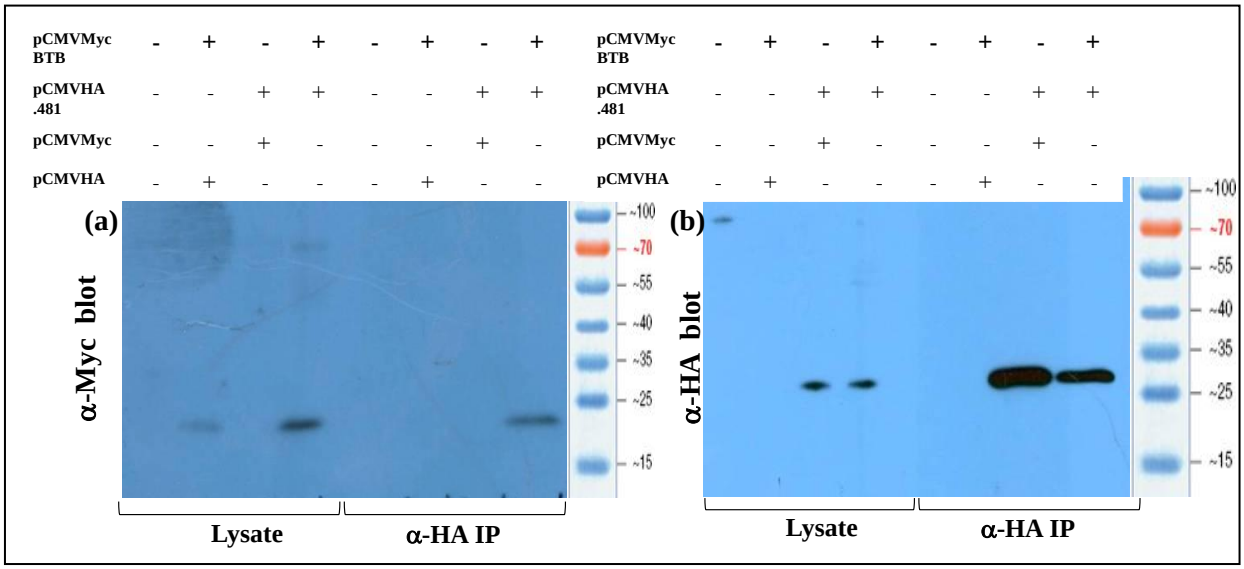


Figure 4.23: Interaction of the clone 481 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP followed by by α-HA (a) and α-Myc (b) blots. In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 528 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 528 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged prey proteins. (Figure 4.25a) To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. The

detection of a 16kDa band in lane 7 and lane 8 indicates that in the anti-Myc blot, there was HA tagged prey protein 528 in these anti-HA immunoprecipitates. This result demonstrates that anti-HA immunoprecipitation specifically brings down Myc epitope tagged BTB proteins only in lysates that contain both proteins. (Figure 4.25b)

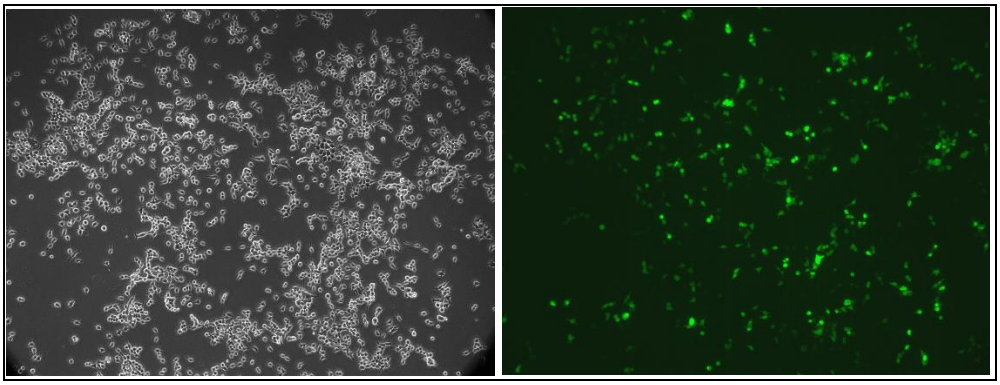


Figure 4.24: Clone 528 IP experiment: HEK 293T cells transfected with the pCDNAGFP plasmid were examined under fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

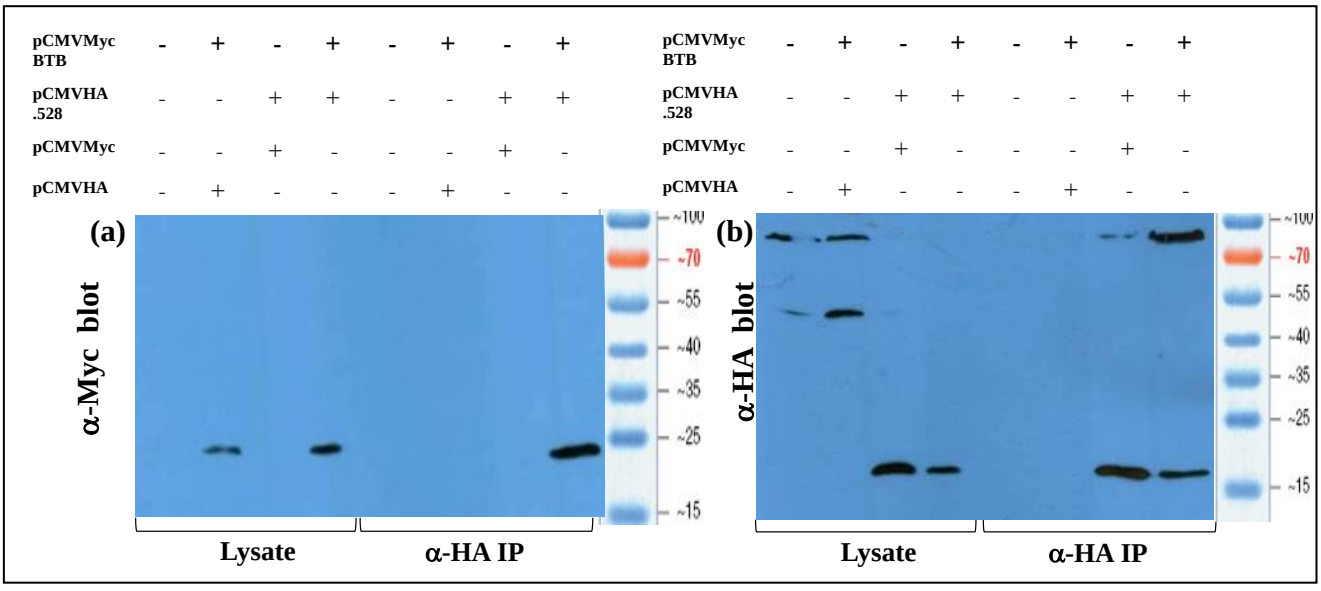


Figure 4.25: Interaction of the clone 528 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP followed by by α-HA (a) and α-Myc (b) blots. In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 88 prey protein was performed with anti-c-Myc beads and protein complexes precipitating with the prey protein 88 were subsequently analyzed with anti-HA blot. Observation of a 41kDa band in lane 3 and lane 4 revealed the expression of the HA tagged prey protein 88. The last lane in this experiment demonstrates that HA tagged clone 88 prey protein can be immunoprecipitated with Myc tagged BTB protein. To demonstrate that this interaction is specific, we re-blotted the same blot with peroxidase coupled anti-c-Myc antibodies. The detection of a 20kDa band in lane 6 and lane 8 indicates that in the anti-Myc blot, there was Myc tagged BTB protein in these anti-Myc immunoprecipitates. This result demonstrates that anti-HA immunoprecipitation specifically brings down Myc epitope tagged BTB proteins only in lysates that contain both proteins. (Figure 4.27)

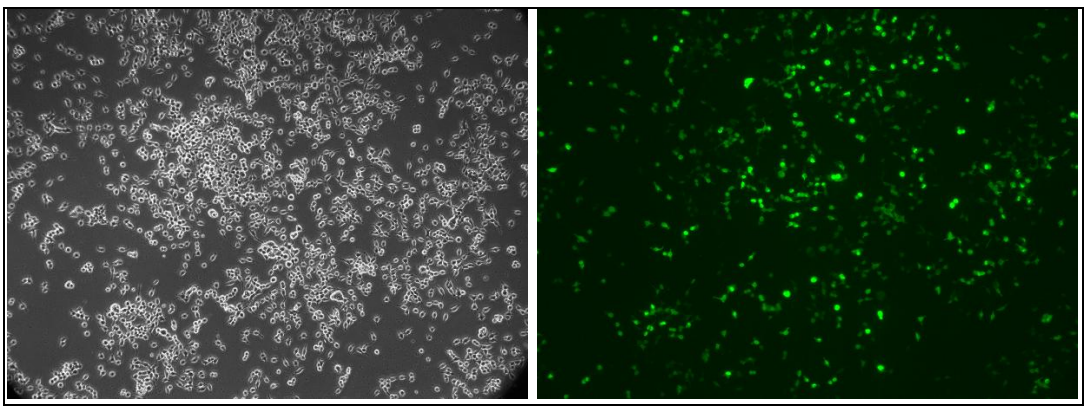


Figure 4.26: Clone 88 IP experiment: HEK 293T cells transfected with the pCDNAGFP plasmid were examined under the fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

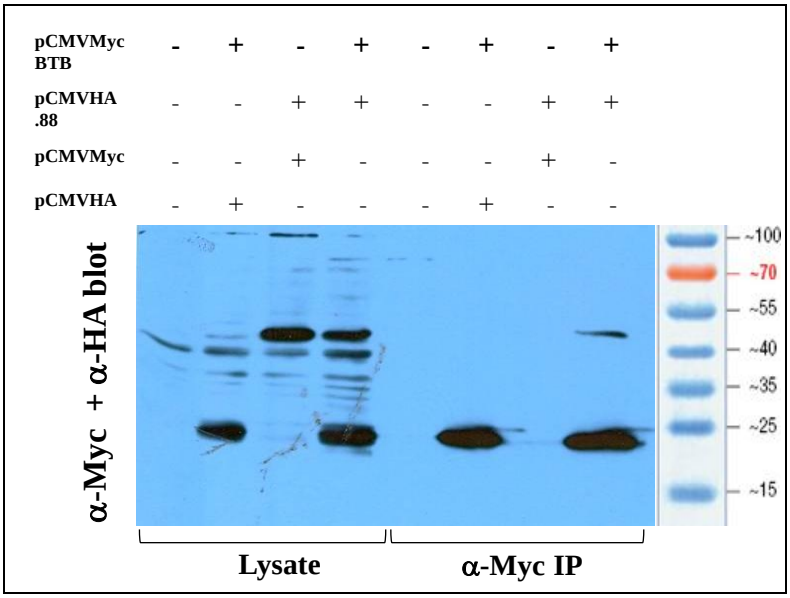


Figure 4.27: Interaction of the clone 88 prey protein with the BTB domain of the ThPOK protein analyzed by α-Myc IP and α-HA and -Myc blots.
In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 20 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 20 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged prey proteins. (Figure 4.29) To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. An expected 20kDa band that corresponds to the HA tagged prey protein 20 could not be detected.

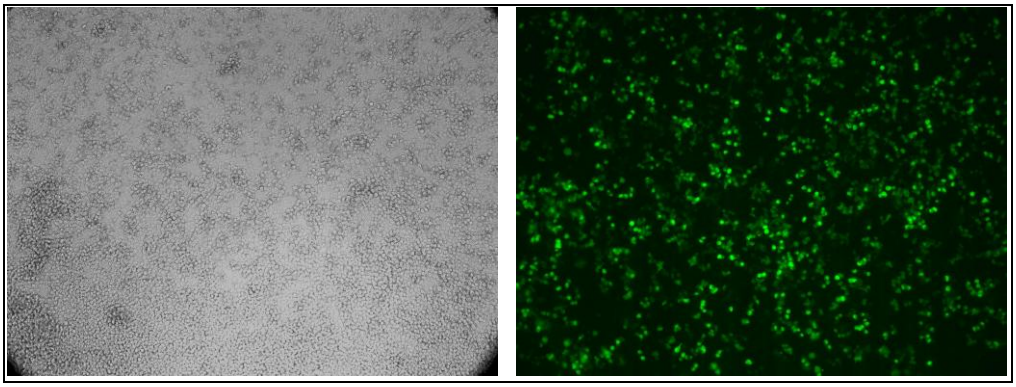


Figure 4.28: Clone 20 IP experiment: HEK 293T cells transfected with the pCDNAGFP plasmid were examined under the fluorescent microscope.
10X magnification, Left: cells under the visible light, right: cells under the fluorescent light using a GFP filter

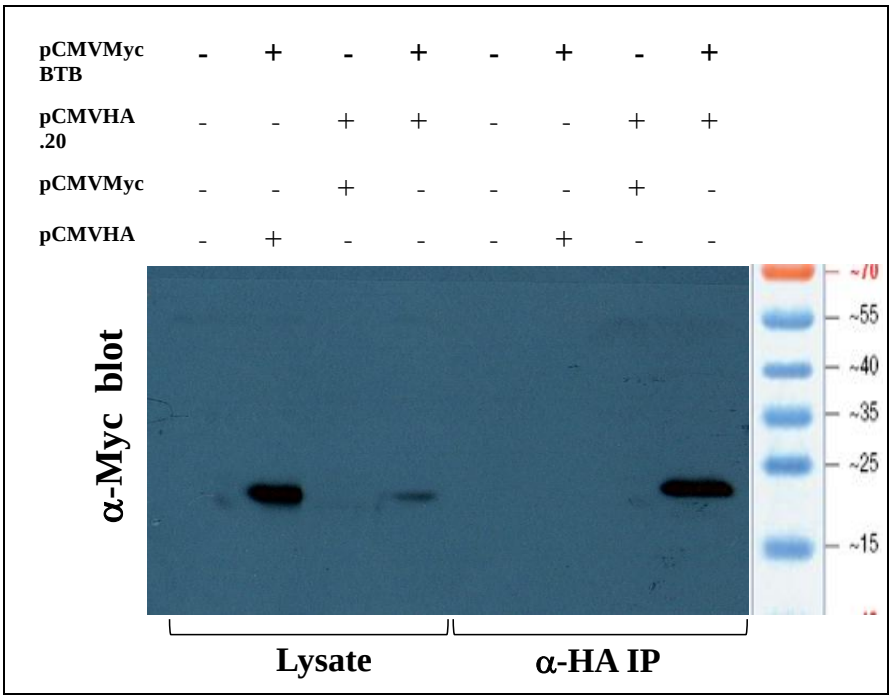


Figure 4.29: Interaction of the clone 20 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP and α-Myc blot.
In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 66 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 66 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged

prey proteins. (Figure 4.30) To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. An expected 12kDa band that corresponds to the HA tagged prey protein 66 could not be detected.

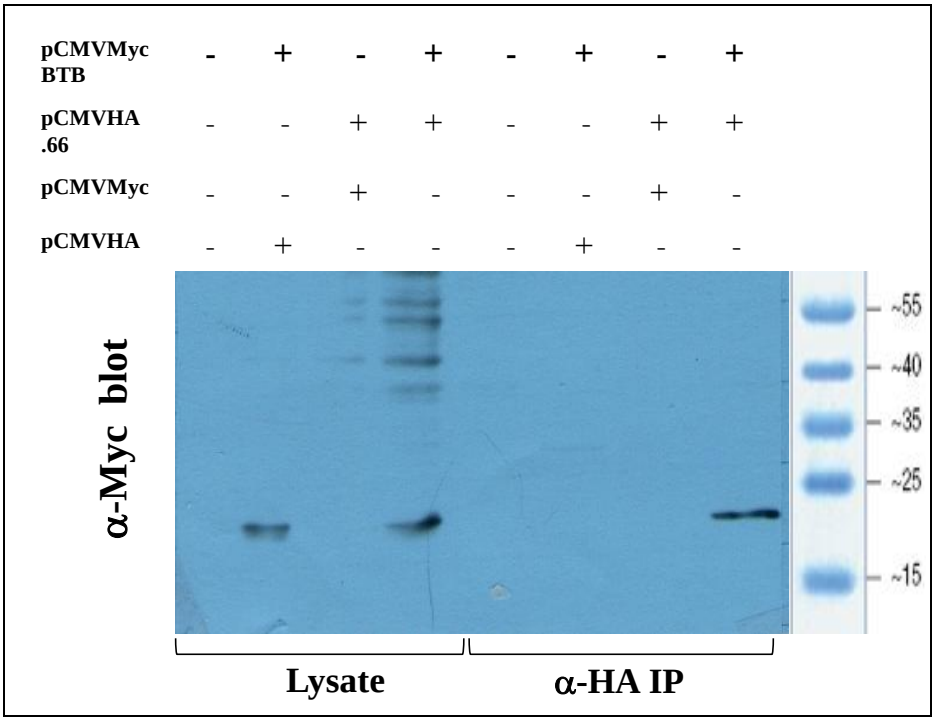


Figure 4.30: Interaction of the clone 66 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP and α-Myc blot.
In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

Immunoprecipitation of the clone 74 prey protein was performed with anti-HA beads and protein complexes precipitating with the prey protein 74 were subsequently analyzed with anti-Myc blot. Observation of a 20kDa band in lane 2 and lane 4 revealed the expression of the Myc tagged BTB protein The last lane in this experiment demonstrates that Myc tagged BTB protein can be immunoprecipitated with HA tagged prey proteins. (Figure 4.31) To demonstrate that this interaction is specific, we also blotted anti-HA immunoprecipitates with peroxidase coupled anti-HA antibodies. An expected 16kDa band that corresponds to the HA tagged prey protein 74 could not be detected.

HEK 293T cells were transfected with pCMV-Myc/BTB, pCMV-HA/74 or co-transfected with pCMV-Myc/BTB and pCMV-HA/74. Lysates from these transfected cells were either directly loaded onto SDS-PAGE for western blotting or immunoprecipitated with anti-HA affinity beads. DNA transfected was kept constant by "filler" DNA which was either the pCMV-HA or pCMV-Myc plasmid. Anti-c-Myc blots of pCMV-Myc/BTB transfected cell lysates revealed the presence of a 20kDa band. This band was absent in cell lysates that were not transfected with pCMV-Myc/BTB (Lanes 1, 3, 5 and 7). Anti-c-Myc western blotting of anti-HA immunoprecipiated lysates revealed a 20kDa Myc tagged BTB band only in lysates containing both Myc tagged BTB domain and HA tagged prey protein 74. We conclude that clone 74 and the BTB domain can interact when overexpressed in HEK293T cells.

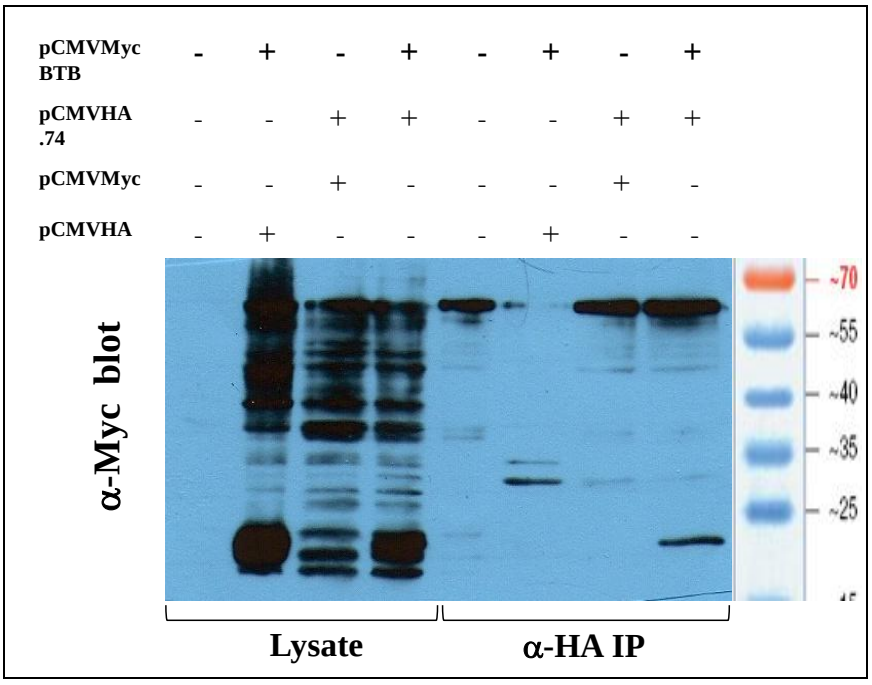


Figure 4.31: Interaction of the clone 74 prey protein with the BTB domain of the ThPOK protein analyzed by α-HA IP and α-Myc blot.
In the table above the figures, '+' sign denotes the plasmids used in transfection of the cells.

5 DISCUSSION

Mature and functional T lymphocytes are the most important line of defense against cancer cells and bacteria or virus infected cells. Maturation of CD4 SP and CD8 SP T cells in the thymus requires the matching of the MHC specificity of the TCR expressed on a thymocyte with the expression of one of the two co-receptors.[8] CD4 SP T cells associated with MHC II are also known as Helper T cells. Once these cells are activated, they rapidly divide and start to secrete cytokines which are involved in the regulation of the immune response. On the other hand, CD8 SP T cells associated with MHC I are also known as Cytotoxic T cells. These cells are involved in the destruction of tumor cells and virally infected cells.[95] However, the actual mechanism of how DP thymocytes differentiate into CD4 or CD8 SP T cells still remains unclear. Understanding the mechanism of this lineage choice is crucial for understanding how T cell mediated immunity occurs. Several models have been proposed to elucidate this process. Furthermore, recent studies have clarified several key molecules having a role in the T cell lineage decision process. ThPOK protein is one of these identified molecules and it is necessary for CD4 lineage commitment. [50, 51]

ThPOK is a C_2H_2-type zinc finger transcription factor, and it contains both a C-terminal Krüppel-like zinc finger domain for DNA binding and an N-terminal BTB-POZ domain for interaction with other transcription factors and protein homo- and hetero-dimerization. The BTB-POZ domain is a protein-protein interaction motif and it is involved in several cellular processes such as protein targeting for ubiquitination, cytoskeleton dynamics, ion channel assembly and gating, and transcriptional regulation.[57, 59] The BTB/POZ domain is crucial for the function of ThPOK during thymocyte lineage decision. Transgenic expression of ThPOK in HD mice was sufficient to disrupt CD8 SP differentiation and promote CD4 lineage; whereas transgenically expressed ThPOK lacking its BTB-POZ domain was not able to favor CD4 lineage differentiation.[58] These findings indicate the necessity of the BTB domain

and the importance of protein-protein interactions in ThPOK function because the upstream regulators and downstream targets of the ThPOK protein are mostly unknown. We decided that the identification of the proteins that interact with this transcription factor would be a good starting point for understanding ThPOK function.

The yeast two hybrid system is a conventional *in vivo* analysis method for identifying protein-protein interactions. As a model organism, *S. cerevisiae* yeast cells are used for trapping eukaryotic proteins that interact. This classical system screens for the interactions between over-expressed bait and prey proteins in yeast nucleus. One disadvantage of the system is that detection is restricted to soluble proteins or soluble portions of membrane proteins. As the system detects the protein interactions indirectly through activated reporter expression, several false-positive interactions could also be detected. [85, 86]

In this study, the classical yeast two hybrid method was used to identify interacting partners of the ThPOK transcription factor. Since the BTB/POZ domain is reported to be crucial for the proper functioning of the ThPOK protein and it is a protein-protein interaction motif, the BTB/POZ domain coding region of *M. musculus* Zbtb7b gene was cloned into the bait plasmid and screened against a human thymic cDNA library. Using bait proteins from the mouse genome and prey proteins from a human thymus cDNA library may have both advantages and disadvantages. The mouse ThPOK-BTB/POZ domain might not interact with the human homologues of truly interacting mouse thymus proteins. On the other hand, the detected interactions between the mouse ThPOK and human thymic proteins might reveal the binding and/or interacting similarities between the mouse and human ThPOK proteins.

After screening the mouse BTB/POZ domain against the human thymic cDNA library, 552 colonies were detected as putative interactors. These colonies were grown on the most stringent yeast growth medium SD (-LTHA). Only a number of these colonies were further tested with β-galactosidase assay in order to increase stringency and get rid of false-positives as much as possible. However, it could have been better to continue to test all 552 colonies with the β-galactosidase assay. Also, mating procedure could have been performed in order to decrease the number of false-positive

interactors. The AH109 yeast strain was used to perform yeast two hybrid screen between the BTB/POZ domain and human thymus proteins. The Y187 yeast strain is an ideal mating partner for the AH109 in a two-hybrid screen. The Y187 yeast cells expressing different bait proteins could have been mated with the AH109 yeast cells expressing prey proteins to eliminate non-specific interactions between bait and prey proteins.

In order to confirm the expression of bait and prey proteins in yeast cells after screening, western blot analysis was performed to detect the Myc-tagged ThPOK/BTB-POZ domain and HA-tagged prey proteins. However, the bait protein ThPOK/BTB-POZ domain could not be detected by anti-Myc blotting. This might be because of the low amount of bait protein expression through its weak promoter. In order to prevent non-specific interactions due to overexpression, the yeast bait expression plasmid was designed with a weak, truncated ADH1 promoter. The expression of bait proteins through this weak promoter might not be adequate to detect the protein expression by western blotting. One other possible reason might be the presence of active proteases in yeast cells that might have caused degradation of the desired proteins in our lysates. This might also explain the observation of many smaller bands below the bands migrating at the expected size, in our western blots. We need to determine the optimum lysis conditions of yeast cells for western blot analysis in their mid-log growth phase, because protein expression is the highest in the log phase of growth and also because most yeast proteases become active when growth enters the stationary phase.

Another possible way of detecting the Myc-tagged BTB/POZ domain expression in yeast cells might be to use anti GAL4-BD antibody instead of anti-Myc antibodies in western blotting experiments. The Myc coding region is between the coding regions of the GAL4-BD and BTB/POZ domains of the pGBKT7 BD plasmid, which encodes a fused protein of GAL4-BD, Myc tag and the BTB/POZ domain. Although western blotting was done under denaturing conditions, the conformational structure of these three protein domains might prevent western blotting antibodies from binding to the Myc epitope properly.

After screening the BTB/POZ domain against the human thymic cDNA library, positive clones were cyro-preserved. As outlined in the methods section, isolated yeast plasmid DNAs were transformed back into bacterial cells in order to select only prey plasmids. Plasmid DNA isolation from yeast cells is more problematic than the one from bacterial cells. As yeast cells have cell walls, it is not easy to destroy the cell wall and isolate the plasmid DNA. Consequently, the yield of DNA mini-preps form yeast cells was consistently lower than those prepared from *E.coli* cells. Due to this reason, transformation of the yeast plasmid DNA into chemically competent bacteria gave small numbers of colonies and sometimes no colonies. Electroporation might have been used instead of the chemical transformation of bacterial cells. Theoretically many more colonies could have been obtained by this method. Furthermore, the low copy number feature of the prey plasmids resulted in lower yields of the plasmid isolation from bacterial cells. Instead of performing restriction enzyme profiling before the sequencing process, colony PCR could have been used to amplify the inserted cDNA regions from yeast colonies. The clones giving more than one PCR bands could have been eliminated from further analysis.

After the sequencing of isolated prey plasmids from positive clones, the sequences of the cDNA regions were compared to human genomic transcripts using NCBI BLAST server. Also, translated sequences of different prey cDNA regions in frame with the activation domains of prey plasmids were aligned to human protein database for correlation between two BLAST results. Nine different clones were selected for confirmation and further analysis among the sequenced clones. These nine clones that correspond to putative interacting proteins are listed in Table 4.1.

BLASTP results of the clones 9, 19, 20, 88, 481 and 528 confirmed that the translated sequences correspond to an expressed protein of our interest. On the other hand, BLASTP results of the clones 66, 74 and 475 indicated that the sequences of these clones corresponded to the untranslated regions in the human genome. Nevertheless, these clones were for further analyzed because of the possibility of previously unknown alternative splicing events resulting in the expression of novel proteins from unidentified exons in their transcripts.

Among the sequenced clones (nearly half of the 552 identified clones), those corresponding to transcription factors, zinc-finger proteins and key proteins involved in T cell development were selected for confirmation and further analysis. Heat shock proteins, ribosomal proteins, mitochondrial proteins, proteosome subunits, elongation factors, cytoskeleton proteins and ferritin were thought to be as common false-positive yeast two hybrid outcomes, and the clones giving false-positive interactions were eliminated from further analysis.

BLASTP alignment of the clone 9 sequence indicated that this clone encoded to the human basic transcription factor 3 (BTF3). BTF3 is required for transcription by RNA polymerase II. Also, it has been shown that BTF3 binds to nascent polypeptide chains.[89, 96, 97] For another putative interactor, clone 19, BLASTP result indicated that this clone encoded to the human proteasome maturation protein (POMP). The proteasome is the key enzyme in the proteolytic cascade required for the generation of peptides presented to CD8 T cells by MHC I molecules. Silencing of POMP expression results in reduced proteasome activity, decreased MHC I surface expression, and induction of apoptosis.[90, 98]

BLASTP alignment of the clone 20 sequence indicated that this clone encoded to the human myocyte enhancer factor 2B (MEF2B). TCR signaling leading to thymocyte apoptosis is mediated through the expression of the Nur77 family of orphan nuclear receptors. MEF2 has been shown to be the major transcription factor responsible for calcium-dependent Nur77 transcription.[99, 100]

BLASTN alignment of the clone 66 sequence indicated that this clone encoded to the untranslated region of the human transcription factor-7 (TCF7). The transcription factor 7 (TCF7) is a downstream effector of the WNT signaling pathway, that is a critical regulator of T cell development in the thymus. TCF7 is found to be expressed not only in thymocytes, but also in mature T cells. TCF7 share common protein motifs, in particular, the C-terminal HMG domain of both proteins is responsible for DNA binding, while a β-catenin-binding domain at the N terminus mediates the interaction with β-catenin. Interestingly, there are multiple TCF7 protein isoforms with distinct functional properties.[101]

BLASTN alignment of the clone 74 sequence indicated that this clone encoded to the untranslated region of the transcription factor ZNF384. It is a nucleocytoplasmic shuttle protein and a transcription factor. It belongs to Krüppel C_2H_2-type zinc finger protein family. Several protein isoforms have been identified.[93, 94] BLASTP alignment of the clone 88 sequence indicated that this clone encoded to the human protein, Dipeptidyl peptidase 7 (DPP7). DPP7 is an aminopeptidase having a role of post-proline cleaving. It is expressed in quiescent lymphocytes. Resting lymphocytes are maintained through suppression of apoptosis, a state which is disrupted by the inhibition of this novel serine protease. It is associated with immune regulation, signal transduction and apoptosis. Its activity is essential for resting T cells, but not for activated T cells. Furthermore, DPP7 inhibition results in increased phosphorylation of Erk-1 and-2 in lymphocytes.[102, 103]

BLASTN alignment of the clone 475 sequence indicated that this clone encoded to the untranslated region of the human host cell factor C1 (HCFC1). This protein is involved in the cell cycle control and transcriptional regulation during herpes simplex virus infection. Alternatively spliced variants which encode different protein isoforms have been described; however, not all variants have been fully characterized.[104-106] BLASTP alignment of the clone 481 sequence indicated that this clone encoded to the human histidine triad nucleotide binding protein 2 (HINT2). Histidine triad proteins represent a small family of nucleotide-binding and -hydrolyzing proteins of uncertain biological function. HINT2 localizes to mitochondria and sensitizes cells to apoptosis. Changes in HINT2 expression alters the expression of the proteins involved in apoptosis. Also, it was shown that HINT2 has tumor suppressive activity and its expression is lost in human hepatocellular carcinoma.[91, 92, 107]

BLASTP alignment of the clone 528 sequence indicated that this clone encoded to the human poly (ADP-ribose) polymerase family member 12 (PARP12). PARP12 belongs to a large family of enzymes that synthesize and transfer ADP-ribose polymers to acceptor proteins, modifying their functional properties. However, the actual role of PARP12 is not yet known. Other PARP family members have been shown to be involved in DNA damage repair, transcriptional regulation and post-translational

modifications. PARP-1 deficiency affects the expression other proteins involved in T cell activation and impairs T cell proliferation. Furthermore, PARP-2 deficiency decreases the survival of DP thymocytes. [108-111] Therefore, PARP12 may be a critical factor for T lymphocyte function and development.

The nine selected clones were re-transformed into yeast cells expressing the bait protein, the ThPOK BTB/POZ domain in order to confirm interactions between the bait and prey proteins. As expected, nine different clones grew on SD (-LT) plates indicating that the sequential transformation was successful. As a confirmation of interaction, these yeast cells growing on SD (-LT) plates were also plated on and grew on SD (-LTHA) plates. As a control, yeast cells containing empty prey plasmid and expressing the BTB protein did not grow on SD (-LTHA) plates. This observation confirmed that the BTB domain is not auto-reactive and can not initiate transcription of the reporter genes in yeast cells without an interacting prey protein. In order to confirm that these different prey proteins are not auto-reactive, the selected nine clones were transformed into yeast cells containing the empty bait plasmid, pGBKT7 BD. As expected, yeast cells did not grow on SD (-LTHA) plates, indicating that the prey proteins fused with GAL4 activation domain do not interact with the GAL4 DNA binding domain without the bait protein.

As further confirmation of interactions in yeast cells, these putative interactors were tested against another zinc finger protein, MAZR which also contains a BTB/POZ domain. The MAZR protein is involved in the regulation of CD8 gene expression during T cell development. There are conserved amino acid residues among ThPOK and MAZR BTB/POZ domains that are required for the interaction with corepressors as well as homo- and hetero-dimerization. As a result, yeast cells expressing both MAZR protein and prey proteins grew on SD (-LTHA) plates. This interaction of identified prey proteins with the MAZR protein might be due to the presence of a BTB/POZ domain in the MAZR structure. Another reason might be that prey proteins interact with bait proteins non-specifically. In order to confirm specific interaction of the BTB/POZ domain with selected prey proteins, further confirmation experiments are required. The interaction of prey proteins could be tested with truncated versions of the ThPOK protein in order to confirm the interactions that occur through the BTB/POZ

domain. Also, an unrelated protein could be used as a bait to test the specificity of interactions with the prey proteins of interest.

Re-confirmation of the putative interactions was performed in mammalian cells. This step was crucial as the actual interactions between the ThPOK protein and other proteins occur in T cells. Although yeast cells are eukaryotic, they differ in post-translational modifications from mammalian cells. HEK293T cells were used for confirmation of interactions in mammalian cells. Transfection of HEK293T cells is much more efficient than the one of T cell lines. Coding regions of the bait and prey proteins were transferred into mammalian expression plasmids and the expression of these proteins were analyzed by western blotting with anti-HA antibodies. The reason why HA-tagged clones 9, 20, 66, 74 and 475 prey proteins were not detected might be related with the small sizes of the prey proteins. Increasing the polyacrylamide gel concentration and/or decreasing the transfer time during western blotting could enhance the specificity of detection.

The prey proteins 19, 20, 66, 74, 481 and 528 were immunoprecipitated with anti-HA beads, and blotted with anti-Myc and -HA antibodies. The prey protein 88 was immunoprecipitated with anti-Myc beads, and blotted with anti-Myc and –HA antibodies. The results of the IP experiments showed that the BTB/POZ domain of the ThPOK protein interacts with prey proteins of interest which are POMP, MEF2B, TCF7, ZNF384, DPP7, HINT2 and PARP12. The interactions detected with immunoprecipitation experiments may be due to the over-expression of bait and prey proteins in HEK293T cells. In order to eliminate this possibility, endogenous co-IP experiments could be performed in T cells. Also, as alternative method to immunoprecipitation, GST pull-down assays could be done in order to detect interactions. These identified and confirmed interactors may be downstream targets of the ThPOK protein. Furthermore, these interacting proteins may regulate the function of the ThPOK protein on its downstream targets. Also, these prey proteins interact with the ThPOK protein may regulate the activation of other key proteins involved in T cell lineage differentiation.

6 CONCLUSION

The aim of this study was to identify putative interacting protein partners of the ThPOK BTB domain. A human thymic cDNA library was screened against the BTB domain of the mouse ThPOK gene by using a classical yeast two hybrid system. Almost half of the 552 colonies that grew on the most stringent selection medium (-LTHA) after library screening were isolated and sequenced to identify putative ThPOK interactors. To identify these proteins, we analyzed the sequencing results using BLAST software to screen human protein databases.

Nine putative prey proteins were selected for confirmation and further analysis according to the BLAST results. These nine clones were either transcription factors or proteins that were previously shown to have a role in T cell development. In order to re-confirm the interactions in yeast, secondary yeast two hybrid screens were carried out by transforming the clones to be further analyzed back into the yeast cells expressing the ThPOK BTB domain containing bait protein. To identify the specificity of the interaction of these nine proteins with the ThPOK BTB domain, another zinc finger protein MAZR, having a BTB/POZ domain and a role in T cell development was used as the bait in another yeast two hybrid screen with putative prey proteins. In this secondary yeast two hybrid screens, all the nine selected prey clones interacted with the ThPOK BTB domain and the MAZR protein. We conclude that these identified interactions were not only specific to the BTB `domain of the ThPOK protein, but also to the MAZR protein through its BTB domain.

The validity of the interactions identified in yeast was next analyzed in mammalian cell lines. The bait and prey clones were transferred into mammalian expression plasmids. We used the HEK 293T cell line to co-transfect bait and prey expression plasmids and to perform co-immunoprecipitation experiments for the re-confirmation of interactions between the bait and prey proteins.

In conclusion, the studies presented herein indicate that the prey proteins encoded by clones 19, 20, 66, 74, 88, 481 and 528 that correspond to the proteasome maturation protein (POMP), the myocyte enhancer factor 2B (MEF2B), the transcription factor-7 (TCF7), the zinc finger protein 384 (ZNF384), the dipeptidyl-peptidase 7 (DPP7), the histidine triad nucleotide binding protein 2 (HINT2) and the poly (ADP-ribose) polymerase family, member 12 (PARP12) respectively, interact with the BTB domain of the ThPOK protein. Additional studies are required to confirm the interactions in mammalian cells and to further characterize the interaction patterns of prey proteins with the BTB domain of the ThPOK protein.

7 FUTURE STUDIES

At the end of this study, the prey proteins 19, 20, 66, 74, 88, 481 and 528 corresponding to POMP, MEF2B, TCF7, ZNF384, DPP-7, HINT2 and PARP-12, were shown to interact with the BTB/POZ domain of the ThPOK protein. The interactions were confirmed by co-immunoprecipitation experiments in HEK293T cells. As these confirmed interactions might be due to the over-expression of the prey proteins in HEK 293T cells, co-immunoprecipitation experiments with endogenously expressed proteins in the relevant T lymphocyte cell lines need to be performed. For this purpose, we already have monoclonal antibodies against some of the identified proteins and these and further monoclonal antibodies could be used for the immunoprecipitating endogenous proteins and confirming their interactions with the ThPOK protein in different T cell lines.

In order to identify which domains of the candidate interactor proteins are responsible for binding the ThPOK protein, truncations or chimeras of these proteins could be constructed and used in further immunoprecipitation experiments to identify minimal interacting domains. In order to be able to study the functional aspects of the interactions on T cell lineage decisions, knock out or over-expression studies can be carried out with the identified proteins. shRNA mediated knock down of the expression of the ThPOK interacting proteins followed by functional studies such as ThPOK transcriptional activity assays, TCR signaling dependent reporter gene activation assays or the determination of in vivo CD4 vs CD8 lineage commitment, will elucidate the biological significance of the identified interactions in this study.

By carrying out the indicated studies, a better understanding of the function and mechanism of the action of the ThPOK protein in T cells can be obtained. Identification of any potential functional relevance may lead to the modification of the T cell lineage decision. Understanding and analyzing the T cell lineage decision is crucial as mature

and functional T lymphocytes are the most important line of immune defense. The long term outcome of this project could be the identification of novel drugs to treat immunodeficiency disorders that arise from problems with lymphocyte development.

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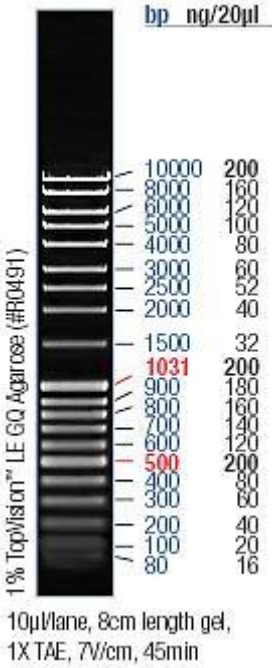
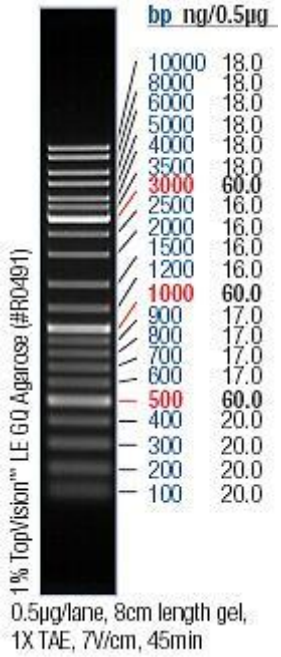
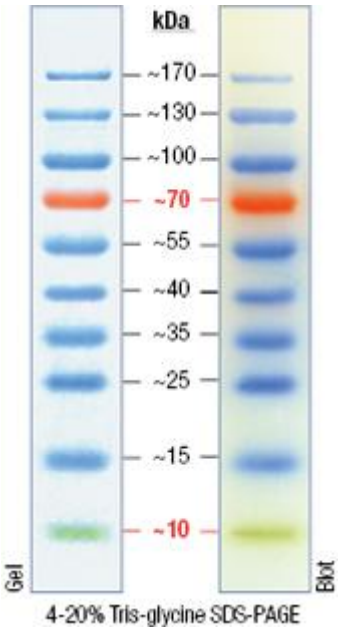
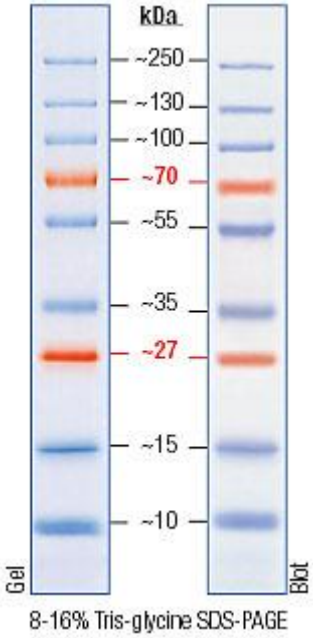
APPENDIX

APPENDIX A- DNA AND PROTEIN MOLECULAR WEIGHT MARKERS

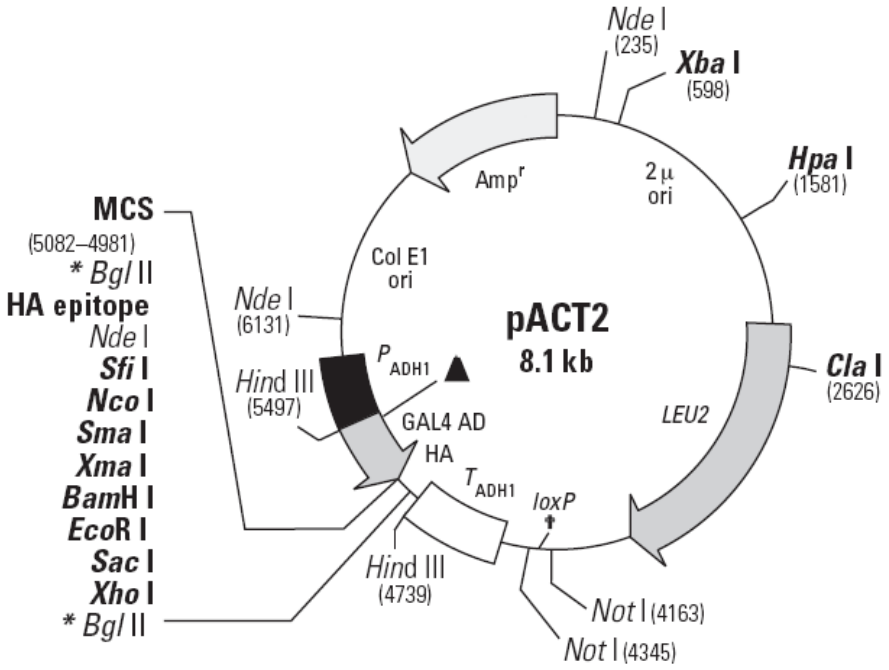
DNA molecular weight markers

MassRuler™ DNA Ladder Mix Fermentas, Germany	GeneRuler™ DNA Ladder Mix Fermentas, Germany
	

Protein molecular weight markers

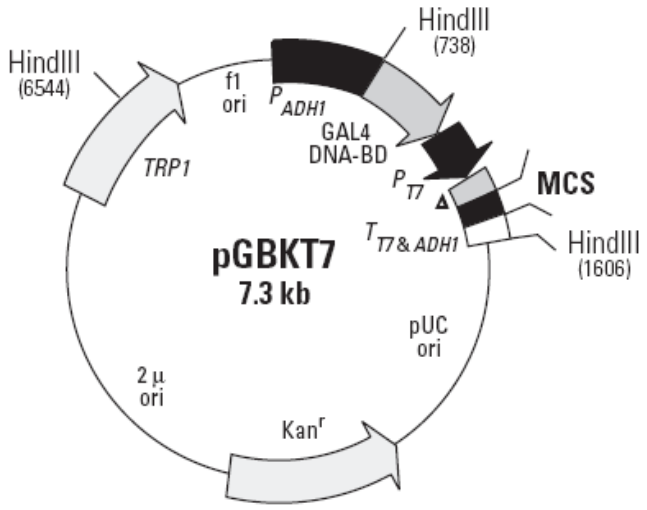
PageRuler™ Prestained Protein Ladder Fermentas, Germany	PageRuler™ Plus Prestained Protein Ladder Fermentas, Germany
	

APPENDIX B –VECTOR MAPS



Location of features:

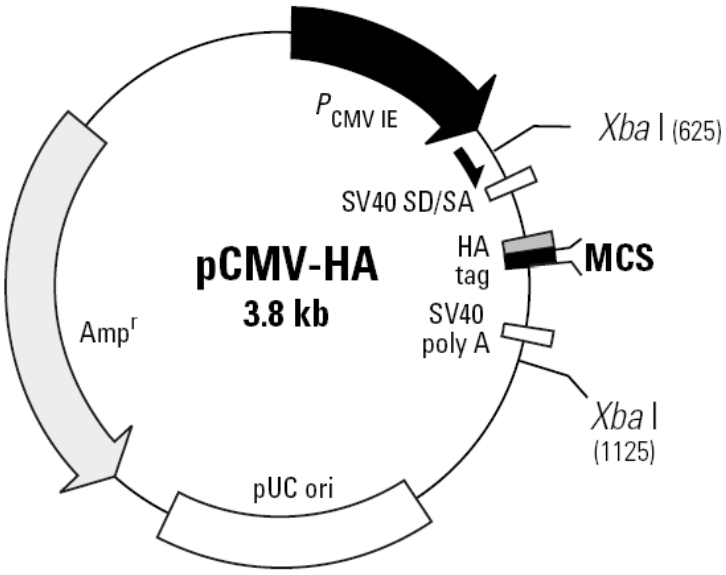
- 2µ origin of replication: 1–2055
- LEU2 coding sequences
  - Start codon (ATG): 2474–2476
  - Stop codon: 3566–3568
- Lox sites: Lox 1: 4268–4327; Lox 2: 4367–4412
- Transcription termination signal
  - Fragment carrying the *S. cerevisiae* ADH1 terminator: 4415–4742
  - Translation stop codon: 4970–4972
- Multiple cloning site: 4927–5079
- Hemagglutinin (HA) epitope: 5042–5068
- GAL4 activation domain polypeptide
  - Start codon (ATG): 5486–5488
  - GAL4 codons 768–881: 5081–5419
  - SV40 T-antigen nuclear localization signal: 5424–5478
- Promoter fragment carrying the *S. cerevisiae* ADH1 promoter: 5504–5901
- pBR322 plasmid replication origin: 6336–6979
- Ampicillin resistance gene
  - Promoter: –35 region: 8052–8057; –10 region: 8029–8034
  - Transcription start point: 8022
  - Ribosome binding site: 7995–7999
  - β-lactamase coding sequences:
  - Start codon (ATG): 7985–7987
  - Stop codon: 7127–7129
  - β-lactamase signal peptide: 7919–7987
  - β-lactamase mature protein: 7130–7918



▲ c-Myc epitope tag

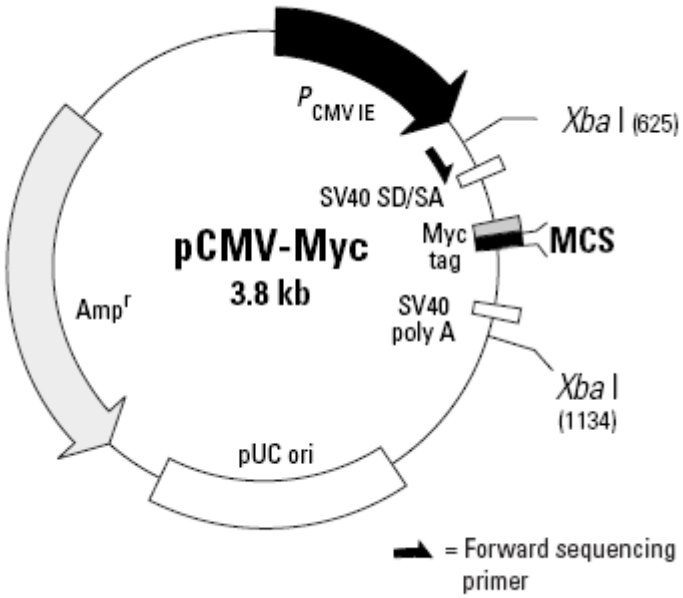
Location of features:

- Truncated *S. cerevisiae ADH1* promoter (P_{ADH1}): 30–736
- GAL4 DNA binding domain (DNA-BD) polypeptide amino acids 1–147: 762–1202
- T7 RNA polymerase promoter: 1212–1235
- c-Myc epitope tag: 1248–1280
- Multiple Cloning Site: 1281–1334
- Transcription termination signals
  T7 terminator: 1335–1381
  ADH1 terminator: 1414–1610
- pUC plasmid replication origin: 1838–2636
- Kanamycin resistance gene: 4144–3222
- Yeast 2 μ replication origin: 4148–5493
- *TRP1* coding sequences
  promoter: 5559–6755
  gene: 6031–6705
- f1 bacteriophage origin of replication: 6756–29



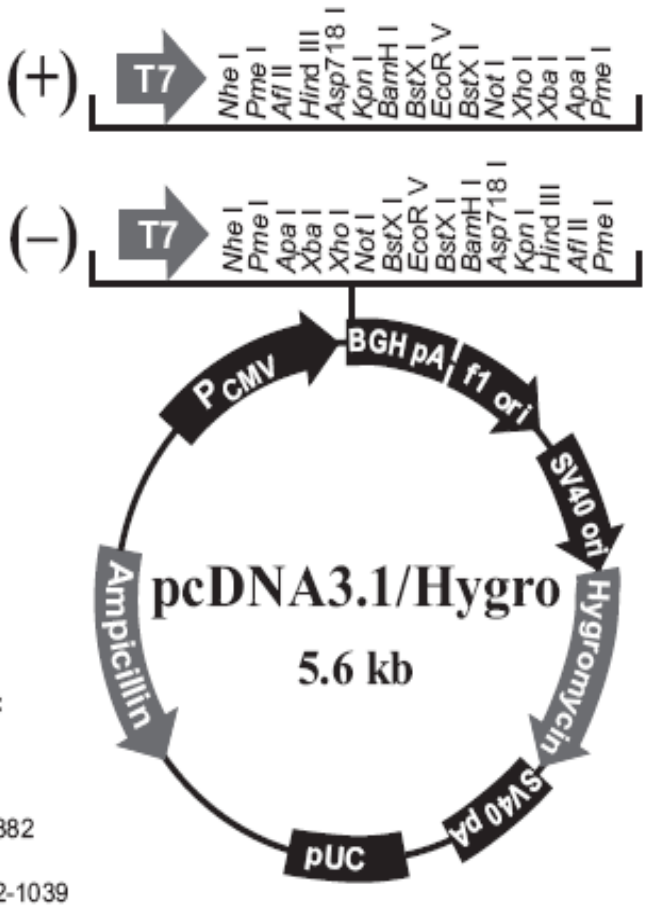
Location of features:

- Immediate early cytomegalovirus promoter ($P_{CMV\ IE}$):
  - Enhancer region: 27–431
  - TATA Box: 520–526
  - Transcription start point: 549
- Intron (SV40 splice donor/splice acceptor):
  - SV40 late 19s mRNA intron: 672–702
  - Modified SV40 late 16s mRNA intron: 672–768
- HA epitope tag with start codon (ATG): 829–858
- Multiple Cloning Site: 872–912
- SV40 polyadenylation signal:
  - Polyadenylation signal: 1044–1049
  - mRNA 3' end:1063
- pUC plasmid replication region: 1536–2179
- Ampicillin resistance (β-lactamase) gene:
  - Promoter:
    - -35 region: 3257–3252
    - -10 region: 3234–3229
  - Transcription start point: 3222
  - Ribosome binding site: 3199–3195
  - β-lactamase coding sequences:
    - Start codon (ATG): 3187–3185
    - Stop codon (TAA): 2329–2327
  - β-lactamase signal peptide: 3187–3179
  - β-lactamase mature polypeptide: 3118–2330



Location of features:

- Immediate early cytomegalovirus promoter ($P_{CMV\ IE}$):
  - Enhancer region: 27–431
  - TATA Box: 520–526
  - Transcription start point: 549
- Intron (SV40 splice donor/splice acceptor):
  - SV40 late 19s mRNA intron: 672–702
  - Modified SV40 late 16s mRNA intron: 672–768
- Myc epitope tag with start codon (ATG): 829–867
- Multiple Cloning Site: 881–921
- SV40 polyadenylation signal:
  - Polyadenylation signal: 1053–1058
  - mRNA 3' end: 1072
- pUC plasmid replication region: 1545–2188
- Ampicillin resistance (β-lactamase) gene:
  - Promoter:
    - -35 region: 3266–3261
    - -10 region: 3243–3238
  - Transcription start point: 3231
  - Ribosome binding site: 3208–3204
  - β-lactamase coding sequences:
    - Start codon (ATG): 3196–3194
    - Stop codon (TAA): 2338–2336
  - β-lactamase signal peptide: 3196–3188
  - β-lactamase mature polypeptide: 3127–2339



Comments for pcDNA3.1/Hygro (+):
5597 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2100
Hygromycin resistance gene: bases 2118-3141
SV40 early polyadenylation signal: bases 3154-3526
pUC origin: bases 3786-4456 (complementary strand)
Ampicillin resistance gene: bases 4601-5461 (complementary strand)

APPENDIX C- PREDICTED PROTEIN SEQUENCES OF THE IDENTIFIED cDNA CLONES

»CLONE 9: BTF3

IRGRVDGKAPLATGEDDDDEVPDLVENFDEASKNEAN

»CLONE 19: POMP

IRGRVDGNGAERLFAELRKMNARGLGSELKDSIPVTELSASGPFESHDLL
RKGFSCVKNELLPSHPLELSEKNFQLNQDKMNFSTLRNIQGLFAPLKLQMEFK
AVQQVQRLPFLSSSNLSLDVLRGNDETIGFEDILNDPSQSEVMGEPHLMVEYKL
GLL

»CLONE 20: MEF2B

IRGRVDGSPRGVMGPPPCPPSPGKCPETTASPSPPKPRPLLTPGGHPWYCA
HFRYGWAAILRPQEGPPPPPFPGSSCQAFAHAVPSPGFLPFLAELTASLSLSPCS
GGRSLGEEGPPTRGASPPTPPVSIKSERLSPAPGGPGDFPKTFPYPLLLARSLAEP
LRPGPALRRLPLADGWPR

»CLONE 66: TCF7

IRGRVDPREVPVRPGQGESAMTEPLSLAGLAPLGPVVAQLWAPRGLGHG
GVWRSEPWDWGLRCTGRLLTALPDPGFLGGESAIPGARQGATATHCILRILSY
HPKD

»CLONE 74: ZNF384

IRGRVDQGARNSQVGHRWLRSVCYGFTDFLEASLLLCLQRPYTLSLPNPP
PRSCGRLCVICETPKQGCCGEGRVQGKRKDWT

»CLONE 88: DPP7

IRGRVNPGQGLCLLPQGCGGVDSTTFPETLGEAGAVPGDPLSTGCSQAQL
GGGCPRGPWGPQHGGQQRAAGPTLPRGKPGGPQQSGPGLAAQTPPTSKQVA
WRAFLTGTYKSQSPKSPAGPFQGGTGWWPEPAVCLCVAAGPQRLSSPGLVYN
ASGSEHCYDIYRLYHSCADPTGCGTGPDARAWDYQACTEINLTFASNNVTDM
FPDLPFTDELRQRYCLDTWGVWPRPDWLLTSFWGGDLRAASNIIFSNGNLDPW
AGGGIRRNLSASVIAVTIQGGAHHLDLRASHPEDPASVVEARKLEATIIGEWVK
AARREQQPALRGGPRLSL

»CLONE 475: HCFC1

IRGRVDGNQASLEAAWEGDCVVSPTREARAMEQATSSSCAAWNSGQAP
RLGDLLGVFCPPPQTSVEEQGLPASQKDLNRAANLSSPPLPTSAWRRSSLCPPG
PLVCCIIVYFAVENVTFSHLGAHSPGPVVLPHPFSRAPIDLFLRRVHRSLLSNPC
DSILP

»CLONE 481: HINT2

IRGRVDRVRGSRGSGKMAAAVVLAAGLRAARRAVAATGVRGGQVRGA
AGVTDGNEVAKAQQATPGGAAPTIFSRILDKSLPADILYEDQQCLVFRDVAPQ
APVHFLVIPKKPIPRISQAEEEDQQLLGHLLLVAKQTAKAEGLGDGYRLVINDG
KLGAQSVYHLHIHVLGGRQLQWPPG

»CLONE 528: PARP12

IRGRVDPPPAEPSCVISGSYFARDAAYSHHYSKSDTQTHTMFLARVLVGE
FVRGNASFVRPPAKEGWSNAFYDSCVNSVSDPSIFVIFEKHQVYPEYVIQYTTS
SKPSVTPSILLALGSLFSSRQ