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Functional Characterization of the B-cell Lymphoma/Leukemia 11A (BCL11A) Transcription Factor

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Functional Characterization of the B-cell Lymphoma/Leukemia 11A (BCL11A) Transcription Factor

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Dedication

To my family

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Previously a t(2;14)(p13;q32) translocation was characterized in four unusually aggressive cases of B cell chronic lymphocytic leukemia (B-CLL). A gene located near the 2p13 breakpoint, *B cell lymphoma/leukemia 11A* (*BCL11A*), was shown to over-express 3 isoforms (BCL11A-XL, L and S). *Bcl11a* knockout mice are severely impaired in B cell development at the early (pro-B) stage.

I have further characterized BCL11A, focusing on the most abundant and evolutionarily conserved isoform, BCL11A-XL (XL). I demonstrated that XL resides in the nuclear matrix, is modified by ubiquitination, and is destabilized by B cell antigen receptor ligation. I identified domains within XL required for its localization within nuclear paraspeckles and for its transcriptional repression.

While BCL11A-XL represses model promoters in non-B cells, its biologically relevant targets in B lymphocytes were unknown. I have identified and confirmed a

number of XL targets which are both up- and down-regulated by XL over-expression in B cell lines. A number of these genes have been implicated in B cell function, including the V(D)J recombination activating (*RAG*) genes. Both RAG1 and RAG2 transcripts were up-regulated by XL. XL binds to the *RAG1* promoter and *RAG* enhancer (*Erag*) *in vivo* as well as *in vitro*. Unexpectedly, XL repressed *RAG1* transcription in non-B cells, indicating that additional B cell-specific factors are required for activation. Over-expression of XL in a V(D)J recombination-competent pre-B cell line markedly induced *RAG* expression and VDJ recombination. IRF4 and IRF8, transcription factors previously shown to be required for early B cell development, were also induced by BCL11A-XL.

I propose that the early B cell progenitor block in *Bcl11a* knockout mice is, at least in part, a direct result of BCL11A-XL regulation of V(D)J recombination. Further experiments are required to establish how other XL targets promote B cell lineage development and how malignant transformation such as in B-CLL may corrupt BCL11A function.

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CHAPTER 1. INTRODUCTION

1.1. Examples of Chromosomal Translocation Involved in B cell Lymphoid Malignancy

Tumor specific chromosomal abnormalities have been identified by cytogenetic markers in human pathogenesis. Chromosomal translocation in B and T cells underlies oncogenesis in addition to structural rearrangements (Blackwell et al., 1990; Haluska et al., 1987; Nowell, 1962). One of the best examples showing the relationship between chromosome abnormality and translocation is chronic myelogenous leukemia (CML) (Hermans et al., 1987). The t(9;2) translocation results in a minute chromosome called the Philadelphia chromosome (Ph1). Ph1 is present in most of patients with CML. The t(9;22)(q34;q11) involves the *Abl* proto-oncogene and the breakpoint cluster region (bcr). The *BCR-Abl* fusion gene is produced by the consequence of this fusion. Nearly all CML cases result in p210 fusion protein. In acute lymphoblastic leukemia (ALL), the Ph1 chromosome produces a p190 fusion protein (Hermans et al., 1987). The normal Abl kinase has weak phosphorylation activity but BCR-Abl fusion protein shows enhanced kinase activity (Lugo et al., 1990). Higher expression levels of BCR-Abl protein display more resistance against apoptotic stimuli (Cambier et al., 1998; Nowell, 1962).

In Burkitt's lymphoma, the *myc* oncogene is introduced into the immunoglobulin heavy –chain (*IgH*) locus by the t(8;14) translocation (Taub et al., 1982). Juxtaposed locations of *c-myc* reside within an active *Ig* gene locus. As a consequence of this translocation, *c-myc* expression is deregulated. Experimental evidence indicates that c-myc is involved in cell growth and differentiation (Cole, 1986). c-Myc also functions as a

transcriptional factor recognizing specific DNA sequences (CACCGTG) (Blackwell et al., 1990).

The t(14;18)(q32;q21) translocation in follicular lymphoma involves the transcriptional unit of *bcl-2* (Yunis, 1987). This is a very common translocation in human lymphoid malignancy. Bcl-2 localizes to the inner mitochondria membrane and blocks programmed cell death (Hockenbery et al., 1990). Unlike other proto-oncogenes which promote cell proliferation, Bcl-2 is involved in carcinogenesis by enhancing cell survival.

The t(11;14)(q13;32) translocation occurs in some B cell chronic lymphocytic leukemias (B-CLL) (Tsujimoto et al., 1984a; Tsujimoto et al., 1984b). The area on chromosome 11q13 was named the *Bcl-1* locus. The *PRAD-1* (parathyroid adenomatosis) gene was identified as a candidate gene for *bcl-1*. *PRAD-1* encodes a novel cyclin and its over-expression by translocation may play an important role for development of B-CLL (Motokura et al., 1991).

The t(1;19)(q23;p13.3) translocation occurs in approximately 30% of acute lymphoblastic leukemias (ALL) (Carroll et al., 1984; Williams et al., 1984). The consequence of this translocation produces an E2A/PBX fusion protein. The *E2A* gene encodes a transcription factor that is a key regulator of B cell development (Mellentin et al., 1989). The chromosome 1 gene, *PBX,* encodes a homeobox protein (Kamps et al., 1990; Nourse et al., 1990).

Another common *Ig* translocation, found primarily in aggressive B cell non-Hodgkin lymphoma (B-NHL), involves a Kruppel-like zinc finger gene, *BCL6*, located on chromosome 3q27 (Miki et al., 1994; Ye et al., 1995). BCL6 is a highly conserved transcriptional repressor, showing 97% identity between the zinc finger domains of frog and man. While its precise functions remain unknown, *BCL6* mutant mice fail to develop germinal centers during a T cell-dependent immune response and a fatal inflammatory

disease distinguished by the presence of TH2 cells (Staudt et al., 1999). The *BCL6* gene and its translocations are unusual in two regards. Firstly, apart from the *IG* translocations, *BCL6* is frequently translocated to at least twenty other non-*IG* sites, including *TTF*, *BOB1* and histone H4 genes where constitutive promoters drive deregulated *BCL6* transcription (Akasaka et al., 2000). Secondly, *BCL6*, in both normal and malignant B cell populations, frequently exhibits mutations, insertions and deletions in the 5' region of the gene, the same region in which translocations occur (Shen et al., 1998). These alterations may arise due to *IG* somatic hypermutation acting on a non-*IG* gene. The mechanisms by which deregulated expression of *BCL6* contributes to the pathogenesis of B-NHL remain unknown (Staudt et al., 1999). Paradoxically, over-expression of *BCL6* under some conditions results in apoptosis rather than transformation (Albagli et al., 1999; Yamochi et al., 1999).

1.2. Isolation and Initial Characterization of B cell Lymphoma/Leukemia 11A (BCL11A)

The chromosomal translocation t(2;14)(p13;q32.3) has been reported in a wide variety of B cell malignancies including B cell precursor acute lymphoblastic leukemia (BCP-ALL), B-NHL, B cell chronic lymphocytic leukemia (CLL) and myeloma; this translocation is frequently the sole cytogenetic abnormality within the neoplastic clone (Mitelman et al., 1997).

We reported the breakpoint cloning of two aggressive cases of CLL in children with t(2;14)(p13;q32.3) (Richardson et al., 1992). Both patients died following unsuccessful attempts at bone marrow transplantation. We showed that in both instances the 14q32.3 breakpoints were within the switch region of the *IgH Cg2* gene, and the 2p13

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breakpoints clustered within 38 bp of each other within a previously uncharacterized CpG island. Cloning the potential gene was accomplished (Satterwhite et al., 2001), and in addition, two adult cases were cloned from the same region using an Ig switch LDI-PCR (Li et al., 1999). All four translocations were associated with a novel zinc finger gene (*BCL11A*) on chromosome 2p13 which was deregulated as a consequence. BCL11A maps closely telomeric to REL in a region selectively amplified, in some instances up to 32-fold, in some subtypes of B-NHL and also in Hodgkin's disease (HD) (Barth et al., 1998; Houldsworth et al., 1996; Joos et al., 2000; Joos et al., 1996; Rao et al., 1998). We have observed that all cases exhibiting 2p13/REL amplification also showed comparable BCL11A amplification (Satterwhite et al., 2001). It therefore seems feasible that over-expression of BCL11A is a pathological consequence of the amplification, although this remains to be demonstrated.

The similarities in structure, function and expression of BCL11A and BCL6 prompted experiments that revealed that all BCL11A isoforms physically associate with BCL6 and two of these isoforms colocalize in nuclear "dots" (Liu et al., 2006). This raises the possibility that these two genes may transform mature B cells via similar pathways. *BCL6* expression in HD appears to define a distinct histological subtype of disease that originates from germinal center B cells (Carbone et al., 1998). However, the possible diagnostic and prognostic significance of *BCL11A* over-expression in both HD and B-NHL and the possible correlation with *BCL6* expression, awaits future studies.

Like *BCL6, BCL11A* shows a high level of conservation. *BCL11A* is the human homologue of mouse *Evi9*, being 94% identical at the nucleotide level, and 98% identical at the protein level. *Evi9* was detected as a gene able to induce myeloid leukemias following proviral integration (Li et al., 1999; Nakamura et al., 2000). It is not known whether deregulated *BCL11A* expression arising from other mechanisms occurs in these

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diseases. Evi9 also was isolated as an interacting partner (CTIP-1) of the orphan nuclear receptor COUP-TF2 (Avram et al., 2000).

Like *BCL11A*, three common isoforms of *Evi9/*CTIP-1 were identified (Nakamura et al., 2000), although the mouse and human isoforms do not correspond exactly. Mouse, chicken, and *Xenopus BCL11A* homologues also have been identified (Figure 1). Such a degree of conservation, comparable to that seen within the PAX gene family for example (Albagli et al., 1999), may indicate a developmentally important role for BCL11A.

Database analysis revealed a human homologue of *BCL11A* mapping to chromosome 14q32.1. This gene (*BCL11B*) is 67% identical to *BCL11A* at the nucleotide level and 61% identical overall at the protein level. *BCL11B*, like *BCL11A*, contains six C_2H_2 zinc fingers and proline-rich and acidic regions with 95% identity in the zinc finger domains. Like *BCL11A*, *BCL11B* is remarkable in having a large 5' CpG island. *BCL11B* is the homologue of mouse *CTIP-2* and is 86% identical at the protein level (Avram et al., 2000). *BCL11B* deficient mice show a block at the CD4$^-$CD8$^-$ double-negative stage of thymocyte development (Wakabayashi et al., 2003).

Together, the translocation and the amplification data implicate deregulated expression of *BCL11A* directly in the pathogenesis of divergent subtypes of aggressive human B-NHL and HD. Additional indirect contributions might be afforded through its heteromeric interactions in the absence of 2p13 loss or gain. *BCL11A* associates with two other highly conserved transcriptional repressors, *BCL6* and *COUP-TFII*, both of which have been implicated in malignancy (Lin et al., 2000; Nakshatri et al., 2000). *COUP-TF1,* for example, is necessary for the induction of growth arrest and apoptosis induced by retinoic acid in malignant cell lines (Lin et al., 2000), while the closely-related COUP-TFII regulates cell cycle progression by modulating expression of *$p21^{WAF1}$, Cyclin D1* and *cdk2* (Nakshatri et al., 2000).

BCL11A and BCL11B were shown to have transcriptional repression activity in GAL4 mammalian one-hybrid studies (Avram et al., 2002; Liu et al., 2006) and interact with the nucleosome remodeling and deacetylase (NURD) complex in Raji cells (Cismasiu et al., 2005). While the GAL4-BCL11B fusion protein showed transcriptional repression activity on a GAL4-luciferase reporter, endogenous BCL11B showed transcriptional activation activity of *interleukin-2 (IL-2)* gene expression (Cismasiu et al., 2006). Prior to the analysis presented in Chapter 4, the true transcriptional targets of BCL11A were unknown.

Figure 1. BCL11AXL is highly conserved in xenopus, chicken, mouse and human.

Alignment among different species shows that BCL11A-XL is highly conserved during evolution. Medline gene accession numbers: Xenopus (BC123944.1), Chicken (AJ851441), Mouse (AK140949), and Human (AJ 404611). The alignment was carried out using a web based software, "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988). Human BCL11A-XL shows 87.1%, 91.3%, and 99% identities to the putative BCL11A proteins of xenopus, chicken, and mouse. The 34 amino acids which are encoded by exon 3 are missing in xenopus and chicken as compared to mouse and human BCL11A.

```
                1        10        20        30        40        50        60        70        80        90        100
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     MSRRKQGKPQHLSKREFSPEPLEAILTDDEPDHGPLGAPEGDHDLLTCGQCQMNFPLGDILIFIEHKRKQCNGSLCLEKAVDKPPSPSPIEMKKASNPVE
      Mouse     MSRRKQGKPQHLSKREFSPEPLEAILTDDEPDHGPLGAPEGDHDLLTCGQCQMNFPLGDILIFIEHKRKQCNGSLCLEKGVDKPPSPSPIEMKKASNPVE
      Chicken   MSRREQGKPQHLSKREFSPEPLEAILTDDEPDHSTLGAPEGDHDLLTCGQCQMNFPLGDILIFIEHKRKQCNGSLCLEKAVDKPPSPSPSELKKASNPVE
      Xenopus   MSRRKQGKPQHLSKREYSPEPVEAILTDDEPDNSTLGAGEGDHDLLTCGQCQMNFPLGDILIFIEHKRKQCNGSLCLEKAGDKPPSPTPSELKKASNPVE
      Consensus MSRRkQGKPQHLSKRE%SPEP1EAILTDDEPDhstLGApEGDHDLLTCGQCQMNFPLGDILIFIEHKRKQCNGSLCLEKavDKPPSPsPsE$KKASNPVE

                101      110       120       130       140       150       160       170       180       190       200
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     VGIQVTPEDDDCLSTSSRRICPKQEHIADKLLHWRGLSSPRSAHGALIPTPGMSAEYAPQGICKDEPSSYTCTTCKQPFTSAWFLLQHAQNTHGLRIYLE
      Mouse     VGIRVTPEDDDCLSTSSRGICPKQEHIADKLLHWRGLSSPRSAHGALIPTPGMSAEYAPQGICKDEPSSYTCTTCKQPFTSAWFLLQHAQNTHGLRIYLE
      Chicken   VGIQVTPEDDDCLSTSSRGICPKQEHIAGK---------------------------------EEPSSYTCTTCKQPFNSAWFLLQHAQNTHGLRIYLE
      Xenopus   VGIQVTPEDDDCLSTSSRGICPKQENAAGK---------------------------------DEPSSYTCTTCKQPFSSAWFLLQHAQNTHGFRIYLE
      Consensus VGIqVTPEDDDCLSTSSRgICPKQEhiAgK..................................#EPSSYTCTTCKQPF.SAWFLLQHAQNTHGlRIYLE

                201      210       220       230       240       250       260       270       280       290       300
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     SEHGSPLTPRVGIPSGLGAECPSQPPLHGIHIADNNPFNLLRIPGSVSREASGLAEGRFPPTPPLFSPPPRHHLDPHRIERLGAEEMALATHHPSAFDRV
      Mouse     SEHGSPLTPRVGIPSGLGAECPSQPPLHGIHIADNNPFNLLRIPGSVSREASGLAEGRFPPTPPLFSPPPRHHLDPHRIERLGAEEMALATHHPSAFDRV
      Chicken   SEHGSPLTPRVGIPSGLGAECPSQPPLHGIHIADNNPFNLLRIPGSVSREASGLGEGRFPPTPPLFSPPPRHHLDPHRIERLGAEEMALATHHPSAFDRV
      Xenopus   SEHGSPLTPRVGIPSGLGAECPSQPPLHGIHIADNNPFNLLRIPGSVSREASGLTEGRFPPTPPLFSPPPRHHLDPHRIERLGAEEMALPTHHPSAFDRV
      Consensus SEHGSPLTPRVGIPSGLGAECPSQPPLHGIHIADNNPFNLLRIPGSVSREASGL.EGRFPPTPPLFSPPPRHHLDPHRIERLGAEEMALaTHHPSAFDRV

                301      310       320       330       340       350       360       370       380       390       400
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     LRLNPMAMEPPAMDFSRRLRELAGNT-SSPPLSPGRPSPMQRLLQPFQPGSKPPFLATPPLPPLQSAPPPSQPPVKSKSCEFCGKTFKFQSNLVYHRRSH
      Mouse     LRLNPMAMEPPAMDFSRRLRELAGNT-SSPPLSPGRPSPMQRLLQPFQPGSKPPFLATPPLPPLQSAPPPSQPPVKSKSCEFCGKTFKFQSNLVYHRRSH
      Chicken   LRLNPMAMEPPAMDFSRRLRELAGNT-SSPPLSPSRPSPMQRLLQPFQPGSKPPFLATPPLPPLQSAPPPSQPPMKSKSCEFCGKTFKFQSNLVYHRRSH
      Xenopus   LRLNPLAMEPPAMDFSRRLRELAGNTGSSPPLSPNRPSPMQRLMQPFPSGNKPPFLATPPLPSLQSVPPPSQPPVKSKSCEFCGKTFKFQSNLVYHRRSH
      Consensus LRLNP$AMEPPAMDFSRRLRELAGNT.SSPPLSP.RPSPMQRL$QPFqpGsKPPFLATPPLPpLQSaPPPSQPPvKSKSCEFCGKTFKFQSNLVYHRRSH

                401      410       420       430       440       450       460       470       480       490       500
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     TGEKPYKCNLCDHACTQRSKLKRHMKTHMHKSSPHTVKSDDGLSTASSPEPGTSDLVGSASSALKSVVAKFKSENDPNLIPENGDEEEEEDDEEEEEEEE
      Mouse     TGEKPYKCNLCDHACTQRSKLKRHMKTHMHKSSPHTVKSDDGLSTASSPEPGTSDLVGSASSALKSVVAKFKSENDPNLIPENGDEEEEEEDDEEEEEEEE
      Chicken   TGEKPYKCNLCDHACTQRSKLKRHMKTHMHKSSPHTVKSDDGLSTASSPEPGTSDLVGSASSALKSVVAKFKSENDPNNIPENGDEEEEEEEEEEEEEEE
      Xenopus   TGEKPYKCNLCDHACTQRSKLKRHMKTHIHKSSPHTVKSDDGLSTASSPEPGTSDLLGSASSVLKSVVAKFKSENDSNLIPENGDEEEEEEEEEEEEEEE
      Consensus TGEKPYKCNLCDHACTQRSKLKRHMKTHnHKSSPHTVKSDDGLSTASSPEPGTSDLvGSASSaLKSVVAKFKSENDpN$IPENGDEEEEE##EEEEEEEE

                501      510       520       530       540       550       560       570       580       590       600
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     EEEE-ELTESE-RVDYGFGLSLEAARHHENSSRGAVVGVGDESRALPDVMQGMVLSSMQHFSEAFHQVLGEKHKRGHLAEAEGHRDTCDEDSVAGESDRI
      Mouse     EEEE-ELTESE-RVDYGFGLSLEAARHHENSSRGAVVGVGDEGRALPDVMQGMVLSSMQHFSEAFHQVLGEKHKRSHLAEAEGHRDTCDEDSVAGESDRI
      Chicken   EEEE-DLTENE-RPDYGFGMNLEAAHHHENNSRA-----GEESRSMPDVMQGMVLSSMQHFSEAFHQVLGEKHKRGHLSEAEVHRDTCDEDSVAGESDRI
      Xenopus   EEEEEDLTETDSRPDYSFGLNLEAARHHENNARS------IEERSMPEVMQGMSGLG-MHHFSDAFHQVLMEKHKRGHLSEPEVQRDTCDEDSVAGESDRI
      Consensus EEEE.#LTE.#.RpDYgFG$nLEAArHHENnsR......g.E.Rs$P#VMQgMvLssMqHFS#AFHQVLgEKHKRgHLsEaEvhRDTCDEDSVAGESDRI

                601      610       620       630       640       650       660       670       680       690       700
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     DDGTVNGRGCSPGESASGGLSKKLLLGSPSSLSPFSKRIKLEKEFDLPPATMPNTENVYSQWLAGYAASRQLKDPFLSFGDSRQSPFASSSEHSSENGSL
      Mouse     DDGTVNGRGCSPGESASGGLSKKLLLGSPSSLSPFSKRIKLEKEFDLPPAAMPNTENVYSQWLAGYAASRQLKDPFLTFGDSRQSPFASSSEHSSENGSL
      Chicken   DDGAVNGRGCSPGESASGGLSKKLLLGSPSSLSPFSKRIKLEKEFDLPATAMPNTENVYSQWLAGYAASRQLKDPFLSFGDSRQSPFASSSEHSSENGSL
      Xenopus   ESGTVNGRGCSPGESASGGLSKKLLLGSPSSLSPFSKRIKLEKEFDLPASAMPNTENVYSQWLAGYAASRQLKEPFLTFGDSRQSPFASSSEHSSENGSL
      Consensus #dGtVNGRGCSPGESASGGLSKKLLLGSPSSLSPFSKRIKLEKEFDLPa.aMPNTENVYSQWLAGYAASRQLK#PFLtFGDSRQSPFASSSEHSSENGSL

                701      710       720       730       740       750       760       770       780       790       800
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
      Human     RFSTPPGELDGGISGRSGTGSGGGSTPHISGPGTGRPSSKEGRRSDTCEYCGKVFKNCSNLTVHRRSHTGERPYKCELCNYACAQSSKLTRHMKTHGQVGK
      Mouse     RFSTPPGELDGGISGRSGTGSGGGSTPHISGPGPGRPSSKEGRRSDTCEYCGKVFKNCSNLTVHRRSHTGERPYKCELCNYACAQSSKLTRHMKTHGQVGK
      Chicken   RFSTPPGELDGGISGRSGTGSGGGSTPHISGPGPGRPSSKEGRRSDTCEYCGKVFKNCSNLTVHRRSHTGERPYKCELCNYACAQSSKLTRHMKTHGQVGK
      Xenopus   RFSTPPGEMDGGISGRSGTGSGGGSTPHISGPGPGRPSSKEGKRSDTCEYCGKIFKNCSNLTVHRRSHTGERPYKCELCNYACAQSSKLTRHMKTHGQVGK
      Consensus RFSTPPGE$DGGISGRSGTGSGGGSTPHISGPGpGRPSSKEGrRSDTCEYCGK!FKNCSNLTVHRRSHTGERPYKCELCNYACAQSSKLTRHMKTHGQVGK

                801      810       820       830       838
                |--------+---------+---------+--------|
      Human     DVYKCEICKMPFSVYSTLEKHMKKWHSDRVLNNDIKTE
      Mouse     DVYKCEICKMPFSVYSTLEKHMKKWHSDRVLNNDIKTE
      Chicken   DVYKCEICKMPFSVYSTLEKHMKKWHSDRVLNNEIKTE
      Xenopus   DVYKCEICQMPFSVYSTLEKHMKKWHSDRVLNNDIKTE
      Consensus DVYKCEICkMPFSVYSTLEKHMKKWHSDRVLNN#IKTE
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Consensus symbols:

! is one of I or V

$ is one of L or M

% is one of F or Y

is one of N, D, E, B, or Z

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1.3. Early B Cell Development

The development of B cells from Hematopoietic stem cells (HSC) is a finely tuned process regulated by multiple transcription factors (described in section 1.4) and cytokines (Medina and Singh, 2005). The activation and silencing of specific subsets of genes appear to control the determination of cell lineages. Transcription factors (PU.1, Ikaros, E2A, BCL11A, EBF, and PAX5) and cytokine receptors (Flk2/Flt3 and IL-7R) have been shown to be key regulators for the development of early B cell progenitors (Medina and Singh, 2005). The pluripotent HSC cells can differentiate into all blood cell types with loss of self-renewal potential. Lymphocytes develop from HSC cells via multipotent progenitors (MPP), and common lymphoid progenitors (CLP) (Akashi et al., 2000). The long term self renewal capacity is lost with up-regulation of the tyrosine kinase receptor Flt3 in MPP cells (Adolfsson et al., 2001).

The expression of *RAG* genes and the start of D_H-J_H rearrangement occur in the earliest lymphocyte progenitors (ELP) differentiated from MPP cells (Igarashi et al., 2002). ELP cells are thought to develop into early T-lineage progenitors (ETP) in the thymus (Allman et al., 2003) and into common lymphoid progenitors (CLP) in the bone marrow, which can give rise to B, T, NK, and DC cells (Akashi et al., 2000; Kondo et al., 2004; Traver et al., 2000). By using cell surface markers, $B220^+$/CD45R and $CD19^-$, pre-pro B cell fractions were isolated (Li et al., 1996). At the early pro-B cell stage, CD19 is expressed and V_H-J_H recombination is completed (Hardy, 1991). Late pro-B cells with successful V_H-DJ_H rearrangement express the Igµ proteins. Assembly of the heavy chain, Igµ and the surrogate light chain, VpreB and ?5 produces the pre-B cell receptor (pre-BCR), which signals allelic exclusion at the *IgH* locus, cell proliferation, and differentiation into small pre-B cells (Meffre et al., 2000).

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1.4. Transcriptional Regulation of Early B Cell Development

Significant progress in the study of the transcriptional control of B cell lineage specification and commitment has been made (Busslinger, 2004) (Figure 2 and 3). Ikaros is a family of zinc finger transcription factors. Dominant negative (DN) Ikaros mice resulted in the failure to produce any B, T, NK, and DC cells, showing that Ikaros is involved in the development of all lymphocytes (Georgopoulos et al., 1994). Ikaros together with Aiolos, which is another zinc finger DNA binding protein, is required in the control of B cell maturation and germinal center formation (Kirstetter et al., 2002; Wang et al., 1998).

The Ets transcription factor PU.1 is required for the generation of multiple lineages of immune cells (DeKoter and Singh, 2000). PU.1 seems to function at the level of myeloid-lymphoid progenitors. B cells are induced at low concentration of PU.1, while macrophage differentiation and blocks of B cell differentiation occur at high concentration of PU.1 (DeKoter and Singh, 2000). PU.1 regulates the expression of IL-7a by binding directly to its 5' region (DeKoter et al., 2002). The lymphoid cytokine IL-7 induces B cell development from the CLP cells and promotes pro-B cell survival (Miller et al., 2002).

Three transcriptional factors E2A, EBF, and Pax5 are pivotal to the differentiation of CLP cells to pro-B cells. The *E2A* gene encodes two isoforms E12 and E47, which are basic helix-loop-helix (bHLH) proteins (Shen and Kadesch, 1995). *E2A* null mutant mice fail to produce mature B cells and are arrested at an early stage indicated by the absence of D_H-J_H rearrangement in those mutant mice (Bain et al., 1994).

The early B cell factor (EBF) is a tissue-specific DNA-binding protein whose expression is detected only in the hematopoietic cells such as CLP, pro-B, and pre-B cells (Hagman et al., 1993). *EBF* knock-out mice show a deficiency in immunoglobulin D_H-J_H rearrangement (Lin and Grosschedl, 1995). A similar developmental arrest in E2A and *EBF* mutant mice suggests that these two transcription factors work together in the early stage of B-lymphopoeisis (Busslinger, 2004). Molecular analysis supports this concept by showing that E2A and EBF bind cooperatively to the promoters of the *?5, VpreB* and *Iga(mb-1)* genes (Busslinger, 2004).

For the complete commitment of B cell progenitors, Pax5 (also known as BSAP) is required in addition to E2A and EBF. *Pax5*$^{-/-}$ mice display developmental arrest at an early pro-B cell phase (Schebesta et al., 2002; Urbanek et al., 1994). Interestingly, *Pax5*$^{-/-}$ pro-B cells are unable to commit to the B-lymphoid lineage, but have potential to differentiate into a broad range of hematopoietic cell types (Nutt et al., 2001). Pax5 represses *MCSF-R, Notch1*, and *Flt3* genes are involved in non-B cell lineage determination, Flt3 (Holmes et al., 2006), Notch (Souabni et al., 2002), and MCSF-R are required for DC, T cell, and myeloid cell development, respectively (Tagoh et al., 2006). Meanwhile, Pax5 activates expression of B cell lineage genes such as *mb-1, CD19, CD79a, ?5* and *BLNK* (Cobaleda et al., 2007). Hence, Pax5 is a crucial B-lineage commitment factor fulfilling its role by activating B cell lineage specific genes, but repressing the expression of non-B cell genes.

BCL11A is also essential for early B cell development(Liu et al., 2003). *BCL11A*$^{-/-}$ fetal livers contain very few B220$^+$ cells, indicating that Bcl11a is required for B cell formation at the pre-pro stage (Figures 2 and 3). B cell lineage genes such as *EBF, Pax5, IL7?, CD19, RAG1*, and *VpreB2* were not expressed in *BCL11A* mutant fetal livers. This suggests that BCL11A functions upstream of EBF1 and Pax5 (Liu et al., 2003).

Figure 2. Transcriptional control of lymphoid development.

Early B cell development from the common lymphoid progenitor (CLP) requires BCL11A, E2A, EBF, PAX5, and Foxp1 transcription factors. Defects of deficiencies of transcription factors result in developmental arrest in mice. Notch signaling is essential for T cell and natural killer (NK) cell development, while ID2 and SpiB signaling is required for dendritic cell progenitors (pDC). The approximate developmental arrest points that arise from deficiencies of these factors are indicated by positions of transcription factors. Cross-suppression among some of these lineage-specific transcription factors (e.g. Notch on E2A, PAX5 on Notch, and ID2 on E2A) is also important for development of lymphoid cells (Busslinger, 2004).
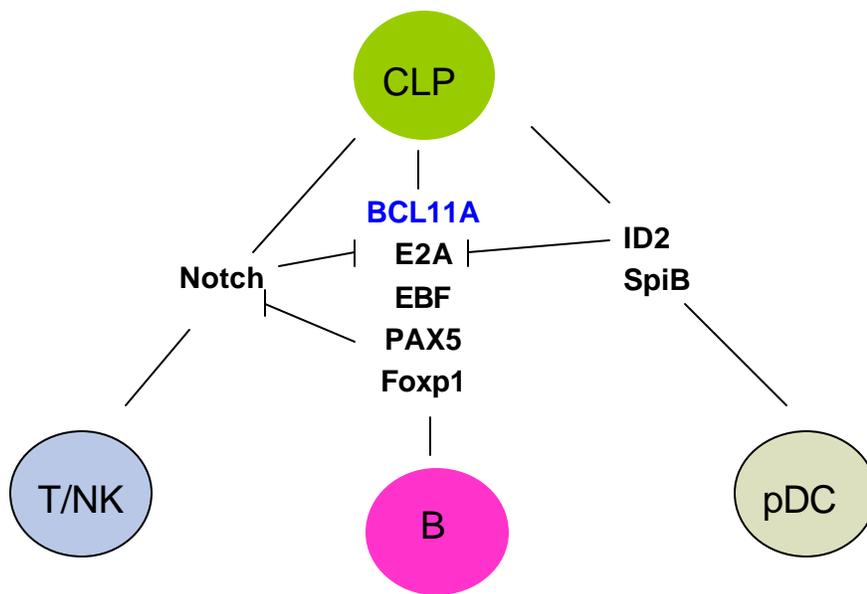
Modified from Annu.Rev. Immunology. 2004.22:55-79

Figure 3. Key events during B cell development.

Expression (+), lack of expression (-) or low levels of expression (+/-) are noted for the recombination activating genes (RAGs), the IL7 receptor, key transcription factors, including BCL11A (in blue), and the rearrangement status of immunoglobulin genes (Busslinger, 2004). *BCL11A* knock-out mice (K/O) show a defect in B cell development at the pre-pro-B cell stage (Liu et al., 2003). Nd, not determined.
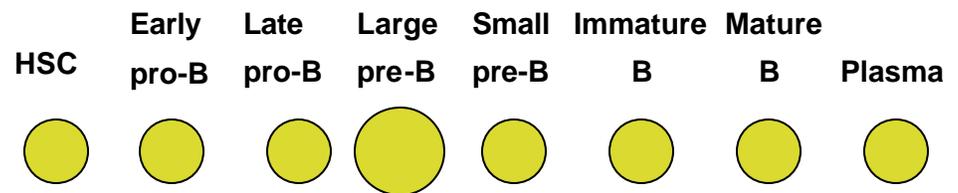
	HSC	Early pro-B	Late pro-B	Large pre-B	Small pre-B	Immature B	Mature B	Plasma
RAG	-	+	+	-	+	low	-	-
VpreB	-	+	+	+	-	-	-	-
?5	-	+	+	+	-	-	-	-
IL7R	-	+	+	+	-	-	-	-
$D\text{-}J_H$	-	+	-	-	-	-	-	-
$V_H\text{-}DJ_H$	-	-	+	-	-	-	-	-
$V_K\text{-}J_K$	-	-	-	-	+	-	-	-
$V_?\text{-}J_?$	-	-	-	-	+	-	-	-
IL7R	-	+	+	+	-	-	-	-
EBF	-	+	+	+	+	+	+	+
Pax5	-	+	+	+	+	+	+	-
Ikaros	+	+	+	+	+	+	+	+
BCL11A	-	+	+	-	nd	-/+	+	-
	K/O							

Modified from Annu.Rev. Immunology. 2004.22:55-79

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1.5. V(D)J Recombination

B and T cells can generate specific immune responses to a huge number of immunogens. The genes coding immunoglobulin and T cell receptor are composed of multiple segments dispersed in the germline. These segments V (variable), D (diversity), and J (joining) are joined together in somatic tissues by the process called V(D)J recombination (Fugmann et al., 2000). Because V(D)J recombination makes double strand DNA breaks, uncontrolled recombination events can result in chromosomal translocation, oncogenesis and cell death.

V(D)J recombination is a site specific and lineage specific recombination process occurring only in immature B and T cells. Site specificity is provided by recombination signal sequences (RSSs) which consist of highly conserved heptamer and nonamer sequences. The heptamer and the nonamer are separated by nonconserved 12 or 23 bp spacers. Efficient recombination is possible only between a 12-RSS and 23-RSS, known as the 12/23 rule (Bassing et al., 2002). V(D)J recombination activity is conferred by recombination activating gene 1 (RAG), RAG2, TdT, DNA-PKcs, Ku70/Ku80, XRCCT, DNA ligase IV, and Artemis (Bassing et al., 2002; Gellert, 2002). RAG1 and RAG2 can confer V(D)J recombination synergistically in nonlymphoid cells (Oettinger et al., 1990).

RAG1 and *RAG2* are convergently transcribed and coordinately controlled in B and T cell progenitors (Nagaoka et al., 2000). The *Erag* enhancer is an evolutionarily conserved transcription enhancer and is required for proper *RAG* expression in early B cell precursors but not in T cells (Hsu et al., 2003). The B cell transcription factor E2A was shown to directly bind to the *Erag* enhancer and transactivate *RAG* expression (Hsu et al., 2003). The forkhead transcription factor, Foxp1, also has Erag binding activity and

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its defect in mice showed diminished expression of RAG proteins as well as other B cell lineage genes (Hu et al., 2006).

1.6. Differentiation of Abelson Virus-Transformed Pre-B Cell Lines and the Abl Kinase Inhibitor, STI-571

The Abelson murine leukemia virus (A-MuLV) can be used to transform adult murine bone marrow or fetal liver cells (Kurosaki, 2003). The retroviral oncogene, v-abl encodes a constitutively active non-receptor tyrosine kinase. Transformed pro-B cells are able to proliferate in culture without interleukin 7 (IL-7) but their maturation halts at the pro-B or early pre-B cell stages. Thus, A-MuLV transformed cell lines contain a productive heavy chain rearrangement but (with few exceptions) no light chain rearrangement. A-MuLV transformed cells resemble large pre-B cells. They proliferate robustly, express low levels of RAG1 and RAG2, and express low levels of "germline" Ig? or Ig? transcripts. Germline transcripts are initiated from cryptic promoters within the J-C intron and correlate strongly with chromatin accessibility of the respective locus prior to its recombination (discussed in Chapter 5) (Sleckman et al., 1996; Van Ness et al., 1981). A-MuLV transformed cell lines, treated with an inhibitor (STI-571/imatinib/Gleevec) of the Abl kinase, are relieved of the differentiation block and progress to a late pre-B cell-like state, displaying *Ig?* or *Ig?* rearrangement (Muljo and Schlissel, 2003) (Figure 4).

Figure 4. Steps in early B cell development subject to Abelson (Abl) transformation and Abl kinase inhibition by STI571.

The Abelson murine leukemia virus tryrosine kinase is constitutively activated. Abl-transformed B cells are arrested at the large pre-B stage of development. Treatment of Abl cells with the Abl kinase inhibitor and chronic myelogenous leukemia therapeutic, STI571/Gleevec, results in G1/S cell cycle arrest, induction of *RAG* expression and immunoglobulin (*Ig*) light chain VJ recombination (Bredemeyer et al., 2006). These features are characteristic of late pre-B cells.

STI571

Abl
Kinase

preBCR

3-5
cycles

VDJ_H

G1 arrest

IgM

V_L-J_L

Large pre-B   Small pre-B   Immature
B cells
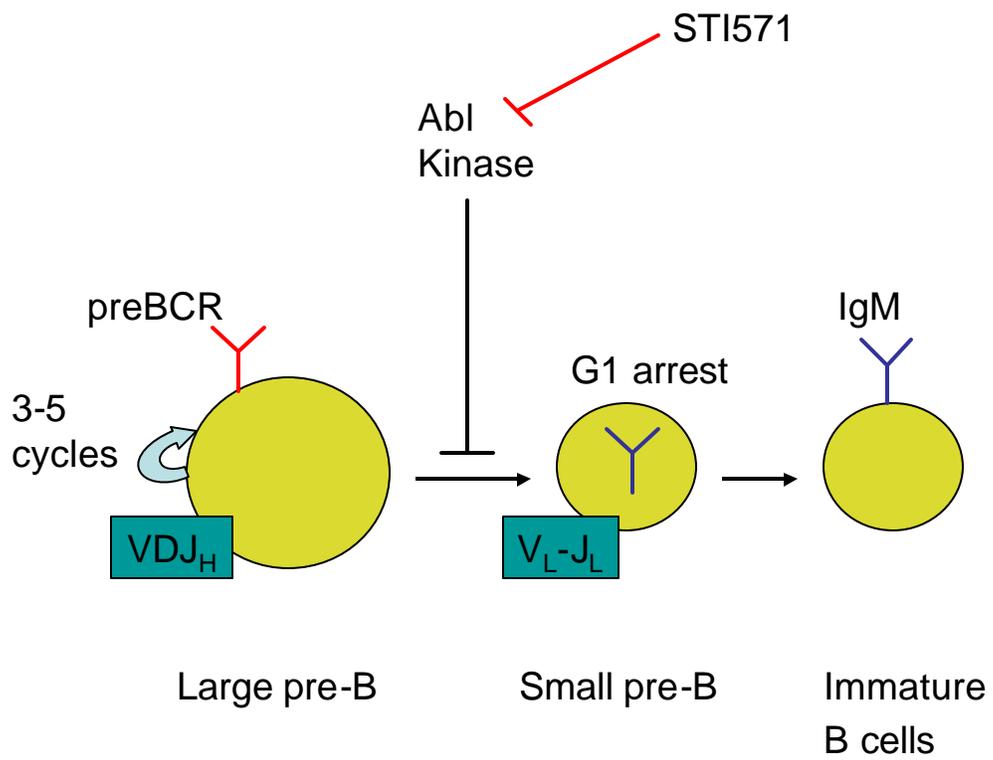
1.7. Control of RAG1 and RAG2 Activity during B Cell Development

RAG expression begins in early lymphoid progenitors (ELP) that can give rise to T cells, B cells, and NK cells (Igarashi et al., 2002). The levels of RAG transcripts are high during early stages of B cell development until rearrangement of immunoglobulin heavy chain (*IgH*) is complete. *RAG* gene expression is suppressed at the large pre-B cell stage where rapid proliferation occurs following the assembly of pre-B cell receptor (pre-BCR) (Nagaoka et al., 2000) (Figure 4). Pre-B cells then exit the cell cycle and the transcription of *RAG* genes resumes for light chain gene assembly. Complete expression of BCR results in the eventual loss of *RAG* expression. Reactivation of RAG activity in peripheral B cells is still controversial.

In addition to the transcriptional regulation discussed in Section 1.4, post-translational regulation of RAG proteins provides another level for control of recombinase activity (Jiang et al., 2005). RAG2 is phosphorylated and degraded through an ubiquitination/proteasome pathway in the S, G2, and M phases of the cell cycle. This explains why V(D)J recombination occurs mainly in the G1 phase. Over-expression of *RAG* genes in non-lymphoid cells could activate recombination of exogenous recombination reporter constructs but not endogenous *Ig* and *TCR* loci (Oettinger et al., 1990). The "accessibility hypothesis" (Stanhope-Baker et al., 1996) proposes that RSS sites normally reside within heterochromatin and have to be "opened" for V(D)J recombination to occur. Germline transcription, DNA methylation, and histone modification have each been implicated in control of chromatin accessibility and V(D)J recombination (Roth and Roth, 2000; Schlissel, 2003).

1.8. Ubiquitination

Ubiquitin (Ub) is a highly conserved 76 amino acid polypeptide that can be covalently attached to another protein through an isopeptide bond. Stability and function of many key cellular proteins are regulated by ubiquitination (Di Fiore et al., 2003). Ub conjugation to the protein substrate is mediated through a cascade of enzymatic reactions. An Ub activating enzyme (E1) forms a thio-ester bond from a cysteine within its C-terminus to a glycine in Ub. Next, an Ub conjugating enzyme (E2) accepts Ub from the E1-Ub conjugate to form the E2-Ub conjugate through a similar thio-ester linkage. Finally, an Ub ligase (E3) transfers Ub to the target protein, resulting in an isopeptide bond between Ub and the substrate lysine (Liu, 2004).

The most prevalent result of ubiquitination is polyubiquitination-mediated protein degradation. If there are at least four Ubs linked through lys48, Ub substrates are recognized and degraded by proteasomes (Conaway et al., 2002). However, a non-proteolytic function of polyubiquitination can be mediated through lys63 on Ub. This mode of ubiquitination is reversible (Spence et al., 2000). Proteins can also be monoubiquitinated. Proteasome independent monoubiquitination is involved in many cellular processes such as protein transport, transcriptional regulation, and DNA repair (Hicke, 2001; Sigismund et al., 2004).

1.9. Localization within the Nuclear Matrix and Nuclear Paraspeckles

The nuclear matrix is a three dimensional structure consisting mainly of protein networks that survive sequential treatment with non-ionic detergents, nucleases and high-salt buffers (Verheijen et al., 1988). The nuclear matrix is thought to be involved in various nuclear activities, including replication, transcription, and RNA processing

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(Verheijen et al., 1988). Recent studies have shown that the nuclear matrix is involved in various nuclear activities such as DNA replication, transcription, RNA processing, viral replication, and hormone activity. Nuclear matrix attachment sites (MARs) often colocalize with replication origins, insulators and other regulatory DNA elements. Several transcription factors have been shown to bind to the nuclear matrix and to MARs. MARs can act as promoters, enhancers, and silencers when bound by transcription factors (Razin et al., 2007). Deletion of nuclear matrix targeting sequence (NMTS) abrogates transcriptional activity in several transcription factors, such as SATB1 and Runx2 (Seo et al., 2005).

Transcription of rRNA genes and the processing of rRNA precursors occur in the nucleolus. Proteomic analysis of purified human nucleoli identified 3 proteins (PSP1, PSP2, and Nono/p54/nrb) (Fox et al., 2002). Indirect immunofluorescence analysis indicated that these proteins also resided in novel non-nucleolar punctuate foci. These foci were termed "paraspeckles" because they localized adjacent to another set of nuclear foci ("speckles") that are involved in storage or recycling of splicing factors (Fox et al., 2002). The biological function(s) of paraspeckles have not been fully discerned. However, paraspeckle proteins are suggested to be involved in RNA metabolism, based on the fact that localization within paraspeckles depends on active transcription and that the 3 PSP proteins contained RNA binding (RRM) domains. Relevant to the studies presented in Chapter 3, it has been shown that BCL11A colocalizes with these proteins and with BCL6 within nuclear paraspeckles (Liu et al., 2006).

CHAPTER 2. MATERIALS AND METHODS

2.1. RNA Isolation

For total RNA isolation, cells were washed twice in PBS and homogenized in TRizol (GibcoBRL). Chloroform was added at 200ul/1ml of TRizol, and phases were separated by centrifugation. RNA was precipitated with isopropyl alcohol and dissolved in DEPC-treated water.

For mRNA isolation, FastTrack 2.0 mRNA isolation kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, cells were homogenized in Lysis buffer and incubated with oligo (dT) cellulose. Oligo (dT) cellulose was washed in Binding buffer and Low Salt Washing buffer. The pellet was transferred into a spin column and washed with Low Salt Washing buffer. mRNA was eluted in elution buffer, precipitated with isopropyl alcohol, and dissolved in DEPC-treated water.

2.2. RT-PCR

For reverse transcription (RT), 1ug of total RNA or 10ng of mRNA, 1ul of oligo (dT) (500ug/ml), 1ul of dNTP mix (1mM each), 4ul of 5X first-strand buffer, 2ul of 0.1M DTT, 1ul of RNAseOut (40 units/ul, GibcoBRL), 1ul of Superscript II (200 units, GibcoBRL) and water were added to each reaction in 20ul of final volume. The reaction mixture was incubated at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 minutes.

For PCRs, 1 to 3 ul of cDNA, 5ul of 10X PCR buffer (200mM Tris-HCl, 500mM KCl), 1.5ul of 50mM $MgCl_2$, 0.4ul of Taq polymerase (5U/ul, Invitrogen), 1ul of

forward primer (10uM) and 1ul of reverse primer (10uM) were mixed and then water was added to a final volume of 50ul.

2.3. Cell Culture

293T, Phoenix, NIH3T3 and COS7 cells were maintained in DMEM (GibcoBRL) containing 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin, and 100 ug/ml streptomycin. Raji, Nalm6, SMS-SB, and A70 cells were maintained in RPMI-1640 (GibcoBRL) containing 10% FBS, $5x10^{-5}$ 2-Mercaptoethanol, 100 U/ml penicillin, and 100 ug/ml streptomycin.

2.4. Retrovirus Preparation and Transduction

Human *BCL11A-XL* cDNA was subcloned into the pXY-IRES-puro (pXY-puro) vector which was a kind gift from Dr. Louis Staudt at Division of Clinical Sciences, National Cancer Institute (Shaffer et al., 2000). Plasmid DNA encoding *BCL11A-XL* was transfected into the Phoenix cell line using Fugene (Roche) to produce retroviral particles. Viral particles were harvested 48hr after transfection and centrifuged to remove cell debris. The viral supernatants were ultracentrifuged at 20000 RPM for 2hr and resuspended in an appropriate amount of serum free Opti-MEM media (Invitrogen). Aliquots of viral supernatants were stored at -80°C.

Viral titers were determined by infecting NIH3T3 cells and counting the number of puromycin-resistant cells. Approximately the same amount of pXY-puro virus or pXY-BCL11A-XL virus was used to produce similar multiplicities of infection (MOIs). The appropriate amount of viral particles were thawed and incubated with 10 ug/ml of DOTAP (Roche) for 10min. Cells were transduced in 24-well culture plates at 25°C by

spin inoculation for 90min (Shaffer et al., 2000). Forty-eight hours after transduction, cells were selected in the presence of 0.5-2ug/ml of puromycin (Sigma). The following concentrations of puromycin were used for each cell line: 293T cells, Nalm6 cells, SMS-SB cells and Jurkat cells – 0.5ug/ml, and BJAB and Raji cells – 1ug/ml.

2.5. Western Blot Analysis

Cells were suspended in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 Mm EDTA) supplemented with protease inhibitors (Roche). Cell lysates were cleared by centrifugation. Loading concentrations of proteins were determined by Bradford assay using the Bio-rad protein assay reagent. Equal amounts of proteins were loaded onto SDS-PAGE gels and transferred to nitrocellulose membranes (Protran BA) using a semi-dry transfer apparatus. Each membrane was blocked with blocking buffer containing 5% milk and 0.1% Tween 20 for 1hr at room temperature. Appropriate concentrations of primary antibodies were used for 1 hr incubations and membranes were washed 4 times in PBST (PBS with 0.1% Tween 20) before incubation with secondary antibodies. Horseradish peroxidase conjugated goat anti-mouse antibody or goat anti-rabbit antibody (Amersham) were used at a 1:8000 dilution for 1hr at RT. After 4 washes, membranes were developed using ECL reagents (Amersham Pharmacia Biotech). The following dilutions were used for each antibody; HA – 1:2000 (Babco), Flag – 1:5000 (Sigma), ß-tubulin- 1:2000 (Santa Cruz), RAG1-1:2000 (Pharmingen, Cal# 554116), PSF-1:2000 and BCL11A-XLc-1:3000 (Bethyl, BL1797).

2.6. Ubiquitination Assays

For in vivo ubiquitination assays, 293T cells were transfected transiently with CMV10-BCL11A-XL or S, pMT-HA-Ub (a kind gift from Dr Huibregtse) and empty CMV10 (Sigma) at the indicated concentrations. Eight hours after transfection, cells were incubated in proteasome inhibitors, MG132 (20uM, Calbiochem) or LLM (50uM, Calbiochem) for an additional 12 hr. The cells were lysed in RIPA buffer containing protease inhibitors and immunoprecipitated using anti-Flag antibody, M2 (Sigma). The lysates were loaded onto SDS-PAGE gels and processed for western blot analysis. Ubiquitin signals were detected using an anti-HA antibody (Babco). For B cell receptor stimulation, goat anti-human IgM polyclonal antibody (Southern Biotechnology) was used at the indicated concentration.

2.7. Nuclear Matrix Fractionation

The procedure was adapted from the method of Reyes et al. (Reyes et al., 1997). For nuclei and cytoplasm isolation, 10^7 cells were resuspended in 200 µl of HNB (0.5 M sucrose, 15 mM Tris-HCl, pH 7.5, 60 mM KCl, 0.25 mM EDTA, pH 8, 0.125 mM EGTA, pH 8, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 5 µg/ml pepstatin, 5 µg/ml leupeptin, and 5 µg/ml aprotinin). Then 100 µl of HNB supplemented with 1% NP-40 was added dropwise. After 5 min of incubation on ice, cells were fractionated into nuclei pellet and cytoplasm supernatant (C) by centrifugation at 6,000 rpm for 3 min. Isolated nuclei were resuspended in 200 µl of nuclear buffer (20 mM Tris-HCl, pH 7.5, 70 mM NaCl, 20 mM KCl, 5 mM MgCl$_2$, and 3 mM CaCl$_2$ supplemented with protease inhibitors) and incubated with 3 U of micrococcal nuclease

(Sigma) for 15 min at room temperature. The reaction was terminated by the addition of EDTA and EGTA to 5 mM each, the mixture was then centrifuged at 5,000 g for 3 min, and the supernatant was designated the nucleoplasm fraction (NP). The nuclear pellet was suspended in CSK buffer and digested with 1 mg/ml of RNase-free DNAase I in CSK buffer plus proteases inhibitors for 15 min at 37°C. Then ammonium sulfate was added to a final concentration of 0.25 M and the mixture was incubated on ice for 5 min. After centrifugation, the soluble material was referred to as the chromatin fraction (CH). The pellet was washed twice with 2 M NaCl and solubilized in urea buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8) to isolate nuclear matrix fraction (NM). The efficiency of the nuclear fractionation was assessed by specific detection of the Lamin B nuclear matrix protein (Seo et al., 2005).

2.8. Electrophoresis Mobility Shift Assays

The minimum promoter region of the mouse *RAG1* gene (Brown et al., 1997) and the *Erag* enhancer region (Hsu et al., 2003) were amplified by PCR using the following primers; RAG1 forward 5'-cattctcagggagggaactg-3', RAG1 reverse 5'-ggcaaagtgtgttctctgctc-3', Erag forward 5'-acaccctaaatgggccgtgaac-3', Erag reverse 5'-cagaacccgagggcttagcatt-3'. R1P-Luc, R2P-Luc, and EragR1P-Luc constructs (described in chapter 5.4) were used as templates for PCRs. Double stranded PCR products were end-labeled with P^{32} using polynucleotide kinase and then purified using Bio-Spin Columns (BIO-RAD). Each reaction mixture was consisted of 5 to 10 ug of nuclear extracts from Nalm6 cells, probes, and a binding buffer (20mM HEPES, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1mM DTT, 0.1% NP40, 3 mg/ml bovine serum albumin, 10% glycerol, 2% Ficoll, 50 ug/ml of sonicated salmon sperm DNA and protease inhibitor

cocktail). The binding mixture was incubated at room temperature for 30 min. For the antibody binding assays, 100ng of BCL11A polyclonal antibody (BL1797) or an equal amount of a control rabbit IgG antibody were added to each binding reaction. Each binding reaction was loaded onto the nondenaturing gels. The gels were dried and analyzed using a Phosphoimager (Molecular Dynamics).

2.9. Luciferase Assays

293T cells were plated at $1x10^6$ cells per well in 12-well plates. Twenty four hr later, cells were transiently transfected with 1ug of *firefly luciferase* constructs, 5 ng of *ranilla luciferase* constructs, and indicated amounts of constructs encoding the *BCL11A-XL* gene. Final trasnsfection concentrations were adjusted to equal amounts with the empty CMV 10 construct. Forty eight hr after transfection, cells were harvested for Dual-Luciferase Reporter Assays (Promega). Cell lysis and luciferase value measurements were performed following manufacture's manual. Values were normalized using Renilla values and expressed in terms of % of control in which only the empty vector (CMV10) was transfected.

2.10. Chromatin Immunoprecipitation (ChIP)

These analyses followed Upstate ChIP kit protocols (www.upstate.com). Nalm6 or Raji cells were cross-linked by incubating at room temperature for 8 min in a final concentration of 1% formaldehyde. Fixed cells were washed twice with ice cold PBS and lysed in SDS lysis buffer (1% SDS, 10mM EDTA and 50mM Tris-HCl, pH 8.1). Lysates were sonicated for thirty sec six times. Sonicated chromatin was diluted 10-fold in a

dilution buffer (0.01% SDS, 1.1% Triton X- 100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl), and then immunoprecipitated with purified rabbit IgG or BCL11A-XL antibodies (Bethyl, BL1797). Antibody/chromatin complexes were washed with a low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), a high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), a LiCl wash buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1) and a TE wash buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA). These complexes were eluted in elution buffer (1% SDS, 0.1 M $NaHCO_3$) and cross links were reversed at 65°C for 6-12 hours in the presence of 0.2 M NaCl. After proteinase K treatment for one hr at 45℃, DNA was recovered by using PCR purification columns (Invitrogen) and then used for PCR analysis. The *RAG1* and *RAG2* promoter and the *Erag* enhancer region were detected using the following primers: RAG1 forward 5'-cattctcagggagggaactg-3', RAG1 reverse 5'-ggagggctaaccacaaatga-3', RAG2 forward 5'-gtggtctctgcttcaggaca-3' and RAG2 reverse 5'-agcaacaatggcaacacaat-3'. Thermal cycling conditions are 95°C for 5 min, then 35 cycles at 95°C for 10 sec, 55°C for 15 sec, 72°C 10 sec and 72 °C for 7 min. Erag forward 5'-tattcaggagggaattaaatgac-3', Erag reverse 5'-gacagaacccgagggcttagcat-3'. PCR conditions used for the Erag enhancer are 95°C for 5 min, then 35 cycles at 95°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 7 min.

2.11. Microarray

A detailed protocol can be obtained on the Iyer lab (University of Texas at Austin) web site (http://mbb3212a1.icmb.utexas.edu/ilcrc/protocols/index.shtml). Briefly, 3ug of mRNA was reverse transcribed using amino-ally UTP/dNTPs. Synthesized cDNA

was hydrolyzed and subjected to PCR purification columns (Invitrogen). Each sample was coupled with Cy3 or Cy5 dyes and mixed samples were hybridized on human array slides. Microarray slides were scanned using a GengePix 400a Microarray Scanner.

2.12. A70-INV Cell Line and STI-571

The A70-INV cell line is described in Chapter 1.5 according to Bredemeyer and others (2006). Briefly, Bone marrow cells from mice were infected with the pMSCV-v-Abl retrovirus to generate v-Abl transformed pre-B cells. These cells were transduced by pMX-RSS-GFP/IRES-hCD4 encoding inverted *GFP* flanked by recombination signal sequences (RSS) and following *IRES-hCD4* (described in Figure 21A). Only after normal rearrangement, *GFP* cDNA is inverted to the sense orientation, which results in GFP expression. pMX-RSS-GFP/IRES-hCD4 retrovirus positive cells were isolated by hCD4 sorting. Cells were transduced by pXY-puro or pXY-BCL11A-XL retroviruses or/and treated with 3uM STI-571 (Novartis) for the indicated time. Rearrangement was quantitated using FACS and genomic DNA PCR analyses.

2.13. Genomic DNA Isolation and Southern Blotting

A DNeasy tissue kit (Qiagen) was used to isolate genomic DNA from A70-INV cells. We used previously published protocols with some modifications for analysis of pMX-RSS-GFP/IRES-hCD4 (Bredemeyer et al., 2006) and *Ig* light chain k (Ramsden, 1994) rearrangements. For genomic PCR, 100-200 ng of genomic DNA was PCR amplified and then subjected to electrophoresis in agarose gels. VJk2 PCR products were

transferred to a nylon membrane for Southern blot analysis. Jk2-2 oligo probes were end labeled using a T4 polynucleotide kinase. Southern blots were pre-hybridized overnight and hybridized with probes for 3 hr at 50°C in Ultra-hyb hybridization solution (Ambion). Blots were washed once at room temperature and twice at 50°C in 2X SSC/0.1% SDS for 30 min each. The blots were exposed to phosphorimager plates for 48-72 hr and the signals were detected using a Phosphorimager (Molecular Dynamics). The detailed strategy and all sequences of used primers are described in Figures 20B and 23.

2.14. Flow Activated Cytometry Sorting (FACS) Analysis

A70-INV cells were incubated in the absence or in the presence of 3uM of STI571 for three days. The harvested cells were washed two times with ice-cold PBS and resupended again in PBS at a concentration of 1 x 10^6 cells/ml. A minimum of 5000 cells per sample was used for FACS analysis. The parental A70-INV cells were used for GFP gating. Flow cytometry (BD FACSCalibur Flow Cytometry System) was used to analyze the samples for GFP positive signals. The collected data were analyzed using the Cell Quest software.

2.15. Inducible shRNA Knock-Down of *BCL11A* and *RAG* Expression in BJAB B Cells

Two complementary shRNA template oligonucleotides targeting a BCL11A gene were synthesized and cloned into the pRSMX-PG vector (Ngo et al., 2006) using HindIII

and BglII sites. pRSMX-PG-BCL11A-shRNA retrovirus was produced in Phoenix cells as described above and used for infection of a Tet-on BJAB cell line (Ngo et al., 2006) which expresses the Tet-repressor. Transduced BJAB cells were selected in the presence of puromycin for 6 days. BCL11A specific shRNA expression was induced with different concentrations of doxycycline for the indicated time. Levels of BCL11A expression were measured by RT-PCR and western blot analysis. The following oligomers were used for the BCL11A shRNA template; forward, 5'-gatcaccccagcacttaagcaaattcaagagatttgcttaagtgctggggtttttttggaaa-3', and reverse, 5'-agcttttccaaaaaaacccagcacttaagcaaatctcttgaatttgcttaagtgctggggtg-3'. BglII and HindIII sites were used for cloning.

2.16. Construction of BCL11A Plasmids

Plasmids CMV10-BCL11A-XL and CMV10-BCL11A-S were constructed as follows: BCL11A-XL and BCL11A-S were amplified by PCR with the same forward primer: 5'-gccaaagcttatgtctcgccgcaagcaaggc-3' and different reverse primers: for XL isoform, 5'-cccaggatcctattcagtttttatatcattattcaac-3', for S isoform, 5'-cccaggatcctcaaattttctcagaacttaag-3'. The PCR products were digested using BamHI and HindIII restriction digestion enzymes and cloned into the CMV10 plasmid (Sigma), which contains a 3xFlag tag. The pXY-BCL11A-XL plasmid was constructed using CMV10-BCL11A-XL as a template and the following PCR primers: forward, 5'-ccgggatccatggactacaaagaccatgac-3' and reverse, 5'-ggacgcggccgctattcagtttttatatcattattc-3'. The PCR products were digested with BamHI and NotI restriction enzymes and cloned into the pXY-IRES-puro plasmid using BglII and NotI sites. pEGFPC1-BCL11A-XL-K5N mutant was generated by site-directed mutagenesis using the Quick Change

site-directed mutagenesis kit and a protocol from Stratagene. The following primers were used; forward, 5'-ccatgtctcgccgcaaccaaggcaaacccccagc-3' and reverse, 5'-gctggggtttgccttggttgcggcgagacatgg-3'. The deletion mutant pEGFPC1-BCL11A-XLdelN80 was constructed using PCR with the following primer pairs: forward, 5'-ggcgtcgacagcgaacacggaagtccc-3' and reverse, 5'-cccaggatcctattcagtttttatatcattattcaac-3'. The PCR products were digested using SalI and BamHI restriction enzymes and cloned into the plasmid vector pEGFPC1. The final clones were verified by sequencing.

CHAPTER 3. CHARACTERIZATION OF BCL11A FUNCTION, STABILITY AND LOCALIZATION

3.1. Overview of BCL11A Domain Structure

The human *BCL11A* locus can produce several isoforms by alternative pre-mRNA splicing (Liu et al., 2006) (Figure 5). Each contains the same N-terminal exon 1 and 2. The N-terminal 12 amino acids within exon 1 are virtually identical with the N-termini of BCL11B, FOG, SALl and Spalt transcription factors (Lin et al., 2004) (Figure 6). This 12 amino acid motif from FOG-2 was fused with the GAL DNA-binding domain and the fusion protein was shown to be sufficient to produce repression activity on a GAL-luciferase reporter. Point mutations of R3, R4, or K5 disrupted the repression activity of the fusion protein (Lin et al., 2004).

The *XL* isoform has five C2H2 zinc finger motifs which are known to be involved in protein-protein interaction, lipid binding, DNA binding, and RNA binding (Laity et al., 2001). The common N-terminal domain contains a rare, C2XX zinc finger, a motif whose function is not well understood. There are three putative nuclear localization signals, NLS1, HKRK at 66-69, NLS2, KHKR at 569-572 and NLS3, PFSKRIK at 631-637. Mutational analysis demonstrated that NLS3 is a bonafide NLS (Liu, 2002). An acidic domain has been shown to have transcriptional activation activity in other proteins including the herpes simplex virus transactivator, VP16 (Shen et al., 1996).

Figure 5. Schematic representation of the functional domains of BCL11A-XL.

The BCL11A "extra long" isoform (BCL11A-XL) has 835 amino acids. This protein migrates at 120kD on a SDS-PAGE gel even though its predicted molecular weight is 91kD. The "long" (BCL11A-L) and "short" (BCL11A-S) isoforms also migrate slower on SDS-PAGE gels (L at 100kD and S at 35kD) than predicted by their sequences. The first 12 amino acids of the N-terminus (shared by all BCL11A isofroms) are highly similar to that of several zinc finger-containing transcriptional factors. There are six zinc fingers and an acidic domain in the XL isoform. The nuclear localization signal (NLS) resides at amino acids 631-637. All isoforms contain the N- terminal region encoded by exon 1 and 2. The exons (boxes) and untranslated regions (lower boxes) are denoted.

Exon 1 — 18 aa

Exon 2 — 110 aa

Exon 3 — 34 aa

Exon 4 — 672 aa

Exon 5
33 aa(S)
29 aa (L)

BCL11A-XL
5.9 kb, 835 aa

H+

BCL11A-L
4.0 kb, 773 aa

H+

BCL11A-S
2.4 kb, 243 aa

Exon

UTR

NLS

C2HC Zinc Finger

C2H2 Zinc Finger

H+ Acidic Domain
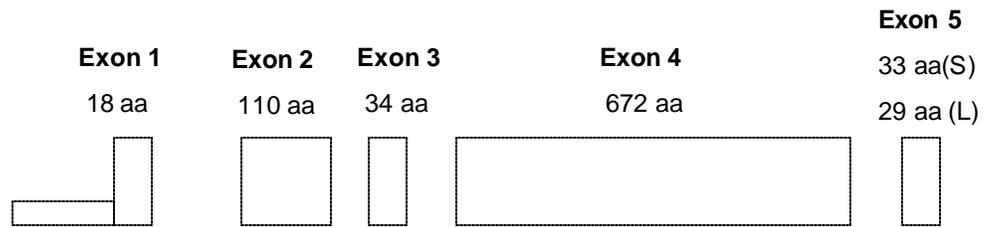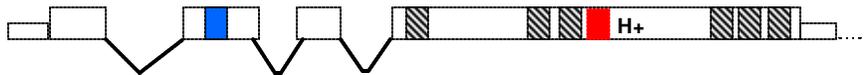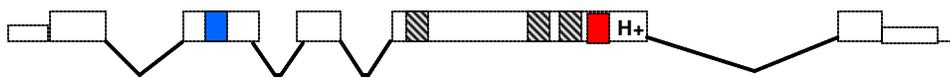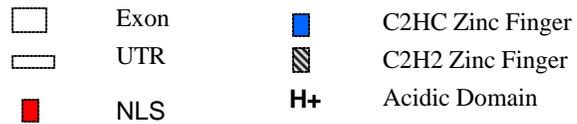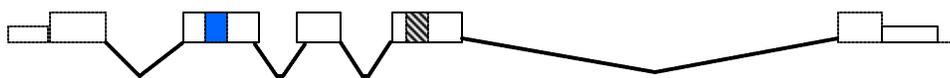
Modified from Molecular Cancer. 2006.5:18

36

Figure 6. Comparison of N terminal sequences and zinc fingers among BCL11 and other highly similar transcription factors.

A. The N-terminal 12 amino-acids are highly similar in several transcription factors that are known to be involved in hematopoeisis. The underlined three amino-acids, RRK, were shown to be essential for transcriptional activity (Hong et al., 2005; Lin et al., 2004).

B. Comparisons of zinc fingers. The highly similar N-termini are followed by a single, canonical C2HC zinc finger, which then is followed by one or more single, double, or triple zinc fingers of the C2H2 type. *BCL11A* and its paralogue, *BCL11B*, as well as early hematopoietic zinc finger (EHZF) and the friend-of-GATA hematopoietic transcription regulators *FOG-1* and *FOG-2*—all encode zinc finger proteins with these conserved features, and all have been implicated in hematological malignancies (Lin et al., 2004). *M, M. musculus*; *H, H. sapiens*; *B, B. taurus*; *R, Rattus norvegicus*; *X. laevis*; *G, G. gallus*.

A
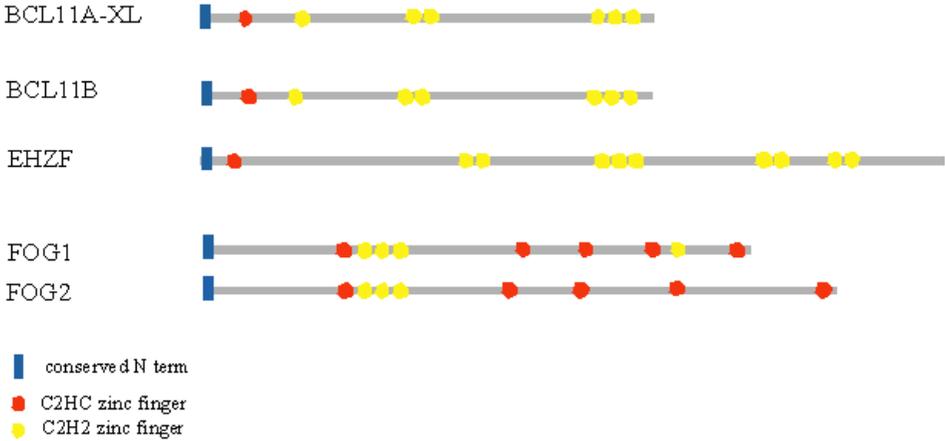
B

Protein	Species	Position											
		1	2	3	4	5	6	7	8	9	10	11	12
FOG-2 (zfpm2, Mm.39496)	M,H,B	M	S	R	R	K	Q	S	K	P	R	Q	I
FOG-1 (zfpm1, Mm.3105)	M,H,B,X	M	S	R	R	K	Q	S	n	P	R	Q	I
Zinc finger protein 423 (EBFAZ, Mm.23452)	M,R	M	S	R	R	K	Q	a	K	P	R	s	v
Zinc finger protein 521 (Evi3, Mm.40325)	M,H,X	M	S	R	R	K	Q	a	K	P	R	s	l
SALL1 (Mm.214361)	M,H,R	M	S	R	R	K	Q	a	K	P	q	h	f
	X	M	S	R	R	K	Q	p	K	P	q	h	f
SALL2 (Mm.39487)	M,H	M	S	R	R	K	Q	r	K	P	q	q	l
	R	M	S	R	R	K	Q	r	r	P	q	q	l
SALL3 (Hs.444836)	H,R	M	S	R	R	K	Q	a	K	P	q	h	l
SALL4 (Mm.256916)	M,H,X	M	S	R	R	K	Q	a	K	P	q	h	I
Spalt 1 (Gga.1817)	G	M	S	R	R	K	Q	a	K	P	q	h	f
Spalt 2 (Gga.167)	G	M	S	R	R	K	Q	a	K	P	q	h	l
Zinc finger protein 64 homolog (Mm.2095)	H,R	M	S	R	R	K	Q	a	K	P	q	h	l
B-cell CLL/lymphoma 11A (BCL11A, Mm.314623)	M,H,X	M	S	R	R	K	Q	g	K	P	q	h	l
B-cell CLL/lymphoma 11B (BCL11B, Mm.116831)	M,H	M	S	R	R	K	Q	g	n	P	q	h	l
Hypothetical Protein LOC55565 (Hs.104788)	M,H	M	S	R	R	K	Q	t	n	P	n	k	v
Conserved		M	S	R	R	K	Q			P			

Adapted from Lin, A. C. et al. J. Biol. Chem. 2004.279:55017

3.2. BCL11A-XL is a Major Isoform Conserved in Evolution

BCL11A-XL was identified initially in human brain and in human B lymphocytes (Liu et al., 2006; Satterwhite et al., 2001). An expressed sequence tag cDNA libraries for XL was observed in the chicken, but surprisingly, no EST for XL was reported in mouse libraries. However, a full-length transcript for mouse BCL11A-XL was cloned from cDNA libraries designed to capture long transcripts more efficiently than do conventional methods (Carninci et al., 2000). Regardless of this and the extremely high homologies among BCL11A-XL orthologues (Figure 1), previous functional studies were performed only on the long (BCL11A-L) isoform (Avram et al., 2000; Avram et al., 2002; Nakamura et al., 2000).

To further address this issue, we generated primers that specifically target the 5' end of the coding region and the 3' UTR of the XL isoform. BCL11A-XL was observed in RNA from both mouse and chicken cell lines (Figure 7A).

Monoclonal (Liu et al., 2006) or commercially obtained polyclonal antibodies specific for both L and XL isoforms of BCL11A were used for immunoblotting nuclear extracts prepared from the DT40 chicken B cell line and from human and mouse B cell lines. We detected only the BCL11A-XL isoform in mouse samples (data not shown). Immunoblots with the XL C-terminal polyclonal antibody (BL1797), which detects the C-terminal region unique to the XL isoform, confirmed the presence of BCL11A-XL in mouse and chicken cell lines (Figure 7B). These results indicate that the highly conserved BCL11A-XL is also the most highly expressed isoform in B cells.

Figure 7. BCL11A-XL expression in chicken, mouse and human cells.

A. Measurement of BCL11A-XL transcript levels by RT-PCR. Primers specific for human BCL11A-XL 5' ends were used to amplify cDNA. RNA was isolated from a human B cell line (Raji), a mouse B cell line (BCL1), mouse whole brain tissue, mouse thymocytes and a chicken B cell line (DT40). BCL11A-XL is expressed in chicken (c), mouse (m) and human (h) cells. The faster migration of chicken BCL11A-XL results from a shorter 3'UTR. PCR primers: forward; 5'-gtggggaaggacgtttacaa-3'; reverse, 5'-atcatgcattcaaacggtga-3'.

B. Western blot identification of BCL11A-XL protein. The polyclonal antibody (Bethyl, BL1797) is specific for the unique BCL11A C-terminus (amino acids 775-835; Figure 5). An *in vitro* translated lysate (IVT) was used as a positive control. Nuclear extracts from a chicken B cell line (DT40), mouse (m) B cell lines (HAFTL-1 and 300.19) and a human (h) B cell line (Raji), express BCL11A-XL. XL and Deg indicate full length and degraded BCL11A-XL, respectively.

A
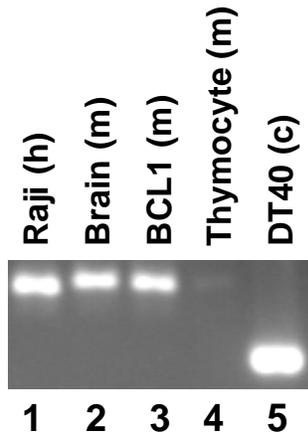
B
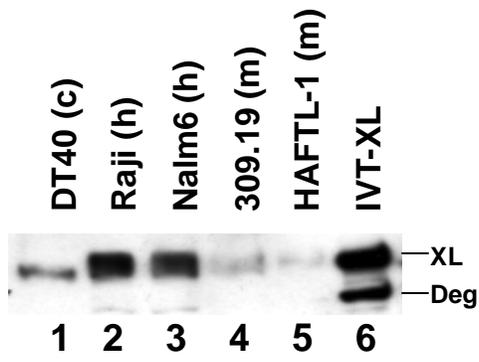
3.3. Ubiquitin Mediated Protein Degradation of Ectopically Expressed BCL11A Proteins

Epitope-tagged expression constructs of BCL11A isoforms were made for *in vitro* and *in vivo* expression. Each of these produced very poor expression levels in transient transfections as compared to epitope-tagged Bright and other factors used routinely in our laboratory (data not shown). This suggested that BCL11A proteins might be subject to ubiquitin-mediated protein degradation. We used the specific proteasome inhibitors LLM and MG132 in an attempt to increase the stability of BCL11A proteins following transfection. Regardless of whether the isoform localized to the nucleus (XL and L) or to the cytoplasm (BCL11A-S), proteasome inhibitors protected BCL11A proteins from degradation (Figure 8A).

293T cells were co-transfected with Flag-tagged BCL11A and HA-ubuiquitin constructs to assess the ubiquitination levels (Figure 8B). In the presence of proteasome inhibitor, typical ladders representing multiple-ubiquitinated BCL11A proteins were observed (lanes 3 and 9). This data suggests that ubiquitination/proteasome mediated degradation pathway may contribute to poor exogenous expression of BCL11A proteins in non-B cells.

3.4. Antigen Receptor Signaling in B cells Induces Instability of BCL11A

It was previously shown that BCL11A-XL and L undergo protein-protein interaction with BCL6, a transcription factor essential for germinal center B cell formation (Liu et al., 2006; Nakamura et al., 2000). BCL6 is expressed in germinal center B cells, but not in plasma cells. Antigen receptor stimulation with anti-IgM was shown to

induce phosphorylation, ubiquitination and degradation of BCL6 in the mature B cell line, Ramos. These authors further reported that ubiquitination-mediated degradation of BCL6 is responsible for rapid down-regulation of BCL6 during post-GC differentiation (Niu et al., 1998).

Because BCL11A-XL expression is also extinguished in post-GC plasma cells (Liu et al., 2006), we tested whether BCL11A proteins are destabilized by antigen receptor stimulation. As shown in Figure 9A, stimulation of the antigen receptor in Ramos B cells resulted in decreased levels of exogenous (lane 2) and endogenous (lane 4) BCL11A-XL, as detected by anti-Flag and BCL11A polyclonal (BL1797) antibodies, respectively. As expected from previous data (Niu et al., 1998), BCL6 showed induced degradation by antigen receptor stimulation (lane 6). Levels of an unrelated nuclear protein, PSF, were not significantly changed after antigen receptor stimulation. Next, Ramos cells were treated with an anti-IgM antibody and/or a proteasome inhibitor, MG132. As shown previously (Niu et al., 1998), proteasome inhibitor treatment blocked BCR-stimulated degradation of BCL6 (Figure 9B, lanes 4, 5 vs 6, 7). Unexpectedly, proteasome inhibitor treatment initially increased the stability (lanes 2 and 6) and later time points decreased the stability of BCL11A-XL (lanes 3 and 7).

We conclude that, as previously demonstrated for the XL-interacting partner, BCL6, human BCL11A-XL is ubiquitinated and proteosomally degraded as a mechanism for controlling expression levels.

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Figure 8. *BCL11A-XL* expression is regulated by ubiquitin-mediated proteosome degradation.

A. Expression of BCL11A isoforms was increased by proteasome inhibitors (LLM or MG132) treatment in 293T cells (lane 2, 4, and 5). Thirty hr after transfection, cells were treated with 40 μM LLM or DMSO (solvent) for an additional 12-14 hr. BCL11A protein expression was detected by anti-Flag antibody following separation by SDS-PAGE and blotting.

B. BCL11A-XL (right) and BCL11A-S (left) are poly-ubiquinated and the modified proteins (adducts) are stabilized by proteosome inhibitors (lanes 3 and 9). Flag-tagged BCL11A-XL and S were transfected into 293T cells together with HA-Ub. Thirty hours after transfection, cells were further incubated in the absence or presence of LLM (40 uM) for 12-14 hr. Harvested cells were lysed and immunoprecipitated by anti-Flag antibodies and loaded onto a SDS-PAGE gel. Ubiquitinated signals were detected by HA-specific antibody immunoblotting. Hc (immunoglobulin heavy chain cross reaction), Lc (immunoglobulin light chain cross reaction).

A
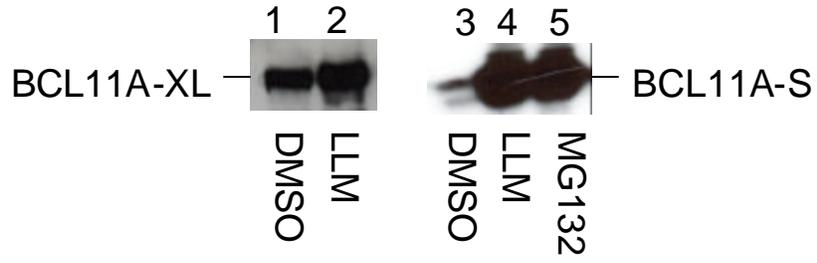
B
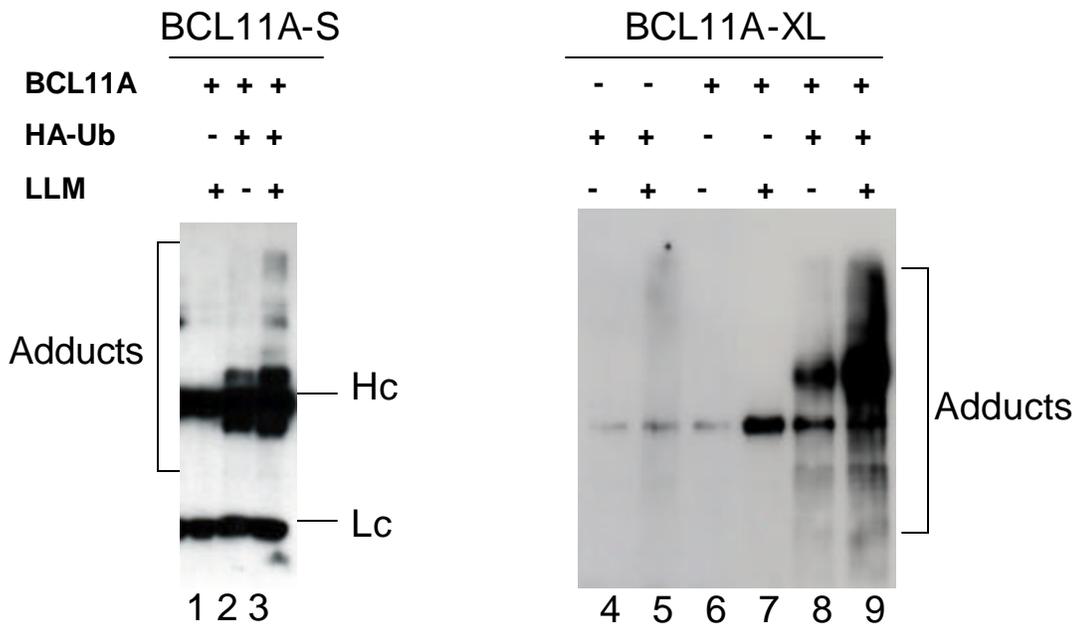
Figure 9. BCL11A instability during B cell receptor stimulation by anti-IgM antibodies.

A. BCL11A transduced Ramos cells were stimulated with anti-IgM polyclonal antibodies at 10ug/ml for 6 hr. Cell lysates were prepared and separated on SDS-gels. Exogenous BCL11A-XL was detected by anti-Flag antibodies (Flag-XL) and total BCL11A-XL by XL polyclonal antibodies (XL). The positive control, BCL6, was destabilized but negative control, PSF, was not affected during BCR stimulation.

B. Anti-IgM induced BCL11A degradation was prevented by a specific proteasome inhibitor, MG132 under short term (3 hr) incubation (lanes 2 and 6). Long term (6 hr) incubation with the proteasome inhibitor resulted in enhanced degradation of BCL11A (lanes 3 and 7). Instability of BCL6 was induced by BCR stimulation (lanes 4 and 5) and blocked by proteasome inhibitor treatment (lanes 6 and 7) regardless of the incubation period. ß-tubulin was not affected by the above treatments.

A

Flag-XL   XL   BCL6

aIgM(10ug/ml)   - +   - +   - +

— BCL11A

— PSF

1 2   3 4   5 6

B

| aIgM (10ug/ml) | - | - | - | 3 | 6 | 3 | 6 | hr |
| MG132 (20uM) | - | 3 | 6 | - | - | 3 | 6 | hr |

— BCL11A

— BCL6

— ßTub

1 2 3 4 5 6 7

48
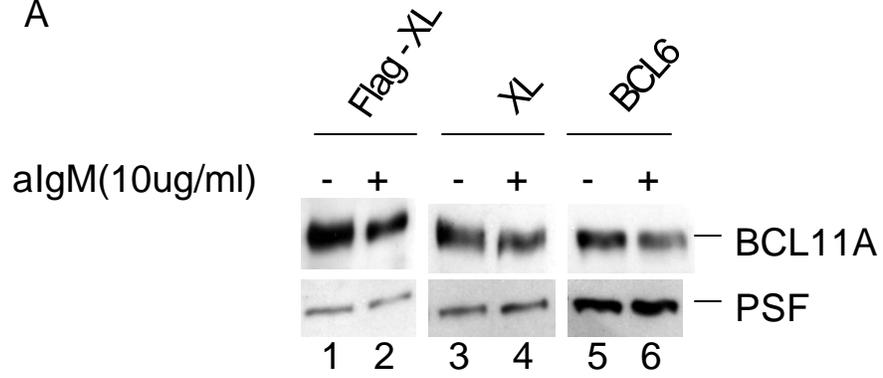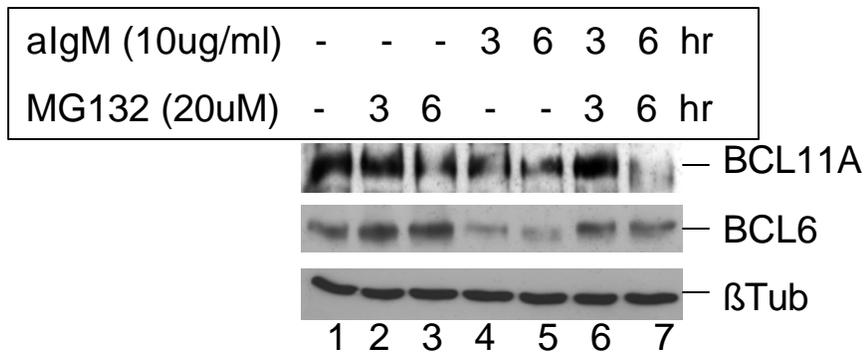
3.5. BCL11A-XL is a Nuclear Matrix Protein

As mentioned above, detecting BCL11A proteins is difficult even in a transient over-expression system. This can be partially explained by the ubiquitin-mediated degradation of BCL11A. We also checked the extraction efficiency of BCL11A with mild lysis buffer which contains only non-ionic detergents because nuclear matrix proteins are known to be resistant to non-ionic detergents (Verheijen et al., 1988). When cells were suspended in mild lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 100 ug/ml PMSF, 1 ug/ml aprotinin), a significant amount of signal resided in the lysis resistant insoluble pellet (data not shown).

Many transcription factors have been identified as insoluble nuclear matrix proteins (described in Section 3.8.2) (Mika and Rost, 2005). To assess solubility of BCL11A proteins, we carried out sub-cellular fractionations of several human B cell lines (Daudi, Raji, and Ramos) that have detectable levels of endogenous BCL11A expression. As shown in Figure 10, BCL11A-XL partitioned almost exclusively within the nuclear matrix fraction (lanes 4, 8, and 12) (Liu et al., 2006).

Thus, BCL11A-XL is another example of a nuclear matrix protein involved in transcription (Verheijen et al., 1988). Unlike nuclear localization signals, there is no consensus nuclear matrix targeting signal (NMTS). The relationship between nuclear matrix localization and function of BCL11A is addressed below.

Figure 10. BCL11A-XL partitions predominantly within the nuclear matrix.

        Daudi, Raji, and Ramos cells were fractionated into cytoplasm (C), nucleoplasm (NP), chromatin (CH), and nuclear matrix (NM). Equivalent volume of each sub-cellular fraction was loaded and resolved on SDS-PAGE gels. Endogenous BCL11A-XL was detected with BCL11A polyclonal antibodies. BCL11A-XL apears predominantly in the insoluble nuclear matrix fractions (lane 4, 8, and 12).

	Daudi				Raji				Ramos			
C	NP	CH	NM	C	NP	CH	NM	C	NP	CH	NM
1	2	3	4	5	6	7	8	9	10	11	12
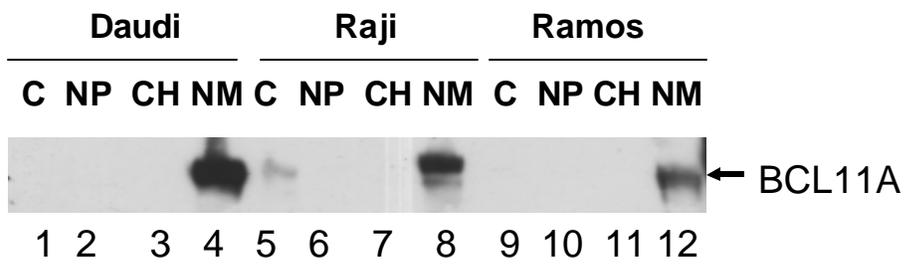
← BCL11A

3.6. The N-terminal Domains of BCL11A is Responsible for Repression Activity and for Paraspeckle Localization

As summarized in Section 3.1, the N-terminal 12 amino acids of BCL11A are virtually identical in several non-paralogous transcriptional factors (Figure 6). To determine whether the N-terminus of BCL11A-XL is also involved in transcription activity, the first 80 amino acids were deleted and the truncated protein was fused to GFP. In transient transfection assays, repression of RAG1-luciferase activity was abolished (Figure 11, lane 5). In addition, the distinct nuclear paraspeckle pattern of XL was significantly diminished to diffuse nuclear (Figure 12A, GFP-XLdelN80), while not affecting nuclear matrix targeting (Figure 12B, lane 8).

Lin and others (2004) observed that the N-terminal K5 of FOG2 was critical for repression activity. Thus, a GFP-BCL11A-XL-K5N mutant was constructed by site directed mutagenesis. As predicted, this point mutation abolished repression of the RAG1P-Luciferase reporter (Figure 11, lanes 7 and 8) but did not affect paraspeckle formation (Figure 12A, GFP-XL-K5N).

These results indicate that the first 80 amino acids of BCL11A-XL contain its transcriptional repression domain and paraspeckle targeting sequence. These two functions can be distinguished by mutation of a highly conserved residue (K5) within the N-terminus.

Figure 11. The N-terminus of BCL11A functions as a transcriptional repression domain.

Wild type (WT), or a N-terminal (80 amino acid) deletion mutant (Del80) or an N-terminal point mutant (K5N) of BCL11A-XL (30, 40, or 60 ng as indicated) was co-transfected with 1ug of RAG1-luciferase (R1P-Luc) and 5ng of Renilla luciferase into 293T cells. Forty hr after transfection, cells were harvested and luciferase activities were measured. The deletion of the first 80 amino-acids (lanes 4 and 5) or site directed mutagenesis of K5 of BCL11A-XL significantly reduced transcriptional repression relative to WT BCL11A-XL (lanes 1-3).

R1P-Luc
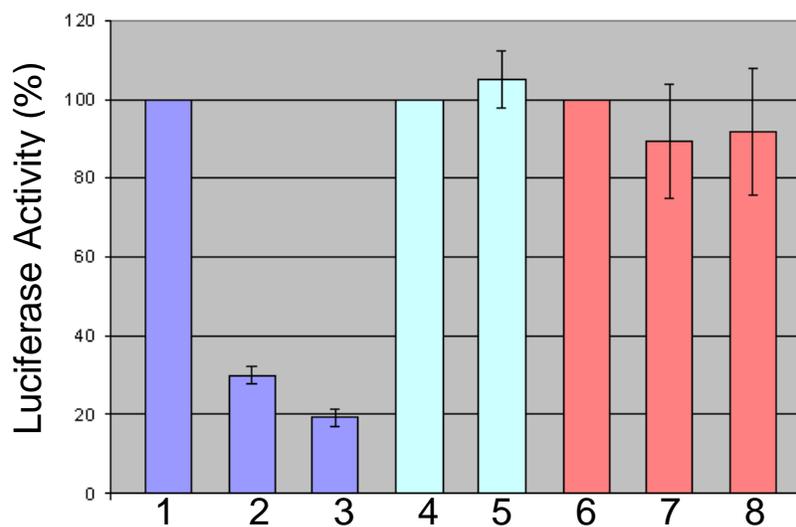
	1	2	3	4	5	6	7	8	
WT	0	30	60	0	0	0	0	0	ng
Del80	0	0	0	0	40	0	0	0	ng
K5N	0	0	0	0	0	0	30	60	ng

Figure 12. The paraspeckle and nuclear matrix targeting motifs of BCL11A.

A. GFP-BCL11A fusions of wild type (GFP-XL), N-terminal deletion (GFP-XLdelN80) or point mutant (GFP-XLK5N) were transiently expressed in 293T cells. Live cells were imaged under an inverted fluorescence microscope. Deletion of the first 80 amino-acids from BCL11A-XL resulted in loss of distinct nuclear paraspeckles (middle panel) while the K5N mutant (left panel) retained distinct paraspeckle pattern (dots, right).

B. Nuclear matrix partitioning of BCL11A-XL was not affected by truncation of its N-terminal 80 amino-acids. Upper, anti-BCL11A immunoblotting; lower, anti-Lamin B immunoblotting.

A

GFP-XL   GFP-XLdelN80   GFP-XL-K5N

B

GFP-XL				GFP-XLdelN80			
C	NP	CH	NM	C	NP	CH	NM

← BCL11A

← Lamin B

1   2   3   4   5   6   7   8
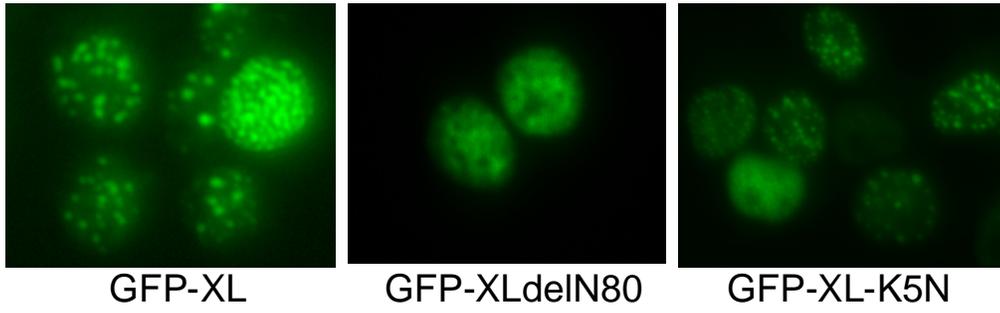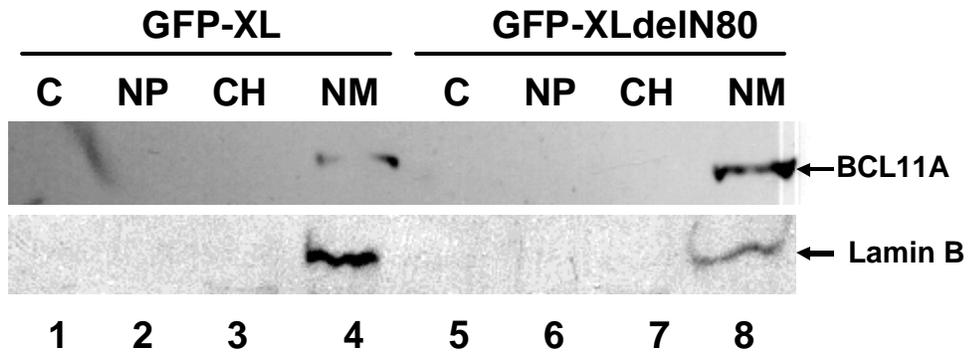
3.7. The Nuclear Localization Signal of BCL11A-XL is Sufficient for Predominant Nuclear Localization of GFP

Point mutation and a fluorescence microscopy demonstrated that NLS (631-PFSKRIK-637) is necessary for nuclear localization of BCL11A (Liu, 2002). To determine whether NLS3 is also sufficient to provide nuclear localization, we constructed NLS3-GFP fusions with wild type (GFP-WT-NLS) and mutant (GFP-MT-NLS) NLS3 and analyzed their localization in transiently transfected NIH3T3 cells. GFP-MT-NLS localized to both the cytoplasm and the nucleus in a pattern indistinguishable from GFP alone (Figure 13). The GFP-WT-NLS fusion localized almost exclusively to the nucleus (Figure 13) in a diffuse pattern.

We conclude that NLS3 is necessary and sufficient for nuclear localization of BCL11A-XL. But as expected from the N-terminal truncation data (Figure 12), NLS3 was insufficient for its sub-nuclear localization within paraspeckles.

Figure 13. The NLS of BCL11A-XL is sufficient to direct nuclear localization of GFP.

Wild type (GFP-WTNLS: PFSKRIK) and mutated (GFP-MTNLS; underlined amino acids mutated; PFSARIA) nuclear localization sequences of BCL11A-XL were fused to GFP, and the fusions were transfected into NIH3T3 fibroblasts. Forty eight hr after transfection, cells were fixed in paraformaldehyde, stained with 4',6-diamideino-2-phenylindole (DAPI) and imaged under an inverted fluorescence microscope. Wild type localized predominantly within the nucleus (overlapping with DAPI staining; right panel), whereas the mutant GFP-fusion localized in a diffuse pattern (left panel) indistinguishable from GFP alone (not shown).

	GFP-MTNLS	GFP-WTNLS
G F P		
D A P I		
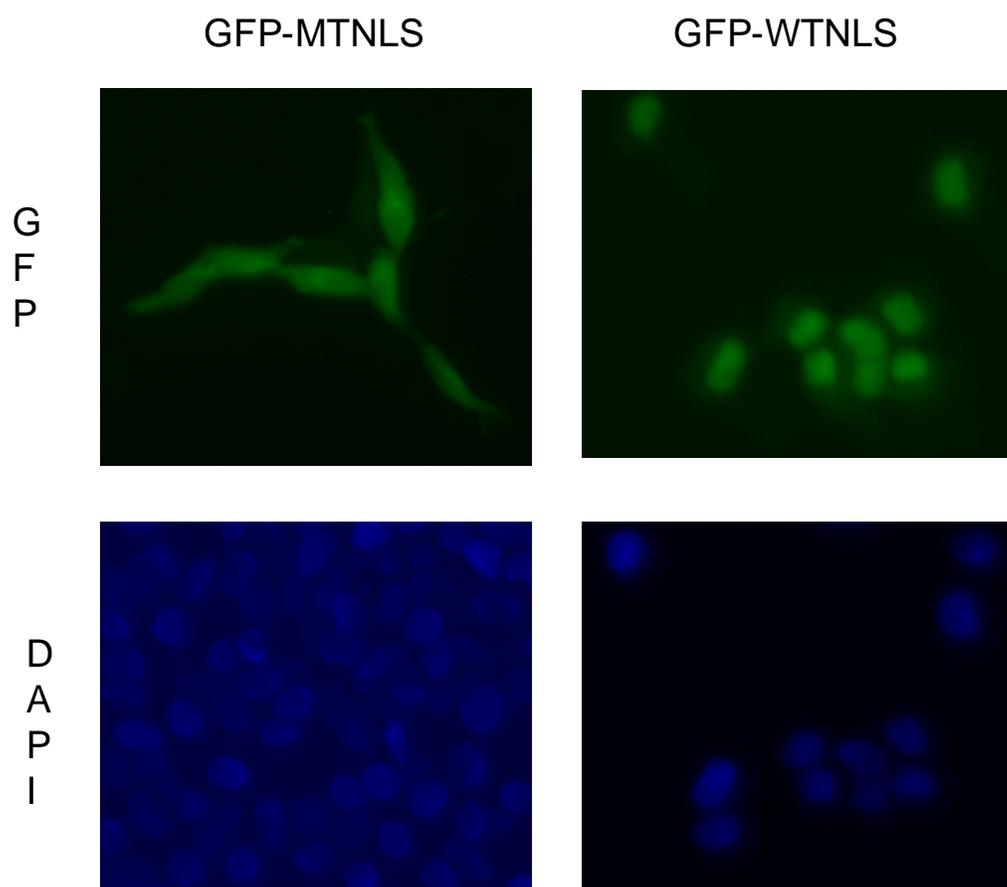
3.8. Discussion

3.8.1. BCL11A-XL IS HIGHLY CONSERVED AND HIGHLY EXPRESSED

BCL11A is highly conserved during evolution. In addition to the high similarity/identity of XL among chicken, frog, mouse and human XL isoform at the protein level (Figure 1), the DNA sequences, even at the third base of codons, are often conserved. For example, the mouse-human DNA identity within XL exons is 88% (data not shown). Three regions within the BCL11A transcription unit have been formally classified among the category of "super-conserved" sequences (Bejerano et al., 2004).

Thus, it seemed reasonable that the XL isoform was the most highly expressed isoform in humans (Liu et al., 2006), but puzzling that XL was not reported to be expressed in mouse B cells (Avram et al., 2002; Nakamura et al., 2000). On re-examining the Northern blot data of Nakamura et al. (2000), it is apparent that these investigators mis-assigned the abundant 5.8 kb species as BCL11A-L-an isoform hardly detectable in human B cells (Liu et al., 2006). This was unfortunate because all previous functional studies in mice were carried out with the L isoform (Avram et al., 2000; Avram et al., 2002; Liu et al., 2003; Nakamura et al., 2000). BCL11A-L lacks 3 of the 5 C2-H2 zinc fingers and likely binds DNA in a different manner than XL (Figure 5).

BCL11A has been strongly implicated in B-CLL and other B cell malignancies (Alizadeh et al., 2000; Satterwhite et al., 2001; Su et al., 2002). In B-CLL patient peripheral blood B cells, follicular lymphomas, and mantle lymphomas, XL is the most highly expressed isoform (Liu et al., 2006; Satterwhite et al., 2001). We have considered these findings in focusing our studies (Chapters 4 and 5) on BCL11A-XL.

3.8.2. BCL11A STABILITY AND UBIQUITINATION, AND PROTEASOME

Protein ubiquitination is involved in a wide array of cellular processes, including cell-cycle control, signal transduction, transcriptional regulation, DNA repair, receptor down-regulation, antigen presentation, and apoptosis (Schwartz and Ciechanover, 1999). Here, we have shown conclusively that ubiquitin-mediated protein degradation is involved in the regulation of BCL11A. All BCL11A isoforms appear to be ubiquitinated, and their stability is greatly increased by proteasome inhibitor treatment. Many transcription factors controlling cell growth are unstable proteins which are degraded through the ubiquitin-mediated proteasome pathway. Many unstable proteins such as p53, Myc, Rel, Fos, and E2F-1 have transcriptional activation domains overlapping with degradation signal sequences (Salghetti et al., 2000). As initial attempts to define ubiquitination target sites, we mutated all the first 10 lysine sites in the repression domain. None of them led to large difference in the sensitivity to proteasome inhibitors (data not shown). Additional mutagenesis efforts will be required to identify the motifs which are responsible for the instability of BCL11A proteins. Because a BCL11A-S isoform also showed ubiquitination/proteasome mediated instability, one would predict that these would reside within the exons shared by S, L, and XL (Figure 5) (Figure 8).

BCL6 expression is regulated by antigen receptor-mediated phosphorylation and polyubiquitination during B cell development in germinal centers (Niu et al., 1998). BCL11A-XL expression was also down-regulated during BCR stimulation. Transient transfection experiments in 293T cells showed an increased stability of BCL11A with proteasome inhibitor treatment. However, BCL11A stability in Ramos cells was decreased by proteasome inhibitor treatment. We suggest that in B cells, the stability of BCL11A may also be regulated by proteasome-uncoupled caspases, because BCR stimulation of Ramos cells with anti-IgM antibodies is also known to induce apoptosis

(Sakata et al., 1995). Ligation of CD40 was shown to be able to rescue antigen receptor stimulated apoptosis (An and Knox, 1996). Co-culturing BCR-stimulated B cells with anti-CD40 antibodies or with caspase inhibitors will allow additional insights into how the stability of BCL11A is regulated during B cell activation

It will also be instructive to determine whether BCL11A has functional PEST sequences. Potential PEST motifs are scattered throughout the XL sequence, but their contribution will require additional mutagenesis. PESTs are known to be involved in proteasome independent regulation of numerous ubiquitinated proteins (Salghetti et al., 2000).

3.8.3. MULTPLE FUNCTIONS OF THE CONSERVED N-TERMINUS OF BCL11A

Deletion and point mutation analysis enabled us to define two apparently non-overlapping functions within the N-terminus shared among all BCL11A isoforms. The first 12 amino-acids were identified as a repression domain. This was predicted from the analysis of FOG2, a transcriptional repressor, which otherwise shares no domains with BCL11 (Lin et al., 2004).

The paraspeckle is a subnuclear body, previously defined by its clustering around speckles and by 3 RRM proteins (PSP1, PSP2, and Nono/p54/nrb) contained within. Partitioning of these proteins between paraspeckles and nucleoli depends on active pol II transcription (Fox et al., 2002). BCL11A-XL and its interacting partner, BCL6, are the only transcription factors shown to localize within paraspeckles (Liu et al., 2006). Very likely, the physical interaction of XL with nonO/p54nrb (Liu et al., 2006) is responsible for localizing the XL-BCL6 complex. However, the significance of this localization is not clear.

We found that sequences within the N-terminal 80 residues of XL are required for paraspeckle targeting. Disruption of sub-nuclear targeting can often affect transcriptional activity. An example of this is the loss of transcriptional repression by SATB1 when its nuclear matrix targeting is abolished (Seo et al., 2005). Thus, we anticipated that a point mutation (K/N5) that eliminated XL repression might eliminate paraspeckle localization and/or nuclear matrix association. This was not the case. While neither function was perturbed, the observation that these functions can be segregated at the primary sequence level provides a means for defining the relationship between subcellular localization and functions of BCL11A by further mutagenesis.

CHAPTER 4. IDENTIFICATION AND ANALYSIS OF BCL11A TARGET GENES

4.1. Introduction

The *BCL11A* gene was identified from patients diagnosed with aggressive B cell chronic lymphocytic leukemia (CLL) with the following translocation t(2;14)(p13;q32.3) (Satterwhite et al., 2001). Dysregulation of this gene may play a crucial role in tumorigenesis of these cases and in other B cell neoplasias where BCL11A is over-expressed.

BCL11A is also critical to early stages of B cell development (Liu et al., 2003). In BCL11A knock-out mice, few B cell progenitors (B220$^+$IgM$^-$) were found in fetal liver or embryonic bone marrow. RT-PCR analysis of cells from mutant fetal livers showed no expression of the genes involved in early B cell development such as *Ebf1, Pax5, Il7r, CD19, Rag1* and *Vpreb2* (Liu et al., 2003).

Avram and her colleagues showed that BCL11A and BCL11B have DNA-binding activity (Avram et al., 2002). Another group showed that the highly conserved N-terminal 12 amino acids (Figure 6) of the transcription factors such as FOG, Sal, Spalt, and BCL11A, have transcriptional repression activity (Lin et al., 2004). However, no bonafide biological targets were identified.

Even though BCL11A is suggested to be involved in the tumorigenesis of mature B cells and the development of early B cells, its role in these processes is still unknown. To have a better understanding of BCL11A functions, we sought to identify target genes that are deregulated by BCL11A-XL over-expression in pre-B and mature B cell lines

using DNA microarray analyses. We validated deregulation of 14 genes which we considered to be of significant immunologic interest. Among these were *RAG1* and *RAG2*. Further characterization of *RAG* regulation by BCL11A-XL and its consequences on V(D)J recombination are presented in Chapter 5.

4.2. Stable Over-Expression of BCL11A-XL in Human B Cell Lines

Three B cell lines were transduced with pXY-puro (mock control) or with the same virus containing an N-terminally, Flag-tagged, full-length BCL11A cDNA. The acute lymphoblastic leukemia pre-B cell lines (Nalm6 and SMS-SB) and the Burkitt's lymphoma mature B cell line (Raji) were infected as described in Chapter 2. Over-expression levels of BCL11A-XL were determined by western blotting. We used an anti-Flag mAb to detect exogenous XL and an anti-BCL11A-XL C-terminal polyclonal antibody (BL1797) to detect total BCL11A-XL protein levels.

As shown in Figure 14, each of these B cell lines over-expressed BCL11A-XL after an initial selection with puromycin for 5-6 days. Over-expression was also confirmed by RT-PCR (data not shown). However, prolonged cell culture of transduced Nalm6 and Raji, which express low levels of endogenous XL, resulted in reduction of total BCL11A-XL expression, even under stringent puromycin selection (data not shown). On the other hand, SMS-SB cells, which have no endogenous BCL11A-XL expression, were quite stable with regard to exogenous BCL11A-XL expression.

Therefore, to collect mRNA for microarray analysis, Nalm6 and Raji cells were harvested 1 week after transduction and SMS-SB cells were harvested 4 weeks after transduction (when total BCL11A-XL over-expression was at its peak).

Figure 14. Stable B cell lines over-expressing BCL11A-XL.

pXY-IRES-puro Nalm6 and SMS-SB (SB), pre-B cell lines, and Raji, a mature B cell line, were transduced with empty retrovirus (pXY-IRES-puro; abbreviated as V) or with a full length, Flag-tagged BCL11A-XL-containing retrovirus (pXY-BCL11A-IRES-puro; abbreviated as XL). Transduced cells were selected in the presence of puromycin (Sigma) at 0.5-1 ug/ml concentration for 5 days. Cells were further cultured for the indicated periods (1 week for Nalm6 and Raji cells and 4 weeks for SMS-SB cells), and cell lysates were prepared for western blotting. Anti-Flag mAb (Sigma) identifies exogenous Flag-BCL11A-XL and polyclonal anti-BCL11A (XLc pAb, Bethyl) identifies both endogenous and exogenous BCL11A-XL expression. ß-tubulin was used as a loading control.

	Nalm6 1wk		SB 4 wks		Raji 1wk		
Cell	V	XL	V	XL	V	XL	
							Flag-XL
							Total XL
							ßTub
	1	2	3	4	5	6	
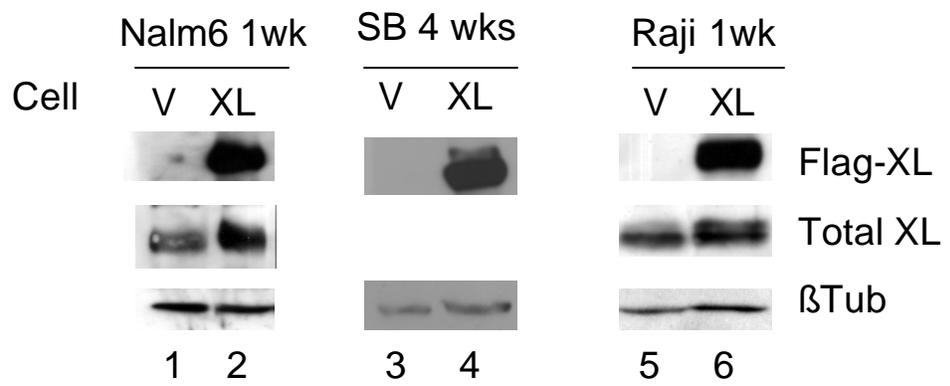
4.3. Putative BCL11A Target Genes Obtained from DNA Microarray Analysis

RNA samples were isolated from these transduced cells and converted into cDNA for DNA microarray analysis. These studies, performed in collaboration with Dr. Iyer's lab (Section 2.11 and http://microarray.icmb.utexas.edu/), utilized chips in which the total human genome (about 50,000 genes) is represented as full length or partial length cDNAs. Based on microarray duplicates of each B cell line, about 1100 gene-spots turned out to be valid array spots after normalization. The SMS-SB microarays showed up-regulation of about 40% of the ~1100 genes and down-regulation of the other 60%. Microarray analyses of Nalm6 and Raji showed up-regulation of ~60% of genes and down-regulation of ~40% of genes. Those genes that showed at least a 1.8-fold difference in expression levels between BCL11A-XL transduced cells (XL) and pXY-puro transduced cells and were considered most relevant to B cell biology are shown in Table 1. RT-PCR results performed on the same mRNA were generally consistent with the microarray data (Figure 15 and data not shown).

A number of membrane-bound molecules were deregulated by BCL11A-XL over-expression. CD9, CD53, and CD82 belong to the tetraspanin family that is involved in cell aggregation, migration, signal transduction and cell survival (Lagaudriere-Gesbert et al., 1997a; Lagaudriere-Gesbert et al., 1997b; Ono et al., 1999). BCL11A-XL activated *CD9* in Nalm6. *CD53* and *CD82* were induced by BCL11A-XL in Raji cells. CD38, a 45-kDa transmembrane glycoprotein, was down-regulated in Nalm6, SMS-SB, and Raji cells by BCL11A-XL over-expression. Stimulation of CD38 was shown to induce cell proliferation in mature B cells and apoptosis in precursor B cells (Silvennoinen, 1996). *CD99* known to be involved in T cell death, (Pettersen et al., 2001), was up-regulated by

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BCL11A-XL over-expression in Nalm6 and Raji cells. B7 (CD80), a co-stimulatory factor, which induces T cell proliferation and the secretion of interleukin 2 (Gimmi et al., 1991) was down-regulated in Raji cells by BCL11A-XL. *LTA* and *TNF*, which are shown to be involved in inflammation, autoimmunity, and the development of follicular dendritic cells and peripheral lymphoid tissues such as spleen, Peyer's patch, and peripheral lymph nodes (Kuprash et al., 2002), were up-regulated by BCL11A-XL in Raji cells.

Among the list of transcription factors potentially deregulated by BCL11A-XL over-expression is *Foxp1*. Foxp1 is required for the transition of pro-B to pre-B cells, potentially by affecting V(D)J recombination through the *Erag* enhancer binding (Hu et al., 2006). *Foxp1* was up-regulated in Nalm6 and SMS-SB cells while it was down-regulated in Raji cells by BCL11A-XL over-expression. Activating transcription factor 5 (*ATF5*), involved in differentiation and cell survival of neuron cells (Angelastro et al., 2006; Angelastro et al., 2003) was down-regulated only in SMS-SB cells by BCL11A-XL.

As further addressed in Chapter 5, BCL11A-XL appears to regulate several genes involved in V(D)J recombination (Table 1). VpreB and ?5 are the surrogate light chain components of pre-B cell receptor (BCR) (Zhang et al., 2004). *VpreB* and *?5* were down-regulated in SMS-SB cells and up-regulated in Nalm6 cells by BCL11A-XL over-expression. *RAG1* and *TdT* genes were also implicated from previous "Lymphochip" data of Liu (2002). The Lymphochip is an array employing oligonucleotides complementary to 3' UTRs of genes involved in the immune system and preferentially expressed in lymphoid cells (Liu, 2002; Shaffer et al., 2000). *RAG1* was up-regulated in Nalm6 and Raji but was down-regulated in SMS-SB cells.

RAG2 expression did not appear to be altered on Lymphochips, and its expression levels were too low to be assessed in the cDNA microarrays (data not shown). Given that the implications of *RAG1* and *Foxp1* findings and that *RAG1* and *RAG2* genes are coordinately regulated by the *Erag* enhancer (Schlissel, 2003) (Figure 16), we checked *RAG2* expression levels by RT-PCR (Figure 15B). As anticipitated, *RAG2* was also activated by BCL11A-XL in Nalm6 cells. However, there was little difference in *TdT* expression between the control and BCL11A-XL transduced cells (data not shown).

To analyze RAG expression levels semi-quantitatively, we carried out RT-PCR with serial dilutions of cDNA samples from Nalm6 cells. Up-regulation of *RAG1* and *RAG2* by BCL11A-XL in Nalm6 was more obvious in these serial RT-PCR assays (Figure 15B).

Table 1. A list of putative BCL11A-XL target genes from microarray assays.

Microarray assays were carried out multiple times using cDNA generated from pXY-puro or pXY-BCL11A-XL transduced Nalm6 and SB pre-B cells or from Raji mature B cells. Of the potential 1100 target genes, whose transcription levels were deregulated by BCL11A-XL at least 1.8 fold, 14 (shown) below were selected for validation and/or further analysis. *TdT* and *RAG1* were initially identified in a lymphochip screen (Liu, 2002).

Gene	Basic Functions
CD9	Differentiation, aggregation and cancer
CD38	Calcium signaling, apoptosis and ADP-ribosyl cyclase
CD53	Sinal transcuction and cell growth
CD82	TCR/CD3 pathway and tumor suppressor
CD99	T cell adhesion and cell death
LTB	Lymphotoxin beta (TNF alpha), inflammation, cell death
TNF	Tumor necrosis factor, inflammation, cell death, B cell development
Foxp1	Transcriptional repressor and cell development, B cell development
ATF5	Transcription factor and brain development
B7	Co-stimulatory factor for T cell activation
VpreB	Immunoglobulin gene rearrangement and B cell differentiation
?5	Immunoglobulin gene rearrangement and B cell differentiation
TdT	Terminal deoxynucleotidyltransferase
RAG1	DNA recombination and cell differentiation

Figure 15. Validation of selected targets identified in microarrays (table 1) by semi-
    quantitative RT-PCR.

A. Total RNA was isolated from Nalm6, SMS-SB, and Raji B cells transduced with pXY-puro (V) or pXY-BCL11A-XL (XL) retroviruses for reverse transcription and RT-PCR analysis. GAPDH was used as internal control. RAG results are highlighted by the box. The primers used for RT-PCR are in Table 2.

B. Template dilution confirms that RT-PCR for *RAG1* and *RAG2* is semi-quantitative. The same Nalm6 cDNA samples from Figure 15A were employed. Lanes 1 and 4, a 1:9 dilution (1/9); lanes 2 and 5, a 1:3 dilution (1/3); and lanes 3 and 6, undiluted samples (1). GAPDH was used as a loading control. Up-regulation of *RAG1* and *RAG2* by BCL11A-XL over-expression can be observed most clearly using a 1:3 dilution (lanes 2 and 5).

A

	10 days	4 wks	10 days	
	Nalm6	SMS-SB	Raji	
Cell	V XL	V XL	V XL	

CD9

CD38

CD53

CD82

CD99

FOXP1

LTA

TNF

ATF5

B7

VpreB

?5

RAG1

GAPDH

1   2   3   4   5   6

B

Nalm6	V			XL		
Dilution	1/9	1/3	1	1/9	1/3	1

RAG1

RAG2

GAPDH

1　2　3　4　5　6
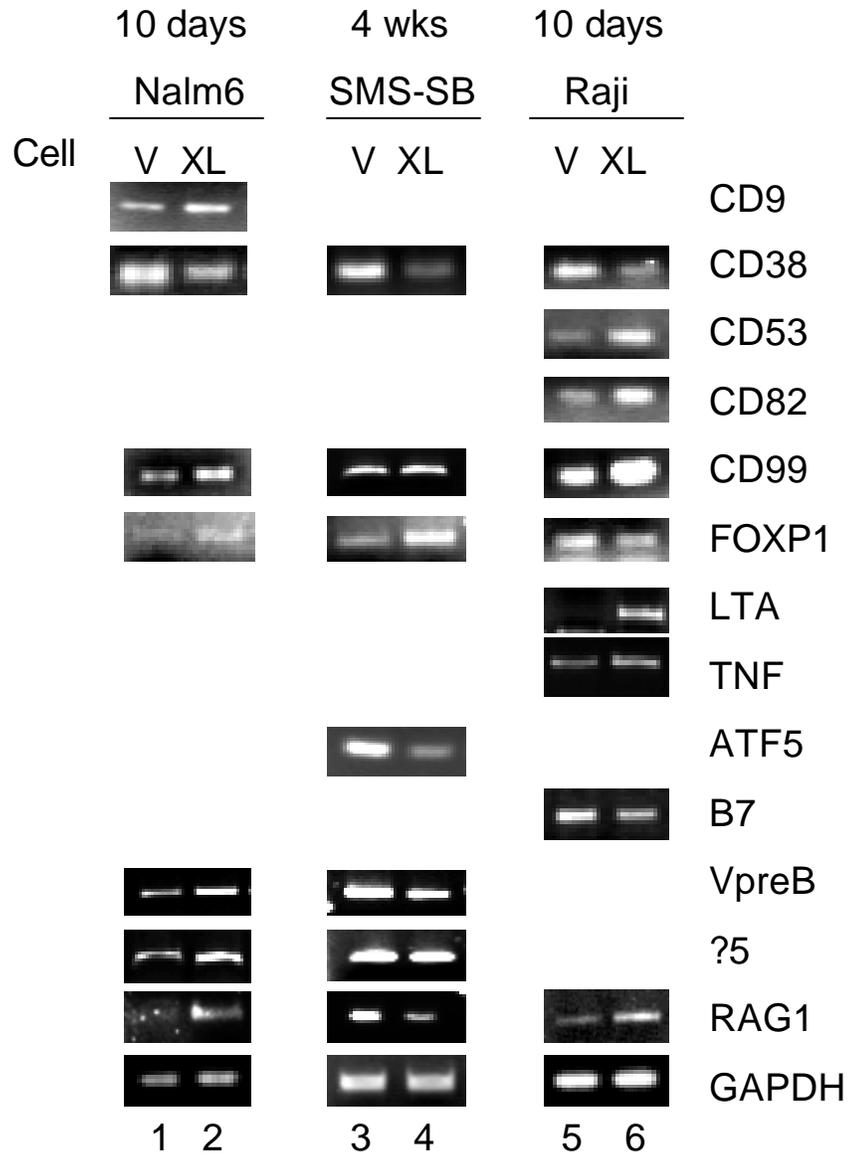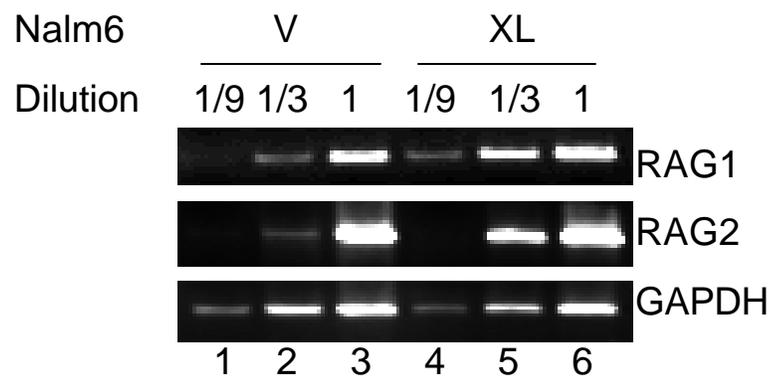
Table 2. Primers used in RT-PCR experiments of Figure 15.

Gene	Oligonucleotide Sequences (5' to 3')
CD9-F	TGCATCTGTATCCAGCGCCA
CD9-R	CTCAGGGATGTAAGCTGACT
CD38-F	GGAGAAAGGACTGCAGCAAC
CD38-R	CATGTATCACCCAGGCCTCT
CD53-F	CAAGAATATCACGGCATGG
CD53-R	CCACAGAACTACTGCAGATCATAG
CD82-F	CAGGATGCCTGGGACTACGT
CD82-R	GACCTCAGGGCGATTCATGA
CD99-F	GATGGTGGTTTCGATTTATC
CD99-R	CTCTTCCCCTTCTTTCCTGTGGCTGCC
LTA-F	CATGACACCACCTGAACGTC
LTA-R	GACCACCTGGGAGTAGACGA
TNF-F	AGCCCATGTTGTAGCAAACC
TNF-R	GGAAGACCCCTCCCAGATAG
Foxp1-F	GTCGGGCGGCAGCAACCACTTACTAGAGTG
Foxp1-R	GAAGAGCTGGTTGTTTGTCATTCCTCTTGGGA
ATF5-F	ACCGCAAGCAAAAGAAGAGA
ATF5-R	GGCCTTGTAAACCTCGATGA

B7-F	AACTCAGGACACTGAGCTTG
B7-R	TTGCTCTCTCAAATTCCAGG
VpreB-F	TTTGTCTACTGCACAGGTTGTGG
VpreB-R	TGCAGTGGGTTCCATTTCTTCC
Nalmda5-F	ACGCATGTGTTTGGCAGC
Nalmda5-R	GGCGTCAGGCTCAGGTA
RAG1-F	ATCCCAATGCTTCCAAAGAG
RAG1-R	CCATTGAATCTTGGCTTTCC
RAG2-F	CACTCTAGGGATTCAAAGATC
RAG2-R	GATGTGTAGCTTTGGAAATCT
GAPDH-F	CATGTTCGTCATGGGTGTGAACCA
GAPDH-R	GTTGCTGTAGCCAAATTCGTTGTC

4.4. Discussion

Using cDNA microarrays and RT-PCR, we identified a number of putative target genes of BCL11A-XL. Unlike the model promoter studies in which BCL11A showed transcriptional repression activity (Avram et al., 2000; Liu et al., 2006), transcriptional up-regulation was noted for several genes (*CD9, CD53, CD82, CD89, LTA*, and *TNF*). Others were down-regulated (*ATF5* and *B7*) by BCL11A-XL over-expression. Another group including *Foxp1, VpreB, ?5, RAG1*, and *RAG2* showed both up-regulated and down-regulated depending on cell lines. Speculations on how BCL11A-XL regulation might correlate the functions of the proteins encoded by some of these genes follows.

4.4.1. REGULATION OF TETRASPANIN PROTEINS BY BCL11A

We observed that tetraspanin family proteins, CD9, CD53, and CD82 were up-regulated by BCL11A. Tetraspanin proteins interact with each other and their associated proteins laterally within membrane microdomains (referred to as "tetraspanin-enriched microdomains") which are different from lipid rafts (Levy and Shoham, 2005). CD9, CD53, and CD82 were shown to link integrins to protein kinase C (PKC). It has been suggested that this tetraspanin-integrin-PKC linkage may be involved in cell migration (Levy and Shoham, 2005; Zhang et al., 2001). CD82 was shown to associate with HLA class I molecules (Lagaudriere-Gesbert et al., 1997b).

There are numerous examples of how cell migration and cell adhesion participate in normal and neoplastic B cell growth and differentiation (Hemler, 2003; Levy and Shoham, 2005). However, beyond the PKC linkage, nothing is known about tetraspanin-

mediated signal transduction in lymphocytes. We noticed that the adhesion properties of several of the BCL11A-XL transduced cell lines were altered (data not shown). Transduced cells clumped together in larger aggregates. We have initiated a collaboration with Dr. Wolfgang Fry (UT Austin, Department of Biomedical Engineering) to determine whether XL-induced tetraspanin over-expression affects lymphocyte rolling, a property critical for their homing within both infected and uninfected tissues.

4.4.2. REGULATION OF TUMOR NECROSIS FACTORS BY BCL11A

Cytokines are essential signals in a variety of immunological, inflammatory and infectious diseases (Ware, 2005). Lymphocyte communications with surrounding cells are often mediated by tumor necrosis factor (TNF) and lymphotoxin (LT)-related cytokines. Lymphotoxin-a (LTA) and TNF are members of the TNF superfamily involved in cell proliferation, death, and differentiation. *LTA*-deficient mice showed defects in the development of lymph nodes, Peyer's patches, natural killer cells as well as dendritic cell migration (Ware, 2005). It was shown that LTA was highly expressed in B-CLL and can enhance B cell proliferation in an autocrine manner (Kulmburg et al., 1998).

BCL11A-XL interacts physically with BCL6, a "master regulator" of germinal center formation. TNF family members were also altered by BCL6 overexpression (Shaffer et al., 2000). Perhaps loss BCL11A plays a redundant role in GC and lymph node formation, or BCL11A-BCL6 interaction is required to modulate a subset of TNF family promoters. This would predict that BCL11A deficiency at the mature B cell stage would lead to similar impairment in peripheral lymphoid centers. Conventional BCL11A knockout leads to perinatal lethality and pre-proB cell block in fetal livers, and thus,

precludes the study of the GC-associated events. Recently we have generated conditional knockout mice that will allow us to test this hypothesis.

These data also predict that up-regulation of LTA and TNF by BCL11A-XL may contribute to the development of B-CLL. Future microarray sampling of t(2;14)-bearing neoplasias will be required to establish this correlation.

4.4.3. REGULATION OF CO-STIMULATORY FACTOR, B7, BY BCL11A

Engagement of the T cell receptor complex (TCR) by antigen is not sufficient to stimulate T cell mediated immune response. B cell surface antigen B7 is a co-stimulatory factor that interacts with the T cell restricted CD28 antigen and induces T cell proliferation and IL2 secretion (Ge et al., 2003; Gimmi et al., 1991). The B7 molecule is a member of immunoglobulin superfamily and its expression is restricted to activated B cells and interferon ?-treated monocytes which can act as antigen presenting cells. B-CLL cells are known to lack significant expression of B7 co-stimulatory factor and to be inefficient at stimulating T cells (Van den Hove et al., 1997; Ward and Kaufman, 2007).

Over-expression of BCL11A-XL led to down-regulation of B7. This suggests that BCL11A-deficient B cells may be hyper-stimulatory in their ability to present antigen. The availability of inducible Cre-expressing lines that allow temporal and lineage-specific elimination of *BCL11A* will allow us to test this idea in our conditional mice. Following *in vivo* immunization, antigen-specific *BCL11A*-deficient B cells can be employed in *in vitro* proliferation and cytokine release assays with Th2 T cell lines specific for epitopes within the same antigen. We further speculate that down-regulation of *B7* expression by BCL11A may enhance B-CLL development by increasing T cell unresponsiveness towards B-CLL cells.

4.4.4. REGULATION OF SURROGATE LIGHT CHAIN GENES BY BCL11A

A successful VDJ_H rearrangement in pro-B cells gives rise to large pre-B cells. The large pre-B cells then undergo several cycles of cell division before exiting the cell cycle. The *Ig* light chain rearrangement occurs in the small resting pre-B cells. The large pre-B cells express pre-B cell receptor (pre-BCR) complexes and are composed of a transmembrane Ig heavy chain, Iga, Igß, and a surrogate light chain (SLC) of VpreB and ?5. The heterodimer of Iga and Igß is non-covalently associated with a pre-BCR and transmits pre-BCR signaling through phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) (Monroe, 2006). It was reported that the pre-BCR positive large pre-B cells show down-regulation of *RAG1* and *RAG2* during their rapid cell division, while the non-cycling pre-BCR-negative small pre-B cells up-regulate both *RAGs* and down-regulate the *SLC* genes (Wang et al., 2002).

Our observation that *RAG* and *SLC* genes were each up-regulated by BCL11A-XL in Nalm6 pre-B cells is inconsistent with the negative correlation of pre-B cell receptor and RAG expression during normal pre-B lineage progression. This might be expected if the XL-transduced Nalm6 cells are in transition between the large and small pre-B; ie, the *SLC* genes are not fully inactivated and the *RAG* genes are beginning to be expressed. Or, because Nalm6 pre-B cells are transformed, they differ in this respect from normal pre-B cells. Data presented in Chapter 5 suggest that the latter interpretation is correct. As with small pre-B cells, Nalm6 cells express high levels of TdT, but they are abnormal in that they secrete detectable levels of μ chain (Smith, 1981). *RAG* genes were down-regulated by BCL11A-XL in SMS-SB cell cells which were cultured for 4 weeks before mRNA preparation. We observed that *RAG* genes were also down-regulated in

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long term cultured Nalm6 and Raji cells (data not shown) while they were up-regulated in short term (1 week) cultured cells. This data suggest that long term over-expression of BCL11A-XL might cause expression waves of some of its target genes.

CHAPTER 5. BCL11A-XL ACTIVATES V(D)J REARRANGEMENT BY INDUCING THE EXPRESSION OF RECOMBINATION ACTIVATING GENES, *RAG1* AND *RAG2*

5.1. Introduction

The *RAG1* and *RAG2* genes are located adjacent to each other and convergently transcribed. DNA-binding elements for several transcription factors have been identified in both *RAG* promoters and in the common *Erag* enhancers (Schlissel, 2003) (Figure 16). Factors that bind and transactivate the *RAG1* promoter (E2A, Ikaros, and NF-Y), and the *RAG2* promoter (LEF-1, Pax5 and c-Myb) have been described (Schlissel, 2003). E2A and Foxp1 bind to the *Erag* enhancer and regulate *RAG* expression and recombination in B cells (Hsu et al., 2003; Hu et al., 2006). The proximal enhancer (*Ep*) and distal enhancer (*Ed*) were identified by mapping DNase I hypersensitive sites(Wei et al., 2005). Ikaros and C/EBP are known as binding factors for the *Ep* enhancer, but no binding factors have been identified for the *Ed* enhancer to date (Wei et al., 2005). Specific interactions between regulatory elements and transacting factors provide lineage specific regulation of RAG expression to B and T cells (Schlissel, 2003).

5.2. Confirmation of Targets by Inducible, shRNA Knock-Down of BCL11A

Up-regulation of *RAG1 and RAG2* was observed following BCL11A-XL over-expression in B cell lines (Figures 14 and 15C). To confirm this, we designed several small hairpin RNAs (shRNA) predicted to target XL specifically or all BCL11A

isoforms. Both standard transfection and unregulated retroviral transductions were unsuccessful (data not shown). Thus, we employed a doxycycline-inducible retroviral vector encoding exon 2 shRNA (common to all BCL11A isoforms) with a Burkitt's lymphoma (mature) B cell line, BJAB, containing an integrated bacterial tetracycline repressor (Ngo et al., 2006).

After establishing a stable shRNA expressing line, we performed a number of experiments to best optimize time and concentration of drug delivery (data not shown). Induction of shRNA expression with doxycyline for 24-48 hr resulted in a robust and specific (as compared to GAPDH and ß-tubulin) knock-down of BCL11A expression as assessed by RT-PCR (Figure 17A) and western blot (Figure 17B) assays. Under these conditions *RAG1* and *RAG2* levels were significantly reduced (Figure 17C).

Figure 16. Schematic of the *RAG* genomic locus and transcriptional factors shown to
bind to promoters and enhancers.

       Arrows denotes the divergent transcriptional initiation sites for RAG1 and RAG2.

The figure is drawn approximately to scale, with the common Erag enhancer located ~22

kb 5' to RAG2 (Schlissel, 2003).

RAG-1   RAG-2 Ep Ed   Erag
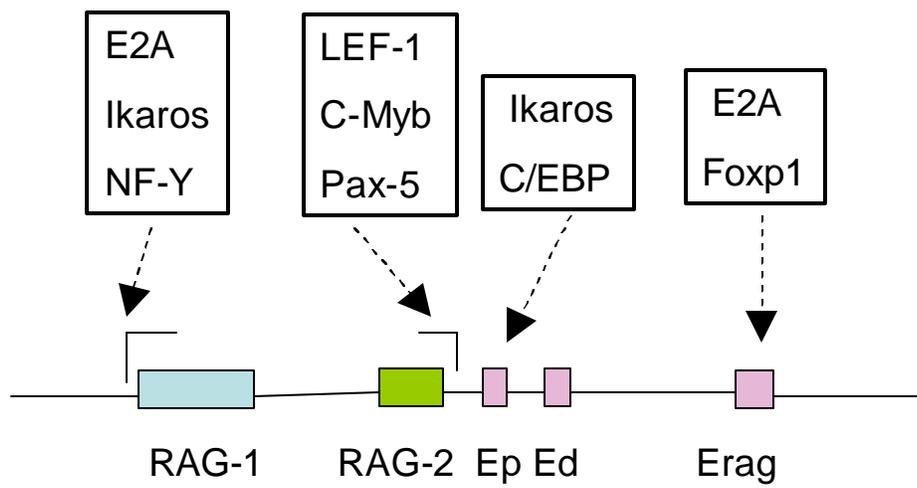
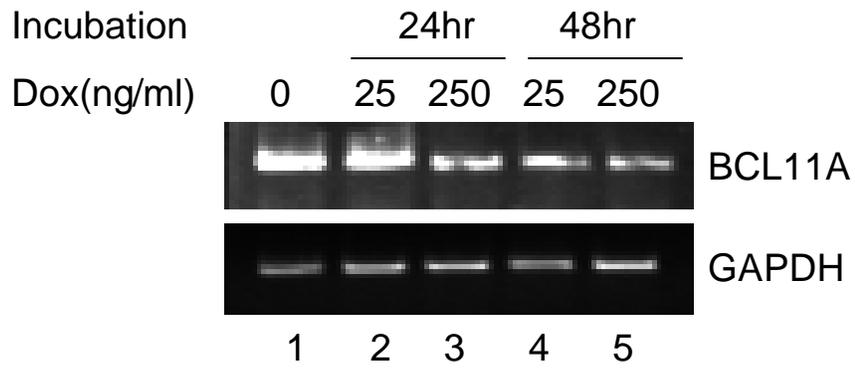Modified from Nature Reviews Immunology 2003. 3:890

Figure 17. Silencing of BCL11A by inducible shRNA knock-down leads to down-regulation of *RAG1* and *RAG2*.

A. Doxycycline (Dox) inducible, stable expression of a small hairpin RNA (shRNA) down-regulated *BCL11A* expression (lanes 3-5) but did not affect GAPDH transcript levels. The vector, pRSMX-GFP, and Tet-repressor-transduced BJAB cells (BJAB-TetR) were described by Ngo et al. (Ngo et al., 2006). The shRNA sequences correspond to exon 2 of all BCL11A and the primers used are described in Materials and Methods. The virus was packaged, transduced as described in previous legends, and infected cells were selected in the presence of 1ug/ml of puromycin. To induce knock-down of BCL11A, doxycycline (Dox) was added in the culture at the indicated concentrations and time periods. The knock-down was confirmed by RT-PCR for the XL isoform of BCL11A.

B. Anti-BCL11A Western blotting confirms *BCL11A* knock-down by Dox-inducible shRNA. BCL11A-XL protein levels were specifically decreased (lanes 2-4) while ß-tubulin protein levels were relatively constant.

C. RAG1 and RAG2 transcript levels are significantly reduced by inducible knockdown of BCL11A-XL. RAG transcript levels directly correlated with BCL11A levels, while GAPDH was not affected by the induction of BCL11A-shRNA.

A

Incubation   24hr   48hr
Dox(ng/ml)   0   25   250   25   250

BCL11A

GAPDH

                1   2   3   4   5

B

Incubation   0hr   48hr   72hr   96hr
Dox(200ng/ml)   -   +   +   +

BCL11A-XL

ß-tubulin

                1   2   3   4

C

                        24hr
Dox(200ng/ml)   -   +

RAG1

RAG2

BCL11A

GAPDH

                     1   2
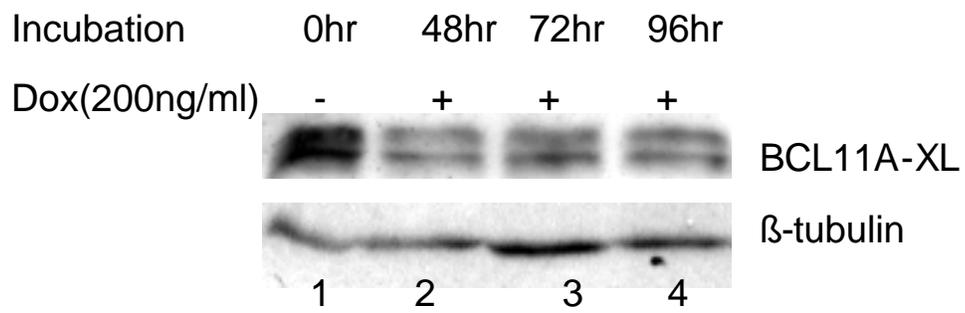
5.3. BCL11A-XL is Recruited to the *RAG1* Promoter and the *Erag* Enhancer *in vivo*

Chromatin immunoprecipitation (ChIP) is a very useful approach to identify and quantitate interactions between proteins and specific regions of DNA. ChIP can be used to detect recruitment of transcription factors to regulatory elements such as promoters and enhancers. We used this tool to test whether BCL11A-XL binds to *RAG* promoters or the *Erag* enhancer *in vivo*. Prior to performing ChIP-PCR assays, the usefulness of the available BCL11A antibodies for im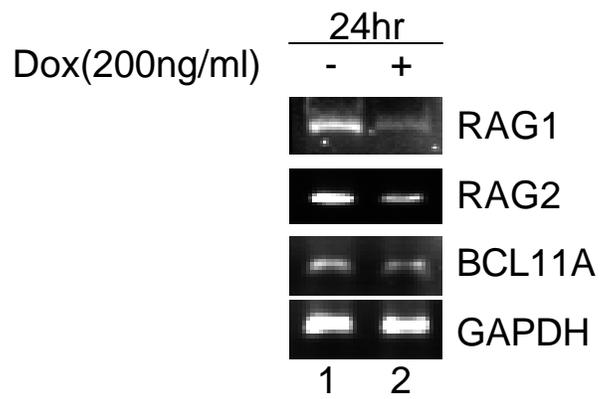munoprecipitation of cross-linked chromatin-protein complexes was established. The polyclonal BCL11A antibody (BL1797), but not the monoclonal antibody (mAb123), could be optimized (Figure 17A).

Specific sets of primers were designed to span the upstream 1kb region of each promoter and for *Erag* (Hsu et al., 2003). ChIP-PCR demonstrated that *RAG1* promoter and *Erag* sequences, but not *RAG2* promoter sequences, were enriched in pull-downs with anti-BCL11A but not with control antibody (Figure 17B). This indicated that BCL11A-XL is recruited either directly or indirectly to the proximal promoter of *RAG1* and to the *RAG* enhancer *in vivo*.

Figure 18. BCL11A is recruited to the *RAG1* promoter and to the *Erag* enhancer in *vivo*.

A. Chromatin immunoprecipitation (ChIP) optimization of anti-BCL11A antibodies. Nalm6 pre-B cells were cross-linked with 1% formaldehyde and were lysed in SDS-lysis buffer. Lysates were immunoprecipitated using a control monoclonal antibody, a BCL11A monoclonal antibody (mAB123), a control polyclonal antibody, or a BCL11A polyclonal antibody. Samples were resolved on SDS-PAGE gels and western blots were carried out with the BCL11A polyclonal antibody. The BCL11A polyclonal antibody was successful and was used in ChIP-PCR experiments below. Immunoglobulin heavy chains (Hc) of the precipitating antibody were also detected by anti-rabbit secondary antibodies.

B. ChIP-PCR identifies the *RAG1* promoter and *Erag* enhancer as targets for BCL11A-XL binding. Immunoprecipitated chromatin samples above, were reverse cross-linked, purified and analyzed by PCR assays using primers that amplify the proximal promoters and the enhancer. Specific PCR products (PCR) were observed in samples targeting the *RAG1* promoter and the *Erag* enhancer (lanes 3 and 9) but not in samples targeting the *RAG2* promoter (lane 6). PCRs from 1% input were used as positive controls (lanes 1, 4, and 7). Purified rabbit IgG antibodies did not immunoprecipitate target DNA significantly (lanes 2, 5, and 8). Detailed procedures are described in Materials and Methods.

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A

1. Input
2. Blank
3. Control mAb
4. BCL11A mAb
5. Control pAb
6. BCL11A pAb

— **BCL11A-XL**

— **IgH cross reaction**

1 2 3 4 5 6

B

RAG1 RAG2 Erag

1% Input | con Ab | XL Ab | 1% Input | con Ab | XL Ab | 1% Input | con Ab | XL Ab

1 2 3   4 5 6   7 8 9

90
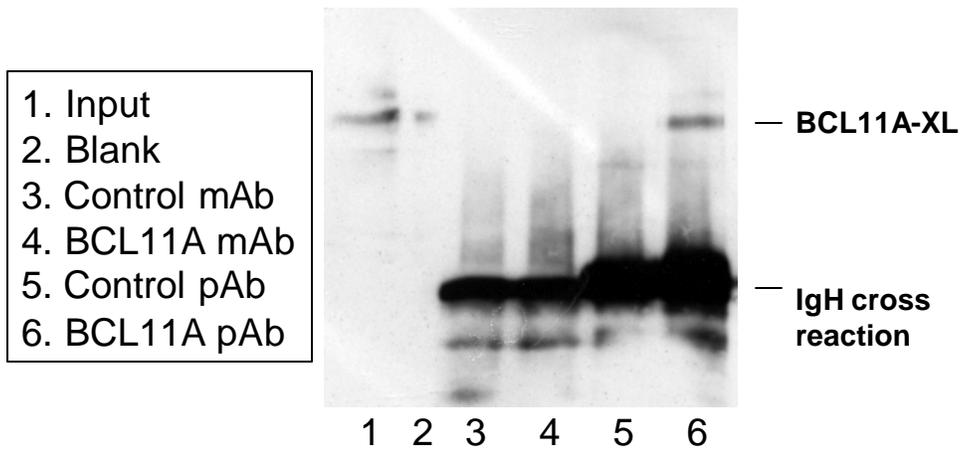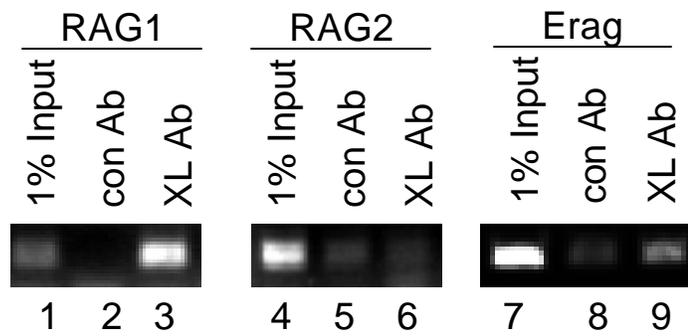
5.4. BCL11A-XL Binds to the *RAG1* Promoter and the *Erag* Enhancer *in vitro*

Electromobility shift assay (EMSA) detects protein:DNA interaction *in vitro*. We performed EMSAs to verify the ChIP results. When P^{32}-labeled DNA fragments from the *RAG1* promoter region were incubated with B cell nuclear extracts, several complexes were observed (Figure 19, A and B). The slowly migrating band (boxed in Figure 19) was specifically abolished by pre- or post-incubation with anti-BCL11A polyclonal or monoclonal antibodies, but not with control antibodies (Figure 19, A and B). A similar sized protein:Erag DNA complex was specifically abolished (Figure 19C). Ablation of complexes as opposed to super-shifted complexes suggests that both anti-BCL11A antibodies interfere with DNA binding, leading to dissociation of BCL11A/DNA complexes. No binding to *RAG2* promoter probes was observed (data not shown).

These results are consistent with previous ChIP-PCR results and indicate that BCL11A-XL binds to the *RAG1* promoter and *Erag*, but not to the *RAG2* promoter, *in vivo* and *in vitro*.

Figure 19. BCL11A-XL binds to the *RAG1* promoter and the *Erag* enhancer *in vitro*.

The *RAG1* promoter and *Erag* enhancer regions were PCR amplified for EMSA probes. Probes were labeled with ^{32}-P and then incubated with nuclear extracts prepared from B cell lines (Nalm6, A; Raji, B) confirmed to express BCL11A-XL. The probe/nuclear extracts mixtures were further incubated with control antibodies (C1; mouse anti-Myc antibody or C2; rabbit IgG antibody) or with BCL11A antibodies (M; mAb123 or P; BCL11A polyclonal, BL1797) and then resolved on native 6% gels. BCL11A/DNA complexes are indicated by arrows and highlighted in boxes. The addition of BCL11A antibodies caused the ablation of the BCL11A/DNA complexes, while control antibodies caused no changes in gel shift complexes.
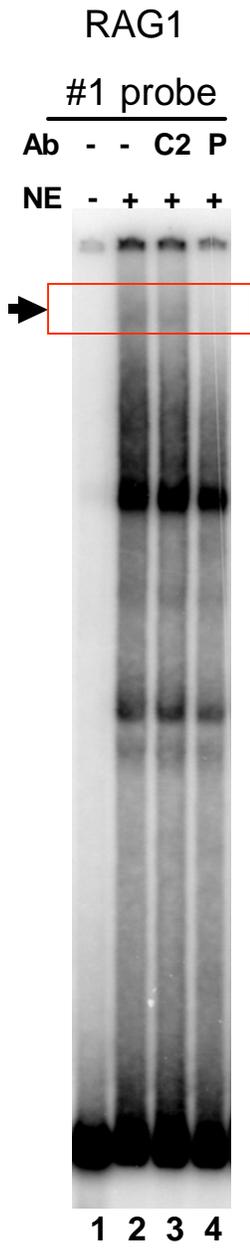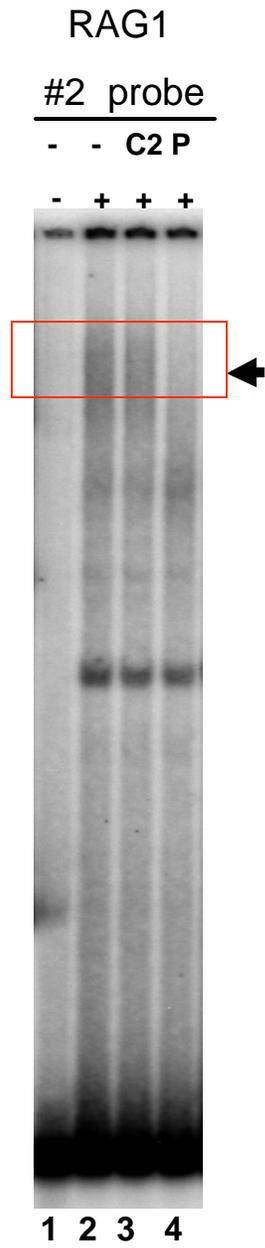
5.5. BCL11A-XL Represses the *RAG1* Promoter in Non-B cells

The expression and knock-down experiments of Figures 15 and 16 indicated that both *RAG1* and *RAG2* are activated by BCL11A-XL over-expression. Thus, it was informative to determine whether BCL11A-XL was sufficient to transactivate *RAG* genes following transient transfection into fibroblast (NIH3T3) or epithelial (293T) cell lines. BCL11A-XL was co-transfected with previously characterized (Hsu et al., 2003) *RAG* promoter-driven luciferase constructs (R1P-Luc and R2P-Luc) or with *RAG* pr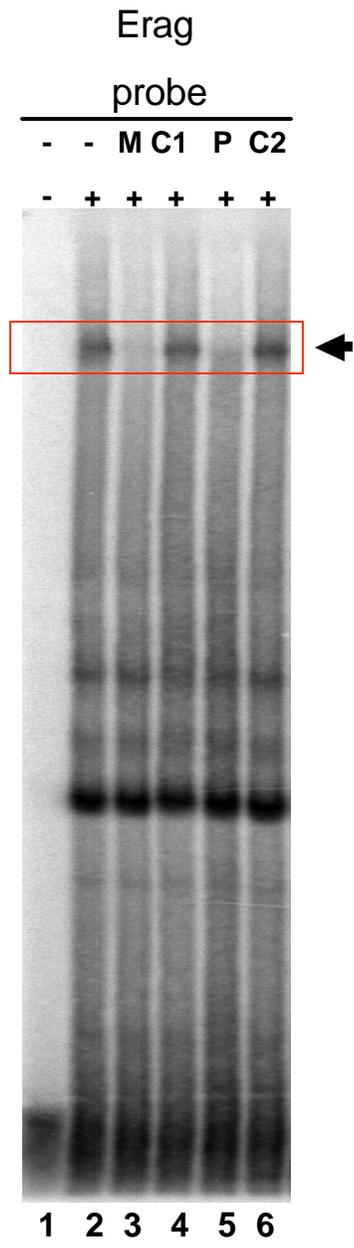omoter + *Erag*-driven constructs (EragR1P-Luc and EragR2P-Luc). Relative luciferase activities were set at 100% with samples from empty CMV10 vector-transfected cells

Unexpected from the B cell expression data, transfection of increasing amounts of BCL11A-XL in 293T cells significantly repressed the *RAG1 (*R1P-Luciferase) promoter (Figure 20A). Consistent with the EMSA and ChIP data, no effect was observed on the *RAG2* (R1P-Luc) promoter (Figure 20B). Even though interaction between BCL11A-XL and the *Erag* enhancer was observed by gel shift and ChIP-PCR assays, EragR2P-Luc activities were not affected and EragR1P-Luc activities were similar to those of R1P-Luc by BCL11A-XL transient co-transfection of 293T cells (data not shown). Identical results were observed for all these constructs in NIH3T3 fibroblasts (data not shown).

BCL11A and its paralogue, BCL11B, were shown to interact with the nucleosome remodeling and histone deacetylase (NuRD) complex (Cismasiu et al., 2005). One of the NuRD components, metastasis tumor antigen (MTA) 1 was shown to enhance BCL11B repression activity synergistically, while MTA2 did not (Cismasiu et al., 2005). Thus, we tested whether there is synergism between BCL11A-XL and MTA proteins on *RAG1*

promoter (R1P-Luc) activity. As shown in Figure 20C, significant augmentation of BCL11A-XL repression activity was observed with MTA1, but not with MTA2, co-transfection. Neither of the other self-associating BCL11A isoforms (BCL11A-L or BCL11A-S) nor other previously characterized BCL11A interacting proteins (BCL6, HIC1 or Foxp1) had significant effect on *RAG1* promoter repression when they were co-transfected with BCL11A-XL (data not shown).

We conclude that BCL11A-XL in non-B cells, under the transient transfection conditions employed, represses transcription of the same target gene which it activates in B cell lines. One interpretation of this result is that stable integration of the target gene is required for activation by the nuclear matrix-associated XL isoform. Alternatively, BCL11A may require, in addition to MTA1, other B cell-specific factors to activate *RAG1*.

Figure 20. BCL11A-XL represses RAG1-driven transcription reporter activity in non-B cells.

A. BCL11A-XL represses RAG1-luciferase in 293T epithelial cells. Cells were transiently transfected with 1ug of *RAG1* promoter luciferase (R1P-Luc), 5ng of Renilla luciferase (Renilla–Luc) and the indicated amounts of BCL11A-XL. The total amount of DNA was adjusted with empty CMV10 plasmid DNA (lane 1). Cells were harvested 40-48 hr after transfection and analyzed for luciferase activity. The efficiency of transfection was normalized to Renilla luciferase activity. Luciferase activity was set at 100% in cells transfected with empty vector alone for reading. Error bars indicate standard deviation of 3-5 independent experiments. The bottom panel shows results of western blots carried out to confirm BCL11A-XL expression levels with the increasing amounts of DNA. GAPDH blots were used as loading controls. Increasing expression levels of BCL11A-XL result in increased repression activity on the *RAG1* promoter (lanes 2-4).

B. BCL11A does not repress the *RAG2* promoter. Cells were transiently transfected with R2P-luciferase, Renilla luciferase, and CMV10-BCL11A-XL. Assays were normalized as in (A) and ectopic BCL11A-XL protein levels were confirmed by western blotting (data not shown).

C. Co-expression of MTA1 enhances RAG1 repression by BCL11A. Reduction of RAG1-driven luciferase activity with co-transfected MTA1 (lane 3) was significantly greater than that of BCL11A-XL alone (lane 2). Co-expression of MTA2 did not affect repression activity of BCL11A on the R1P-luciferase reporter.

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A

R1P-Luc

B

R2P-Luc

C
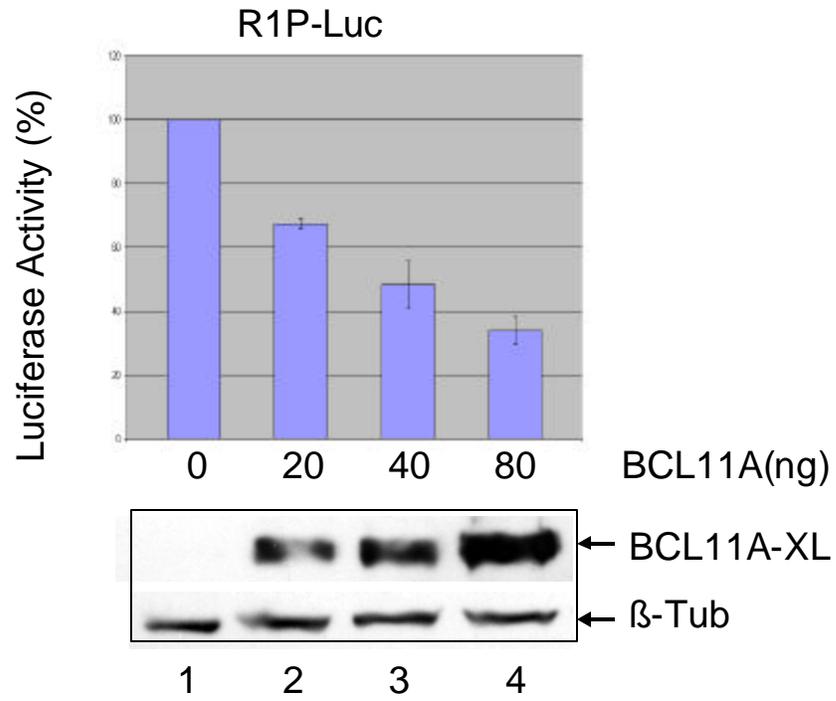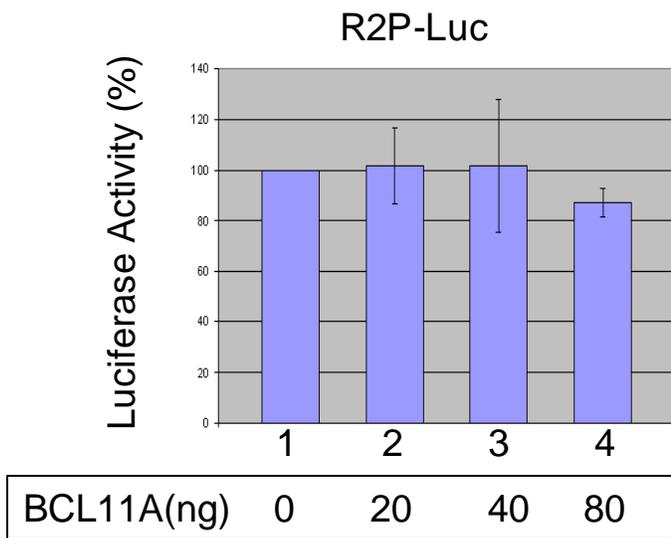
R1P-Luc
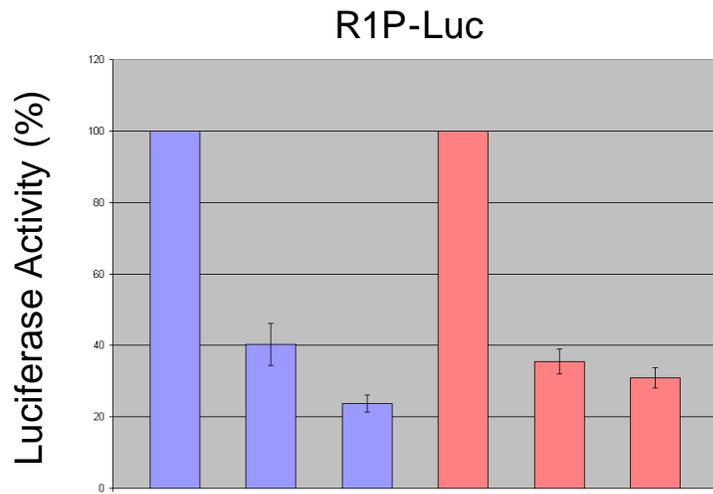
BCL11A	0	50	50	0	50	50	ng
MTA1	0	0	100	0	0	0	ng
MTA2	0	0	0	0	0	100	ng

5.6. BCL11A-XL Transduction into a V(D)J-Competent Pre-B Cell Line, A70-INV

Transformation of murine fetal liver or bone marrow B cell progenitors by the Abelson (Abl) murine leukemia virus arrests these cells at the early (large) pre-B cell stage (low to negligible RAG1 and RAG2 expression). The A70-INV Abl-transformed B cell line retains these properties, but it has been engineered for inducible V(D)J recombination analysis by integration of a recombination substrate, pMX-INV, which on inversion activates expression of GFP (Muljo and Schlissel, 2003). Induction of recombination is achieved by treatment of A70-INV cells by the Abl kinase inhibitor, STI571 (Muljo and Schlissel, 2003). It has been suggested that STI571 causes differentiation of early pre-B cells to a late pre-B cell-like state through regulation of *BLNK, Syk*, and other genes (Muljo and Schlissel, 2003). *RAG1* and *RAG2* expression is induced, followed by V-J recombination of the ? light chain locus, and accordingly in A70-INV cells, inversion of the integrated pMX-INV (Bredemeyer et al., 2006).

We transduced A70-INV cells with empty virus (pXY-puro) or with pXY-BCL11A-XL. Puromycin-selected bulk (uncloned) cells were screened by RT-PCR (Figure 25) and western blot (Figure 22) assays to assess BCL11A-XL over-expression. As shown in Figure 21, BCL11A protein is barely detectable in untransduced or mock-transduced A70-INV cells, whereas transduced cells express significant levels. RT-PCR analysis shows that BCL11A-XL over-expression induces *RAG1* and *RAG2* transcription in the A70-INV cells (Figure 25).

Figure 21. Strategy for the detection of the RSS-mediated inversion of an ectopic V(D)J recombination substrate and for the detection of endogenous V?-J? rearrangement.

A. pMX-INV encodes anti-sense-oriented green fluorescence protein (GFP) cDNA flanked by V(D)J recombination signal sequences (*RSS*). The Abelson (Abl) kinase-transformed pre-B cell line, A70, was transduced with the pMX-INV retrovirus to produce A70-INV (Bredemeyer et al., 2006). The Abl kinase inhibitor, STI571, can induce rapid *RAG* gene expression and the recombination of the recombination target genes. Human *CD4 (hCD4)* cDNA was placed downstream of internal ribosome entry site (IRES) (upstream of the anti-sense *GFP* cDNA) to allow screening of pMX-INV positive cells by anti-hCD4 FACS. After RSS-mediated recombination, *GFP* will be inverted to sense orientation, allowing GFP expression. Arrows indicate primers used to amplify rearranged pMX-INV substrates. Without rearrangement, both primers will be in the same orientation so that no PCR products are generated. Only after successful rearrangement of pMX-INV will PCR products be detected.

B. Schematic of PCR amplification to detect V?-J? (Ramsden, 1994). Only after successful rearrangement, will the V? and J? segments get close enough to allow PCR amplification under typical reaction conditions. Primer locations are indicated by arrows. The Vcon (forward) and J?2-1 (reverse) primers can detect VJ?1 and VJ?2 rearrangements and the J?2-2 primer can be used to make a probe for Southern blot assays later. The Vcon (forward) and J?5 (reverse) primers will be used to detect VJ?4 and VJ?5 rearrangements.

100

A

pMX-INV rearrangement

Rearrangement
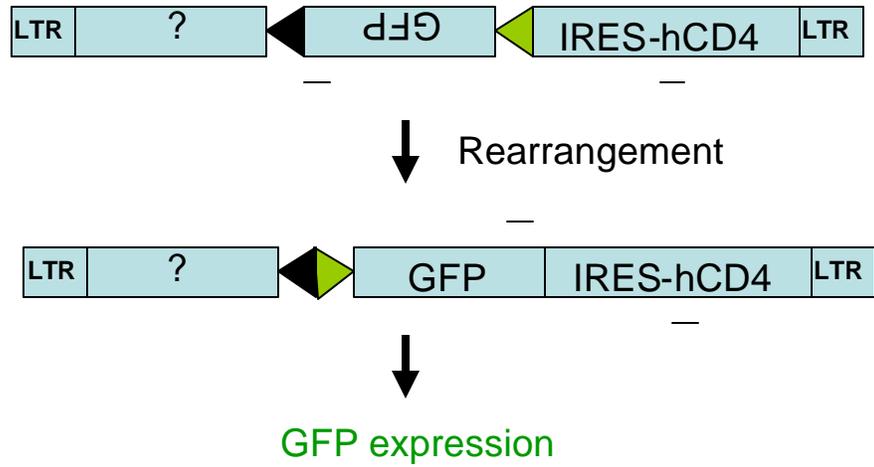
GFP expression

Modified from Nature.2006. 442:466

B

VJk Rearrangement

Vcon

Jk2-1

Jk2-2

Jk5-1
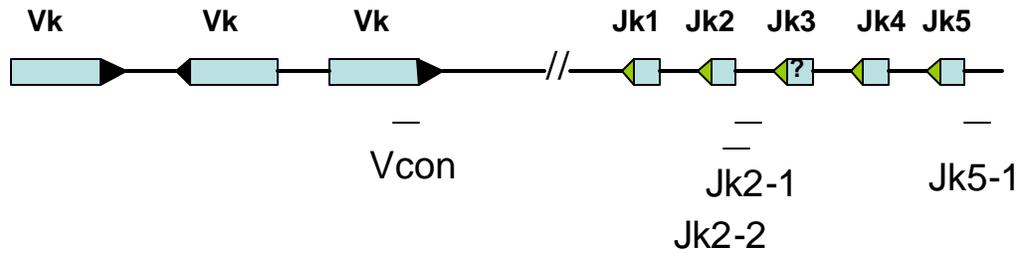
Modified from The Journal of Immunology 1994. 153:1150

Figure 22. Stable over-expression of BCL11A-XL in A70-INV pre-B cells.

A70-INV cells were transduced with pXY-IRES-puro (V) or pXY- BCL11A-XL (XL) and selected with 1ug/ml of puromycin for 6 days. Western blot assays show over-expression of Flag-BCL11A-XL in A70-INV-BCL11A-XL cells (lane 3) as compared to in the parental A70-INV (lane 1) and A70-INV-pXY-puro (mock) transduced cells (lane 2). Anti-Flag immunoblotting detected ectopic BCL11A-XL and anti-BCL11A immunoblotting detected total expression of BCL11A-XL. ß-tubulin bands were used as loading controls.
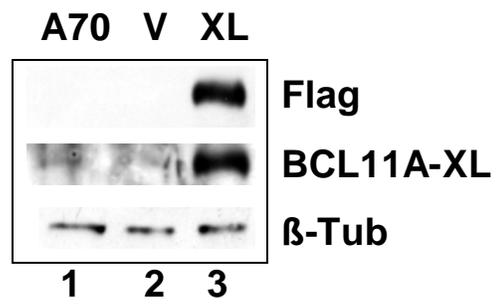
5.7. BCL11A-XL Activates an Integrated V(D)J Recombination Substrate, pMX-INV, Independently from the Abelson Kinase Inhibitor, STI571

The strategy used to detect the inversion of pMX-INV and V-J recombination of the endogenous ? light chain locus is described in Figure 21 (Bredemeyer et al., 2006). Mock viral transduction produced 0.7% and 18.1% GFP-positive cells with or without STI571 treatment, respectively (Figures 22, A and B). This level of "leaky" GFP expression is similar to previously published data (Bredemeyer et al., 2006). BCL11A-XL transduced cells produced 7% and 34.5% GFP-positive cells with or without STI571 treatment, respectively (Figures 22, C and D).

This ~10-fold increase in recombination substrate inversion by BCL11A-XL in the absence of STI571 treatment suggests that BCL11A acts independently. Consistent with this interpretation, the level of substrate inversion by XL in the presence of STI571 was consistently additive. In addition, unlike the G1/S arrest induced by STI571 treatment, XL transduction was accompanied by no alteration in cell cycling (as measured by DNA content of propidium iodide stained cultures by FACS; data not shown).

Figure 23. Flow cytometry detection of enhanced V(D)J recombination substrate
inversion in A70-INV pre-B cells over-expressing BCL11A-XL or treated with
the Abelson kinase inhibitor, STI571.

Cells were transduced as described in previous legends and then were incubated in the absence (STI -) or in the presence (STI +) of STI571 at 3uM for 72 hours. Inversion of pMX-INV resulted in GFP expression, which could be assessed by FACS. The x axis indicates GFP green fluorescence intensity, and the y axis indicates cell numbers. Percentages of GFP-positive cells are given in each plot.

A Mock   STI -
   200   1%

B Mock   STI +
   100   18%

C BCL11A   STI -
   100   7%

D BCL11A   STI +
   100   34.5%

106
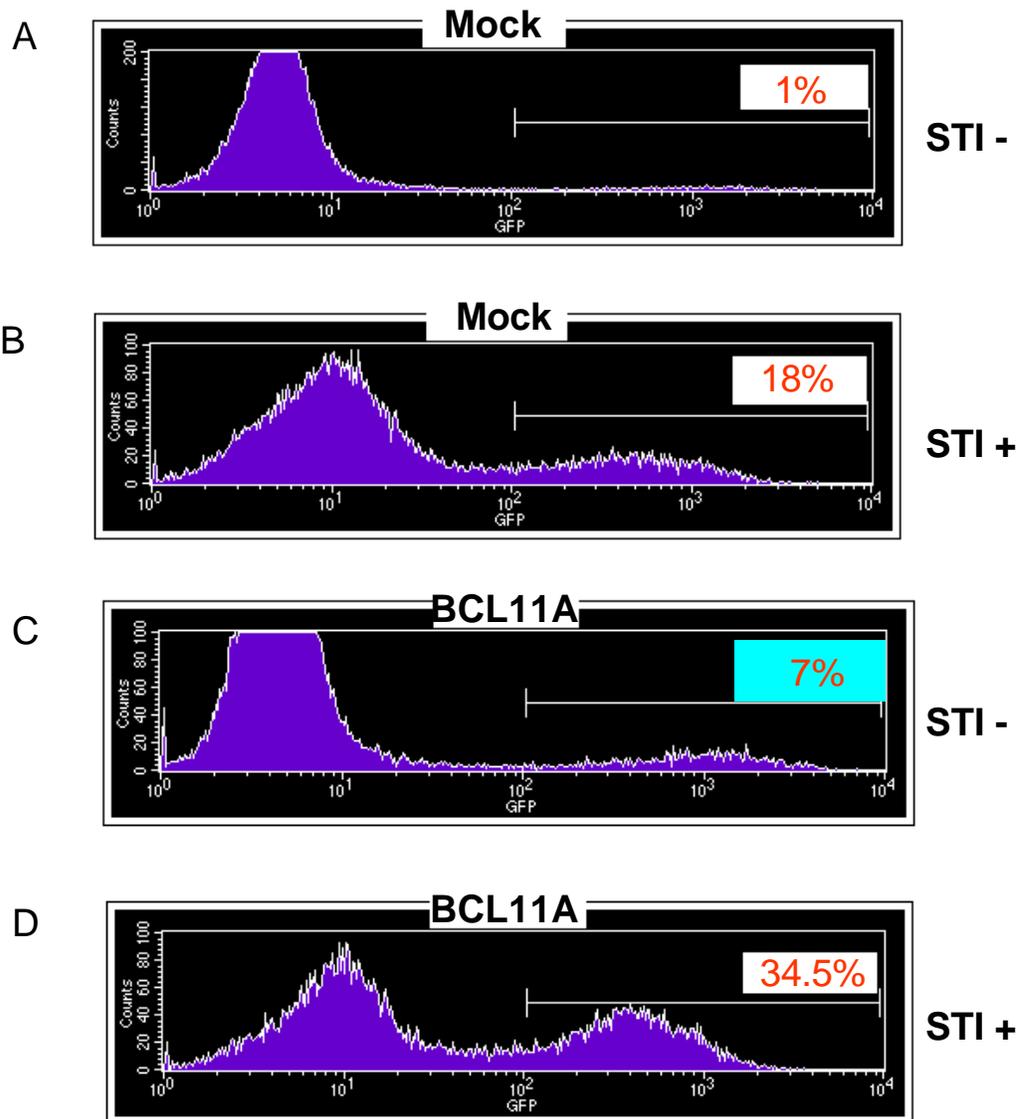
5.8. Confirmation of pMX-INV Rearrangement by Genomic PCR

To verify that GFP expression resulted from inversion of pMX-INV, we carried out PCR assays on genomic DNA using primers complementary to hCD4 (a gene encoding the CD4 surface marker, which can be used for selection) and inverted GFP. pMX-INV inversion allows successful PCR product formation by providing the reverse primer with the correct orientation of GFP cDNA (Figure 21).

As shown in Figure 24A, a significant increase in the intensities of inverted GFP-hCD4 PCR products was observed in STI571-treated or BCL11A-XL-transduced cells as compared to STI571-untreated or mock virus transduced cells, respectively (Figure 24A). These results confirm that the induction of GFP represents authentic RSS-mediated V(D)J inversion of the integrated recombination substrate.

5.9. Induction of Endogenous VJ? Rearrangement in BCL11A-XL Transduced Pre-B Cells

Although integrated as a single copy, the pMX-INV reporter locus may not display the equivalent heterochromatin structure as the endogenous light chain loci do in pre-B cells. To assess whether the endogenous locus can also be induced to rearrange by BCL11A-XL over-expression, genomic DNA was analyzed by PCR using a "universal" V? forward primer and J?2-1 and J?5-1 reverse primers (indicated in Figure 21). Efficient PCR can occur only if VJ? rearrangement deletes the long intervening sequence between V? and J? elements. Details of this strategy were described previously (Ramsden, 1994). Assessment of VJ?2 rearrangement required Southern blotting of the

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PCR products because there were significant amounts of non-specific PCR products (Figure 24B).

As shown in Figure 24B, increased levels of J? rearrangement (lanes 2, 4, 6, and 8) were observed in both STI571-treated and BCL11A-XL-transduced cells as compared to control cells (lanes 1, 3, 5, and 7). Since more VJ?4 rearrangement was observed in BCL11A-XL-transduced cells than in STI571 treated cells (lanes 6 and 8), this result suggests that there are differences in mechanism and outcome of VJ? rearrangement when induced by STI571 or by BCL11A-XL.

Figure 24. Genomic confirmation of BCL11A-XL induction of recombination substrate and endogenous *Ig?* light chain rearrangement.

A. PCR amplification of RSS‑mediated inversion of pMX-INV substrates. Genomic DNA was isolated from the indicated cell lines for PCR following the strategy described in Figure 21. STI571 treatment (3µM, 48hr) or transduction with BCL11A-XL retrovirus induced inversion of GFP, allowing PCR amplification (lane 2 and 4). PCR amplification of the endogenous RAG1 gene (which is unaffected by recombination signal sequence‑mediated recombination) used as a DNA loading control. The following primers were used for PCR; pA; 5'-cacaacatcgaggacgg-3', hCD4R; 5'-gcaccactttctttccctga-3'. The primers for RAG1 are described in Chapter 2.10.

B. Detection of endogenous Ig? light chain rearrangement induced by STI571 or BCL11A-XL. A degenerate V? consensus primer (Vcon) and J?2-1 or J?5 reverse primers were used to amplify rearrangements from genomic DNA. When J?2-1 was used as a reverse primer, 190 bp (VJ2) and 540 bp (VJ1) PCR products are expected. Amplified PCR products (arrows) were resolved on agarose gels and then subjected to Southern blot analysis using the J?2-2 probe (left panel). PCRs using Vcon and J?5 primers produced 270 bp (VJ5) and 600 bp (VJ4) rearrangement products (arrows, right panel). The 100 bp DNA ladder is depicted. The following primers were used for PCR and blotting; Vcon forward; 5'-ccgaattcgsttcagtggcagtggrtcwggtac-3', ?2-1 reverse; 5'ggttagacttagtgaacaagagttgagaa-3', ?2-2 probe; 5'-caagagttgagaagactacttacgtttt-3', and ?5 reverse; 5'-tgccacgtcaactgataatgagccctctc-3'. The amplification conditions were 30 cycles of 30 s at 94 °C, 90 s at 60 °C, and 60 s at 72 °C.

A

pMX-INV

STI   -   +   -   -

Vector   -   -   +   -

BCL11A   -   -   -   +

GFP-hCD4

RAG1

1   2   3   4

B

Kappa Light chain

Jk2-2 blot of
Jk2-1 PCR

STI   - + - -

Vector   - - + -

BCL11A   - - - +

Jk5 PCR

        - + - -

        - - + -

        - - - +

600 —
400 —

200 —

100 —

VJ1

VJ2

1 2 3 4

800 —

600 —
400 —
200 —
100 —

VJ4

VJ5

5 6 7 8

110
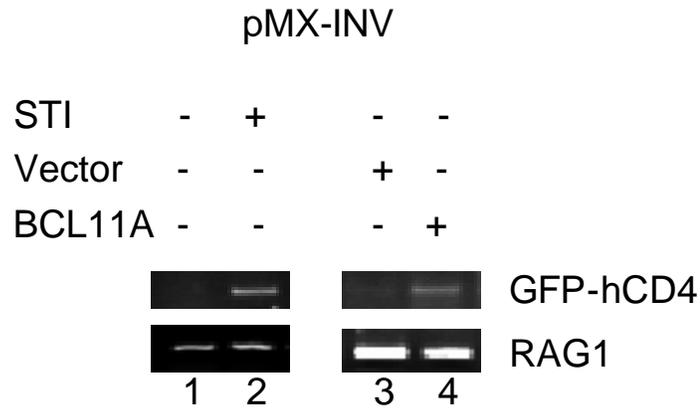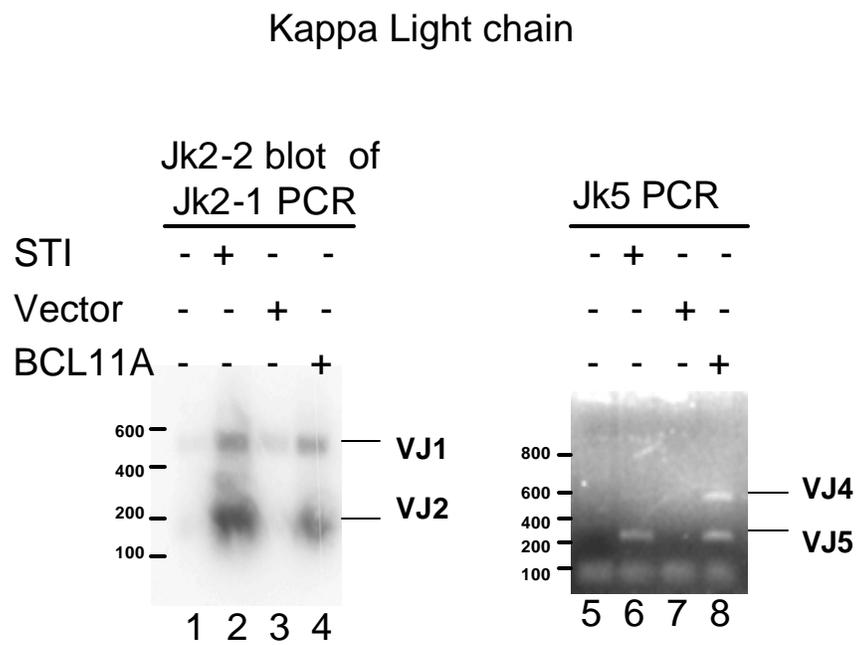
5.10. BCL11A-XL Over-Expression Regulates *RAG1*, *RAG2*, and Additional Genes Implicated in V(D)J Recombination.

The results of the functional experiments reported above could be ascribed exclusively to up-regulation of *RAG* genes, since ectopic introduction of *RAG1* and *RAG2* in non-B cells is sufficient for V(D)J recombination substrate rearrangement (Angelin-Duclos and Calame, 1998; Romanow et al., 2000). As expected, the extremely low levels of *RAG1* and *RAG2* transcripts in A70-INV pre-B cells were significantly induced by STI571 treatment or by BCL11A-XL transduction (Figure 25).

In addition to *RAG*s, several key genes (Schlissel, 2003) known to encode proteins involved in V(D)J recombination were modulated. Both *IRF-4* and *IRF-8* were up-regulated by BCL11A-XL over-expression (Figure 25). Up-regulation of these transcription factors has been reported to be essential for *Ig* light chain gene recombination (Lu et al., 2003; Ma et al., 2006).

STI571 treatment of Abl pre-B cell lines was shown to induce expression of *IRF-4, Spi-B*, and *Ig?* germline transcription (Muljo and Schlissel, 2003). This was observed in STI57I treatment of A70-INV cells (g?, Figure 25). Surprisingly, *Ig?* germline transcription, a consequence of chromatin accessibility of the *J?* locus, was down-regulated in BCL11A-XL-transduced cells (Figure 25).

As we observed in other B cell lines (Figure 15A), the Erag-binding factor and pro-B-pre-B regulator, Foxp1, was induced slightly in A70-INV cells by BCL11A-XL over-expression (Figure 25). However, expression of pre-B cell receptor encoding genes, *?5* and *VpreB*, which were unexpectedly up-regulated in Nalm6 pre-B cells were not modulated in A70-INV cells.

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Figure 25. BCL11A-XL over-expression or STI571 treatment induce *RAG1, RAG2*, and other V(D)J recombination-associated genes.

STI571 treatment at 3uM for 20 hr induced up-regulation of *RAG1, RAG2,* and *Ig?* germline transcripts in A70-INV cells (lane 2) relative to untreated cells (lane 1) Transduction of A70-INV with BCL11A-XL (alone) up-regulated *RAG1, RAG2, IRF-4* and *IRF-8* (lane 4) relative to mock-transduced cells (lane 3). GAPDH was the loading control. The primers used for these RT-PCR amplifications are listed in Table 3.

STI(3uM)	-	+	-	-
Vector	-	-	+	-
BCL11A	-	-	-	+

RAG1
RAG2
IRF-4
IRF-8
gVk
?5
VpreB
Foxp1
BCL11A
GAPDH

1 2   3 4
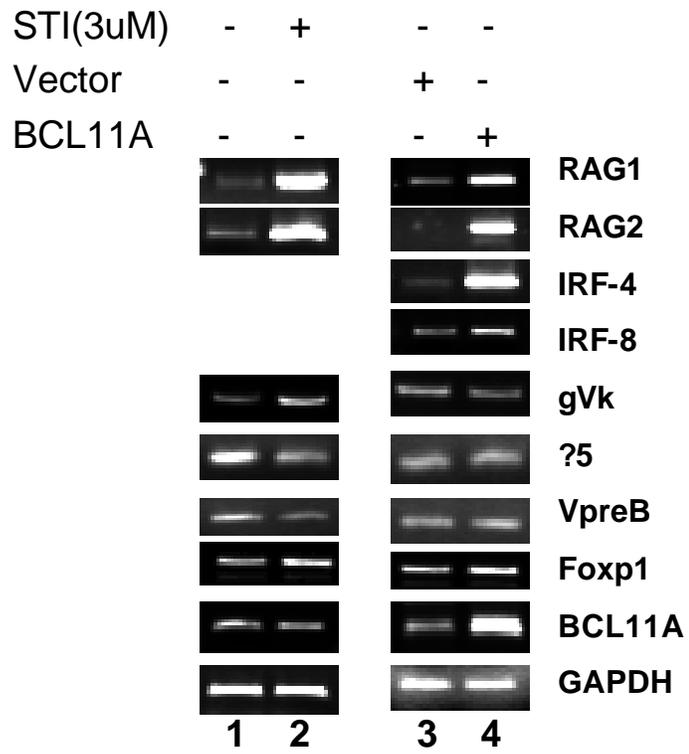
Table 3. Primers used in RT-PCR analysis of Figure 25.

RAG1-F	CCAAGCTGCAGACATTCTAGCACTC
RAG1-R	CTGGATCCGGAAAATCCTGGCAATG
RAG2-F	CACATCCACAAGCAGGAAGTACAC
RAG2-R	GGTTCAGGGACATCTCCTACTAAG
IRF4-F	CCACGGACACACCTATGATG
IRF4-R	GGTCTGGAAACTCCTCACCA
IRF8-F	GGGCTGCCTAAGTTGTATG
IRF8-B	ACCACCCTGCTGTCAGGTAG
Spi-B-F	AGAGGACTTCACCAGCCAGA
Spi-B-R	TGAGTTTGCGTTTGACCTTG
gVk-F	CCACATGCCTTTCTTCAGGGACAAGTGGGA
gVk-R	GTTATGTCGTTCATACTCGTCCTTGGTCAAC
Foxp1-F	AAGGGGCAGTATGGACAGTG
Foxp1-R	CCCAGAGGTTCACTCCATGT
BCL11A-3'E-F	CCCAGAGTAGCAAGCTCACC
BCL11A-BamH1-R	CCCAGGATCCTATTCAGTTTTTATATCATTATTCAAC

5.11. Discussion

Microarray and RT-PCR analyses provided us with several potential target genes of BCL11A-XL. In particular, the *RAG* genes were validated as BCL11A-XL targets using several approaches, including shRNA knock-down, ChIP, EMSA, and luciferase assays.

To assess the functional consequence of *RAG* up-regulation, we employed an Abl transformed, V(D)J-competent pre-B cell line, A70-INV. Over-expression of BCL11A-XL in A70-INV cells up-regulated *RAG1, RAG2, IRF-4*, and *IRF-8,* which led to the induction of V(D)J rearrangement of exogenous and endogenous targets. Further work will be required to identify the molecular mechanisms by which BCL11A modulates the transcription levels of these potential target genes and to assess the biological consequences of this regulation. Some of the issues we face and how we plan to address them are discussed below.

5.11.1. REGULATION OF RECOMBINATION ACTIVATING GENES (RAG)S BY BCL11A

Our data indicate that BCL11A-XL binds to *RAG* regulatory elements within the *RAG1* promoter and the *Erag* enhancer to regulate RAG transcription. Ectopic expression of BCL11A-XL activated RAG1 and RAG2 in Nalm6 and Abl pre-B cells. However, BCL11A-XL repressed a luciferase reporter driven by the *RAG*1 promoter in non-B cells. In addition, XL repression of *RAG1* or *RAG2* promoter-driven luciferase reporter was not affected by *Erag*.

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How can we explain the difference outcomes in non-B transient transfections with the expression assays in B cells? One possible explanation for the contradictary data is that activity of the regulatory elements might require a specific chromatin structure in the genome. It was reported that *Erag* activity was far more potent in stable transfection assays than in transient transfection assays (Hsu et al., 2003). BCL11A might also require other B cell-specific transcription factors to activate the *RAG* promoter and the *Erag* enhancer properly. We have attempted to address these issues by carrying out luciferase assays in untransduced or BCL11A-XL transduced A70-INV pre-B cells. However, the poor transfection efficiency of this line has given us inconsistent results (data not shown). To further address this issue, we will carry out stable transfection in B cells employing *RAG* regulatory elements in their germline context with the open reading frames of *RAG1* and *RAG2* replaced with GFP.

5.11.2. BCL11A CONSENSUS DNA BINDING SITES

Two putative DNA binding consensuses have been reported. One is a GC-rich sequence, 5'-GGCCGG-3', which was identified for BCL11A-L by a random repeat oligomer binding and amplification approach (Avram et al., 2002). We identified a CCC-(T/ A)-GC sequence as a putative consensus for BCL11A-XL using the same approach (Liu et al., 2006). Neither was confirmed for endogenous targets *in vivo*. Nor is it clear whether the differences in L and XL structure (Figure 5) underlie these differences.

Sequence alignments (data not shown) reveal several examples of consensus sequences within *RAG1* promoter and the *Erag* enhancer that are conserved within mice and humans. We have begun to determine the precise BCL11A-binding sites. Employing duplexed oligonucleotides which span these regions as competitors in EMSAs, although

unsuccessful to date (data not shown), is a logical approach to continue. Neither the ChIP or EMSA results rule out the possibility that BCL11A does not bind DNA directly, but is recruited there in a complex. We have not succeeded in producing recombinant, full-length XL in quantities required to test this possibility. Finally, it may be informative to use "ChIP on ChIP" assays (Ren et al., 2000) to construct genome-wide maps of the interaction between the BCL11A protein and DNA.

5.11.3. REGULATION OF V(D)J RECOMBINATION BY BCL11A

BCL11A-XL induced transcription of *RAG1, RAG2, IRF-4* and *IRF-8* in the Abl pre-B cells. In V(D)J recombination, there are at least two levels of control: RAG expression and chromatin accessibility (Schlissel and Stanhope-Baker, 1997). Up-regulation of *RAG* genes can account for the rearrangement of an exogenous recombination target, pMX-INV, because RAG expression alone was shown to be sufficient to induce rearrangement of exogenous target genes with a strong promoter even in fibroblasts (Angelin-Duclos and Calame, 1998).

Chromatin accessibility is required for the rearrangement of the endogenous *Ig* loci. V(D)J recombination and B cell development are blocked in *IRF4* and *IRF8* double knock-out mice at the pre-B cell stage (Lu et al., 2003). The results presented here, along with the phenotype of *BCL11A* knock-out mice, indicate that *BCL11A* acts upstream of both *IRF-4* and *IRF-8*. Histone modifications were shown to be involved in the accessibility and activation of the light chain loci (Schlissel and Stanhope-Baker, 1997). Ectopic expression of IRF-4 in *IRF-4,8$^{-/-}$* pre-B cells increased acetylation of histone H3 and H4, trimethylation of H3K4 (modifications typically associated with euchromatin) within the *Ig?* light chain enhancer, and ? germ line transcription (Ma et al., 2006). Even

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though there was up-regulation of *IRF-4* and *IRF-8* in BCL11A-XL transduced A70-INV cells, germ line ? transcription was not affected. It has been reported that germ line transcription is not required for V(D)J recombination (Angelin-Duclos and Calame, 1998). Therefore, we suggest that an open chromatin structure of ? loci in BCL11A-XL transduced A70 cells might be provided by other mechanisms, such as histone modifications rather than germ line transcription. It will be informative to study how histone modifications are affected in BCL11A-XL transduced A70 cells using anti-histone chromatin immunoprecipitation.

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