AN ABSTRACT OF THE THESIS OF

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Title: REGULATION OF BCL11B BY POST-TRANSLATIONAL MODIFICATIONS.

Abstract approved

Mark E. Leid

Bcl11b (B-cell lymphoma/leukemia 11b), also known as Ctip2 (Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2), is a C2H2 zinc finger transcriptional regulatory protein, which is an essential protein for post-natal life in the mouse and plays crucial roles in the development, and presumably function, of several organ systems, including the central nervous, immune, craniofacial formation and cutaneous/skin systems. Moreover, inactivation of Bcl11b has been implicated in the etiology of lymphoid malignancies, suggesting that Bcl11b may function as a tumor suppressor.
Bcl11b was originally identified as a protein that interacted directly with the orphan nuclear receptor COUP-TF2. Later studies revealed that this C2H2 zinc finger protein can bind DNA directly in a COUP-independent manner, and it has been studied mostly as a transcription repressor. In T cells, gene repression mediated by Bcl11b involves the recruitment of class I HDACs, HDAC1 and HDAC2, within the context of the Nucleosome Remodeling and Deacetylation (NuRD) complex. The hypothesis that Bcl11b functions as a transcriptional repressor has been supported by transcriptome analyses in mouse T cells and human neuroblastoma cells. However, approximately one-third of the genes that were dysregulated in the double positive (DP) cells of Bcl11b-null mice were down-regulated relative to control T cells, suggesting that Bcl11b may act as a transcriptional activator in some promoter and/or cell contexts. We have also found that Bcl11b functions as a transcriptional activator in a promoter context-dependent manner. However, how Bcl11b and its transcription regulatory activity is regulated still remain largely unknown.

It has become evident that post-translational modifications (PTMs) play essential roles in modulating activity of transcription regulators. By sensing extracellular signals, cells initiate a series of signaling cascades, which eventually transduce to transcription factors by PTMs, leading to changes of gene expression profile.

Here, we study the reversible, covalent modification of Bcl11b by phosphorylation and small ubiquitin-like modifier (SUMO). We have identified
K679 and K877 as the two major Bcl11b SUMOylation sites by mutagenesis study. We have shown that phosphorylation and SUMOylation of Bcl11b are likely mutually exclusive processes, and phosphorylation of Bcl11b inhibits its SUMOylation by promoting the recruitment of SUMO specific protease SENP1. To study the function of Bcl11b SUMOylation, we fused SUMO1 to the amino terminus of Bcl11b. This generated a form of Bcl11b that was constitutively sumoylated without the complications of indirect effects associated with overexpression of SUMO1. Our data presented using the constitutive SUMO-Bcl11b demonstrated that SUMOylation compromises the transcription repression mediated by Bcl11b. Interestingly, when Bcl11b is fused to a cleavable form of SUMO, Bcl11b is targeted to ubiquitination pathway and it is degraded through proteasome machinery, suggesting that SUMOylation targets Bcl11b to the ubiquitination-proteasome machinery and deSUMOylation of SUMO conjugated Bcl11b is required for this process.

These results described herein provide a framework for understanding the mechanisms underlying the transcription regulatory activities of Bcl11b, and how Bcl11b is regulated by post-translational modifications, including phosphorylation and SUMOylation. These studies may contribute to a better understanding of the molecular and cellular basis for Bcl11b function in vivo.
REGULATION OF BCL11B BY POST-TRANSLATIONAL MODIFICATIONS

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Xiao Liu, Author
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CONTRIBUTION OF AUTHORS

Dr. Mark Leid designed research. Xiao Liu performed research. Chapter 3: Most of the experiments were performed by Xiao Liu. Dr. Lingjuan Zhang performed the experiments described in Fig. 3.7A, 3.7B and 3.7C. Dr. Theresa Filtz and Dr. Walter Vogel provided valuable suggestions for this project.
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REGULATION OF BCL11B BY POST-TRANSLATIONAL MODIFICATIONS

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW
1.1 History of the Bcl11b/Ctip proteins — Bcl11a (also known as Ctip1) and Bcl11b (also known as Ctip2) were originally cloned by the Leid laboratory in 2000 based on the ability of these proteins to interact with and mediate transcriptional repression of COUP-TF orphan nuclear receptors in a yeast two-hybrid screening system (Avram et al., 2000). Therefore, the Leid lab named these two proteins as COUP-TF-interacting proteins 1 and 2 (CTIP1 and CTIP2, respectively). Bcl11a/Ctip1 was later identified by Neal Copeland’s lab as a site of retroviral integration that was causally associated with murine myeloid leukemia (Nakamura et al., 2000). Subsequently, several reports were published implicating Ctip1/Bcl11a in human B cell leukemias (Dyer and Oscier, 2002; Kuppers et al., 2002; Martin-Subero et al., 2002; Martinez-Climent et al., 2003; Satterwhite et al., 2001).

Ctip2 is named by NCBI as Bcl11b because of its close sequence identity to Bcl11a (Avram et al., 2002), even though Bcl11b is not expressed in B cells and has never been implicated in B cell leukemia. In 2003, Bcl11b was recloned by Kominami’s group, and referred to as radiation-induced transcript-1 (Rit-1) because they found that Bcl11b was mutated in the mouse thymus in the process of tumorigenesis induced by gamma radiation (Wakabayashi et al., 2003a). This observation led Kominami’s group to hypothesize that Bcl11b may function as a tumor suppressor gene in T cells (Wakabayashi et al., 2003), and this has been supported by reports of a link between loss of homozygosity at the human Bcl11b locus and T cell leukemia (Bezrookove et
al., 2004; MacLeod et al., 2004; MacLeod et al., 2003; Nagel et al., 2003; Okazuka et al., 2005; Su et al., 2004), and the recent finding that 20% of childhood T-ALL exhibit abnormalities in Bcl11b expression (Przybylski et al., 2005; Su et al., 2006). Recurrent chromosomal rearrangements at the Bcl11b locus as well as point mutations of Bcl11b have also been found in human T-cell leukemias (Nagel, 2003; Przybylski et al., 2005). It has been shown that lack of Bcl11b results in vulnerability to DNA replication stress, and damage and mutation of Bcl11b leads to an increase of the proliferation rate of hematopoietic progenitor cells (Kamimura et al., 2007; Karlsson et al., 2007). These reports strongly support the hypothesis that Bcl11b is a tumor suppressor in T cells. Two other splice variants of Bcl11b have also been cloned by Kominami’s group, defined herein as Bcl11b-long (containing all exons) and Bcl11b-short (containing exons 1 and 4; see Fig. 1.1). In the present study, we focused on the Bcl11b splice variant containing exon 1, 2 and 4, because this is the original splice variants cloned by Dr. Leid’s lab and it appear to be one of the dominant splice variants expressed in T cells.

1.2 Bcl11b is a transcriptional regulatory protein — Earlier studies from the Leid lab found that Bcl11b repressed transcription of reporter genes in transiently transfected cells, either by interacting with other promoter-bound transcription factors, such as COUP-TFs, or by direct, sequence-specific DNA binding activity (Avram et al., 2000; Avram et al., 2002; Satterwhite et al., 2001; Topark-Ngarm et al., 2006). By a binding-site selection technique, Bcl11b was
shown to bind to GC-rich target sequence, suggesting the regulatory element to which Bcl11b binds is reminiscent of a GC-box (Avram et al., 2002). The transcriptional repression mediated by Bcl11b has been shown to be mediated through the recruitment of the class III histone deacetylase (HDAC) SIRT1 (Senawong et al., 2003) and/or the Nuclleosome Remodeling and Deacetylation (NuRD) complex, including class I HDACs, HDAC1 and HDAC2 (Topark-Ngarm et al., 2006), to the promoter template (Cismasiu et al., 2005; Topark-Ngarm et al., 2006). Bcl11b has also been reported to sequester and target transcriptional activators, such as HIV Tat, to heterochromatic loci, which may represent a third mechanism of Bcl11b-mediated transcriptional silencing (Marban et al., 2005). The hypothesis that Bcl11b functions as a transcriptional repressor has been supported by transcriptome analyses in mouse T cells (Kastner et al., 2010) and human neuroblastoma cells (Topark-Ngarm et al., 2006), because most of the dyregulated genes upon Bcl11b ablation were up-regulated derepressed in the absence of Bcl11b. However, approximately one-third of the genes that were dysregulated in the thymocytes of Bcl11b-null mice were down-regulated relative to control T cells, suggesting that Bcl11b may act as a transcriptional activator in some promoter and/or cell contexts. Recent reports from the Leid lab have also shown that Bcl11b functions as a transcriptional activator in a promoter context-dependent manner (Golonzhka et al., 2009a; Golonzhka et al., 2009c; Kastner et al., 2010). The underlying mechanism(s) for Bcl11b-mediated transcriptional activation remains unknown.
1.3 Role of Bcl11b in T-cell Development — The multiple specification and differentiation steps in T cell development in the thymus are regulated through the concerted action of a series of transcription factors, including Bcl11b. Germline deletion of Bcl11b results in a blockade of the DN3→DN4 transition (β selection) with the complete absence of αβ T cells, and this block is phenotypically similar to that seen in NCoR- (Jepsen et al., 2000), E2A- (Bain et al., 1997), and HEB- (Bain et al., 1997; Barndt et al., 1999; Zhuang et al., 1996) null mice, conditionally null c-myb mice when crossed with the appropriate deleter strain (Bender et al., 2004), and transgenic mice ectopically expressing Id2 (Morrow et al., 1999) or a dominant negative form of the E-protein HeLa E-box binding protein (HEB) (Barndt et al., 1999) in thymocytes. To bypass the early T cell development stages and study the role of Bcl11b in DP stage, mice with Bcl11b deletion (Bcl11b^{dp/-}) at the DP stage were generated by crossing Bcl11b^{L2/l2} mice with a CD4-cre transgenic mice. Studies using Bcl11b^{dp/-} mice revealed that Bcl11b is also required for positive selection and survival of double-positive thymocytes (Albu et al., 2007; Kastner et al., 2010). Thus, Bcl11b plays an important role in at least two stages of T cell development: β selection (DN3 → DN4 transition) and in the DP → SP transition.

1.4 Functions of Bcl11b during development of the CNS and cutaneous systems — Bcl11b is expressed early during mouse development as well as in the adult animal, and in both cases; expression is most predominant in the
CNS, thymus, and skin/epithelial structures (Golonzhka et al., 2007; Leid et al., 2004). Mice null for expression of Bcl11b exhibit perinatal lethality, which is reminiscent of the COUP-TFI−/− phenotype (Qiu et al., 1997; Yamaguchi et al., 2004), and severe phenotypes in all tissues that express the gene. For example, Jeff Macklis’ lab described defects in axonal projection by corticospinal neurons in the CNS in Bcl11b-null mice (Arlotta et al., 2005), and the Leid lab has observed severe defects in axonal path-finding in this and other regions of the CNS (Golonzhka et al., unpublished observations).

In collaboration with Indra’s lab, the Leid lab has recently reported that ablation of Bcl11b affected skin development: Bcl11b null mice exhibit a compromised barrier formation, leading to dehydration, electrolyte imbalances, and dysregulated proliferation and differentiation of epidermal keratinocytes during development (Golonzhka et al., 2009c). In addition, lack of Bcl11b in the oral ectoderm leads to a differentiation block in the ameloblast lineage, cells which are responsible for enamel secretion in the developing tooth (Golonzhka et al., 2009b).

1.5 Regulation of transcription factor by Post-translational modifications

— Post-translational modifications (PTMs) of proteins have essential roles in creating a highly dynamic system that receives and responds to changes in the cellular microenvironment. PMTs regulate the function of a protein by adding a modifying chemical group or other modifier protein to one or more of
its amino acid residues. Some PTMs, such as glycosylation and disulfide bridge formation, are stable and are required for proper folding of newly synthesized protein. Others, such as phosphorylation, ubiquitination and SUMOylation are more transient and reversible, and have crucial roles in relaying rapid message in the cells.

1.5.1 Phosphorylation — Protein phosphorylation is the most common type of post-translational modification used in signal transduction. It affects every basic cellular process, including growth, metabolism, proliferation, differentiation, organelle trafficking, motility, membrane transport, immunity, muscle contraction, learning and memory. Protein kinases catalyze the transfer of the γ-phosphate from ATP to Ser, Thr or Tyr residues of protein targets in eukaryotes (Ubersax and Ferrell, 2007).

One of the most studied and also the most understood pathways is initiated by activation of growth factor receptors (Hazzalin and Mahadevan, 2002), which harbor intrinsic tyrosine-kinase activity. Upon ligand binding, growth factor receptors autophosphorylate themselves, which subsequently activate many downstream kinase cascades, including the phosphatidylinositol 3-kinase pathway, the protein kinase C pathway, the pathways that regulate small GTPases, such as Rho, Rac and Cdc42, and the extracellular-signal-regulated kinase / mitogen-activated protein kinase (ERK/MAPK) pathway (Hazzalin and Mahadevan, 2002). Activated kinases, such as PKCs and ERK1/2 can enter
the nucleus and phosphorylate Ser or Thr residues of a variety of resident transcription factors that bind to the upstream regulatory elements of target genes. For example, it has been shown that epidermal growth factor (EGF) by activating EGF receptor, induces full activation of ERK1/2 within one minute of treatment (Hazzalin et al., 1997). Activated ERK1/2 then translocates from the cytosol to the nucleus, and phosphorylates Elk1 (Ets-like protein-1), a member of the Ets family of transcription activators, at Ser383 and Ser389 residues, leading to increase of its transcription activation activity. It has been shown that phosphorylation not only enhances Elk1’s DNA binding (Yang et al., 1999), but also leads to the recruitment of the CBP/p300 coactivator complex through direct protein-protein interaction between Elk1 and p300, to target promoter of Elk1 (Li et al., 2003).

In some other cases, dephosphorylation also plays crucial role in regulating transcription factor function. One well known example is calcium signaling and NFAT activation. NFAT (nuclear factors in activated T cells) molecules are heavily phosphorylated in resting T cells and localize primarily in the cytosol (Okamura et al., 2000). Upon activation of T cell receptors, intracellular Ca^{2+} concentration increases, leading to activation of calcineurin, a phosphatase that dephosphorylates NFAT. Dephosphorylated NFAT then translocates into the nucleus, resulting in activation of genes responsible for generating immune responses (Rao et al., 1997).
1.5.2 SUMOylation — SUMO proteins are Small Ubiquitin-like Modifiers which are covalently attached to and detached by SUMO-specific isopeptidases of the sentrin-specific protease (SENP) from other cellular proteins in cells to modify their function. SUMOylation is a post-translational modification, and it is also a major regulator of protein function that is involved in multiple cellular processes.

Yeast SUMO Smt3 was identified as a suppressor to the centromeric protein Mif2 which contained one SUMO protein. (Meluh and Koshland, 1995), but the mechanism of this suppression remained obscure. Subsequently, mammalian SUMO-1 was identified in yeast two-hybrid screens as an interaction partner of the RAD51/52 (Shen et al., 1996) nucleoprotein filament proteins, which mediate DNA repair, and a component of multiprotein nuclear complexes the promyelocytic leukaemia protein PML (Boddy et al., 1996), and the proapoptotic protein Fas (Okura et al., 1996). The nuclear pore protein RanGAP1 (Ran-GSTase-activating protein1) was the first reported target which is covalently modified by SUMO1, a process described as SUMOylation (Mahajan et al., 1997; Matunis et al., 1996).

There are four SUMO paralogues, SUMO1-4, and a large numbers of proteins are being identified as SUMO substrates. SUMO-1 is only 18% identical to ubiquitin. SUMO-2 and -3 are more highly related to each other; however, they share only approximately 50% sequence with SUMO-1. This difference is also
reflected in the specificity of SUMOylated targets. SUMO-1 does not form multimeric chains, although the related SUMO-2 and -3 do. The specificity of SUMO-4, and its ability to be conjugated to target proteins, remains an open question (Guo et al., 2004; Owerbach et al., 2005).

1.5.3 The SUMO conjugation cycle — SUMO attachment to its target is similar to that of ubiquitin (Fig. 1.2). First, a C-terminal peptide is cleaved from SUMO by a protease family called SENP by using ATP to reveal a di-glycine motif that allows SUMO to be conjugated to lysine residues in target proteins. The cleaved SUMO then becomes bound to an E1 enzyme which is a heterodimer of SAE1 (SUMO Activating Enzyme1). Next, SUMO is then passed to an E2 which is a conjugating enzyme Ubc9 (ubiquitin-conjugating 9). Ubc9 is the only known SUMO E2 conjugating enzyme and Ubc9 directly binds to the consensus SUMOylation motif on the substrate proteins. The SUMOylation consensus motif is ψKxD/E (ψ is a large hydrophobic amino acid) (Yang et al., 2006). Finally, one of a small number of E3 ligating proteins attaches SUMO to the target protein. In yeast, there are four SUMO E3 proteins, Cst9, Mms21 (Branzei et al., 2006), Siz1 and Siz2. While in ubiquitination an E3 is essential to add ubiquitin to its target, evidence suggests that the E2 is sufficient in SUMOylation as long as the consensus sequence is present. It is thought that the E3 ligase promotes the efficiency of SUMOylation and in some cases has been shown to direct SUMO conjugation onto non-consensus motifs. E3 enzymes can be largely classed into PIAS
proteins, such as Mms21 (a member of the Smc5/6 complex) (Dietrich et al., 1997) and Piasgamma and HECT proteins. Recent evidence has shown that PIAS-gamma is required for the SUMOylation of the transcription factor yy1 but it is independent of the zinc-RING finger (identified as the functional domain of the E3 ligases).

**1.5.4 DeSUMOylation** — Sumoylation is reversible and is removed from targets by specific SUMO proteases in an ATP dependent manner. SUMOylation is a highly dynamic process that can be readily reversed by SENP (SUMO-specific isopeptidases of the sentrin-specific protease). In yeast, Ulp1 was first identified as a SENP homologue that specifically cleaves Smt3 at the C-terminus. Either pro-Smt3 or mature Smt3 cannot be rescued when Ulp1 yeast deletion strains undergo cell cycle arrest. Ulp2 is another Smt2-specific protease act to cleave Smt3 from substrate proteins but does not involved in the maturation of pro-Smt3. In addition, lack of yeast strains show an accumulation of Smt2 polymers, which suggest Ulp2 functions in the processing and editing of Smt3 chains (Wilkinson and Henley, 2010).

There are six SENPs in mammals (SENP1-3 and SENP 5-7; (Di Bacco et al., 2006; Gong and Yeh, 2006), which are also required for production of the mature forms of SUMO. Mammalian SENPs can be classified in to three groups. SENP1/2 has a broad specificity for SUMO-1 and SUMO-2/3 and functions both in their conjugation and deconjugation (Hang and Dasso, 2002;
Zhang et al., 2002). SENP2 and SENP5 are prefer SUMO2/3 over SUMO1 (Di Bacco et al., 2006; Gong and Yeh, 2006). SENP6 and SENP7 also act preferentially on SUMO2/3 (Mukhopadhyay et al., 2006; Shen et al., 2009). Moreover, both SENP6 and SENP7 are not involved in maturation of pro-SUMO proteins and they have less activity in the monomeric SUMO2/3 deconjugation from substrate proteins. However, SENP6 and SENP7 can deconjugate poly SUMO2/3 efficiently (Mukhopadhyay et al., 2006; Shen et al., 2009). Thus the distinctions between the functions of different SENP enzymes are clear. SENP1/2 is primarily responsible for cellular maturation of the SUMO proteins and act as deconjugation of both SUMO1 and SUMO 2/3 from substrates. The function of SENP3/5 is to remove monomeric SUMO2/3 from substrates and SENP6/7 act as editors of SUMO2/3 chains.

1.6 Cross-talk between PTMs — During the past few years, a compelling amount of data has shown cross-talk between PTMs and the involvement of a sequential series of PTMs in transmitting and regulating signaling. Protein phosphorylation is a regulator of SUMOylation where it can either enhance or inhibit substrate SUMOylation. A phosphorylation-dependent SUMOylation motif (PDSM) in target protein has been identified to induce phosphorylation-mediated enhancement of substrate SUMOylation. This motif is found in many transcription factors, including GATA-1, MEF2A and HSF1 (Zimnik et al., 2009). SUMOylation can also be negatively regulated by phosphorylation. Hyperphosphorylation of PML by phosphatase inhibitor treatment significantly
decreased its SUMOylation. Similarly, phosphorylation of transcription factor Elk-1 represses its SUMOylation, leading to loss of its transcriptional repressive activity (Yang et al., 2003a). Similar to phosphorylation, there is a complex direct interplay between the SUMOylation and ubiquitination machineries. For example, SUMOylation of E2-25K, an E2 enzyme in the ubiquitin pathway, inhibits its enzymatic activity, leading to down-regulation of the ubiquitination system (Pichler et al., 2005). Conversely, a novel family of RING-finger ubiquitin ligases that recognize SUMOylated substrates has been recently identified, known as STUbLs (SUMO targeted ubiquitin ligases). STUbLs directly target SUMOylated substrate to the ubiquitination system, promoting target proteins for degradation by the 26S proteasome (Geoffroy and Hay, 2009).

1.7 Research Objectives — Previous studies from our lab found that when primary thymocytes were stimulated with the protein kinase C (PKC) activator PMA and the calcium ionophore A23187, a combination that has been shown to bypass the T cell receptor (TCR) signal and activate T cell signaling pathways, Bcl11b was dynamically regulated by PTMs including phosphorylation and SUMOylation, by the MAP kinase pathways, and these PTMs influence the transcriptional regulatory activity of Bcl11b on target promoters (unpublished work). Therefore, in the present study, we investigated (1) the major Bcl11b SUMOylation sites; (2) the mechanisms
regulating the molecular switch between Bcl11b phosphorylation and SUMOylation; and (3) the role of Bcl11b SUMOylation.
**Fig 1.1 Mouse Bcl11b locus.** Schematic representation of mouse Bcl11b genomic locus and splice variants (upper part). The lower part shows the relationship of exonic structure to the protein. Single letter is the amino acid abbreviation. Box highlights the region that is enriched with indicated amino acids.
Fig. 1.2. SUMO conjugation pathway. The immature form of SUMO is first cleaved by SUMO specific protease SENPs at the C-terminal GG cleavage site to expose the di-glycine motif (1). This cleaved form of SUMO is then activated by and transferred to the E1 SUMO conjugation enzyme (2). SUMO is then passed to the active site cysteine of the E2 conjugating enzyme, Ubc9 (3), which then catalyses the transfer of SUMO to the target protein, in conjunction with an E3 enzyme (4, 5). DeSUMOylation is catalyzed by SENP family proteases (6). SUMO is then released and is then available to undergo next round of conjugation to target proteins.

(Wilkinson and Henley, 2010)
REGULATION OF BCL11B BY POST-TRANSLATIONAL MODIFICATIONS

CHAPTER 2

METHODS AND MATERIALS
2.1 Cell culture — Primary thymocytes were isolated from 4–8 week-old C57BL/6 wild-type mice. Thymocytes were cultured at 37 °C in RPMI1640 (VWR) with 5% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin (Invitrogen) for 4 hr prior to drug treatment. Jurkat, the human leukemia T cell line, was grown under the same conditions except that 10% FBS was used. HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (VWR) with 10% FBS and 1% penicillin/streptomycin.

2.2 Chemicals and antibodies — Phorbol 12, 13-dibutyrate (PMA) and anti-Flag antibody was purchased from Sigma (St. Louis, MO). A23187, U0126, SB202190, MG132, zVAD were purchased from EMD Biosciences (Gibbstown, NJ). Calyculin A was purchased from Cell Signaling (Danvers, MA). Antibodies to Bcl11b (ab18465), SUMO1 (ab32058), and SUMO2/3 (ab3742) were purchased from Abcam (Cambridge, MA). Anti-phospho-threonine (9386) was purchased from Cell Signaling. Antibodies against SUMO1, and SUMO2 were purchased from Abcam (Cambridge, MA). The anti-hemagglutinin (HA) antibody was purchased from Aves Labs Inc (Tigard, Oregon). The anti-ubiquitin antibody was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

2.3 Compound treatments — Primary thymocytes were cultured for 4 hrs before treating with vehicle (DMSO) or a P/A cocktail consisting of PMA (100 nM) and A23187 (500 nM) for indicated times. In some studies, cells were
pretreated with indicated kinase inhibitors for 30 mins prior to P/A treatment. Jurkat cells were serum starved overnight before stimulation with P/A.

2.4 DNA constructs — Flag-tagged Bcl11b (F-Bcl11b) was made as previously described (Senawong et al., 2003). F-Bcl11b lysine to arginine mutant constructs including K607R, 805R, and K607/805R (2R) were made using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). HA-SUMO1 and HA-SUMO2 DNA constructs were purchased from Addgene Inc (Cambridge, MA). HA-SENP1 and HA-SENP2 constructs were generously provided by Dr. Grace Gill (Department of Pathology, Harvard Medical School). HA-F-Bcl11b was generated by PCR amplification of HA-SUMO1 with primers containing appropriate restriction sites for insertion into the N-terminal of F-Bcl11b. All constructs were verified by complete DNA sequencing. The tk promoter was removed from pBLCAT2 vector using BamH I and Bgl II or BamH I and Xho I restriction enzymes. The BamH I/Bgl II fragment of pBLCAT2 was re-ligated to reconstitute a promoterless-CAT reporter construct. The BamH I/ Xho I promoterless-vector was used for subsequent cloning as described below. The –2.9 kb Id2-CAT reporter constructs were prepared by amplification of a 5´ region of mouse Id2 gene ranging from –2.9 kb to +152 bp, relative to TSS using appropriate primers and a DNA template from mouse ES cells (Expand long template PCR system, Roche). These amplified fragments were cloned into pCR 2.1 vectors using a TA cloning kit (Invitrogen). An insert in the pCR 2.1 vector was verified by
sequencing before being removed and cloned into BamH I/Xho I sites of the promoterless-CAT reporter construct.

2.5 Transfections — 293T cells (2 X 10^6 cells) were plated onto a 10 cm plate and transfected 24 h later using the calcium phosphate method. Cells were transfected with indicated amount of DNA and an empty expression vector to standardize the total amount of DNA transfected. The medium was changed, and the cells were harvested 24 and 48 hr after transfection, respectively. Jurkat cells (2 X 10^6 cells) were plated onto a 35 mm dish and transfected with wild-type F-Bcl11b or F-Bcl11b mutants using TransIT-Jurkat reagent (Mirus Bio LLC). 24 hr later, cells were serum starved overnight followed by P/A treatment for 60 mins prior to harvest.

2.6 Immunoprecipitation, co-immunoprecipitation and immunoblotting — To preserve phosphorylation and SUMOylation of Bcl11b, cells treated with indicated compounds were harvested, lysed in a denaturing buffer containing 250 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 5 mM N-ethylmaleimide, 0.1 mM hemin chloride and 1% SDS, boiled for 5 mins, sonicated and centrifuged to remove DNA. The resulting whole cell extracts were then diluted to 0.1% SDS and 1% Triton X-100, and Bcl11b was immunoprecipitated (IP) using Bcl11b antibody-coupled sepharose beads. IP assays and immunoblotting (IB) were conducted as described previously (Zhang et al., 2009). To study protein-protein interaction, whole cell extracts
were prepared using a lysis buffer containing 250 mM NaCl, 2 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 5 mM NEM, 0.1 mM H.C., and 0.05% NP40 under non-denaturing conditions. IP and IB analyses were performed as described above.

2.7 Reporter gene assays — HEK293T cells were transfected and harvested 48 hr after transfection. A β-galactosidase expression vector (pCMV-Sport-βGal, Life Technologies) was co-transfected as an internal control, and β-galactosidase activity and total protein concentration were used for normalization across all samples. The relative chloramphenicol acetyltransferase was determined as described previously (Dowell et al., 1997).
CHAPTER 3

RESULTS
3.1 Identification of Bcl11b SUMOylation sites — Putative Bcl11b SUMOylation sites were identified using a search algorithm (SUMOplot™; http://www.abgent.com/tools/ SUMOplot_login), and four sites bearing SUMO consensus motif have been identified, including K215, K679, K713 and K877 (Fig. 3.1A). Bcl11b K679 was confirmed as a site of SUMO1 and SUMO2 adduction by site-directed mutagenesis studies in HEK293 (Fig. 3.1C), and Jurkat (Fig. 3.1D) cells. These mutagenesis studies revealed that K877 was also a site of Bcl11b SUMOylation (Figs. 3.1C and 3.1D). Both SUMO1 (Fig. 3.1C, lanes 4 and 6) and SUMO2 (lanes 9 and 11) chains were evident at the K679 site, as observed with the wild-type protein and the Bcl11b K877R mutant, respectively. However, SUMO1/2 chains were less evident at the K877 site, as revealed by the Bcl11b K6797R mutant (lanes 5 and 10 of Fig. 3.1C). Interestingly, mutation of either of the primary SUMOylation sites alone resulted in hyper-SUMOylation of the remaining residue (compare lane 4 to lane 5 and 6 of Fig. 1C and lane 9 to lane 10 and 11 Fig. 3.1C; lane 2 to lane 4 and 6 of Fig. 3.1D), suggesting that SUMOylation of these two primary sites may be linked.

As SUMOylation is often associated with transcriptional repression (Long et al., 2004; Sapetschnig et al., 2002; Yang et al., 2003b), and Bcl11b is known as a transcriptional repressor in various promoter contexts (Topark-Ngarm et al., 2006), we assessed the ability of SUMO-site mutants of Bcl11b to repress target gene expression. For this purpose we used the Id2 promoter, a direct
target of Bcl11b-mediated transcriptional repression in mouse thymocytes (manuscript in preparation; see also below). Surprisingly, the transcriptional repressive activity of Bcl11b on a heterologous reporter construct harboring the Id2 promoter upstream of a CAT reporter was not compromised by single or compound mutation of the two sites of SUMOylation, indicating that SUMOylation does not likely play a role in Bcl11b-mediated repression.

3.2 The molecular nature of the Bcl11b phospho-SUMO switch — SUMOylation is a dynamic process that is readily reversed by a family of highly active SUMO-specific proteases, the SENP family of proteins (Drag and Salvesen, 2008). Previous study from our lab found that phosphorylation of Bcl11b induced by either 5 min of P/A (PMA and A23187) or continuous calyculin A (phosphatase inhibitor) treatment leads to rapid deSUMOylation of Bcl11b, suggesting that phosphorylated Bcl11b may be a substrate for SENP-mediated deSUMOylation. To test this hypothesis, we investigated whether SUMOylated Bcl11b is a substrate for SENP1 and SENP2. When co-expressed with Bcl11b and HA-SUMO1 or HA-SUMO2, SENP1, and SENP2 efficiently deSUMOylated SUMO1-Bcl11b and SUMO2-Bcl11b (Fig. 3.2A upper blot) without affecting total Bcl11b protein levels (lower blot). We also found that SENP1 stably interacted with Bcl11b or the Bcl11b complex (Fig. 3.2B), and this interaction was not dependent on the SUMOylation status of Bcl11b because the SUMO-deficient mutant K607/805R interacted with SENP1 in a manner indistinguishable from that of wild-type Bcl11b (Fig. 3.2C).
To map the interaction domains between Bcl11b and SENP1, HEK cells were co-transfected with SENP1 with full length or indicated truncation mutant of Bcl11b (Fig. 3.2D). As shown in Fig. 3.2E, the N-terminal fragment (amino acid 1-428) of Bcl11b efficiently interacted with SENP1 (Fig. 3.2E, lane 6), whereas the C-terminal fragment (amino acid 733-885) of Bcl11b failed to be co-immunoprecipitated with SENP1 (Fig. 3.2E, lane 12). Therefore, the interaction domain between Bcl11b and SENP1 is mapped to N-terminal region (aa 1-428) of Bcl11b.

3.3 Next, we studied how phosphorylation may influence the interaction between Bcl11b and SENP1 — PP6, one of the PP2A family phosphatases, has been identified as a Bcl11b phosphatase from a proteomic screen of Bcl11b complexes in native mouse thymocytes (data not shown). Basal phosphorylation of Bcl11b was almost completely eliminated when cells were co-transfected with expression vectors encoding Bcl11b and PP6 (Fig. 3.3A). Bcl11b, when dephosphorylated by PP6, failed to interact with SENP1 (compare lanes 2 and 4 of bottom blot of Fig. 3.3B), and SUMO1-Bcl11b could not be deSUMOylated by SENP1 under these conditions (compare lanes 2 and 4 of top blot of Fig. 3.4C). These findings are indicative of physical and functional lack of SENP activity in the Bcl11b complex upon Bcl11b dephosphorylation. Therefore, phosphorylation of Bcl11b appears to be required for its interaction with SENP1. To confirm this finding in a native context we performed co-IP experiments using primary mouse thymocytes that
had been treated with P/A for 5 and 60 min to achieve maximal Bcl11b phosphorylation and dephosphorylation, respectively. Bcl11b co-immunoprecipitated with SENP1 weakly under basal conditions (Fig. 3.3C, lane 5), and this interaction was further enhanced by Bcl11b phosphorylation after 5 min of P/A treatment (lane 6). The amount of Bcl11b co-immunoprecipitating with SENP1 returned to low levels upon dephosphorylation of Bcl11b after 60 min of P/A treatment (Fig. 3.3C, lane 7). These findings indicate that the phosphorylation status of Bcl11b dictates the interaction of SENP1 with the Bcl11b complex (Fig. 3.3D).

3.4 Bcl11b interacts with SUMO E2 conjugation enzyme Ubc9 — We hypothesized that Bcl11b also interacts with the SUMO-conjugating enzyme Ubc9, because it is the only SUMO E2 enzyme in the mammalian genome. Indeed, we found that Bcl11b interacted with Ubc9, as expected (Fig. 3.4, lane 6 of upper blot). Next we wanted to map the interaction domains between Bcl11b and Ubc9. By using the Bcl11b truncated mutants in the co-immunoprecipitation experiments with HA-Ubc9, we identified Ubc9 preferentially interacting with the C-terminal region of Bcl11b. As shown in Fig 3.4, the C-terminal domain interacted strongly with Ubc9 (aa. 733-885; Fig. 4, lane 12 of the lower blot), whereas the N-terminal region of Bcl11b failed to co-immunoprecipitate with Ubc9 (aa. 1-428; Fig. 4, lane 8 of the upper blot).
Considered all together, these findings suggest that: (1) modification of Bcl11b by PTMs is a highly dynamic series of linked events, (2) phosphorylation and SUMOylation of Bcl11b are likely mutually exclusive processes, (3) phosphorylation of Bcl11b inhibits its SUMOylation by promoting the recruitment of SUMO specific protease SENP1, (4) Bcl11b also interacted with the SUMO E2 conjugation enzyme Ubc9, and whether this interaction is also influenced by the phosphorylation status of Bcl11b needs to be determined in the future.

3.5 Covalent attachment of SUMO-1 abrogates Bcl11b-mediated repression — To overcome the low efficiency of Bcl11b SUMOylation in HEK cells under basal conditions, we fused SUMO1 to the amino terminus of Bcl11b (see cloning strategy in Fig. 3.5). This generated a form of Bcl11b that was constitutively SUMOylated without the complications of indirect effects associated with overexpression of SUMO1. This SUMO fusion assay has been used in many reports to demonstrate the role of SUMO1 in modulating the transcriptional activity of transcription factors, such as Sp3 and Smad4 (Ref, 2004, BJ-Smad4). In our studies, we generated a noncleavable SUMO1-Bcl11b by fusing amino acid 1-95 of SUMO1 to Bcl11b (Fig. 3.5C, upper panel), by passing the GG cleavage sites located at aa 96-97 of SUMO1 (Fig. 3.5B). A cleavable SUMO1(c)-Bcl11b has also been made by fusing amino acid 1-101 of SUMO1 to Bcl11b (Fig. 3.5C, lower panel). The non-cleavable SUMO1-Bcl11b did not overtly activate the Id2 promoter in HEK cells but fusion to
SUMO1 did reduce the transcriptional repressive activity of Bcl11b by approximately 2-fold (Fig. 3.6B) without influencing expression levels (Fig. 3.6B lower panel) or the nuclear/sub-nuclear distribution of the protein. Moreover, co-expression of SUMO1-Bcl11b attenuated repression of the Id2 promoter by WT Bcl11b (Fig. 3.6C), possibly by hetero-complex formation, as revealed by co-IP analyses (Fig. 3.6D; upper panel).

Bcl11b harbors a putative SUMO-interaction motif (SIM) within its C-terminal acidic domain. However, we have been unable to detect direct interaction of Bcl11b with SUMO1 in vitro (data not shown), suggesting that the association between Bcl11b and SUMOylated Bcl11b is not mediated through a direct interaction between the SUMO1 moiety and the putative SIM of Bcl11b. The Leid lab has previously observed Bcl11b self-association in a SUMO-independent manner (Topark-Ngarm et al., unpublished data), and it seems conceivable that this homotypic interaction may underlie the SUMO1-Bcl11b/Bcl11b heterocomplex formation that we observed in Figs. 3.6C and D.

We have shown previously that SUMO1-Bcl11b can be deSUMOylated by SENP1. Therefore, next we determined how deSUMOylation influenced Bcl11b-mediated repression. In contrast to constitutive fusion to SUMO1, deSUMOylation of wild-type Bcl11b by SENP1 further enhanced Bcl11b-mediated repression (Fig. 3.6E). In contrast, the transcriptional repressive activity of the double SUMO-acceptor site mutant Bcl11b K607/805R (Bcl11b
2R) on the Id2 promoter reporter was not significantly affected by co-transfection with the SENP1 expression vector (Fig. 3.6E). Collectively, these findings indicate that SUMOylation compromised the transcriptional repressive activity of Bcl11b, which may allow for induction of the Id2 promoter downstream of cell signaling pathways, including T cell receptor activation. In addition, SUMOylation of Bcl11b would appear to result in recruitment of the transcriptional coactivator p300 (unpublished work) to the Bcl11b complex present on the Id2 promoter template, further suggesting that SUMOylation of Bcl11b may actively contribute to transcriptional induction of the Id2 promoter in thymocytes.

3.6 Targeting SUMOylated Bcl11b by the ubiquitin machinery — The Leid lab has shown that SUMOylated-Bcl11b appeared stable and functionally involved in promoting Id2 mRNA induction at least within a few hours after T cell stimulation. However, prolonged treatment of mouse thymocytes with P/A resulted in degradation of SUMOylated Bcl11b, as well as unSUMOylated Bcl11b, and the appearance of proteolytic cleavage products (compare lanes 4 and 6 to other lanes in Fig. 3.7A). This finding suggests that SUMOylation of Bcl11b selectively targets the protein for degradation. However, we had not previously noted stability issues with SUMO1- (Fig. 3.6B, D) or SUMO2- (data not shown) Bcl11b fusion proteins, indicating that SUMOylation per se did not negatively influence the stability of Bcl11b.
However, the SUMO1-Bcl11b fusion protein used in Fig. 3.6 was engineered to lack a SUMO hydrolase cleavage (Fig. 3.5B and 3.5C). To determine if SUMO1-Bcl11b hydrolysis may be coupled to degradation of the SUMOylated protein, a second SUMO1-Bcl11b construct, SUMO1(c)-Bcl11b, was prepared that harbored a natural SENP cleavage site (Fig. 3.5B and 3.5C). First, we tested the expression of the SU(c)-Bcl11b fusion protein compared to wild-type Bcl11b. Surprisingly, SUMOylated Bcl11b was barely detectable in transfected HEK cells using an anti-Bcl11b antibody (Fig. 3.8B, right blot). This lack of detection was not due to the quality of the SUMO-Bcl11b construct, because it was expressed as efficiently as Flag-Bcl11b using an in vitro transcription/translation system (Fig. 3.8A), suggesting SUMO-Bcl11b may be preferentially degraded in HEK cells. In contrast to the non-hydrolyzable SUMO1-Bcl11b fusion protein, SUMO1(c)-Bcl11b was extremely labile, and this instability was rescued by incubation of the transfected cells with MG132, but not zVAD, inhibitors of the proteosome and caspases, respectively (Fig. 3.8C). Indeed, inhibition of the proteosome with MG132 resulted in a very high molecular weight version of SUMO1c-Bcl11b, which was cross-reactive with an anti-ubiquitin antibody (data not shown) suggesting the ubiquitination preceded proteolytic degradation of the SUMO1c-Bcl11b fusion protein. This was again confirmed in native, mouse thymocytes in which substantial Bcl11b ubiquitination was observed at 6 hrs after initiation of P/A treatment (Fig. 3.7B). The coupling of SUMO hydrolysis and proteosomal degradation of Bcl11b is
reminiscent of the mode of action of the SUMO-targeted ubiquitin ligase (STUbL) class of proteins, such as RNF4 (Lallemand-Breitenbach et al., 2008).

The stability of the SUMO1(c)-Bcl11b fusion protein was rescued by co-expression of wild-type Bcl11b, and maximal SU-Bcl11b stability was achieved when wild-type Bcl11b and SUMO1c-Bcl11b was transfected in the HEK cells at 1:1 ratio (Fig. 3.8D). Moreover, wild-type Bcl11b robustly co-immunoprecipitated with SUMO1c-Bcl11b (Fig. 3.8D), suggesting that wild-type Bcl11b may interact with SUMO(c)-Bcl11b to maintain the stability of the latter.
Figure 3.1.

A

SUMO consensus motif: $\Psi^KXE$
$(\Psi=\text{large hydrophobic aa}; X=\text{any aa})$

\[
\begin{array}{c}
\text{Bcl11b} \\
213 \text{ IA} \ GKDE \ \text{PS} \ 290 \ (K215) \\
678 \text{ KR} \ I \ KVE \ KD \ 683 \ (K679) \\
710 \text{ HF} \ MKDF \ FL \ 717 \ (K713) \\
874 \text{ NDVKE} \ QA \ 881 \ (K877)
\end{array}
\]

B

Wild Type  

\[
\begin{array}{c}
\text{1} \ F \ Bcl11b \ 885 \\
\text{K215} \ K215 \\
\text{K679R} \ K679 \\
\text{K713R} \ K713 \\
\text{K877R} \ K877
\end{array}
\]
Figure 3.1.

C

| pCDNA   | + | + |
| Bcl11b wt | + | + |
| Bcl11b K679R | + |
| Bcl11b K877R | + |
| Bcl11b 2R | + |
| HA-SUMO1 | + | + | + | + |
| HA-SUMO2 | + | + | + | + |

MW (kDa)
- 250
- 180
- 130
- 250
- 180
- 130

1 2 3 4 5 6 7 8 9 10 11 12
**Figure 3.1.**

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Figure 3.1. Identification of Bcl11b SUMOylation sites (A), Diagram of the four predicted lysine SUMOylation sites in Bcl11b. The SUMO consensus sequence is shown at the top. The predicted lysine (K) for SUMOylation is highlighted in red. “ψ” refers to any large hydrophobic amino acid, and “X” can be any amino acid. The fourth amino acid is usually glutamic acid (E). The lower panels indicate amino acid sequences around each predicted SUMOylation lysine. (B), Schematic representation of the location of the predicted Bcl11b SUMOylation sites. (C) Identification of K679 and K877 as the major Bcl11b SUMOylation sites by site-directed mutagenesis. HEK cells were cotransfected with expression vectors encoding Bcl11b, two point mutants (K679R and K877R), or the double mutant (2R) and HA-SUMO1 or HA-SUMO2 as indicated. Whole cell extracts from transfected HEK cells were immunoprecipitated with the anti-Bcl11b antibody, and the immunoprecipitates were probed with an anti-HA antibody to detect HA-SU-Bcl11b (the upper blot). The blot was then stripped and re-probed with the anti-Bcl11b antibody (lower blot). (D). Jurkat cells were transfected with expression vectors encoding Bcl11b, two point mutants (K679R and K877R), or the double mutant (2R). Whole cell extracts from transfected Jurkat cells were immunoprecipitated with the anti-Flag antibody, and the immunoprecipitates were probed with an anti-Bcl11b antibody to Bcl11b and SUMOylated Bcl11b (indicated by arrows). Note that Jurkat cells, which do express high levels of Bcl11b, carry out the SUMOylation reaction efficiently and without the need of over-expressed SUMO-1.
Figure 3.2.

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MW (kDa)

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MW (kDa)

Flag(SENP1)
Figure 3.2.

D

WT

1-428

1-746

733-885

E

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SEN1

Input

SEN1-IP

130 90 72 54 26 90

Bcl11b

Flag(Bcl11b)

SEN1
Figure 3.2. SENP1 preferentially interacts with phosphorylated Bcl11b. (A), SENP1 and SENP2 efficiently deSUMOylate both HA-SU1-Bcl11b and HA-SU2-Bcl11b. HEK cells were cotransfected with expression vectors encoding wild-type Bcl11b and HA-SUMO1 or HA-SUMO2 and SENP1 or SENP2 as indicated. Whole cell extracts from transfected HEK cells were immunoprecipitated with an anti-Bcl11b antibody, and the immunoprecipitates were probed with an anti-HA antibody to detect SUMOylated Bcl11b (the upper blot). The blot was then stripped and probed with an anti-Bcl11b antibody as shown in the lower blot. (B), SENP1 interacts with Bcl11b complexes. HEK cells were transfected with an expression vector encoding Bcl11b with or without an expression vector encoding Flag-tagged SENP1 (F-SENP1). Bcl11b was immunoprecipitated using an anti-Bcl11b antibody, and the immunoprecipitates were probed with anti-Bcl11b (upper blot) or anti-Flag (SENP1; lower blot) antibodies. (C), SUMOylation is not required for the interaction between Bcl11b and SENP1. HEK cells were transfected with an expression vectors encoding wild-type Bcl11b or the double mutant (2R). Whole cell extracts from transfected HEK cells were immunoprecipitated with an anti-Bcl11b antibody and the immunoprecipitates were probed with an anti-Bcl11b (the upper blot) or anti-Flag (SENP1; lower blot) antibodies. (D), Schematic diagram of the full-length wild type (WT) or truncations of Bcl11b used for mapping the interaction between Bcl11b and SENP1. Locations of the individual zinc finger motifs are indicated by a cross mark. (E), Mapping the interaction domains between Bcl11b and SENP1. HEK cells were cotransfected with expression vectors encoding wild-type Bcl11b or truncation mutants and SENP1 as indicated. Whole cell extracts from transfected HEK cells were immunoprecipitated with an anti-SENP1 antibody, and the immunoprecipitates were probed with an anti-Bcl11b or anti-Flag antibodies or to detect co-IPed Bcl11b (the upper and middle blots). The blot was then stripped and probed with an anti-SENP1 antibody as shown in the lower blot.
Figure 3.3.

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Figure 3.3.

C

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MW (kDa)

72 - 90 - 130 - 180 - 250 -

1  2  3  4  5  6  7

Bcl11b
SENP1

D

SU
SENP

P P P

Bcl11b
Figure 3.3. SENP1 preferentially interacts with phosphorylated Bcl11b. (A), Bcl11b is dephosphorylated by PP6. HEK cells were cotransfected with expression vectors encoding Bcl11b in the presence or absence with PP6 as indicated. Bcl11b was immunoprecipitated, and the immunoprecipitates were first probed with anti-phospho-threonine/-serine antibody (upper blot), then the blot was stripped and reprobed with an anti-Bcl11b antibody (lower blot). The asterisks represent non-specific anti-phospho-serine/threonine immunoreactivity in the input lanes. (B), SENP1 does not interact with Bcl11b complex when Bcl11b is dephosphorylated by PP6. HEK cells were cotransfected with expression vectors encoding Bcl11b, F-SENP1 and HA-PP6 as indicated. Bcl11b was immunoprecipitated, and the immunoprecipitates were probed with anti-SUMO1 antibody (upper blot), and anti-Flag (SENP1) antibody (lower blot), then the upper blot was stripped and reprobed with Bcl11b (middle blot). (C), Native SENP1 preferentially interacted with phosphorylated Bcl11b in primary thymocytes. Primary thymocytes were treated with P/A for 5 min to achieve maximum Bcl11b phosphorylation or 60 min to induce dephosphorylation of Bcl11b as indicated. SENP1 was immunoprecipitated and the immunoprecipitates were probed with an anti-Bcl11b antibody (upper blot) or an anti-SENP1 antibody (lower blot). (D), a model for the interaction between phosphorylated Bcl11b and SENP1. SENP1 preferentially interacts with phosphorylated form Bcl11b and catalyzes its rapid deSUMOylation.
**Figure 3.4.**

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MW (kDa):

- 130
- 90
- 72
- 55
- 26

Flag(Bcl11b)
Figure 3.4. The interaction between Bcl11b and SUMO E2 ligase Ubc9
Mapping the interaction domains between Bcl11b and Ubc9. HEK cells were
cotransfected with expression vectors encoding wild-type Bcl11b or truncation
mutants and HA-Ubc9 as indicated. Whole cell extracts from transfected HEK
cells were immunoprecipitated with an anti-HA antibody, and the
immunoprecipitates were probed with an anti-Flag antibody or to detect co-
IPed Bcl11b (the upper blot).
Figure 3.5.

A

B

...YQEQTGHHSTV......

SUMO1

C

Non-cleavable

Cleavable
Figure 3.5. Constructions of the SUMO-Bcl11b fusion plasmids (A), Strategy to clone a SUMO-Bcl11b fusion construct. An insert of HA-SUMO1 with HindIII digestion sites on both ends was generated by PCR using Sra-HA-SUMO1 (ordered from addgene) as template. After digestion with HindIII, HASUMO1 was ligated to HindIII digested Bcl11b in pcDNA3.1 vector (HindIII site is located 5' of the Flag tag) to generate the SUMO-Bcl11b fusion construct. (B), Residue sequences around the GG cleavage site in the C-terminal of SUMO1. (C), Schematic representation of the cleavable or non-cleavable SUMO-Bcl11b fusion constructs. Amino acid 1-95 of SUMO1 (without the GG cleavage site) was fused to Bcl11b to generate the non-cleavable form, whereas amino acid 1-101 of SUMO1 (including the GG cleavage site) was fused to Bcl11b to generate the cleavable form.
Figure 3.6.

A

Mouse ld2 promoter

B

Fold Repression

1 2 3 4 5 6 7
Figure 3.6.

C

```
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Figure 3.6.

E

Fold Repression of kd2 promoter

Vector ctrl
WT
K679/877R

SENPI
Figure 3.6. Fusion to SUMO1 abrogates Bcl11b-mediated repression (A), Diagram of the CAT reporter construct harboring the mouse ld2 promoter. Approximately a 3 kb fragment of mouse ld2 promoter, from -2919 bp upstream to +152 bp downstream of TSS, were cloned to a promoter-less CAT reporter plasmid. The TSS site is denoted as +1. Predicted binding sites for transcription factors in this region are boxed: E box (the binding site for the basic helix-loop-helix factor), RFX1 (R) and GATA1 (G). (B), Fusion to SUMO1 abrogates Bcl11b-mediated repression. HEK cells were co-transfected with the reporter construct Id2-CAT and increasing amount of wild-type Bcl11b or HA-SUMO1-Bcl11b fusion construct as indicated. Similar levels of Bcl11b and SU1-Bcl11b protein were expressed as shown in the blot below the figure. Relative CAT expression was measured as described previously (C) Co-transfection of HA-SU1-Bcl11b impairs transcriptional repression mediated by wild-type Bcl11b. HEK cells were cotransfected with ld2CAT reporter and wild type Bcl11b or a combination of half WT Bcl11b and half SU1-Bcl11b. Relative CAT expression was measured as described previously. (D), SU-Bcl11b interacted with wild type Bcl11b. HEK cells were cotransfected with indicated amount of SU1-Bcl11b and wild type Bcl11b. SU1-Bcl11b was immunoprecipitated using anti-HA antibody and the immunoprecipitates were probed with anti-Bcl11b antibody. 10% input signal is shown in the lower blot. (E), DeSUMOylation by SENP1 enhanced Bcl11b-mediated repression. Wild type Bcl11b or the SUMO deficient mutant K679/877R was cotransfected with ld2-CAT reporter plasmid with or without SENP1 as indicated. Relative CAT expression was measured as described previously. Statistical significance was determined using Student’s T test (*, p<0.05; **, p<0.01).
Figure 3.7.

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P/A (min)

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B

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| 250| 250|
| 180| 180|
| 130| 130|
| 90 | 90 |
| 72 | 72 |

C

<table>
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| 180| 180|
| 130| 130|
| 90 | 90 |

Bcl11b
Figure 3.7. Dynamic interplay between SUMOylation and ubiquitination of Bcl11b in primary thymocytes stimulated with PMA and A23187. (A), Bcl11b is SUMOylated in T cells stimulated with P/A. Thymocytes were treated with P/A for indicate times prior to preparation of a whole cell extract, which was then subjected to immunoprecipitation using anti-SUMO1 antibody. Immunoprecipitates were probed with an anti-Bcl11b antibody (B), prolonged stimulation of primary thymocytes leaded to Bcl11b degradation. Primary thymocyte stimulated with P/A for times indicated were analyzed by immunoblotting using anti-Bcl11b antibody. HDAC1 blot was shown as loading control. (C), Time course of Bcl11b ubiquitination in stimulated primary thymocytes. Primary thymocytes were treated with P/A for times indicated, and Bcl11b was immunoprecipitated using anti-Bcl11b antibody prior to immunoblotting analyses using anti-ubiquitin antibody (upper blot), and the blot was then stripped and reprobed with anti-Bcl11b antibody (lower blot).
Figure 3.8.

A

SU(c)-Bcl11b
Bcl11b

B

SU(c)-Bcl11b
Bcl11b

HA

1 2

Bcl11b

3 4
Figure 3.8. Su(c)-Bcl11b forms protein complex with non-SUMOylated Bcl11b. (A), Expression of Su(c)-Bcl11b in vitro. DNA constructs encoding SU(c)-Bcl11b or wild type Bcl11b were used to express $^{35}$S-labeled protein using the transcription and translation coupled reticulocyte lysate system. Protein expression was detected by autoradiography. (B), Expression of the constitutive SUMO1(c)-Bcl11b protein in cells. HEK293T cells were transfected with the same amount of SU(c)-Bcl11b or wild type Bcl11b, and the resulting whole cell extracts were subjected to immunoblotting using anti-Bcl11b antibody (the left blot), and the blot was then stripped and reprobed with anti-HA antibody (the right blot). (C), SU(c)-Bcl11b is degraded through Ub-proteasome pathway. HEK293T cells transfected with SU(c)-Bcl11b were treated with MG132 (5uM) or zVAD (5uM) for indicated hours before harvest for immunoblot analysis using anti-HA antibody. (D), Interaction between non-SUMOylated Bcl11b and SU(c)-Bcl11b prevents SU(c)-Bcl11b from Ub-proteasome dependent degradation. HEK293T cells were transfected with various amount of SU(c)-Bcl11b and wild type Bcl11b as indicated, and the resulting whole cell extracts were subjected to immunoprecipitation experiments using anti-HA antibody. Then the purified SU(c)-Bcl11b complex was analyzed by immunoblotting using anti-Bcl11b antibody. 10% input signal of SU(c)-Bcl11b (HA blot) and total Bcl11b (Bcl11b blot) were shown in the upper panels. HDAC1 blot was shown as loading control.
Figure 3.9 Model of Bcl11b regulation by post-translational modifications. A model for regulation of Bcl11b by dynamic phosphorylation and SUMOylation in thymocytes stimulated with PMA and A23187. Bcl11b is basally phosphorylated and SUMOylated and it is further phosphorylated by both ERK1/2 and p38 MAPKs in thymocytes treated with P/A for 5 mins. Phosphorylated Bcl11b attracts phosphatase PP6, which then dephosphorylates Bcl11b at 30 mins of P/A treatment. The dephosphorylated Bcl11b interacts with SUMO E2 ligase Ubc9, leading to Bcl11b SUMOylation at 60 mins of P/A treatment. Prolonged treatment of P/A eventually leads to Bcl11b degradation by the STUbLs proteins recruited through the SUMO moiety on Bcl11b.
REGULATION OF BCL11B BY POST-TRANSLATIONAL MODIFICATIONS

CHAPTER 4

DISCUSSION AND CONCLUSION
Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2 (Ctip2/Bcl11b), a C2H2 zinc finger transcriptional regulatory protein, is an essential protein for post-natal life in the mouse, and plays crucial roles in the development, and presumably function, of several organ systems, including the central nervous (Arlotta et al., 2005), immune (Wakabayashi et al., 2003b), craniofacial formation and cutaneous/skin (Golonzhka et al., 2009a; Golonzhka et al., 2009c) systems. Moreover, inactivation of Bcl11b has been implicated in the etiology of lymphoid malignancies, suggesting that Bcl11b may function as a tumor suppressor. Here, we study the reversible, covalent modification of Bcl11b by phosphorylation and small ubiquitin-related modifier (SUMO). We have identified K607 and K805 as the two major Bcl11b SUMOylation sites by mutagenesis study. We have shown that phosphorylation and SUMOylation of Bcl11b are likely mutually exclusive processes, and phosphorylation of Bcl11b inhibits its SUMOylation by promoting the recruitment of SUMO specific protease SENP1. Sumoylation may affect protein stability and/or activity, protein localization, most likely through altered protein-protein interactions. Although protein SUMOylation was originally implicated in transcriptional repression or extinguishing activation by several factors, including p300, HDAC1, KAP1, and PPARγ, a number of more recent reports have described a role for SUMOylation in transcriptional activation. Our data presented herein demonstrated that SUMOylation compromises the transcription repression mediated by Bcl11b and a cleavable form of SUMOylated Bcl11b is targeted to
ubiquitination pathway and it is eventually degraded through proteasome machinery.

4.1 Dynamic Interplay between Bcl11b Phosphorylation, Dephosphorylation, and Sumoylation — Protein phosphorylation is critical in the control of multiple cellular processes, including SUMOylation, and it can either promote or inhibit substrate SUMOylation, depending on the substrate protein. The first example shown for phosphorylation-dependent regulation of protein SUMOylation is the nuclear protein PML, hyperphosphorylation of which significantly decreased its SUMOylation (Muller et al., 1998). Similarly, phosphorylation inhibits the SUMOylation of transcription factor PR-B, Elk1 and KAP1 (Daniel et al., 2007; Li et al., 2007; Yang et al., 2003a), whereas phosphorylation of MEF2C, GATA1 and Pc2 is required for its SUMOylation (Kang et al., 2006; Roscic et al., 2006).

Treating primary thymocytes with PMA and A23187 (P/A) leading to activation of MAPK kinases induces Bcl11b phosphorylation accompanied with deSUMOylation of Bcl11b between 5-10 mins of treatment. Interestingly, this phosphorylation/deSUMOylation process is transient, and it is followed by a profound dephosphorylation of Bcl11b followed by an increase of Bcl11b SUMOylation between 30~60 mins of kinase activation. We have showed that Bc11b SUMOylation is antagonistically regulated by protein phosphorylation. First, blockage of Bcl11b dephosphorylation by ERK inhibition also blocked
Bcl11b SUMOylation at 60 mins of P/A treatment (Zhang 2011 paper in preparation). Second, the increase of Bcl11b phosphorylation by phosphatase inhibitor CalA treatment is well correlated with the decrease of Bcl11b SUMOylation (Zhang 2011 paper in preparation).

All these results indicate the existence of a phospho-SUMO switch that is governing the antagonistic relationship between Bcl11b phosphorylation and SUMOylation. We found that SUMO-specific protease SENP1 interacts with Bcl11b and catalyzes its deSUMOylation. Many interactions between enzymatic SENPs and their substrates have been reported to be transient, and often a catalytically dead version of SENP1 is used to replace wild type SENP1 in co-immunoprecipitation assays to potentially trap the interaction between SENP1 and its substrates, such as ELK1 (Wilkinson and Henley, 2010) and KAP1. In contrast, we observe a stable interaction between SENP1 and Bcl11b when co-expressed in HEK cells, indicating that Bcl11b is a bona fide substrate of SENP1. In addition, phosphorylation of Bcl11b is required for its interaction with SENP1 because dephosphorylated Bcl11b failed to interact with SENP1 (Fig. 3.2B). In addition, we also found that while phosphorylation promotes the recruitment of SENP1 to Bcl11b. Bcl11b also interacted with the SUMO E2 conjugation enzyme Ubc9, and whether and how this interaction may be also influenced by the phosphorylation status of Bcl11b has yet to be determined. Therefore, we propose that upon activation of MAPK kinases in T cells, the initial Bcl11b phosphorylation acts to recruit SENP1, which in turn
deSUMOylates Bcl11b, and the subsequent Bcl11b dephosphorylation blocks the interaction between Bcl11b and SENP1, thus allows stable SUMOylation of Bcl11b.

4.2 SUMO-targeted degradation of Bcl11b — Recently, a subset of ubiquitin ligases, known as SUMO-Targeted Ubiquitin Ligases (STUbLs), have been identified to be responsible for ubiquitin conjugation to SUMOylated proteins. The STUbL protein binds SUMO chains on substrate proteins, leading to ubiquitination of the substrate protein and, ultimately, its proteasomal degradation. For example, the protein PML is SUMOylated upon arsenic treatment, and SUMOylated PML recruits RNF4, a SUMO-dependent E3 ubiquitin ligase, leading to polyubiquitination and proteasome-dependent degradation of PML (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008).

We have found that P/A treatment in T cells induces Bcl11b SUMOylation (~1 hr), but later Bcl11b is deSUMOylated and became heavily ubiquitinated then degraded (> 6 hrs) (Fig. 3.7B). Interestingly, when a cleavable form of SUMO1 is covalently attached to Bcl11b through gene fusion (SUc-Bcl11b), Bcl11b becomes highly unstable and is degraded through a proteasome-dependent pathway (Fig. 3.7C). In contrast, no stability issue is noticed when Bcl11b is attached with the noncleavable form of SUMO1, indicating that deSUMOylation is required for the degradation process. In addition, co-expressed wild type Bcl11b protects SUc-Bcl11b from degradation and thus
restores SUc-Bcl11b expression, suggesting that the interaction with wild type Bcl11b may prevent the recruitment of STUbLs to SU-Bcl11b. Taken together, we propose that in primary thymocytes, SUMOylated Bcl11b may interact with unSUMOylated Bcl11b, which in turn protect it from SENP-mediated deSUMOylation and proteasomal degradation during the initial period of P/A treatment. Similarly, it has been reported that SUMO1 modified RanGAP1 forms a high affinity complex with RanBP2 that protects RanGAP1 from SENP-mediated deSUMOylation (Zhu et al., 2009). Furthermore, Bcl11b is dephosphorylated during the initial period of P/A treatment, providing another mechanism to prevent the recruitment of SENP to the complex. However, later in the time course, when Bcl11b phosphorylation gradually returns to basal level, SUMOylated Bcl11b is deSUMOylated and the interaction between Bcl11b/SU-Bcl11b may be compromised, leading to recruitment of Ub-proteasome system and ultimately Bcl11b/SU-Bcl11b degradation.

Based on the data presented herein, we propose the following model for the role of SUMOylation and phosphorylation in regulating Bcl11b transcription regulatory activity and stability. Bcl11 phosphorylation inhibits its SUMOylation by recruiting SUMO specific protease SENP1. Sumoylation compromises the transcription repression mediated by Bcl11b. Sumoylated Bcl11b is eventually targeted to ubiquitination pathway, possibly through the recruitment of SUMO-Targeted Ubiquitin Ligases (STUbLs), leading to degradation of SU-Bcl11b by the proteasome machinery. It appears that deSUMOylation of SUMO
conjugated Bcl11b is required for the STUbL-mediated degradation because only the cleavable form of SUMO-Bcl11b fusion protein, but not the noncleavable fusion, is instable in cells and can be degraded through the proteasome.

In summary, mounting evidence continues to imply that Bcl11b may be an important component of T cell signaling pathway, and we demonstrate here that Bcl11b phosphorylation, dephosphorylation and SUMOylation switch triggered by signaling pathway could be a central regulatory circuit in mediating the de-repression of Bcl11b-repressed genes, such as Id2. With this caveat, regulation of Bcl11b activity through the phospho-SUMO switch may represent a novel strategy of gene regulation with implication for treating T cell leukemia/lymphoma. The intricate interplay between MAPK signaling activation and the Bcl11b phospho-SUMO switch is likely to be critical for the dynamic regulation of cellular response upon T cell activation.


malignancies with t(2;14)(p13;q32) translocation involving the BCL11A gene. Leukemia 16, 937-939.


Muller, S., Matunis, M.J., and Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. EMBO J 17, 61-70.


