

Involvement of PP6 in Dephosphorylation of Bcl11b,

a Tumor Suppressor Transcription Factor

by

Chelsea R. Parker

A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for
the degree of

Honors Baccalaureate of Science in Biochemistry & Biophysics

Presented April 20, 2012
Commencement June 2012

AN ABSTRACT OF THE THESIS OF

Chelsea R. Parker for the degree of Honors Baccalaureate of Science in Biochemistry & Biophysics from Oregon State University presented on blank date. Title: Involvement of PP6 in Dephosphorylation of Bcl11b, a Tumor Suppressor Transcription Factor.

Abstract Approved: _____

Theresa M. Filtz

The purpose of this honors thesis research project is to investigate the molecular causes of childhood and infant T-cell acute lymphoblastic leukemias. T-cells are the work horses of the immune system, and like any organ, must develop properly to be fully functional. Improper development of thymocytes into T-cells can lead to development of T-cell leukemias and lymphomas. Regulation of thymocyte development is coordinated by transcription factors, of which Bcl11b is essential for proper T-cell development. Bcl11b is regulated by phosphorylation and dephosphorylation. Previously, a correlation was found between dephosphorylation of Bcl11b and increased expression of Id2 in developing T-cells. Regulation of the tumorigenic Id2 gene is crucial for proper T-cell development. Based on preliminary studies, we hypothesized that PP6 is the phosphatase responsible for dephosphorylating Bcl11b and altering transcriptional activity of the Id2 gene. DNA constructs for PP6 and Bcl11b proteins were transfected into HEK-293T cells, and then cells were lysed and assayed for Bcl11b phosphorylation. Our results indicated that PP6 co-expression increased Bcl11b dephosphorylation in HEK-293T cells. However, reporter gene assays showed that PP6 co-expression has no effect on Bcl11b-dependent repression of the Id2 promoter in transfected HEK-293T cells. The

next step is to investigate whether PP6 down-regulation of Bcl11b affects Id2 expression in native thymocytes.

Key Words: Transcription Factor, Bcl11b, T-ALL, PP6

Correspondence: parkercr.27cp@gmail.com

© Copyright by Chelsea R. Parker
April 20, 2012
All Rights Reserved

Involvement of PP6 in Dephosphorylation of Bcl11b,
a Tumor Suppressor Transcription Factor

by

Chelsea R. Parker

A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for
the degree of

Honors Baccalaureate of Science in Biochemistry & Biophysics

Presented April 20, 2012
Commencement June 2012

Honors Baccalaureate of Science in Biochemistry and Biophysics project of Chelsea R. Parker presented on April 20, 2012.

APPROVED:

Mentor, representing Pharmaceutical Sciences

Committee Member, representing Biochemistry and Biophysics

Committee Member, representing Pharmaceutical Sciences

Department Chair, Pharmaceutical Sciences

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Chelsea R. Parker, Author

ACKNOWLEDGEMENTS

I would like to thank the Howard Hughes Medical Institute and the Department of Pharmaceutical Sciences for funding my wonderful experience in the HHMI summer research program. I also appreciate the time and mentorship of Dr. Theresa Filtz and Dr. Kevin Ahern. I would like to thank Dr. Lingjuan Zhang, Xiao Liu, Dr. Walter Vogel and Dr. Mark Leid for the provision of preliminary data for this project. Special thanks to Andrew Hau and Dr. Jane Ishmael for the HEK-293T cells, and to Dr. David Brautigan, who graciously provided me with PP6 constructs and antibodies. Lastly I would like to recognize Connie Shen, my undergraduate lab partner, for support and assistance.

TABLE OF CONTENTS

	<u>Page</u>
THESIS STATEMENT	1
INTRODUCTION	2
T-ALL	2
T-cell development	2
Transcription factors	4
Bcl11b	6
Research goals	8
MATERIALS AND METHODS	10
Cell lines	10
Plasmids and transfection	10
Phosphorylation assays and Western blotting	10
Reporter gene assays	11
RESULTS	13
DISCUSSION	18
WORKS CITED	22

LIST OF FIGURES

Figure	Page
1. The process of thymocyte development	3
2. PP6 co-expression reduced basal phosphorylation of Bcl11b but not after PMA stimulation for 4 hours	13
3. Dephosphorylation of Bcl11b is observed when co-expressed with PP6 or H114A, with and without PMA stimulation for 30 min.....	14
4. CAT Reporter Assay shows PP6 co-expression with Bcl11b has no effect on Id2 repression.....	15

DEDICATION

My UHC Thesis is dedicated to my parents, who taught me to always do my best.

**Involvement of PP6 in Dephosphorylation of Bcl11b,
a Tumor Suppressor Transcription Factor**

THESIS STATEMENT

The transcription factor Bcl11b is dephosphorylated by PP6 in HEK-293T cells, ultimately causing de-repression (expression) of the Id2 gene.

INTRODUCTION

T-ALL

T-cell Acute Lymphoblastic Leukemia, or T-ALL, is an aggressive hematopoietic cell cancer. Unfortunately, T-ALL disproportionately affects children, and according to the American Cancer Society, T-ALL represents 15%-18% of all childhood acute leukemias (“What Are the Key Statistics for Childhood Leukemia?”). Childhood T-ALL is associated with a moderate rate of relapse (Hoang and Hoang 2010). The tumor is not actually a solid mass because the cancer is the result of uncontrolled proliferation of improperly differentiated thymocytes (T-cell stem cells). T-cells are a crucial component of the adaptive immune system; T-ALL cancer patients are also immune-compromised and at risk of developing infections and anemia. Genetic analysis of patients with T-ALL has yielded many insights as to the mutations and mechanisms involved in disease progression, but there is still much to learn. Current research on the molecular causes of T-ALL hopes to shed light on possible treatments to help improve the prognosis for many young patients.

T-cell development

In a healthy individual, thymocytes are initially formed in the bone marrow as pluripotent hematopoietic stem cells. Instead of completing their development in the bone marrow, thymocytes migrate to the thymus and undergo an extensive maturation and proliferation process. This process requires carefully orchestrated genetic expression

changes to form functional T-cells for the immune system. Many more thymocytes are produced than survive; as the cells develop and commit to the different possible lineages, they are deleted from the repertoire if they do not function correctly (Murphy 2008).

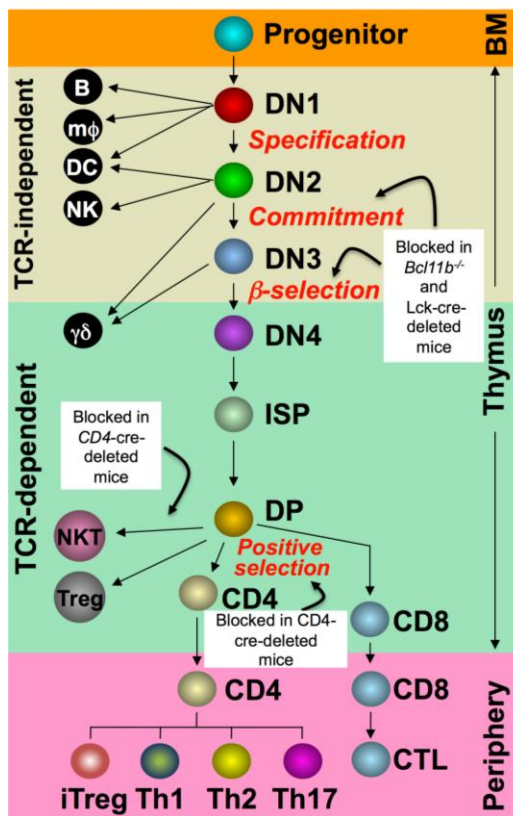


Figure 1. The process of thymocyte development, as described by E. V. Rothenberg and modified by M. Leid and T. Filtz (Rothenberg and Taghon 2005).

The surface of differentiating T-cells is embedded with many different receptors (TCRs) and cell markers (Th1, Th2, CD3, CD8, CD4, etc). CD4 and CD8 TCRs are multi-subunit proteins comprised of α and β chains. The expression of these surface proteins changes over time as the cell matures and rearranges its genes to produce TCR combinations that will be unique to each cell. When cells express neither CD4 nor CD8 T-cell co-receptors, they are said to be at the double-negative or DN stage (there are

several subsets of the DN stage) (Figure 1). Thymocytes at this stage have not yet committed to the T-cell lineage; they may still differentiate to become NK cells or other myeloid cells (Li, L et al. 2010; Di Santo 2010). Cells that express Kit and CD44 but not CD25 are called DN1 cells. DN2 cells express CD25, and later, CD44 and Kit expression is reduced in the DN3 stage. At the DN3 stage, the cell is also expressing a pre-T-cell β -receptor and exhibits full T-lineage-specific gene expression. If the pre-T-cell β -receptor is properly formed, the cell passes through the commitment checkpoint known as β -selection. After β -selection, the cell has committed to the $\alpha\beta$ lineage. This particular assortment of cell surface receptors leads to T-cell proliferation and the expression of both CD4 and CD8 to form double-positive (DP) cells. This is the start of TCR-dependent selection. At this stage, cells test the efficacy of the α -chain TCR, and undergo positive selection if they are able to recognize self-peptide presented on self-Major Histocompatibility Complex (MHC) markers. After a cell has been positively selected, it becomes a single positive (SP) cell - it will only express CD4 or CD8 co-receptors, but not both. SP cells then migrate through the thymus for additional selection. In this stage, known as negative selection, T-cells that react too strongly to self peptides are deleted (Murphy 2008; Rothenberg et al. 2010; Rothenberg and Taghon 2005).

Transcription factors

Cells must use the information encoded in their DNA to make the proteins that are necessary for cellular function and development. To do this, cells first transcribe their DNA into messenger RNA (mRNA) using an enzyme called RNA Polymerase, which

reads the DNA template and builds an RNA chain complementary to it. The mRNA molecule contains the genetic information needed to synthesize polypeptide chains from amino acids. The process of making mRNA is called transcription and the process for producing polypeptide chains is called translation. Transcription is a highly regulated process that occurs in the nucleus of eukaryotic cells. Transcription factors, sometimes called sequence-specific DNA-binding proteins, bind to certain DNA sequences to regulate gene expression. By binding to promoter or silencer DNA sequences adjacent to (but sometimes distant from) genes, transcription factors either activate or repress the recruitment of RNA Polymerase proteins, thus affecting gene transcription (Latchman 2008). Transcription factors can perform these functions alone but most require a complex of other proteins. There are a variety of transcription factor families which organize the different proteins into groups based on function and protein structure. Transcription factor function is often affected and altered by other regulatory proteins, e.g. kinases and phosphatases, which relay information signals from the cell surface to the transcription machinery inside the nucleus.

The entire process of thymocyte development requires the combined effort of a variety of proteins including transcription factors and their regulators to coordinate the expression of genes necessary for thymocyte maturation (Rothenberg et al. 2010). Some proteins are crucial for the gene rearrangements that produce the T-cell receptors, and others regulate or induce cellular changes required for proliferation. Sometimes, the same transcription factor is activated at more than one point in development, or performs different functions in different stages. Since the process of thymocyte development is so

complicated, it is critical that all of the signaling cascades, transcription factors, and other cellular machinery function properly.

Bcl11b

T-ALL occurs when there are errors in the thymocyte maturation and proliferation process; thymocytes reach a certain point in the process and continue to proliferate without any further maturation or specialization. The body's circulatory system and immune system become clogged with useless, non-functioning cells. Although it is unknown exactly what goes wrong during thymocyte development to cause T-ALL, researchers have hypothesized that one transcription factor called Bcl11b (also known as COUP-TF Interacting Protein 2, or CTIP-2) plays a crucial role in the differentiation and specification process. Previous research has shown that Bcl11b is required for positive selection and survival of double positive thymocytes (Albu et al. 2007; Wakabayashi et al. 2003; Kastner et al. 2010; Rothenberg et al. 2010). Bcl11b was originally isolated and cloned in the laboratory of Dr. Mark Leid at Oregon State University (Avram 2000). Bcl11b has never been crystallized, but its sequence contains homology to several domains whose structures are generally known. It is a Krüppel-like zinc finger transcription factor encoded on human chromosome 14q32.2 with six zinc finger domains (Satterwhite 2001). Zinc finger proteins contain polypeptide chains that bind four zinc atoms coordinated by two cysteine and two histidine (C₂H₂) residues which act as a DNA binding site (Latchman 2008).

In thymocytes, mutation of loss of Bcl11b results in dysregulation (too much or too little expression) of genes, while overexpression of wildtype Bcl11b can reduce cellular proliferation (Kastner et al. 2010; Karlsson et al. 2007). Like many transcription factors, Bcl11b proteins regulate the expression of over 1,000 different genes as thymocytes develop and are necessary for T-cell lineage commitment, especially in the DN2 stage of development (Kastner et al. 2010; Wakabayashi et al. 2003). Mutations or deletions of the Bcl11b gene have been reported in 16% of human T-ALL cases (Przybylski et al. 2005; De Keersmaecker et al. 2010). Researchers report that, in mouse models, missense and frameshift mutations of Bcl11b were found in 15% of induced lymphomas, and all mutations were located in the region coding for the three C-terminus DNA-binding zinc fingers (Karlsson et al. 2007). In murine models of T-ALL, thymocytes never develop past the DN2 stage if Bcl11b is knocked out, will not progress to the DN3 stage, and maintain NK-cell lineage potential (Ikawa et al. 2010; P. Li et al. 2010; Li, L. et al. 2010). Research has also shown that Bcl11b is crucial for positive selection of DP to SP cells and CD8⁺ T-cell immune function (Kastner et al. 2010; Zhang, S. et al. 2010). If Bcl11b is knocked out at the DP stage, positive selection does not occur. Microarray assays in mice show that Bcl11b is a co-regulator that can act as both a repressor and activator, depending on the gene and developmental stage (Kastner et al. 2010). Thus far, research indicates that Bcl11b is always associated with the NuRD complex, which is comprised of twelve or more polypeptides and is important for repression across the genome (Topark-Ngarm et al. 2006).

Of the genes that Bcl11b is known to regulate, cKrox and Runx3 are genes that determine whether a DP cell will become either CD4⁺ or CD8⁺ SP cells. CD9, CD160,

and integrin are cell surface proteins whose expression is regulated by Bcl11b. When surface levels of these proteins change it affects which environmental signals are sensed by the cells. Bcl11b is also a known suppressor of the tumorigenic Id2 gene. Id proteins play important roles in lymphocyte development and CD8⁺ T-cell immunity, but dysregulation can be problematic (Rivera and Murre 2001; Cannarile et al. 2006). Over-expression of Id2 can result in tumor growth and T-cell lymphomas like T-ALL (Lasorella et al. 2001).

Researchers in Dr. Mark Leid's lab discovered a correlation between dephosphorylation of Bcl11b and increased expression of Id2 in thymocytes (developing T-cells). Data from Dr. Theresa Filtz's lab in the OSU College of Pharmacy has shown that in stimulated mouse thymocytes, Bcl11b undergoes several kinetically concerted changes, first undergoing rapid phosphorylation over a period of 5 min, followed by dephosphorylation at 30 min, then a slower return to basal phosphorylation levels over 2 hours. Unpublished data from Dr. Filtz's and Dr. Leid's labs also indicates that other post-translational modifications of Bcl11b include sumoylation and ubiquitination (Zhang, L. et al. 2012). All of these alterations are part of the pathways that regulate Bcl11b and ensure that its expression and function is properly maintained throughout thymocyte development. It is important to understand these processes to obtain a better understanding of the molecular causes of childhood T-ALL and that will hopefully lead to the development of better treatments against the disease.

Research goals

One of the primary goals of this project was to identify the phosphatase involved in the dephosphorylation of Bcl11b. Based on studies of proteins that interact with Bcl11b, we hypothesized that PP6 is the phosphatase responsible for dephosphorylating Bcl11b upon stimulation of thymocytes. A secondary component of the project was to investigate the role that dephosphorylation of Bcl11b plays in the regulation of transcription of the Id2 gene promoter in a Bcl11b over-expression system (Human Embryonic Kidney cells (HEK-293T)). Identification of the phosphatase that affects Bcl11b is important because it may be possible to target that molecule for treatment of these aggressive childhood cancers and other diseases in humans.

MATERIALS AND METHODS

Cell lines

HEK-293T cells (1×10^6 cells/plate) were grown in DMEM media supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin from Mediatech. Cells were grown at 37°C with 5% CO₂ humidity in 10 cm tissue culture plates. Cells were rinsed with 1x Trypsin EDTA (0.23% Trypsin, 2.21mM EDTA in HBSS) from Mediatech for passage.

Plasmids and transfection

Id2 reporter gene constructs, β -galactosidase, and Bcl11b “F-CTIP-2 Mt20” cDNA-containing mammalian expression plasmids purified by CsCl₂ were kindly provided by Lingjuan Zhang and Dr. Mark Leid. Wild-type PP6 and H114A (mutant PP6) mammalian expression plasmids were kindly provided by Dr. David Brautigan of the University of Virginia. To control for transfection efficiency, control samples received pcDNA3 mammalian expression vector without inserts. HEK-293T cells were transfected using the Calcium-Phosphate method (Five Photon Biochemicals protocol) with 5 μ g of DNA or less per 10 cm plate and allowed to incubate 48 hours before treatments.

Phosphorylation assays and Western blotting

Cells were treated with 1 μ M PMA (Sigma-Aldrich) and 500 nM A23187 (EMD Biosciences) in 100% DMSO vehicle at 48 hours post transfection to stimulate phosphorylation. For time-course experiments, cells were incubated with PMA-A23187 treatment for 4 hours, 1 hour, 30 min, 5 min, and 0 min before harvesting. Invitrogen NUPAGE BisTris 4-12% gradient gels were used for Western blots. Anti-CTIP2 polyclonal antibodies were obtained from Abcam. The anti-phospho-Thr antibodies were purchased from Cell Signaling and anti-PP6 antibodies were a generous gift from Dr. David Brautigan. Blots were developed with standard ECL reagents.

Reporter gene assays

HEK-293T cells were transfected with Id2-reporter, β -galactosidase, Bcl11b, and PP6 constructs. To create the Id2 reporter construct, a -2.9 to +0.152 kb fragment from the mouse Id2 locus was subcloned into the pCR2.1 vector in front of the chloramphenicol acetyl transferase gene as described by Lingjuan Zhang (Zhang, L. et al. 2012).

The β -galactosidase expression vector (pCMV-Sport- β Gal, Life Technologies) was co-transfected as an internal control for normalization of transfection efficiency and protein expression. β -galactosidase activity was measured by addition of 0-nitrophenyl- β -D-galactopyranoside, and reactions proceeded at 30°C until a visible color change was evident (~20 min). The colorimetric A_{240} absorption of each sample was then measured. Protein assays were conducted on lysates as described elsewhere (Dowell et al.).

Cells were harvested 48 hours post transfection and lysed by repeated freezing and thawing cycles. Cellular debris was removed by centrifugation, at which point the lysate was incubated with [¹⁴C] chloramphenicol and acetyl-coenzyme A. Relative chloramphenicol acetyl transferase activity was determined by monitoring the acetylation of chloramphenicol by autoradiography following thin-layer chromatography (TLC). The percent conversion of non-acetylated to acetylated products was measured by excising the radioactive spots corresponding to substrate and product from the TLC plate and quantifying β -emission in a scintillation counter (Carey, Peterson, and Smale).

RESULTS

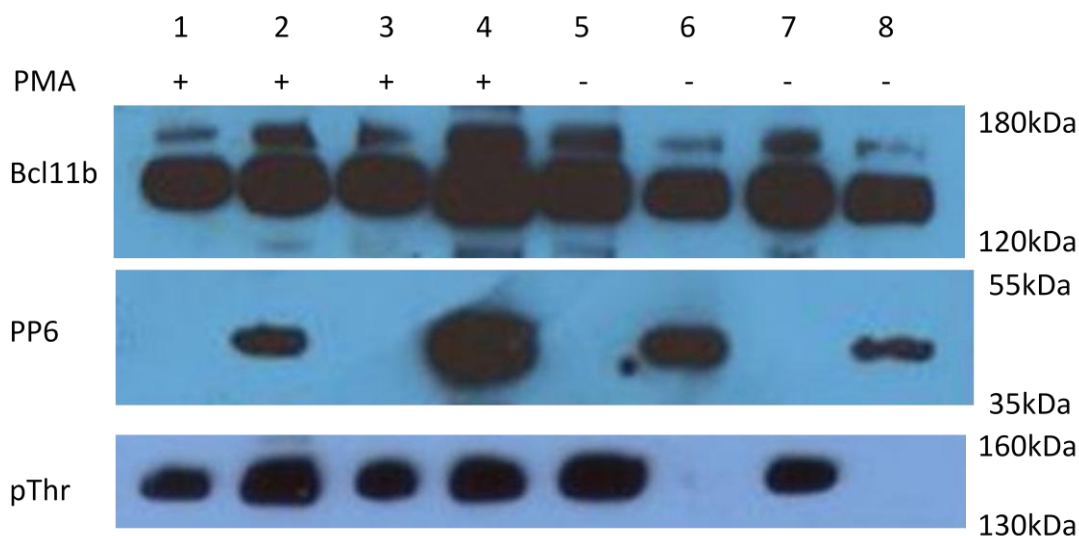


Figure 2. PP6 co-expression reduced basal phosphorylation of Bcl11b but not after PMA stimulation for 4 hours. HEK-293T cells were transfected with plasmids containing Bcl11b (all lanes) or PP6 (lanes 2, 4, 6, and 8) as described. 48 hours post-transfection, cells were treated with PMA (1.0 μ M) or DMSO as indicated above blots. Samples were harvested after 4 hours and immunoprecipitated with anti-Bcl11b antibodies prior to separation by SDS-PAGE and immunoblotting with antibodies indicated at left. Shown are immunoblots of the 100-150kDa bands for blots with anti-Bcl11b and anti-pThr antibodies, and the 35-55kDa bands for anti-PP6. This experiment was repeated at least twice.

The results in Figure 2 are representative of the outcome of several repeated experiments designed to test the ability of PP6 to dephosphorylate Bcl11b with and without PMA stimulation. 48 hours after transfection with plasmids containing PP6 and Bcl11b, cells received treatment with PMA as indicated in Figure 2. After 4 hours of PMA exposure, the cells were lysed and immunoprecipitated with Bcl11b antibodies to detect phosphorylation by Western blot with anti-phosphothreonine antibodies. The immunoblots show that Bcl11b was expressed in every lane as expected, and PP6 was expressed in samples that were transfected with PP6. In groups that were transfected with PP6 and not stimulated with PMA (Figure 2 Lanes 6 and 8), it appears that PP6 was able

to decrease basal levels of Bcl11b phosphorylation. However, samples that were transfected with PP6 and stimulated for 4 hours with PMA (Figure 2 Lanes 2 and 4) showed high levels of phosphorylation, suggesting that PP6 could not overcome the phosphorylating effects of PMA at the 4 hour time point.

We repeated the experiment described by Figure 2, but limited PMA treatment to 30 min. The results are shown in lanes 1-4 of Figure 3. When PMA stimulation is limited to 30 min, PP6 reduced basal phosphorylation and blocks PMA-stimulated phosphorylation of Bcl11b.

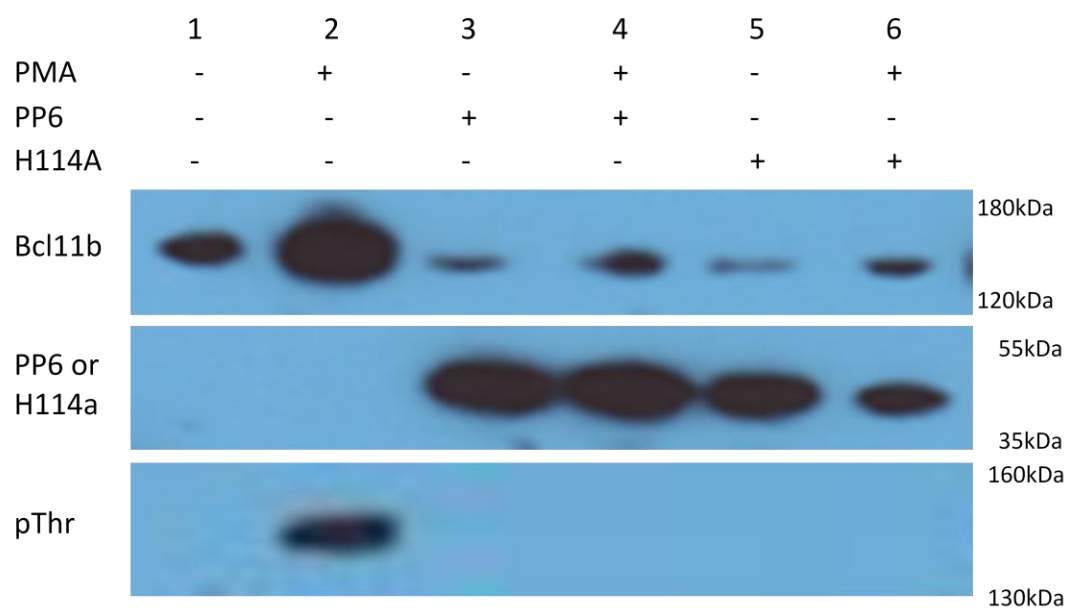


Figure 3. Dephosphorylation of Bcl11b is observed when co-expressed with PP6 or H114A, with and without PMA stimulation for 30 min. HEK-293 cells were transfected with plasmids containing Bcl11b (all lanes), PP6 (as indicated above blots), or H114A (as indicated above blots). Cells were treated with PMA or DMSO as indicated for 30 min at 48 hours post-transfection. Samples were harvested and immunoprecipitated with anti-Bcl11b antibodies prior to separation by SDS-PAGE and immunoblotting with antibodies against Bcl11b, pThr, or PP6. Shown are immunoblots of the 100-150kDa bands for blots with anti-Bcl11b and anti-pThr antibodies, and the 35-55kDa bands for anti-PP6/H114A. This experiment was repeated at least twice.

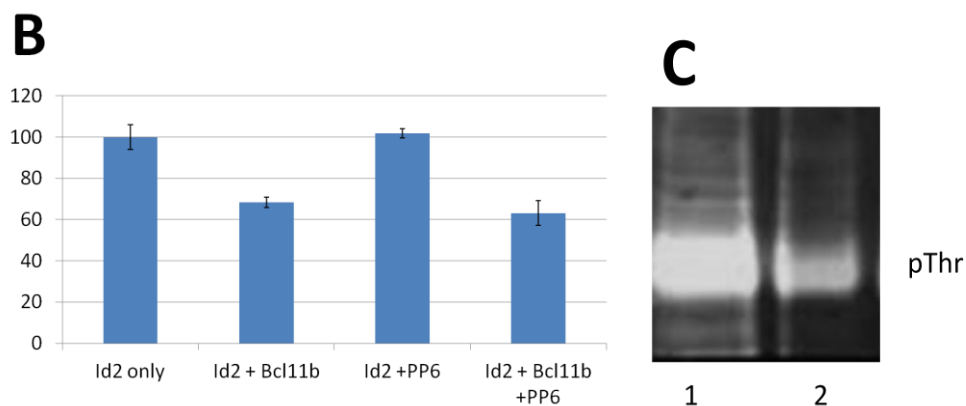


Figure 4. CAT Reporter Assay shows PP6 co-expression with Bcl11b has no effect on Id2 repression. **A)** HEK-293 cells were transfected with plasmids containing an Id2 promoter-CAT reporter gene construct (all lanes), Beta-galactosidase (all lanes), Bcl11b (lanes 3, 4, 7, and 8), or PP6 (lanes 5, 6, 7, and 8) as indicated. Beta-gal quantification was used to normalize DNA expression across all samples (not shown). Radioactive C^{14} was used to tag the CAT substrate to quantitate regulation of the Id2 promoter by Bcl11b in the transfected cells in the absence and presence of PP6. **B)** Basal levels of Id2 promoter-CAT reporter expression without co-expression of Bcl11b is shown in the first column. Expression levels decrease by approximately 32% when co-expressed with Bcl11b, as shown in the second column. When co-expressed with PP6, the Id2 promoter-CAT reporter construct shows no repression. Id2 promoter-CAT reporter expression levels decrease by approximately 37% when co-expressed with Bcl11b and PP6. **C)** Western blots were conducted on samples shown in Figure 4A corresponding to sample 4 (Lane 1) and sample 8 (Lane 2) following immunoprecipitation of Bcl11b (method as described in Figure 2). Shown are immunoblots of the 100-150 kDa bands for blots anti-pThr antibodies detected by near-IR fluorescent secondary antibodies and quantitated using a Licor Odyssey near-IR scanner. Phosphorylation of Bcl11b was reduced by approximately 50% in the presence of co-transfected PP6.

Our second hypothesis was that co-expression of PP6 and Bcl11b results in de-repression (increased expression) of the reporter gene controlled by the Id2 promoter. After discussing the overexpression issues mentioned earlier with members of collaborating labs, the transfection concentration of PP6 constructs was halved. Since PP6 reduced basal phosphorylation of Bcl11b in HEK-293T cells, stimulation by PMA was eliminated for the secondary hypothesis experiments. HEK-293T cells were again transfected via the calcium-phosphate method, this time with plasmids containing cDNAs

for PP6, Bcl11b, Beta-galactosidase, and an Id2 promoter-CAT reporter construct. CAT assays were used to quantitate regulation of the Id2 promoter by Bcl11b in the transfected cells in the absence and presence of PP6. The Beta-galactosidase was used to normalize for DNA expression among samples. The results of the CAT assay demonstrate no significant difference between Id2 repression by Bcl11b in the absence or presence of PP6 (Figure 4A and Figure 4B).

DISCUSSION

Thymocytes are very difficult to culture and notoriously hard to transfect. HEK-293T cells were chosen as a model cell line because they have very low endogenous levels of PP6. Researchers in collaborating laboratories have also found that Bcl11b is easily phosphorylated when expressed in these cells. Initially, experimental results with co-transfection of Bcl11b and PP6 in HEK-293T cells were promising (Figure 2). PP6 is capable of dephosphorylating Bcl11b in HEK-293T cells in the absence of PMA stimulation (decreasing basal phosphorylation). The basal levels of phosphorylation of Bcl11b are quite high in HEK-293T cells. In some experiments, the basal phosphorylation level was so high that PMA treatment was not very effective at further increasing Bcl11b phosphorylation. Variable phosphorylation was an issue in obtaining consistent data over several experiments.

Figure 2 further shows no decrease in phosphorylation for samples treated with PMA for 4 hours that also express PP6. It appears that PP6 cannot overcome the 4 hour stimulation with PMA to dephosphorylate Bcl11b. Thus, PP6 may be part of the regulatory mechanism controlling basal levels of Bcl11b phosphorylation, but may be inhibited by extended treatment with PMA.

We hypothesized that stimulation of phosphorylation would recreate the phosphorylation and dephosphorylation cycle of Bcl11b noted in previous experiments with thymocytes. To test this, we completed several time course experiments with PMA treatment of varying times (4 hours, 1 hour, 30 min, 5 min, and 0 min) to determine the treatment dependent kinetic pattern of changes in phosphorylation levels of Bcl11b in the

absence of PP6 in HEK-293T cells (results not shown). We compared the phosphorylation kinetic pattern of Bcl11b in HEK-293T cells with the pattern seen in thymocytes. The time course experiments indicated that phosphorylation in the model cell line follows a somewhat similar cycle to that seen in thymocytes with a slight delay. The highest levels of phosphorylation of Bcl11b occurred at 30 min of PMA treatment rather than 5 min as in thymocytes. Unfortunately we had some reproducibility issues in these experiments with varying levels of phosphorylation in samples that received no PMA treatment. Much of this was due to varying levels of basal phosphorylation.

Based on evidence of elevated Bcl11b phosphorylation after 30 min of PMA incubation, we decided to use this treatment time for further experiments to discern the effect of PP6 on Bcl11b phosphorylation levels. As shown in Figure 3, PP6's ability to dephosphorylate Bcl11b when PMA stimulation is limited to 30 min is quite evident (Figure 3 Lane 4); basal levels of Bcl11b phosphorylation were also eliminated by PP6 co-expression in this experiment.

To investigate the specificity of PP6's effect on Bcl11b phosphorylation levels, we used H114A as a negative control for PP6. H114A was designed to be a catalytically inactive mutant of PP6 phosphatase. However, in our hands, H114A was a poor negative control for PP6 seeing as Bcl11b was dephosphorylated in Figure 3 Lane 6, a sample which expressed H114A (without PMA treatment). The ability of a catalytically inactive mutant to reduce Bcl11b phosphorylation was unexpected and requires alternative hypotheses to explain the action of PP6 co-expression in reducing Bcl11b phosphorylation levels in HEK-293T cells. Perhaps PP6 stimulates dephosphorylation of Bcl11b by recruiting other phosphatases into a complex. In this scenario, it is possible

that PP6 and H114A are nucleating factors for Bcl11b dephosphorylation by multiple phosphatases. Another unexpected problem arose in the experiments with PP6 and H114A co-transfection with Bcl11b. Co-transfection of moderately high levels of PP6 or H114A with Bcl11b drastically reduces the expression of Bcl11b. Low levels of Bcl11b may explain why phosphorylation is not evident in lanes containing the negative control H114A. This co-expression problem of reduced Bcl11b levels in the presence of PP6 or H114A turned out to be an on-going issue.

We used Id2-CAT reporter assays to investigate the effect of Bcl11b dephosphorylation on Id2 gene induction or repression. The Id2-CAT reporter assays were also affected by Bcl11b-independent actions of PP6 to reduce overall expression of transfected genes in HEK-293T cells (Figure 3). Results from the Id2-CAT reporter gene assays were inconsistent throughout the project, most likely because of this effect of PP6. For the experiment portrayed in Figure 4, we used half of the amount of PP6 plasmid for transfection in an attempt to avoid confounding problems with suppression of protein expression. In this experiment, PP6 co-expression had less effect on Bcl11b expression levels and no effect on the transcriptional regulatory activity of Bcl11b towards the Id2 gene promoter. Even so, Bcl11b levels were reduced in the PP6-cotransfected samples, confounding our results.

The unanticipated effects of PP6 on Bcl11b expression in transfected HEK-293T have probably doomed this approach to studying the effects of Bcl11b dephosphorylation on Id2 gene regulation. In the future, Dr. Filtz will continue to pursue other avenues to study the importance of PP6 in regulation of Bcl11b, potentially by using Bcl11b phosphosite mutants and PP6 knockdown experiments.

Further, Dr. Filtz and Dr. Leid have some new data suggesting that Bcl11b is not simply regulated by phosphorylation and dephosphorylation cycles. In thymocytes, recent unpublished data show that Bcl11b is regulated by MAPK-mediated phosphorylation followed by sumoylation and ubiquitination (Zhang, L. et al. 2012). Sumoylation of Bcl11b is only minimal in HEK-293T cells, and it may be that Bcl11b must have sumoylation concurrent with dephosphorylation to increase de-repression of the Id2 gene. Eventually, findings from this research could be used to develop small molecules to target against phosphatases and interfere with Bcl11b's activity in T-cells, perhaps changing its function in disease.

WORKS CITED

- Albu, D. I. et al. "BCL11B Is Required for Positive Selection and Survival of Double-positive Thymocytes." *Journal of Experimental Medicine* 204.12 (2007): 3003–3015.
- Avram, D. "Isolation of a Novel Family of C2H2 Zinc Finger Proteins Implicated in Transcriptional Repression Mediated by Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) Orphan Nuclear Receptors." *Journal of Biological Chemistry* 275.14 (2000): 10315–10322.
- Cannarile, Michael A et al. "Transcriptional Regulator Id2 Mediates CD8 T Cell Immunity." *Nature Immunology* 7.12 (2006): 1317–1325.
- Carey, Michael, Craig L Peterson, and Stephen T Smale. *Transcriptional Regulation in Eukaryotes : Concepts, Strategies, and Techniques*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 2009.
- Dowell, P. "P300 Functions as a Coactivator for the Peroxisome Proliferator-activated Receptor Alpha." *Journal of Biological Chemistry* 272.52 (1997): 33435–33443.
- Hoang, Trang, and Thu Hoang. "The T-ALL Paradox in Cancer." *Nature Medicine* 16.Number 11 (2010): 1185–1186.
- Ikawa, T. et al. "An Essential Developmental Checkpoint for Production of the T Cell Lineage." *Science* 329.5987 (2010): 93–96.
- Karlsson, Anneli et al. "Bcl11b Mutations Identified in Murine Lymphomas Increase the Proliferation Rate of Hematopoietic Progenitor Cells." *BMC Cancer* 7.1 (2007): 195.
- Kastner, Philippe et al. "Bcl11b Represses a Mature T-cell Gene Expression Program in Immature CD4+CD8+ Thymocytes." *European Journal of Immunology* 40.8 (2010): 2143–2154.
- De Keersmaecker, Kim et al. "The TLX1 Oncogene Drives Aneuploidy in T Cell Transformation." *Nature Medicine* 16.11 (2010): 1321–1327.

- Lasorella, A, T Uo, and A Iavarone. "Id Proteins at the Cross-road of Development and Cancer." *Oncogene* 20.58 (2001): 8326–8333.
- Latchman, David S. *Eukaryotic Transcription Factors*. Amsterdam; Boston: Elsevier/Academic Press, 2008.
- Li, L., M. Leid, and E. V. Rothenberg. "An Early T Cell Lineage Commitment Checkpoint Dependent on the Transcription Factor Bcl11b." *Science* 329.5987 (2010): 89–93.
- Li, P. et al. "Reprogramming of T Cells to Natural Killer-Like Cells Upon Bcl11b Deletion." *Science* 329.5987 (2010): 85–89.
- Murphy, Kenneth, Paul Travers, and Mark Walport. *Janeway's Immunobiology*. 7th ed. New York and London: Garland Science, Taylor & Francis Group, LLC, 2008.
- Przybylski, G K et al. "Disruption of the BCL11B Gene Through Inv(14)(q11.2q32.31) Results in the Expression of BCL11B-TRDC Fusion Transcripts and Is Associated with the Absence of Wild-type BCL11B Transcripts in T-ALL." *Leukemia* 19.2 (2005): 201–208.
- Rivera, R, and C Murre. "The Regulation and Function of the Id Proteins in Lymphocyte Development." *Oncogene* 20.58 (2001): 8308–8316.
- Rothenberg, Ellen V., and Tom Taghon. "Molecular Genetics of T Cell Development." *Annual Review of Immunology* 23.1 (2005): 601–649.
- Rothenberg, Ellen V., Jingli Zhang, and Long Li. "Multilayered Specification of the T-cell Lineage Fate." *Immunological Reviews* 238.1 (2010): 150–168.
- Di Santo, J. P. "A Guardian of T Cell Fate." *Science* 329.5987 (2010): 44–45.
- Satterwhite, E. "The BCL11 Gene Family: Involvement of BCL11A in Lymphoid Malignancies." *Blood* 98.12 (2001): 3413–3420.

Topark-Ngarm, A. et al. "CTIP2 Associates with the NuRD Complex on the Promoter of p57KIP2, a Newly Identified CTIP2 Target Gene." *Journal of Biological Chemistry* 281.43 (2006): 32272–32283.

Wakabayashi, Yuichi et al. "Bcl11b Is Required for Differentiation and Survival of A β T Lymphocytes." *Nature Immunology* 4.6 (2003): 533–539.

"What Are the Key Statistics for Childhood Leukemia?" Web. 24 Jan. 2012.

Zhang, Ling-juan, Walter K. Vogel, et al. "Coordinate Regulation of Bcl11b Activity in Thymocytes by the MAPK Pathways and Protein Sumoylation." *Manuscript in Submission* (2012).

Zhang, S., M. Rozell, et al. "Antigen-specific Clonal Expansion and Cytolytic Effector Function of CD8⁺ T Lymphocytes Depend on the Transcription Factor Bcl11b." *Journal of Experimental Medicine* 207.8 (2010): 1687–1699.

