Factors that Modify and Control Bcl11b,

a Tumor Suppressor Protein

by

Connie Guo Shen

A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
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the degree of

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Commencement June 2013

AN ABSTRACT OF THE THESIS OF

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Theresa M. Filtz

The experiments explained in this Honors thesis are focused on finding a cellular model for analyzing Bcl11b, a transcription factor dysregulated in 20% of cases of T-cell Acute Lymphoblastic Leukemia (T-ALL). Acute Lymphoblastic Leukemia is the most common childhood cancer. Because T-ALL is a result of incorrect thymocyte development, research into how T-cells mature is essential. A key factor in T-cell maturation is the transcription factor Bcl11b. Two key Bcl11b post-translational modifications include phosphorylation and sumoylation, of which Bcl11b has 23 and 2 sites, respectively. The first experiments attempted to identify an ideal model for studying Bcl11b; however both cellular models tested had negative characteristics. Mouse thymocytes could not be transfected using the Invitrogen Neon® Electroporation System. P2C2 cells post-translationally modified Bcl11b differently than thymocytes. The next experiment was to identify which of Bcl11b's phosphorylation sites contribute more towards the composite phosphorylation level and was more important in cell signaling. This was done with Bcl11b mutants that had key phosphorylation sites eliminated. While initial data suggested that mutating phosphorylation sites affected overall phosphorylation levels, subsequent experiments resulted in conflicting data suggesting that the number of phosphorylation sites eliminated had little effect on the expressed composite phosphorylation level of Bcl11b.

Key Words: T-ALL, Bcl11b, P2C2, phosphorylation, sumoylation

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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.

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Connie Guo Shen, Author

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DEDICATION

My Honors thesis is dedicated to my mother, Yayu Guo, because she's awesome.

Factors that Modify and Control Bcl11b, a Tumor Suppressor Protein

INTRODUCTION

Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia is a white blood cell cancer, and is the most common leukemia found in children (LeMaistre, Shaughnessy, & Stein, 2013). Around 4000 new cases of ALL appear in the United States annually (LeMaistre et al., 2013). In cases of ALL, the bone marrow creates white blood cells that never fully mature and undergo uncontrolled proliferation. These lymphoid cells replace normal marrow tissue and hematopoietic cells through proliferation (Rytting, 2012). They cannot aid in immunity.

There are several ways to treat ALL including chemotherapy, radiation, or bone marrow or cord blood transplants. The overall survival rate with chemotherapy is 80% for children. Destruction of 99% of thymocytes means that the patient has entered remission (LeMaistre et al., 2013). The surviving 1% of cells can quickly multiply, however, which makes relapse quite common. These relapsed patients are typically treated with bone marrow transplants, but these procedures are risky, so it is not always used.

T-cell Acute Lymphoblastic Leukemia (T-ALL) is a cancer that accounts for 10-15% of all childhood cases of acute lymphoblastic leukemia (Goldberg et al., 2003). Relative to other ALL patients, children with T-ALL have a worse prognosis (Goldberg et al., 2003). For adults, a study that examined past literature compiled a cohort of nearly

1000 adult T-ALL patients. Of those patients, the weighted mean rate of complete remission was 88%, and the weighted mean survival rate was 40% with a wide variation of 25%-77% (Hoelzer & Gökbuget, 2009).

T-cell Maturation Process

T-cell maturation starts when progenitor hematopoietic cells migrate from the bone marrow to the thymus. T-cells and progenitors express a variety of cell surface proteins such as T-cell receptor (TCR) and cell markers (Th1, Th2, CD3, CD4, CD8, etc.). Cells starting to mature in the thymus are called double negative (DN) thymocytes because they lack the CD4 and CD8 protein markers (Di Santo, 2010).

Development starts when DN thymocytes proliferate while under the influence of Notch1's signaling interactions and a variety of ligands expressed by thymic epithelial cells (Di Santo, 2010). The cells pass from the first stage, DN1, to DN2a, then to DN2b then to DN3a, and finally then to DN3b. During each stage, the types of surface proteins change. While passing through steps DN2b and DN3a, these cells slow their proliferation and rearrange the β, δ, and γ genes of their pre-TCR (Rothenberg, Zhang, & Li, 2010). This arrangement creates a unique combination for each cell. Prior to arriving at stage DN3b, the cell has the option of becoming either a γδ T-cell or a αβ T-cell.

The cell commits to the αβ T-cell lineage by expressing a properly formed pre-T-cell β-receptor and passing through a checkpoint called β-selection. After committing to this lineage, the cell continues to proliferate and soon expresses both CD4 and CD8 markers becoming double positive (DP) cells. DP cells then begin the TCR-dependent selection of development known as positive selection (Rothenberg et al., 2010).

The CD4 and CD8 TCRs are composed of multi-subunit proteins of α and β chains. At this stage, the α chains are tested to recognize a self-peptide presented on self-Major Histocompatibility Complex (HMC) markers. If they successfully recognize self, then they are positively selected and will become a single positive (SP) cells. This means they will express either the CD4 or CD8 marker, but not both. During positive selection, a vast majority of cells are destroyed because they are either too under-reactive to foreign particles or over-reactive to self. Destroying cells because they over-react to self is called negative selection. If the cells survive selection, they migrate into the blood stream to await final specification (Rothenberg et al., 2010). Figure 1 shows thymocyte development.

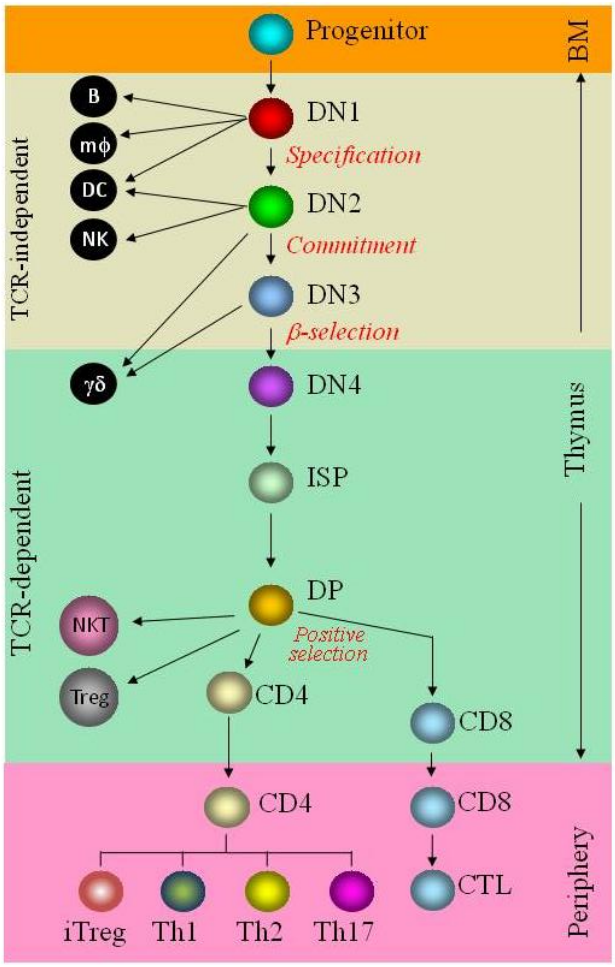


Figure 1. Thymocyte development. Created by E.V. Rothenberg, and modified by M. Leid and T. Filtz.

Transcription factors

Molecules of deoxyribonucleic acid (DNA) carry genetic information. This information is coded in heredity units called genes. In a process called transcription, these genes are transcribed into messenger RNA (mRNA) by a variety of proteins. Transcription factors are among those proteins, and they control the expression of genes by activating or repressing transcription. The mRNA is then coded into a polypeptide in a process called translation. Following translation, the polypeptide undergoes more

modification to become a fully functioning protein. These proteins can do a variety of activities within the cell, including acting as transcription factors (Berg, Tymoczko, & Stryer, 2012). The proteins can undergo post-translational modification such as phosphorylation and sumoylation, which will be discussed in further detail later.

Transcription factors themselves contain two regions of interest. The first is the DNA binding region and other regions responsible for producing or silencing the target gene. The DNA binding regions can be of different shapes, and transcription factors are grouped into families based on these shapes. An example is the homeobox domain which binds to DNA with a helix-turn-helix motif. Another domain shape is the cysteine-histidine zinc finger domain. Factors with this domain shape typically have multiple copies. For review see (Latchman, 1997).

The other region of interest in transcription factors is the one that controls the activation or deactivation of the DNA of interest. These activation domains are grouped based on what type of acidic amino acids they are rich in – glutamine or proline. This domain interacts with components of the basal transcriptional complex. The complex contains RNA polymerase II and other transcription factors, and these proteins must assemble at the gene promoter for transcription to occur. The complex forms when the DNA binding domain binds to the DNA and the activation domain binds to the transcriptional complex. They also helps pull apart the double helix to expose the template strand and launch the RNA polymerase to begin transcribing (Latchman, 1997).

Transcription factors can also bind to silencer regions and prevent the targeted DNA sequence from being transcribed. This can be performed by binding to a DNA site

that deters attempts at transcription or by quenching the effects of a positive acting factor by blocking the activity of its activation domain (Latchman, 1997).

Transcription factors can also activate more long term regulation by helping acetylate or deacetylate the lysine residue on histones organizing the DNA of interest. The proteins that perform the acetylation and deacetylation are co-factors, proteins that cannot bind to DNA on their own, and they are helped by transcription factors. Acetylation of histones increases DNA's chances of being transcribed. Acetylation of lysine in the histone tail lessens its positive charge which in turn, decreases its attraction towards negatively charged DNA (Berg et al., 2012).

Bcl11b

Bcl11b is a transcription factor isolated and cloned in Dr. Mark Leid's laboratory at Oregon State University (Avram et al., 2000). Bcl11b is also called COUP-TF Interacting Protein 2 (CTIP-2). Because the protein has never been crystallized, the 3D structure of Bcl11b is unknown. What is known is that Bcl11b is coded on chromosome 14q32.1, and that it's DNA-binding domain contains 6 C_2H_2 zinc fingers with proline-rich and acidic regions (Satterwhite et al., 2001). Zinc fingers are DNA binding sites on transcription factors, and they contain two cysteine and two histidine residues (Latchman, 1997). Bcl11b's activation domain, like others in the COUP-TF family, frequently recruits nuclear receptor co-repressor (NCoR), the silencing mediator for retinoid and thyroid hormone receptor (SMRT), and the nucleosome remodeling and histone deacetylase complex (NuRD) (Kominami, 2012).

There is likely a relationship between Bcl11b and T-ALL. Bcl11b is absolutely crucial for thymocyte development, and T-ALL occurs when thymocytes proliferate without fully maturing. Although it is unknown exactly how Bcl11b is implicated in leukemia, there is evidence the two are related. Bcl11b was highlighted in a series of papers published in Science in July 2010 that emphasized the importance of Bcl11b as "A Guardian of T-cell Fate"(Di Santo, 2010; Ikawa et al., 2010; Li, Leid, & Rothenberg, 2010). When Bcl11b is knocked out in mice, thymocytes do not develop past the DN2 stage, and these cells differentiate into the natural killer cell lineage (Li et al., 2010). When Bcl11b is eliminated artificially, cells that have originally committed to the T-cell fate start activating genes related to natural killer cells (Li et al., 2010). Bcl11b is also crucial later in the thymocyte developmental pathway for β-selection and positive selection of human thymocytes (Albu et al., 2007; Kastner et al., 2010; Rothenberg et al., 2010; Wakabayashi et al., 2003). Another study cultured murine hematopoietic progenitors in the presence of a cocktail of cytokines including interleukin-7 (IL-7) and on immobilized Notch ligand DLL4 protein (Ikawa et al., 2010). This study determined that these progenitors stopped development and continued to proliferate, and that when IL-7 levels were reduced, there was stimulated robust T-cell development. Since there was a similar stop in development in cells deficient in Bcl11b, this study suggested that Bcl11b-linked cytokine signaling thresholds and T-cell lineage commitment in early thymocyte development were connected (Di Santo, 2010).

Bcl11b regulates the expression of over 1000 different genes important for the development and differentiation of thymocytes (Kastner et al., 2010). In mouse studies, Bcl11b can act as both a repressor or activator, depending on the genes involved and the

developmental stage of the thymocytes (Kastner et al., 2010). Genes of interest that Bcl11b is known to regulate include the cKrox and Runx3 genes that determine whether a DP cell becomes a CD4+ or CD8+ SP cell. Bcl11b also regulates the genes CD9, CD160, and integrin which code for cell surface proteins and the proto-oncogene Id2 which important is for lymphocyte development (Cannarile et al., 2006). When Id2 is overexpressed, tumor growth and T-cell lymphomas may result (Lasorella, Uo, & Iavarone, 2001). Research in Dr. Mark Leid's laboratory has found that different sumoylation of Bcl11b causes it to have a different regulatory effect on Id2.

There are also statistics that suggest that Bcl11b is linked with leukemia. In 16% of cases of human T-ALL, Bcl11b is dysregulated (De Keersmaecker et al., 2010; Przybylski et al., 2005). Research has also found that in 15% of induced lymphomas in mice models, there was a missense or frameshift mutation of Bcl11b, and of the 15% of Bcl11b related tumors, all of them contained mutations in the region of the three C-terminal DNA-binding zinc fingers domains of Bcl11b (Karlsson, Nordigaarden, Jönsson, & Söderkvist, 2007).

Phosphorylation and Sumoylation

In the College of Pharmacy at OSU, Dr. Filtz's laboratory is collaborating with Dr. Mark Leid's laboratory to study Bcl11b. They have preliminary data showing that Bcl11b is modified in two ways when stimulated to mature. Bcl11b is quickly phosphorylated, a common mechanism of protein regulation in cells. After phosphorylation, Bcl11b is massively de-phosphorylated and then finally sumoylated. This sequence occurs within 60 minutes of thymocyte stimulation.

Phosphorylation is the addition of highly charged phosphate groups to the serines, threonines, and tyrosines of a protein. Once phosphorylated, the function of the protein changes. In fact, phosphorylation is a common mechanism of protein regulation in cells. Molecules that add phosphate groups are called kinases, and molecules that remove phosphate groups are called phosphatase.

Sumoylation is the addition of SUMO (small ubiquitin-related modifier) peptides to a protein. For a more detailed summary of SUMO peptides and sumoylation, see (Geiss-Friedlander & Melchior, 2007). A short summary follows. SUMO peptides are roughly 100 amino acids long, and while they resemble the physical structure of ubiquitin, they share less than 20% of the amino-acid sequence, and they display a different range of surface amino acids.

Most organisms have several SUMO proteins: SUMO1-SUMO4 with SUMO1, SUMO2, and SUMO3 being the ones ubiquitously expressed. SUMO4 is expressed in only select regions in eukaryotes. Mature forms of SUMO2 and SUMO3 are 97% identical, but both are only 50% identical to SUMO1. Thus, SUMO1 and SUMO2/3 act differently.

Sumoylation of a target protein requires many intermediate steps. SUMO peptides contain a Gly-Gly motif at the C-terminal extension. Thus, the first step is that this motif must be removed by SUMO-specific isopeptidases (sentrin-specific proteases, SENPs) which remove different amounts of amino acids depending on the type of SUMO peptide. Then, in an ATP-dependent reaction, the SUMO peptide is attached to a protein called UBA2, and is then moved to another protein called UBC9. Finally, the SUMO peptide is covalently attached to a lysine residue of the target protein by SUMO E3

ligases. Usually, only one SUMO peptide is attached during sumoylation, but SUMO peptides can stack.

The SUMO peptides can also be removed from a substrate. Removing is performed by sentrin-specific proteases (SENP1-3 and SENP5-7). SENP family members differ in various components including their activity towards different SUMO types. For example, SENP3 and SENP5 remove SUMO2/3 from substrates.

Sumoylation has a range of effects. It can change localization, alter activity, and sometimes stabilize a modified protein. SUMO is a reversible protein modifying, and it can change its target's localization by altering protein interactions.

Sumoylation is also commonly associated with changes in the activity of transcription factors. These transcription factors have increased repression or increased expression of target genes. Specifically for Bcl11b, sumoylation correlates with a change in the activity from a repressor to an activator of gene expression at a target oncogene (Zhang et al., 2012). Preliminary data also suggested that dephosphorylation of Bcl11b promoted sumoylation (Zhang et al., 2012).

General Research Goal

We had two general research goals for this Honors thesis project. The first was to obtain more generalized information about the kinetic phosphorylation and sumoylation changes of Bcl11b. Because Bcl11b is absolutely critical for successful T-cell maturation, it is a potential target for future pharmaceutical therapy for child-onset leukemia, but more information is needed before advanced research can commence. To find more generalized information, we need find a cellular model in which Bcl11b can be

manipulated and studied. This way, we can better understand the activity of Bcl11b in native thymocytes. Currently, we are using thymocytes extracted from 3 week old mice. These thymocytes are resistant to transfection, unable to be propagated in culture, and generally difficult to work with.

Having a cellular model that mimics native thymocytes well and is easy to transfect is important. One such experiment that would be more meaningful with such a model would be Bcl11b mutant work. Information about Bcl11b's modification sites and Bcl11b mutants will be provided in greater detail in Chapter 3, but to quickly summarize, Bcl11b has 23 phosphorylation sites and 2 sumoylation sites. Dr. Mark Leid's lab has created mutants of Bcl11b by removing groups of phosphorylation sites. These Bcl11b mutants have key phosphorylation and sumoylation sites mutated to alanine and arginine residues, respectfully. This eliminates places the mutant Bcl11b molecule could be phosphorylated and sumoylated. At the moment, the mutant work is done with HEK-293T cells, which may be easy to transfect and propagate in culture but may not mimic how thymocytes post-translationally modify Bcl11b as accurately enough for future work. Being able to create a good thymocyte model for these Bcl11b mutants will allow us to analyze key components of how the transcription factor works naturally.

CHAPTER 1

Introduction

Access to a good cellular model to study Bcl11b would be extremely beneficial. This ideal model would be easy to transfect and manipulate, would be homogenous, and be easily propagated. A good cellular model would also be one that has Bcl11b in behaving normally post T-cell activation. Typical post-translational modification of Bcl11b in thymocytes follows: Bcl11b is phosphorylated within 5 minutes, dephosphorylated within 30, and sumoylated within 60 minutes. Further, Bcl11b phosphorylation and sumoylation appear to be mutually exclusive.

One possible good model system would be murine thymocytes. While these cells would need to be constantly extracted from mice thymi and impossible to propagate in culture, they undoubtedly phosphorylate, dephosphorylate, and sumoylate Bcl11b as thymocytes should. There are already projects in Dr. Mark Leid's and Dr. Filtz's labs in which the endogenous mouse Bcl11b is studied. However, the main drawback to using a murine model is that they are difficult to transfect. Any study interested in over-expressing Bcl11b, mutants of Bcl11b, or proteins associated with Bcl11b would be tricky to accomplish. Likewise, a study investigating removal of endogenous proteins through the transfection of siRNA would be similarly difficult to accomplish. Thus, in this first chapter, I will discuss an attempt at creating a protocol to transfect genes of interest into mice thymocytes.

The instrument for transfection was the Invitrogen Neon® Electroporation System. Dr. Filtz's lab had co-purchased the instrument with Dr. Arup Indra's lab,

another pharmaceutical science lab at OSU. The Neon® uses electroporation to transfect T-cells; one can optimize the voltage, pulse width, and pulse number for maximal transfection efficiency for different cell types. The original research goal was to optimize the transfection settings of the Invitrogen Neon® Electroporation System to transfect thymocytes at a high efficiency to allow for expression of regulatory enzymes in thymocytes as they are stimulated to mature. As the Neon® had given instructions on how to optimize the transfection settings of various different types of blood cells, we had confidence that this type of transfection would be successful.

Methods and Materials

Cell Lines

Thymocytes were extracted from the thymi of 3 week old mice. Following extraction, the thymi were ground using the coarse-sides of glass slides while submerged in RPMI medium including 10% FBS. The media containing dispersed thymocytes settled for 15 minutes, after which the supernatant was pipetted into a sterile tube, leaving behind connective tissue and debris. More debris from the thymi was allowed to settle before the supernatant was pipetted into another sterile tube, and centrifuged at 90xg for 10 minutes. The pellet following centrifugation was resuspended in fresh RPMI medium including 10% FBS, and was allowed to incubate at 37°C in 5% CO_2 for 4-5 hours prior to transfection.

Plasmids

To determine successful transfection, a reporter construct of the cDNA sequence for GFP (green fluorescent protein from *Aequorea victoria;* Tsien 1998) was inserted into the multiple cloning site of the pcDNA3 vector (ClonTech) previously by used Dr. Filtz.

Transfection

In a 24-well plate, each well was prepared with 0.5 mL of RPMI medium, 2×10^5 thymocytes and 1 ug of GFP-pcDNA3 DNA as a reporter construct. The cells were electroporated using various settings according to the Neon instruction manual. After 24 hours, the media was supplemented with 100 U/mL of penicillin and 100 µg/mL of streptomycin from Mediatech. After 48 hours, the cells were examined under a fluorescent microscope to detect green fluorescent T-cells which would indicate successful transfection with the GFP-pcDNA3 construct.

Results

In trying to optimize the Neon for optimal transfection of mice thymocyte for future Bcl11b transfection work, three parameters were systematically varied according to the manufacturer's recommendations. We varied pulse voltage between 850 and 2500, pulse width between 10 and 30, and number of pulses from 1 to 3. All of the recommended optimization settings for the Neon electroporation system failed to produce green fluorescent thymocytes, indicating a lack of transfection. As a positive control for the plasmid and the Neon® Nucleofector, Shreya Battacharya in Dr. Arup Indra's lab (College of Pharmacy) used the Neon® transfection instrument and our GFP-pCDNA3 plasmid preparation to transfect her primary keratinocytes. The primary keratinocytes

were successfully transfected by standard protocols, suggesting that our DNA and the instrument worked sufficiently.

Discussion

For reasons that are not clear, the Neon® Electroporator does not appear to be a good system for transfection of primary thymocytes. Consultation with technical service revealed that there were no reports in their extensive database of prior transfection of primary thymocytes with their system. Technical support did not offer any new suggestions for us. We used the most highly recommended parameters and did not succeed. We used the parameters suggested for a similar cell line, Jurkat T-cells, a transformed human T-cell line, which should be very similar to the mouse thymocytes, but were similarly unsuccessful. A decision was made that the cost of investigation of more exotic parameters was not within the budget at this time.

Chapter 2

Introduction

While thymocytes are a good cell model, they are difficult to transfect. Thymocytes also do not survive past 48 hours post extraction. An ideal cell model would propagate easily in culture and be easy to manipulate. It would also closely mimic thymocytes accurately in how Bcl11b is post-translationally modified. (In thymocytes, Bcl11b is phosphorylated within 5 minutes, dephosphorylated within 30 minutes, and sumoylated within 60 minutes.)

The previous chapter discussed using the thymocytes from 3 week old mice as cellular models. Even discounting the difficulty of transfecting murine models, it would be beneficial to find a sustainable human cell line that can mimic thymocytes' Bcl11b changes post activation and could be easily propagated in culture.

The cell line chosen was a DN3-like leukemic cell line P2C2 created by Dr. Ellen Rothenburg and Dr. Long Li of the California Institute of Technology (Long, 2010). We have limited information about P2C2 cells. At the moment, our only information is that P2C2 cells are a DN3 like T-cell line cell (Long, 2010). In this chapter, I define how Bcl11b was modified upon activation in this cell line and compare that to the status of normal thymocytes. If the phosphorylation and sumoylation status of Bcl11b in P2C2 cells occurred to the same degree at the same time as the modification of Bcl11b in thymocytes, P2C2 cells could be used as an immortal and more easily manipulated substitute for thymocyte cells. This would reduce the need for live rodents to perform this work.

Methods and Materials

Cell lines

The P2C2 cells were given by Dr. Ellen Rothenburg and Dr. Long Li (California Institute of Technology) on 25 Sept 2009. P2C2 cells were grown in RPMI media with 5% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin from Meditech at a density of 0.1-1 × 10^6 cells / mL. The cells were incubated at 37°C with 5% CO_2.

Phosphorylation assays and Western blotting

P2C2 cells were treated with 1 μM PMA from Sigma-Aldrich and 500 nM A23187 from EMD Biosciences in 100% DMSO (which will be referred to as P/A) to mimic stimulation of the maturation process in thymocytes (Ohoka et al., 1996; Takahama & Nakauchi, 1996). The cells were treated 5 minutes, 30 minutes, 1 hour, or 2 hours prior to harvest. The cells were lysed, and the lysate was run on Invitrogen NuPage BisTris 4-12% gradient gels. The lysate was immunoprecipitated with goat anti-CTIP2 antibody-linked sepharose. After the protein was transferred to a membrane, the membrane was immunoblotted with anti-Bcl11b (#25B6) and anti-phosphothreonine (Cell Signaling #9386) primary antibodies followed by HRP-linked secondary antibodies. Antibodies were detected with enhanced chemiluminescent (ECL) substrate and exposed to X-ray film.

Stripping Primary Antibodies and Re-Immunoblotting

After Western blotting with anti-phosphothreonine antibodies, the immunoblots were placed in 40°C stripping buffer (0.2 M Glycine, 2.5 pH, 0.1% Tween) for 3 washes,

each 15 minutes long. Afterwards, the membrane was immunoblotted with anti-SUMO1 (ab32058) antibody purchased from Abcam (Cambridge, MA) followed by HRP-linked secondary antibodies and ECL detection.

Results

P2C2 are a DN3-like T-cell line. They are easily propagated in culture and are promising cell models to study Bcl11b in situ. However, relatively little is published about them. The purpose of this experiment was to identify how P2C2 cells phosphorylate and sumoylate endogenous Bcl11b following activation. To test for the presence of phosphorylated endogenous Bcl11b, anti-phosphothreonine antibodies were used for immunoblots. The results are shown in Figure 2 displaying results from P2C2 cells treated with P/A for 5 minutes, 30 minutes, 1 hour, or 2 hours prior to harvest. The experiment also included a non-treated control. After treatment for the indicated time, the cells were lysed and immunoprecipitated with Bcl11b antibodies. As seen in the figure, each lane that contained samples treated with P/A expressed phosphorylated Bcl11b, which can be seen expressed at the 130 kDa region. This indicates that Bcl11b was present endogenously in P2C2 cells. Lanes with sample treated with P/A demonstrate a seemingly linear increase in the amount of phosphorylated Bcl11b with time of treatment.

For examining how sumoylation levels of Bcl11b change over time in P2C2 cells, I stripped the anti-phosphothreonine antibodies from the Western blot membrane, and re-immunoblotted with anti-SUMO antibodies. Figure 3 represents a blot created in such a manner. As stated earlier in the introduction, SUMO peptides stack, and sumoylated

Bcl11b appears up as a dark ladder between 135 and 250 kDa. This figure, similar to the previous one, shows a seemingly linear increase in the amount of expressed sumoylated Bcl11b following P/A treatment.

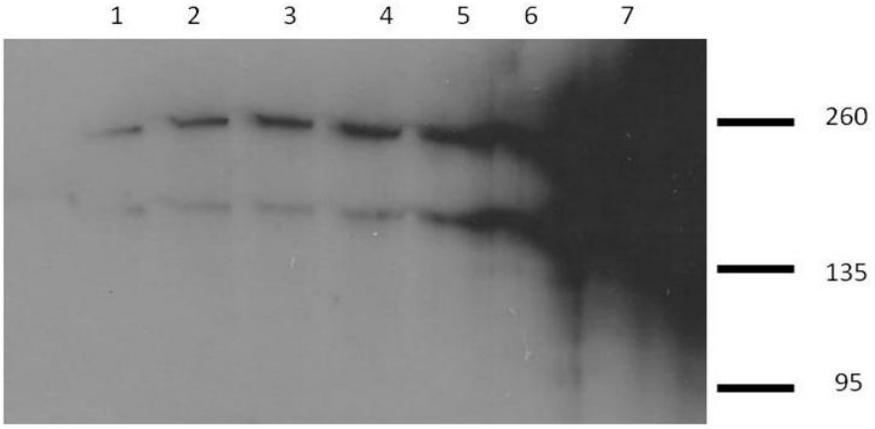


Figure 2. Phosphorylation of endogenous Bcl11b extracted from P2C2 cell samples following P/A treatment. The blot was incubated with anti-phosphothreonine (anti-pThr) antibody. The 260, 130, and 95 marks depict the migration of protein standards in kilodaltons (kDa). The P2C2 samples in each of the lanes were treated with P/A, however at varying times. Lanes 1 and 2 were not treated with P/A, lane 3 was treated with P/A for 5 minutes, lane 4 for 30 minutes, lane 5 for an hour, and lane 6 for 2 hours. Lane 7 is an input control lane showing total protein in the cells. Phosphorylated Bcl11b protein runs at approximately 135 to 150 kDal

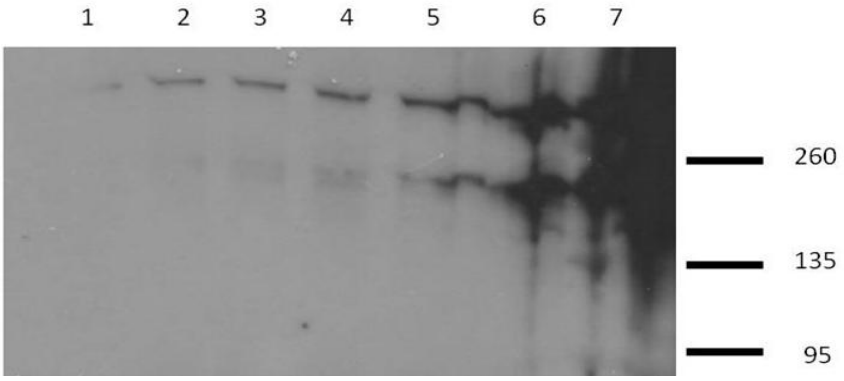


Figure 3. Sumoylation of endogenous Bcl11b extracted from P2C2 cell samples following P/A treatment. The blot was incubated with anti-SUMO antibody. The 260, 130, and 95 marks depict the migration of protein standards in kilodaltons (kDa). The P2C2 samples in each of the lanes were treated with P/A, however at varying times. For both blots, lanes 1 and 2 were not treated with P/A, lane 3 was treated with P/A for 5 minutes, lane 4 for 30 minutes, lane 5 for an hour, and lane 6 for 2 hours. Lane 7 is an input control lane showing total protein in the cells. Sumoylated Bcl11b is much larger, running at 150 to 260 kDal (bottom blot).

Discussion

P2C2 cells are a DN3-like transformed pre-leukemic cell line developed by Dr. Ellen Rothenberg (personal communication, California Institute of Technology) chosen as a model to study Bcl11b in situ in a thymocyte-like cell. Ideally, a cell model for studying Bcl11b would be easy to transfect, easy to propagate in culture, and would mimic how Bcl11b is modified post-translationally in thymocytes. P2C2 cells are easy to propagate in culture, and this part of the project involved determining whether or not they also mimicked how thymocytes modified Bcl11b following translation.

Immunoblotting with anti-Bcl11b antibodies demonstrated that there was endogenous Bcl11b present in P2C2 cells. This is important because Bcl11b appears at the DN2 stage of thymocyte development at the earliest (Di Santo, 2010). While P2C2 cells are derived from the DN3 stage of thymocyte development, it is still prudent to check for Bcl11b.

However, several observations of the immunoblots suggest that the P2C2 cells would be a poor cell model to study Bcl11b. First, the P2C2 cells only responded to P/A treatment after 60-120 min (Figure 2, lanes 5 and 6). This is incredibly delayed compared to thymocytes which respond within 5 min. Second, the P2C2 endogenous Bcl11b was not dephosphorylated at the 30 minute mark. Comparing the two figures, the samples in Figure 2 demonstrates a high level of phosphorylated Bcl11b 60-120 minutes following cell activation. In normal thymocytes, this would not occur. Similarly, there is a continuous increase in sumoylated Bcl11b post P/A treatment as seen in Figure 3. In natural thymocytes, sumoylation occurs only after dephosphorylation. The P2C2 do not demonstrate the mutual exclusivity of phosphorylation and sumoylation that normal thymocytes demonstrate.

In summary, the response of P2C2 cells to P/A treatment in terms of phosphorylation and sumoylation of Bcl11b is sufficiently different from native thymocytes to not be an acceptable substitute. Unfortunately, harvesting mice thymocytes appear to be the best source for studying thymocytes at the moment.

CHAPTER 3

Introduction

The previous two chapters discussed finding a good model for future Bcl11b post-translational modification research. This chapter will be focused on research on Bcl11b post-translational modifications.

Bcl11b undergoes post-translational modifications, two of which are phosphorylation and sumoylation. Common to other transcription factors, Bcl11b has multiple phosphorylation sites (phosphosites). Previous data collected by Dr. Walter Vogel with mass spectrometry reveals that Bcl11b contains 23 phosphosites. As demonstrated in Figure 4, these phosphosites were grouped into clusters based on similar kinetic changes in phosphorylation following thymocyte stimulation (Zhang et al., 2012). Bcl11b also has 2 sumoylation sites. To better study the effects these sites have on each other and on general Bcl11b as a whole, mutants were designed by Ling-Juan Zhang of Dr. Mark Leid's laboratory and created by Xiao Liu. Multiple mutant constructs of Bcl11b were created with three different clusters of 1 to 6 phosphorylation sites altered to alanines. For mutants that had multiple phosphosites mutated, the chosen phosphosites were grouped because they showed similar kinetic changes. Also included in the mutants is a double sumoylation site mutant called MT26 in which all SUMO-sites were deleted. These mutants are listed in Table 1.

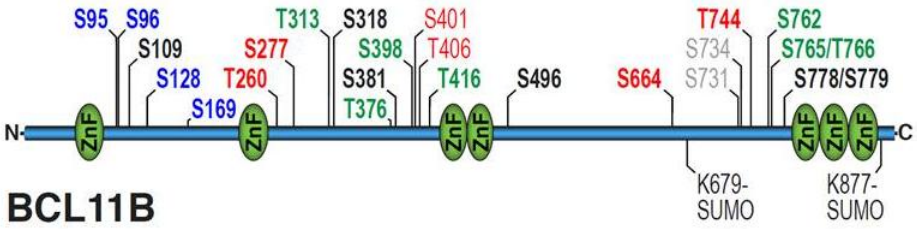


Figure 4. Schematic of Bcl11b. This figure shows the location of the 23 phosphorylation sites (phosphosites) and 2 sumoylation sites (SUMO-sites) on Bcl11b. The phosphosites are color coordinated so each color represents a specific type of kinematic change in phosphorylation activity. Also shown are the 6 zinc finger domains (ZNF).

Mutant Name	Amino Acid(s) Mutated
N1	S496A
N3	S318A, S381A, S664A
N4	S401A, S405A, S406A, T416A
N5	T260A, S277A, S318A, S381A, S664A
C1	S664A
C6	T744A, S762A, S765A, T766A, S778A, S799A
MT26	K679R, K877R

Table 1. Bcl11b mutants. The first six mutants contained one or more phosphosites mutated to alanine. The last mutant (MT26) is the double sumo-site mutant; both sites were mutated to arginine. For phosphomutants with multiple mutation sites, the phosphosites were chosen using preliminary data suggesting that the phosphosites possessed similar kinetic changes in phosphorylation level following thymocyte stimulation.

This type of mutant research requires over-expression of non-endogenous DNA, namely mutant Bcl11b and other associative proteins. Thus, cell transfection is required. Because thymocytes were difficult to transfect, HEK-293T (Human Embryonic Kidney) cells were chosen as the cell model. These cells are easy to manipulate, and they mimic thymocytes in Bcl11b post-translational modification to an acceptable degree for this level of work. As there was preliminary data suggesting that dephosphorylation of Bcl11b promoted sumoylation, MT26 and its phosphorylation and sumoylation levels were also of interest (Zhang *et. al.*, 2012 and personal communication).

This experiment sought to test two hypotheses. The first was whether or not phosphorylation and sumoylation levels were mutually exclusive; when one level increased, the other was predicted to decrease. This hypothesis would be tested with Bcl11b DNA with mutant phosphosites and MT26. The second hypothesis is that there are certain phosphosites or groups of phosphosites that are modified to a greater degree during the phosphorylation and dephosphorylation processes. Perhaps these phosphosites are more important to Bcl11b during its interactions within the cell and could be a potential target for future pharmaceuticals. This experiment would attempt to identify these phosphosites.

The relationship between Bcl11b phosphorylation and sumoylation was analyzed initially by transfecting HEK-293T cells with Bcl11b wild type and mutant DNA and co-transfecting with a sumo protease called SENP1. Previous work in Dr. Leid's lab had indicated that phosphorylation attracted SENP1; this experiment will also test this hypothesis. Prior to harvest, the cells would be treated with a chemical phosphatase inhibitor to examine the effect of an artificially high level of phosphorylation of the wild

type and mutant Bcl11b. The sumoylation levels of Bcl11b would also be quantitatively measured at this time.

In work performed later, HEK-293T cells were again transfected with Bcl11b wild type and mutant DNA. This time, however, they were cotransfected with SUMO1. One of the drawbacks of the previous experimentation is that there seemed to be a low level of sumoylated Bcl11b in HEK-293T cells. The intent was to magnify over-expression of sumoylated Bcl11b to analyze how Bcl11b's phosphorylation and sumoylation were impacted when sites were mutated.

Both of these experiments had the intent of identifying which individual or group of phosphosites experienced more change in phosphorylation. The earlier experiments also sought how changes in phosphorylation would change Bcl11b's association with SENP1. Attention to Bcl11b's interaction with SENP was eventually dropped, and we refocused our attention to examine how phosphorylation levels were changed when SUMO sites were mutated and vice versa.

Methods and Materials

Cell lines

HEK-293T cells were grown in 10% FBS DMEM medium. Cells were split into 6-well plates with 0.25 million cells / 2 mL of medium / well. They were incubated under the same conditions as the mouse thymocytes and P2C2 cells.

Plasmids and transfection

Bcl11b "F-CTIP-2 Mt20" cDNA-containing mammalian expression plasmids and the other Bcl11b mutant cDNA-containing plasmids purified by $CsCl_2$ were designed by Lingjuan Zhang, created by Xiao Liu, and provided by Dr. Mark Leid. Similarly Dr. Mark Lied supplied the SENP1 and HA-SUMO1 cDNA plasmids.

Phosphorylation assays and Western blotting

At 30-35% confluency, cells were transfected with Bcl1b wild type and mutant cDNA (N4, N5, and C6 mutants only), and SENP1 (sumo protease) plasmids using calcium phosphate (FIVEphoton Biochemicals protocol; ("Calcium Phosphate Transfection Kits," 2009)). 24 hours post transfection, media was changed to include antibiotics (Chapter 1 protocol) and 2.5% FBS. 48 hours post-transfection and thirty minutes prior to harvesting, cells were treated with 2 uL of 50 mM Calyculin A, a phosphatase inhibitor or 2 uL DMSO as a vehicle control. Cells were lysed with nuclear extraction buffer (25 mM Hepes, pH 7.1, 1 mM EDTA, 400 mM NaCl, 15% glycerol, 50 mM NaF, 0.1% NP40, 0.2 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, supplemented with complete protease inhibitor cocktail (Fermentas) and 1 mM PMSF) to preserve protein-protein interactions. Cells were then sonicated using a Branson 450 sonicator using 25% amplitude for 5 seconds, 6-8 times with 5 second intervals on ice. Immunoprecipitation was done using goat anti-CTIP2 antibody-linked sepharose. Western blots performed on cell lysates using the same phosphothreonine and Bcl11b antibodies mentioned in the protocol of Chapter 2. The SENP1 antibodies came from Santa Cruz. Using near-

infrared fluorescent secondary antibodies, the fluorescent intensity of bands on the immunoblot was quantitated using a Licor/Odyssey® instrument.

In a separate experiment, cells were transfected with Bcl1b wild type and mutant cDNA (all of the mutants) using calcium phosphate. Some of the cells were co-transfected with HA-SUMO1 cDNA. Cells were harvested, and western blots performed on cell lysates using anti-Bcl11b antibody (#25B6), anti-phosphothreonine antibody (Cell Signaling #9386), and anti-SUMO1 antibodies (Abcam). Because the SUMO1 cDNAs constructs were HA tagged, the anti-HA-tag antibody (Aves Lab, Inc.) was occasionally used to detect sumoylation. Quantitative Western blot data was collected using the Licor Odyssey Infrared Imaging System.

Results

The initial purpose of the experiment was twofold. First, we sought to identify how mutating phosphosites would affect Bcl11b's composite phosphorylation level. We also wanted to analyze how removing groups of phosphosites would affect Bcl11b's interaction with SENP1. Ideally, the resulting data would suggest which phosphosites were more influential to composite phosphorylation level and to interactions with other proteins.

To evaluate the basal and total stimulated phosphorylation levels of Bcl11b mutants relative to wild-type, we performed immunoprecipitations on samples from HEK-293T cells that had been co-transfected with SENP1 and Bcl11b (either wild-type or mutant constructs) in a manner to preserve protein-protein interactions. We then ran the immunoprecipitants on SDS-PAGE and performed Western blotting analysis with

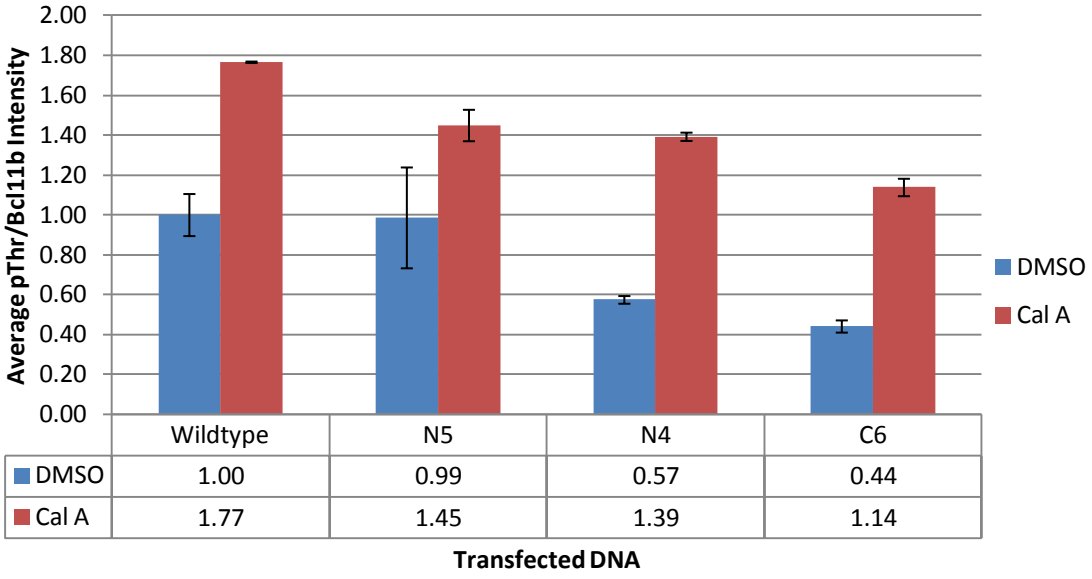
anti-Bcl11b, anti-pThr, or anti-SENP1 antibodies and fluorescent secondary antibodies for quantitation. For each sample, we calculated the amount of pThr relative to total Bcl11b levels and the amount of SENP1 relative to total Bcl11b levels for each sample. We obtained quantitative data on the amount of Bcl11b protein and on phosphorylated threonine in the lysate samples. We then calculated the relative amount of phosphothreonine on Bcl11b to total Bcl11b in each sample (Figure 5, y axis). The levels of SENP1 relative to Bcl11b following quantitative Western blot analysis were taken as an indication of the extent of interaction of SENP1 with Bcl11b.

An evaluation of the Western blots revealed that relative to wild-type Bcl11b, the N4 and C6 mutants had reduced basal levels of phosphorylation (Figure 5; DMSO bars). Unfortunately, the basal phosphorylation levels of the N5 mutant were too variable across samples to draw any definitive conclusion.

Calyculin A (Cal A) is a phosphatase inhibitor of PP1 and PP2a, and by treating HEK-293T cells, the overall level of phosphorylation increases. When looking at samples treated with the phosphatase inhibitor, Calyculin A (Figure 5; Cal A for 30 min), the overall phosphorylation of wild-type Bcl11b was increased as expected. Further, following treatment with Cal A, all of the samples expressing Bcl11b phosphosite mutants had reduced phosphorylation compared to wild type.

Previous work had shown that increased phosphorylation of Bcl11b resulted in increased interactions with the sumo protease SENP1 (Zhang et al, 2013). Therefore, we next sought to investigate whether loss of phosphorylation sites would reduce the interaction with SENP1. The interaction of SENP1 with N5 and N4 mutants relative to wild-type Bcl11b was relatively unchanged (Figure 6) in samples from untreated cells.

However, the C6 mutant showed less interaction with SENP1 than wild-type Bcl11b. Further, treatment of the transfected cells with Cal A reduced the interaction of all the mutant Bcl11b constructs with SENP1 relative to vehicle treatment as seen in Figure 6. This is in contrast to wild type Bcl11b where phosphorylation increases did not affect SENP1 interaction.



	Wildtype	N5	N4	C6
DMSO	1.00	0.99	0.57	0.44
Cal A	1.77	1.45	1.39	1.14

Figure 5. Phosphothreonine phosphorylation of Bcl11b relative to total protein in the absence and presence of Calyculin A treatment. HEK-293T cells were transfected with wild type Bcl11b cDNA or one of three mutants (N5, N4, C6) corresponding to three different groups of phosphosite mutants. Cells were treated with vehicle (blue bars) or Calyculin A (red bars) for 30 min prior to harvest and immunoprecipitation with anti-Bcl11b antibodies. Western blots with anti-phosphothreonine and anti-Bcl11b antibodies were performed and quantitated as described in Methods. Error bars display the range of two replicates in a single experiment. This experiment was performed twice.

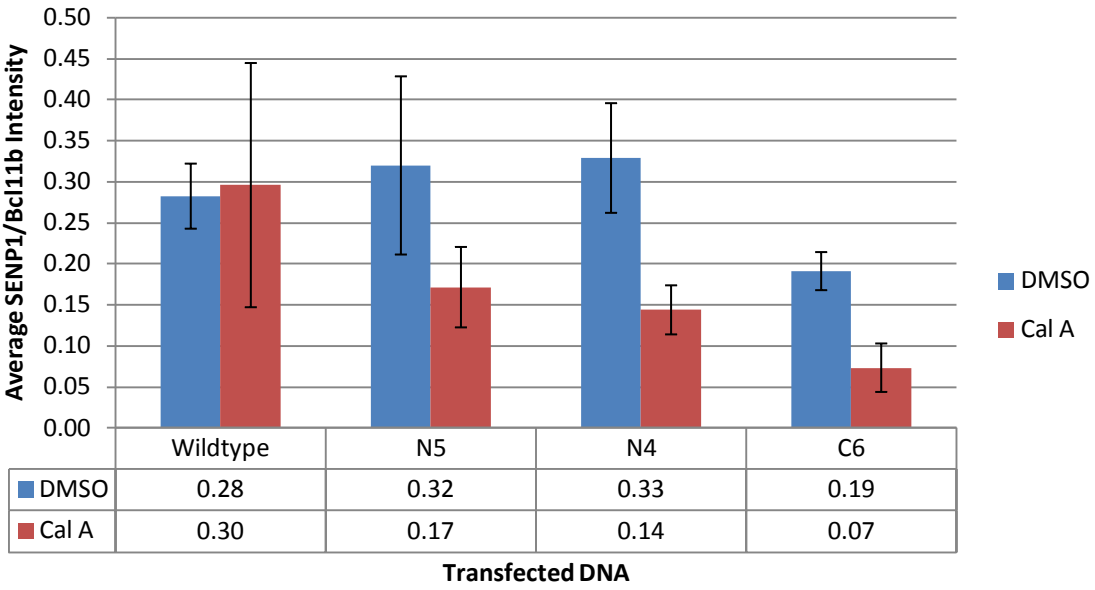


Figure 6. SENP1 co-immunoprecipitation with Bcl11b relative to total Bcl11b levels in the absence and presence of Calyculin A treatment. HEK-293 cells were transfected with SENP1 cDNA and co-transfected with either wild type Bcl11b cDNA or one of three mutants (N5, N4, C6) corresponding to three different groups of phosphosite mutants. Cells were treated with vehicle (blue bars) or Calyculin A (red bars) for 30 minutes prior to harvest and immunoprecipitation with anti-Bcl11b antibodies. Western blots with anti-SENP1 and anti-Bcl11b antibodies were performed and quantitated as described in Methods. Error bars display the range of two replicates in a single experiment. This experiment was performed twice.

In separate experiments, we sought to quantitate the phosphorylation and sumoylation status of several more phospho- and SUMO-site mutants. We quantified basal phosphorylation of Bcl11b in HEK-293 cells transfected with wild type and all of the mutants described in Table 1. A Western blot from cell lysates was immunoblotted with anti-Bcl11b and anti-pThr antibodies (Figure 7). Analysis of the immunoblot data normalized to Bcl11b levels revealed that none of the phosphomutants' phosphorylation state was different from wild type Bcl11b. In contrast, MT26, the double sumo-site mutant, had a higher significant pThr/Bcl11b ratio compared to wild type Bcl11b.

Similar to quantitating the phosphorylation levels of Bcl11b, we sought to quantitate the sumoylation levels of Bcl11b. To do so, we sought to over-express sumoylated Bcl11b by co-transfecting HEK cells with Bcll1b (wild type or mutant) and a cDNA construct of SUMO1 tagged with HA. Samples from these cells were immunoblotted with anti-Bcl11b and anti-SUMO antibodies and quantified using fluorescent secondary antibodies. The resulting immunoblot was examined for evidence of sumoylation. Sumoylated Bcl11b has a unique appearance. It is heavier than non-sumoylated Bcl11b and is seen as a ladder migrating at higher molecular weights on anti-Bcl11b immunoblots. An example of an endogenous sumoylated Bcl11b ladder made from samples from Jurkat cells in an experiment performed by Kimberly Rodriquez appears in Figure 8B. Figure 8A shows immunoblots created with anti-Bcl11b and anti-SUMO1 antibodies using samples from the transfected HEK cells. A comparison of the two blots shows that the HEK-293T cells co-transfected with the HA-SUMO1 DNA construct failed to increase the amount of sumoylated Bcl11b. This is illustrated by the fact Figure 8A fails to show the same red ladder seen in Figure 8B.

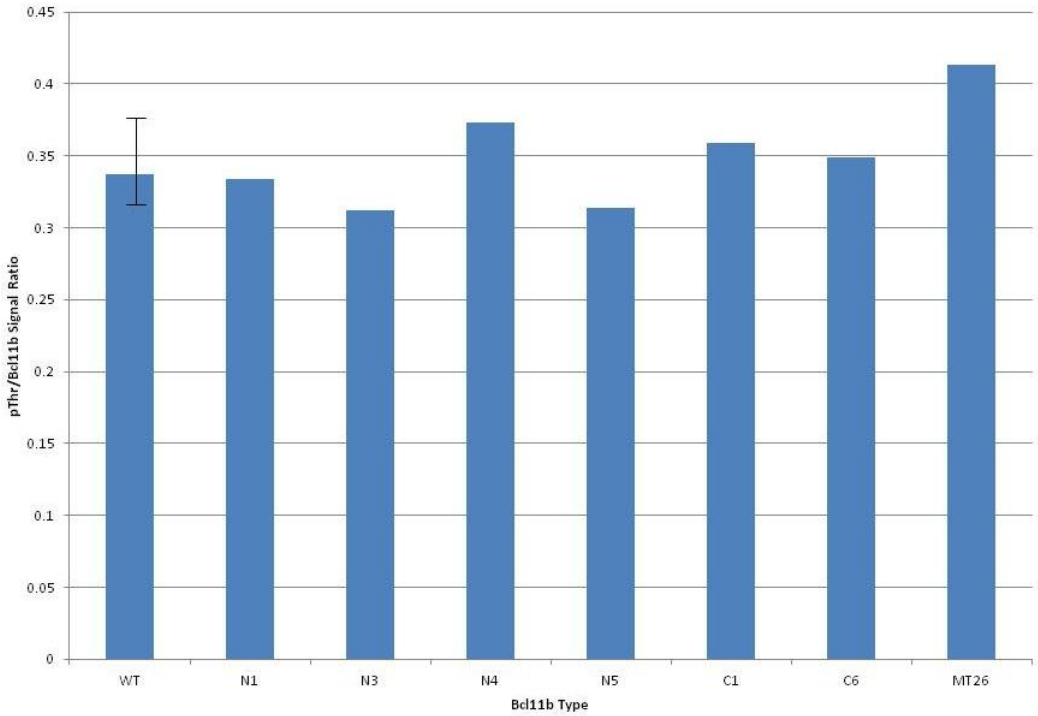


Figure 7. Ratio of threonine-phosphorylated Bcl11b to total in transfected HEK-293T-cells overexpressing wild type Bcl11b (WT), a variety of phosphosite mutants, and MT26. HEK cells were transfected with Bcl11b wild type (in duplicate) or mutant cDNA (in singlet). Anti-Bcl11b antibody (#25B6) and anti-phosphothreonine antibody (Cell Signaling #9386) were used to detect threonine phosphorylated and total Bcl11b respectively. Fluorescently-tagged secondary antibodies for the Licor Odyssey imager were used to quantitate relative levels of phopho and total Bcl11b in each sample. For wild type, the error bar represents a range of duplicate samples. The mutant constructs are as indicated in Table 1. MT26 is the K679R and K877R double sumo-site mutant. This experiment was performed five times.

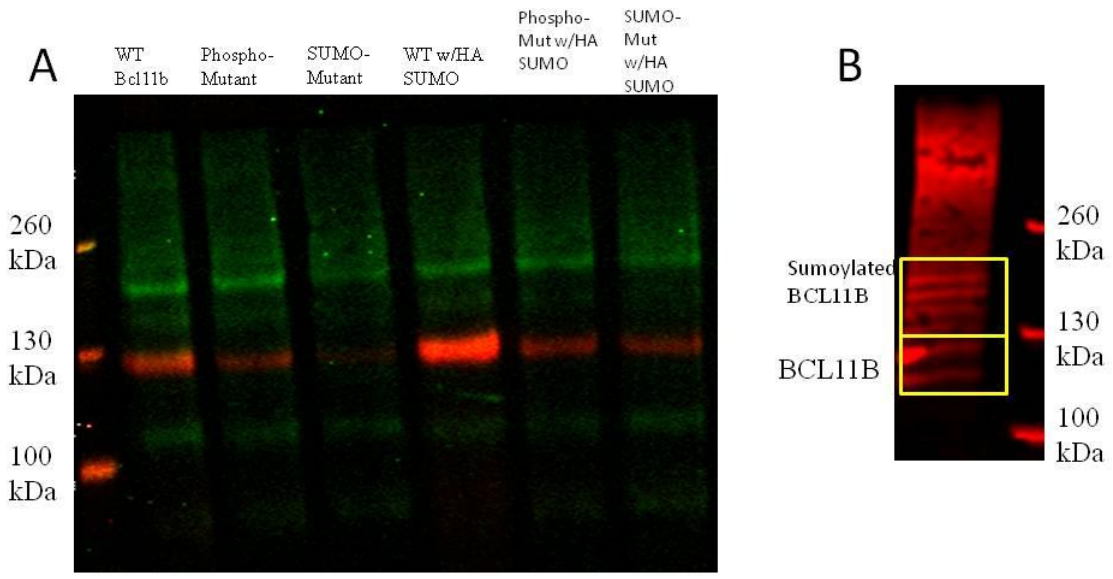


Figure 8. HEK-293T-cells co-transfected with HA-SUMO1 DNA and wild-type or mutant BCL11B constructs. A.) A representative immunoblot. The three molecular weight markers are labeled to the left. Lanes depict HEK-293T-cells transfected with wild type Bcl11b (lane 1), a representative phosphomutant (lane 2), and the double sumo mutant (lane 3). Lane 4, 5, and 6 depict the same Bcl11b constructs in the same order co-transfected with HA-SUMO1 DNA. The blots were immunoblotted with anti-Bcl11b antibody (#25B6) which showed up in the red channel, and anti-SUMO1 antibodies which showed up in the green channel B.) BCL11B endogenous to Jurkat cells. (Figure courtesy of Kimberly Rodriquez) For comparison, an immunoblot of BCL11B and sumoylated BCL11B as endogenously expressed in Jurkat cells. Jurkat cells were lysed and nuclear extracts run on SDS-PAGE for separation by size. The gel was transferred and immunoblotted with anti-Bcl11b antibody (#25B6; red channel). BCL11B naturally appears as a doublet of two splice variants at molecular weights of approximately 130 and 133 kDa. The top yellow box shows sumoylated BCL11B appearing as a higher molecular weight ladder above non-sumoylated BCL11B. The three molecular weight markers are labeled to the right of the blot.

Discussion

We sought to identify the effects of phosphorylation on the interaction of Bcl11b (wild type and mutants) with the sumo protease, SENP1. Focusing on Figure 5, data from this experiment revealed that all Bcl11b phosphomutants from samples treated with

Calyculin A were phosphorylated some degree. We had anticipated that one cluster or another would be differentially phosphorylated relative to the others to reveal the most highly phosphorylated cluster and to help pinpoint the most important phosphorylation sites. Unfortunately, the roughly uniform loss of phosphorylation from each mutant relative to wild-type Bcl11b does not help to narrow the important sites. However, these results were encouraging as we had anticipated that mutation of phosphosite amino acids identified by mass spectrometry would lead to overall reduced phosphorylation, providing verification that some of the phosphosites identified by mass spectrometry were also phosphosites in the context of transfected HEK-293 cells and were phosphorylated to a significant level in the context of the whole protein.

Previous work suggested that the phosphorylation of Bcl11b attracts SENP1. Had that held true, more SENP1 should have co-precipitated with the Calyculin A treated samples than from vehicle treated. We found the opposite, suggesting a correlation between SENP1 interaction and less phosphorylation of Bcl11b. Unfortunately, this correlation is opposite to that seen in thymocytes and suggests that HEK-293T cells do not completely mimic Bcl11b regulation in thymocytes. Furthermore, more experiments are need with less sample to sample variability to reach definitive conclusions.

In another set of experiments, we focused out attention away from SENP1 and more onto the effect of phosphorylation on sumoylation and vice versa. In these experiments, HEK cells were co-transfected with Bcl11b (wild type and mutant) and SUMO1 in an attempt to increase the level of sumoylated Bcl11b. These samples were immunoblotted with anti-Bcl11b, anti-pThr, and anti-SUMO1 antibodies. While there was an increased focus on sumoylation, one of the purposes of this experiment was to

analyze the correlation between the number of phosphosites mutated and total phosphorylation of Bcl11b. Data from Figure 7 show that this correlation is small and that nearly all of the pThr to Bcl11b ratios collected for the mutants fall within wild type's range of pThr/Bcl11b. This suggests that none of the phosphosites removed are phosphorylated to a significant level, and therefore a difference in composite phosphorylation levels is not easily observed. For example, the C6 mutant shows a pThr/Bcl11b ratio that is close to wild type. Perhaps this is because that the phosphosites mutated into alanine for C6 were phosphorylated to a low level in the first place.

This does not explain why we were able to detect differences in one series of experiments (Figure 5) and not another (Figure 7). The effect of an intervening year between experiments may be important, but we don't know why.

Another purpose of this experiment was to explore the relationship between phosphorylation and sumoylation. There were two ways to analyze this. The first was to analyze how mutating sumoylation sites affected phosphorylation. The right-most column of Figure 7 depicts the double sumoylation mutant, MT26. As shown, MT26 possesses higher phosphorylation levels than the others. In this case, loss of sumoylation sites leads to an increased level of phosphorylation. This data is also consistent with our hypothesis that increased phosphorylation and sumoylation are mutually exclusive.

The second way to explore the relationship between phosphorylation and sumoylation is to analyze how mutating phosphosites affect sumoylation. However, because we could not detect sumoylated Bcl11b in the HEK-293T samples, we cannot make any general conclusions about the removal of phosphosites on sumoylated Bcl11b. We do not know why we could not detect sumoylated Bcl11b in our HEK cells; this

finding was perplexing because others have detected sumoylated Bcl11b using the same protocol. Regardless, we cannot support or refute our hypothesis that phosphorylation and sumoylation are mutually exclusive with these data.

CONCLUSION

The experiments explained in this Honors thesis are focused on finding a cellular model for analyzing Bcl11b, a transcription factor dysregulated in 20% cases of T-ALL. Because T-ALL is a result of incorrect thymocyte development, research into how T-cells mature is essential. A key factor in T-cell maturation is the transcription factor Bcl11b.

We attempted to identify an ideal model for studying Bcl11b; however both cellular models that we tried had negative characteristics. Mouse thymocytes could not be transfected using the Invitrogen Neon ®Electroporation System. P2C2 cells post-translationally modified Bcl11b differently than thymocytes. The next experiment attempted to identify which of Bcl11b's phosphorylation sites contribute more towards the composite phosphorylation level and which were more important in cell signaling. This was done with Bcl11b mutants that had key phosphorylation sites removed. While initial data suggested that mutating phosphorylation sites affected overall phosphorylation levels, subsequent experiments resulted in conflicting data suggesting that the number of phosphorylation sites removed had little effect on the expressed composite phosphorylation level of Bcl11b. Further, the inability to detect sumoylation in transfected HEK-293T cells made this system inappropriate to study the effects of phosphorylated Bcl11b on sumoylation. Nevertheless, we showed that loss of sumoylation sites altered the phosphorylation of Bcl11b, further substantiating the phospho-sumo link.

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