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The CIITA regulation during B cell differentiation to plasma cell

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Doctor of Philosophy

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ABSTRACT

The CIITA Regulation during B cell differentiation to plasma cell

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CIITA, the transcriptional activator of MHC class II expression, is controlled by at least three distinct promoters, pI, pIII, and pIV in a cell type and developmental specific manner. The promoter III regulatory regions that control constitutive CIITA expression in B cells have been characterized; however, other distal regulatory regions that may be required for CIITA regulation have not been identified. By applying a number of approaches, HSS1 (hypersensitive site 1) located 11kb upstream of pIII, was identified as a distal regulatory element involved in CIITA activation B cells. PU.1, an ETS domain containing transcriptional factor, which is essential for lymphocyte development, binds to HSS1 and directs long-range chromosomal interactions between HSS1 and CIITA pIII in B cells. Additionally, several CTCF (CCCTC binding factor) binding sequences on CIITA genes were found. These sites displayed an enhancer-blocking insulator activity, suggesting the possibility that these CTCF sites may be also involved in chromosomal interaction with HSS1 and pIII. CIITA is regulated by cell-type specific transcription factors. ZBTB32/ROG(repressor of GATA3) was identified as a novel repressor of class II transactivator (CIITA) and MHC-II gene expression during the early phase of plasma cell differentiation. ZBTB32 knock out mice showed delayed repression of CIITA and BCL6 mRNA, and lower levels of Blimp-1 mRNA expression compared to wild-type mice during B cell differentiation. Knockdown of ZBTB32 in plasma cells increased CIITA expression, as well as other B cell fate regulators, such as Pax5 and SpiB. In plasma cells, ZBTB32 and the Polycomb repressor complex bound directly to the CIITA promoter prior to Blimp-1 binding. ZBTB32 and Blimp-1 coimmunoprecipitated and co-localized within the nucleus. These results suggest that

ZBTB32 initiates CIITA silencing during B cell differentiation and may important role in plasma cell formation. In summary, the data reported in this thesis introduce novel cis-regulatory elements and transcriptional factors that are required for CIITA regulation during B cell differentiation.

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INTRODUCTION

MHC class II molecules are expressed on professional antigen presenting cells (APC) where they present antigens (Ag) that are recognized by CD4+ T cells (Benacerraf, 1981; Long, 1992). This process is critical to develop adaptive immune responses as it initializes proliferation and differentiation of antigen specific CD4+ T cells. The master MHC class II transcriptional regulatory factor, CIITA is constitutively expressed in antigen presenting cells, such as B cells, macrophages, and dendritic cells (Pai et al., 2002). CIITA is also induced in non-antigen presenting cells in response to IFN-γ stimulation (Steimle et al., 1994). CIITA binds to the MHC class II transcriptional enhancesome and activates MHC class II transcription by interacting with other regulatory factors, RFX5 (regulatory factor X), CREB (c-AMP response element- binding), NF-Y (Nuclear transcription factor-Y), as well as chromatin remodeling factors (Boss and Jensen, 2003; Hake et al., 2000). When B cells differentiate into plasma cells, the expression of CIITA is silenced, as are MHC class II genes (Green et al., 2006; Silacci et al., 1994). This process is tightly regulated to mediate precise control of adaptive immune responses.

This dissertation will focus on the regulation of CIITA in B cells and the mechanisms that silence CIITA expression in plasma cells.

Antigen processing and presentation

There are two classes of MHC class molecules: MHC class I and II. MHC molecules possess different structures to present antigens. Whereas MHC class I molecules are constitutively expressed in nearly all cell types, MHC class II molecules are expressed in selected cell types (Boss and Jensen, 2003; Muhlethaler-Mottet et al., 1997; Pai et al., 2002). Both MHC class molecules are also induced by the cytokine interferon gamma (IFN-γ) (Steimle et al., 1994).

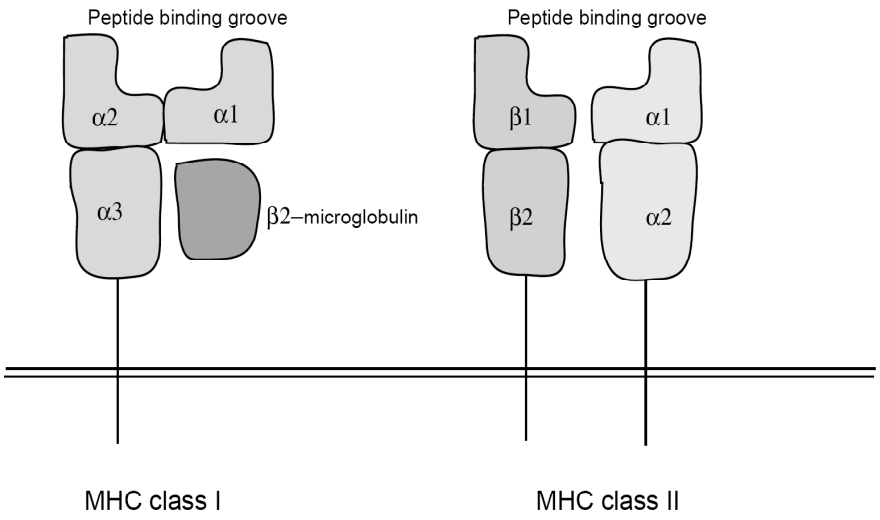


Figure1. The structure of MHC class I and II molecules
The peptide-binding grooves of MHC class I and II molecules that are composed of heterodimeric transmemebrane glycoproteins are illustrated (modified from Janeway's Immunology, 7th edition).

MHC class I

MHC class I presents intracellular antigens, such as those from viral infection, to cytotoxic CD8+ T cells (Viret and Janeway, 1999; Vyas et al., 2008). MHC class I molecules are composed of one α chain encoded on chromosome 6 and a non-covalently associated smaller peptide called β2 microglobulin, which is encoded in chromosome 15 in humans (Figure 1). MHC class I molecules in humans are termed HLA-A, B and C and are encoded in chromosome 6q21. In mice, these molecules are located on chromosome 17 and are termed H2-K, D, and L (Figure 4). α1 and α2 domains of MHC class I generate a peptide-binding cleft that binds short peptides of 8-10 amino acid in the length (Figure 4) (Biddison and Martin, 2001; Peaper and Cresswell, 2008; Vyas et al., 2008). The first step of antigen presentation by MHC class I molecules is to

ubiquitylate antigenic proteins by the proteasome complex in the cytosol. The proteins are proteolysed to produce small peptides, which are delivered into the endoplasmic reticulum (ER) by the TAP transporter, where the peptides are loaded into the antigen binding cleft of MHC class I molecules (Figure 1). The complex of peptide and MHC class I molecules are expressed on the cell surface where they can be recognized by CD8+ T cells. This recognition induces the cytotoxic effect function of CD8+ T cells, resulting in the death of the infected cells (Vyas et al., 2008).

MHC class II

MHC class II functions to present antigens to CD4+ T cells that originate from extracellular events. Then CD4+ T cells regulate other effector cells of the immune system (Benacerraf, 1981; Viret and Janeway, 1999; Vyas et al., 2008). MHC class II molecules are heterodimeric transmembrane glycoproteins composed of α (34kDa) and β (29kDa) chains (Figure1). In contrast to MHC class I, the expression of MHC class II is limited to professional antigen presenting cells, such as B-lymphocytes, dendritic cells, and macrophages (Pai et al., 2002). MHC class II is synthesized in the endoplasmic reticulum (ER) and associated with the invariant chain (Ii) that stabilizes the hetermodimer of MHC class II. The bulk of Ii is released by proteolysis, leaving a residual peptide termed CLIP (class II associated invariant chain peptide). CLIP occupies the peptide-binding cleft and is ultimately replaced with antigenic peptides through a process that involve HLA-DM. Peptide and the MHC class II complex are delivered to the cell surface where the complex can be recognized by antigen-specific CD4+ T cells (Figure 3) (Krensky, 1997). In humans, MHC class II genes are encoded by HLA-DR, HLA-DP, and HLA-DQ; in mice, they are encoded in the locus of IA and IE (Figure 4).

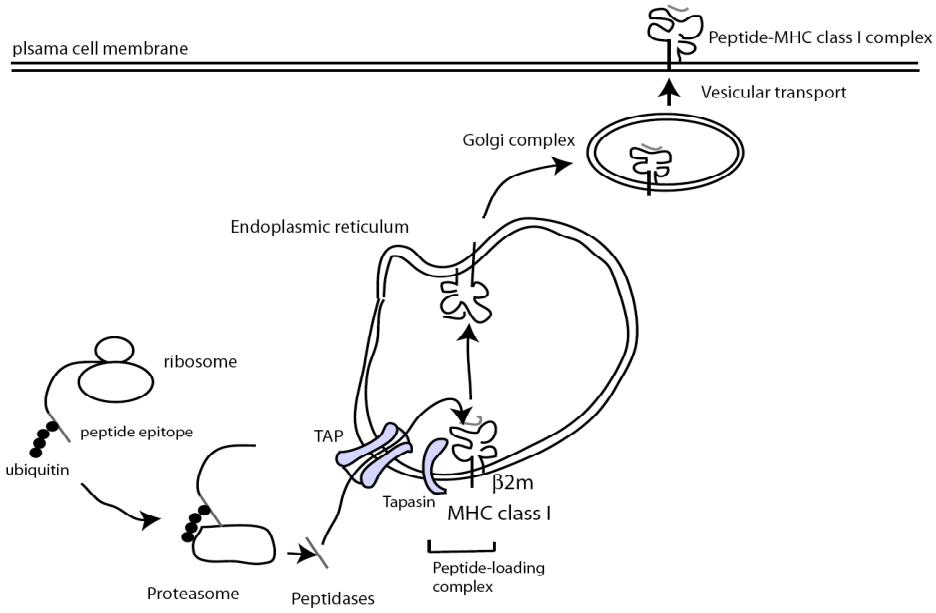


Figure 2. The pathway of MHC class I antigen presentation
The antigen presentation by MHC class I for intracellular antigens is illustrated (modified from *Nature Reviews Immunology* 9, 503-513, 2009).

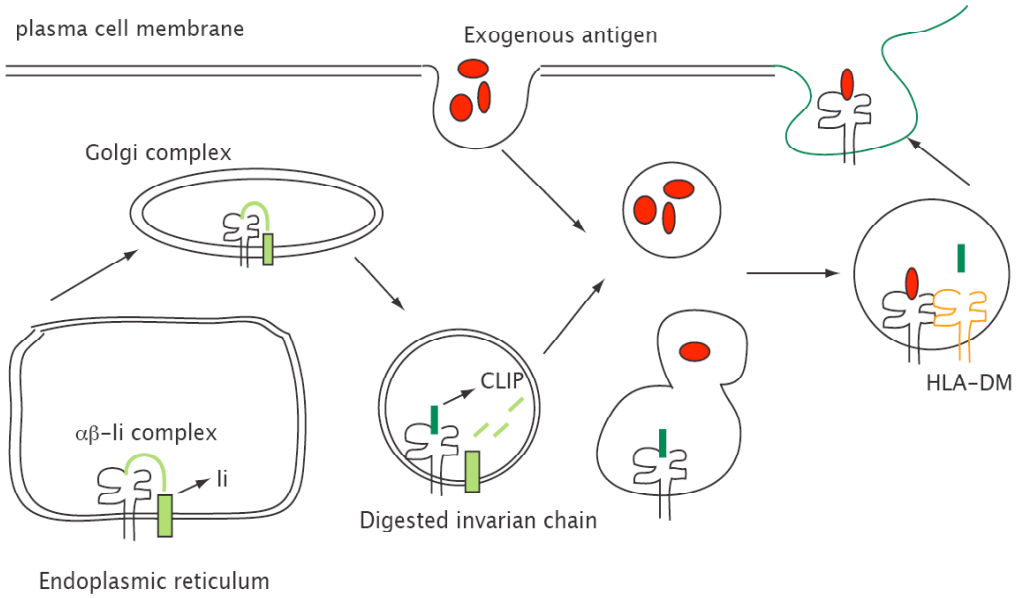
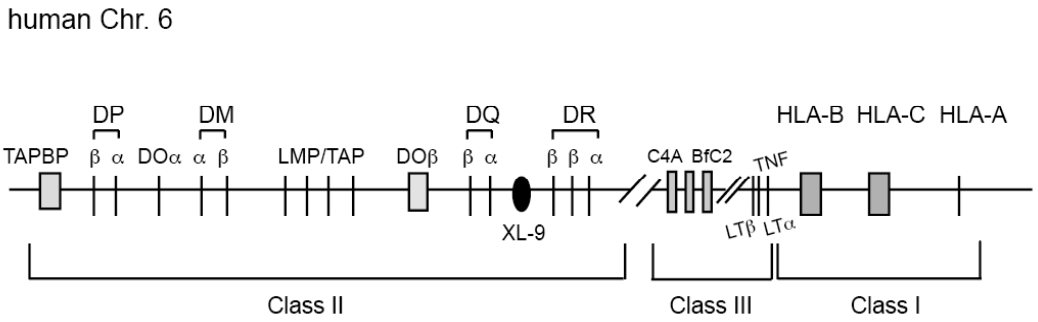
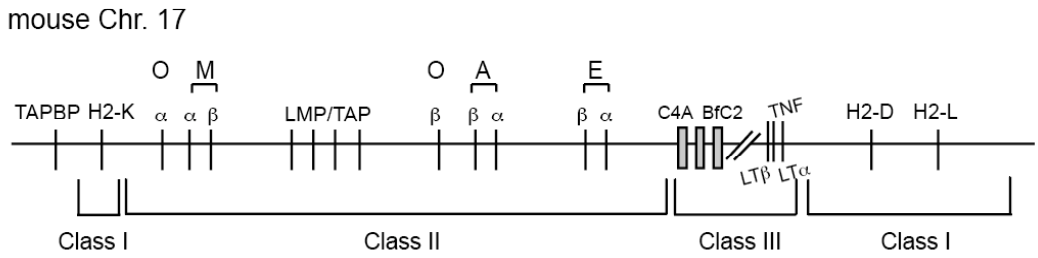


Figure 3. The pathway of MHC class II antigen presentation
The antigen presentation by MHC class II for exogenous antigens is described (modified from Janeway's Immunology, 7th edition).





	MHC class I	MHC class II	TAP1/TAP2	MHC class III
Human	HLA-A HLA-B HLA-C	HLA-DR HLA-DP HLA-DQ	Peptide transporter LMP - encode proteasome subunits	Encode various other protein with immune function
Mouse	H2-K H2-D H2-L	H2A H2E		

β chain encoded in different chromosome 15 (human), 2 (mouse)

Figure 4. Gene structure of the human and mouse Major histocompatibility complex
The structure of MHC class complex and genes encoding MHC class complex is shown. The MHC complex is encoded on chr 6 in humans and chr17 in mice. XL-9 is an insulator element located between HLA-DQ and HLA-DR in humans. A table composing the human and mouse MHC genes is shown (modified from Janeway's Immunology 7th Edition).

Regulation of MHC-II gene expression

MHC class II gene proximal regulatory elements are located 150 bp upstream of the transcription initiation site (Reith and Mach, 2001). This promoter sequence is conserved across species. This promoter proximal regulatory region contains S (W), X1, X2, and Y box motifs that bind RFX, CREB, and NF-Y protein complexes, respectively (Boss, 1999; Reith and Mach, 2001). These factors are required for constitutive or cytokine-induced MHC class II expression (DeSandro et al., 2000; Masternak et al., 1998; Steimle et al., 1995). However, RFX, CREB, and NF-Y binding are not sufficient to activate MHC class II expression. Activation requires the MHC class II transcriptional activator CIITA, which recruits other chromatin remodeling factors, coactivators, and general transcription factors to MHC class II promoters. CIITA therefore serves as a scaffold protein linking DNA binding proteins, chromatin remodeling factors, and the transcriptional machinery (Figure 5) (Boss, 1997).

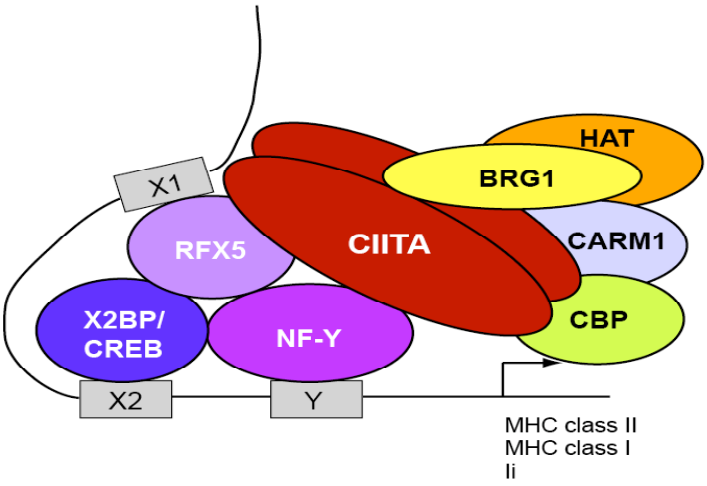


Figure 5. MHC class promoters and transcriptional factors binding to promoters
The transcription factors that bind to MHC class II promoters are depicted. CIITA serves as an adaptor protein that mediates protein-protein binding to MHC class II promoters.

CIITA related disease (Bare lymphocyte syndrome)

Bare lymphocyte syndrome (BLS) is a rare immunodeficiency disorder. It is inherited as an autosomal recessive disease and exhibits severe defects in T cell activation and reduced levels of CD4+ T cells in response to infectious agents (Saleem et al., 2000; Waldburger et al., 2000). BLS patients do not express MHC class II genes (Mach et al., 1996; Reith and Mach, 2001). Induction of MHC-II expression by IFN-γ is also abolished in fibroblast from BLS patients (Mao et al., 1993). Genetic complementation approaches identified four complementation groups among BLS patients (Steimle et al., 1993). B cell lines from these patients were used to identify the defected genes by either complementation cloning or through biochemical protocols (Griscelli et al., 1989; Nekrep et al., 2000; Steimle et al., 1995). CIITA was the defective gene in complementation group A (Table 1).

BLS group	Cell	CIITA activity	Defective genes	MHC promoter Occupancy
A	RJ225	No	CIITA	Yes
B	Ramia	Yes	RFXB	No
C	SJO	Yes	RFX5	No
D	6.1.6	Yes	RFXAP	No

Table 1. The BLS complementation groups and characteristics of each group

Transcriptional factors binding to MHC class II promoters

RFX (Regulatory Factor X)

The RFX complexes consist of three proteins, RFXB (RFXANK), RFX5, and RFXAP (Durand et al., 1997; Moreno et al., 1997). Deficiency of these factors is found in bare lymphocyte syndrome (BLS) patient complementation groups B, C, and D that also have a defect in MHC class II expression (Masternak et al., 1998; Nagarajan et al., 2000; Steimle et al., 1995). All RFX proteins are ubiquitously expressed and bind to DNA of the X1 box of all MHC class II promoters (Moreno et al., 1997). Additionally, Jabrane et al. showed that the RFX5 complex also binds the S element on MHC class II promoters (Jabrane-Ferrat et al., 1996). All MHC class II promoters are unoccupied in RFX mutant BLS patient cell lines (Kara and Glimcher, 1991), indicating that RFX proteins are critical for MHC class II promoter function. The RFX proteins interact with NF-Y (see below), CREB, and CIITA both in solution and on DNA (Louis-Plence et al., 1997; Moreno et al., 1997; Reith et al., 1994a; Reith et al., 1994b; Westerheide et al., 1997).

CREB (c-AMP response element binding)/ CBP/ p300

Using biochemical purification from nuclear extracts, CREB (c-AMP response element binding) was identified as the factor that binds at the X2 element (Moreno et al., 1999). CREB interacts with RFX5 and CIITA to mediate MHC class II transcription (Moreno et al., 1999). Importantly, the C terminal domain of CREB was fully functional to activate MHC class II and was also involved in interaction with other factors (Lochamy et al., 2007). Phosphorylation of CREB at Ser133 leads to recruitment of the co-activator CREB binding protein (CBP) to its target sequence, and the enhancement of transcription (Vo and Goodman, 2001). CBP/p300 does not bind directly to DNA; instead factors like CREB recruit CBP/p300 to the promoter regions of genes (Chrivia et al., 1993).

NF-Y (Nuclear factor-Y)

NF-Y is the ubiquitously expressed CCAAT binding protein and binds to the Y box at MHC class II promoter and other genes (Kern and Woodward, 1991; Louis-Plence et al., 1997; Zhu et al., 2000). NF-Y is a heterotrimer protein consisting of NF-YA, B, and C subunits (Louis-Plence et al., 1997; Mantovani, 1999). NF-Y interacts with RFX and CREB forming the MHC class II enhancesome (Figure 5) (Zhu, 2000 #212). This tri-molecular (RFX, CREB, NF-Y) complex is extremely stable when bound to DNA, and serves as a highly specific target for the recruitment of CIITA.

CIITA (MHC class II transcriptional activator)

CIITA was identified by complementation cloning of the RJ2.2.5 cell line (Steimle et al., 1993) that has a defect in the expression of MHC class II. RFX factors and other factors that bind at MHC class II promoters are not sufficient for MHC class II expression (DeSandro et al., 2000; Masternak et al., 2000). As stated above, CIITA is not a DNA binding protein, but CIITA binding at MHC class II promoters with other factors is required for MHC class II expression (Chang et al., 1994; Hake et al., 2000; Jabrane-Ferrat et al., 1996). Ectopic expression of CIITA in MHC class negative cells is sufficient to initiate MHC class II expression, suggesting that CIITA expression is the only limiting factor for expression of MHC class II (Chang et al., 1994). CIITA recruits co-activators and chromatin remodeling factors, such as arginine methyltransferase (CARM1) and CBP (Zika et al., 2005). Additionally, the ATP-dependent chromatin-remodeling complex, BRG1 (Brahma-related gene 1) is recruited by CIITA for MHC-II expression (Mudhasani and Fontes, 2002; Ni et al., 2008). BRG1-deficient cells exhibited a loss of CIITA-dependent MHC class II expression, indicating that BRG1 is required for MHC-II expression (Mudhasani and Fontes, 2002). Unlike RFX or NF-Y, CIITA expression is limited to specific cell types, developmental stages, or through IFN-γ stimulation (Muhlethaler-Mottet et al., 1997).

Other sequences with similarity to the W, X, Y box elements are important for MHC-II expression (Krawczyk M et al., 2004). Gomez et al. identified several X-like (XL) sequences within the MHC class II region that had properties similar to the conventional HLA-DR WXY box promoter sequences, including transcriptional activity and the binding of RFX5 and CIITA (Gomez et al., 2005). One XL element that was different was XL9 in that it did not bind RFX and CIITA. XL9, which is located in intergenic region between HLA-DQ and HLA-DR (Figure 4), was identified as a novel enhancer-blocking element through interaction with the factor CTCF. Further study has shown that the XL9 element also interacts directly with MHC class II promoters, implying that the regulation of MHC class II is mediated by broadly located distal regulatory elements rather than just the local promoter region W, X, Y sequence elements (Majumder et al., 2008).

CIITA structure, MHC-II expression

CIITA is a member of the CATERIPLLER (CARD, Transcription enhancer, R (purine) binding, pyrin, lots of leucine repeat) family of proteins along with other proteins, Nod1, Nod 2, Monarch1, and Apaf1 (apoptotic protease activating factor) (Harton et al., 2002). A schematic of CIITA's domain structure is shown in the figure 6. The N-terminal of CIITA contains an acidic amino acid domain and a Proline, Serine, and Threonine rich domain. The acidic domain is a key domain for CIITA as it functions as a transcriptional activation domain and interacts with general transcription factors (Figure 6). While the N-terminal of CIITA plays a role in transactivation for MHC class II expression, the C-terminal determines the specificity of CIITA for three MHC class II genes HLA-DR, DQ, and DP (Zhou and Glimcher, 1995). CIITA also contains a GTP binding domain that is required for nuclear import (Harton JA et al., 1999). The GTP domain is responsible for CIITA self-association together with its C-terminal leucine rich repeat domains (Hake et al., 2000; Linhoff MW et al., 2001). Self-associated CIITA is then recruited to MHC

class II promoters for transcriptional activation. It was thought that CIITA self-association stabilizes the MHC class II enhanceosome complex (Linhoff MW et al., 2001; Sisk et al., 2001; Tosi G et al., 2002), but it is not clear why CIITA must self-associate for function. Additionally, CIITA is ubiquitylated in a manner that is independent of the proteasome degradation pathway. This modification is required for transcriptional activation (Greer SF et al., 2003). Again, the mechanism by which this modification facilitates transcription is not known.

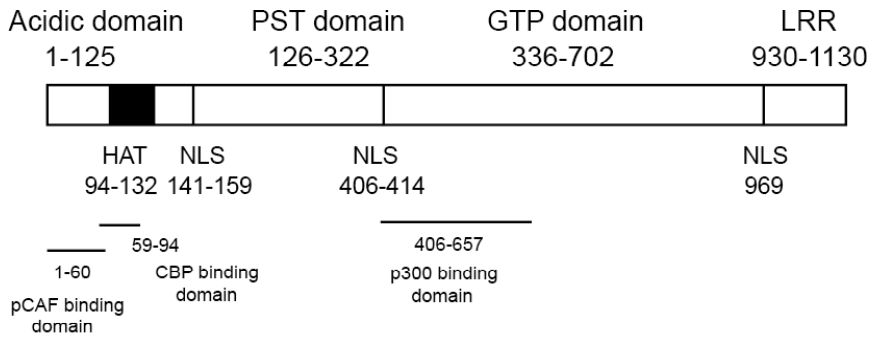


Figure 6. The structure of CIITA protein that is produced from CIITA III and IV
CIITA protein structure is illustrated with specific domains that are required for CIITA transcriptional activation and protein-protein interaction. CIITA isoform 1 has an N-terminal CARD domain not found the other isoforms (not shown).

CIITA gene locus

In humans, CIITA transcription is controlled by four independent promoters termed pI, pII, pIII, and pIV (Muhlethaler-Mottet et al., 1997). Mice have a deletion of pII and therefore use only three of the four promoters. Because each promoter transcribes a unique first exon, distinct isoforms are formed. The unique exon I for each promoter is spliced into a common exon 2 located 18 kb downstream from pI. Each promoter isoform is expressed in a cell type or developmental specific manner (Muhlethaler-Mottet et al., 1997; Nickerson et al., 2001; Pai et al., 2002) or in response to cytokine stimulation (Figure 7) (Steimle et al., 1994; Zhao et al., 2007).

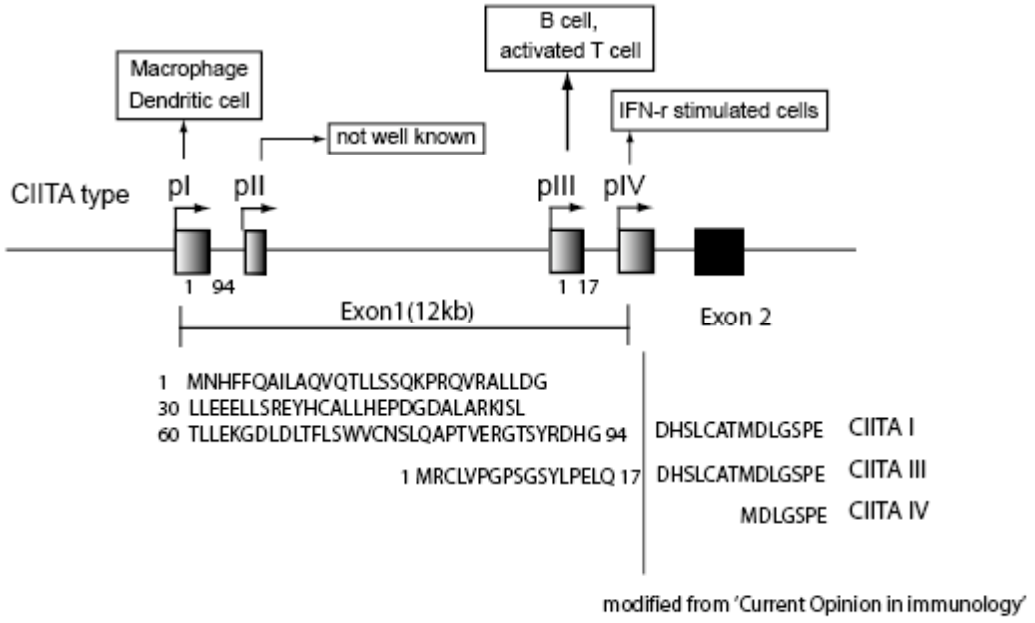


Figure 7. The schematic diagram of CIITA promoters
Each CIITA isoform that is expressed in cell type specific manner is encoded by unique exon 1 from each cell type specific promoter, pI, pIII, and pIV. The N-terminal amino acid sequences produced by each CIITA isoform is illustrated.

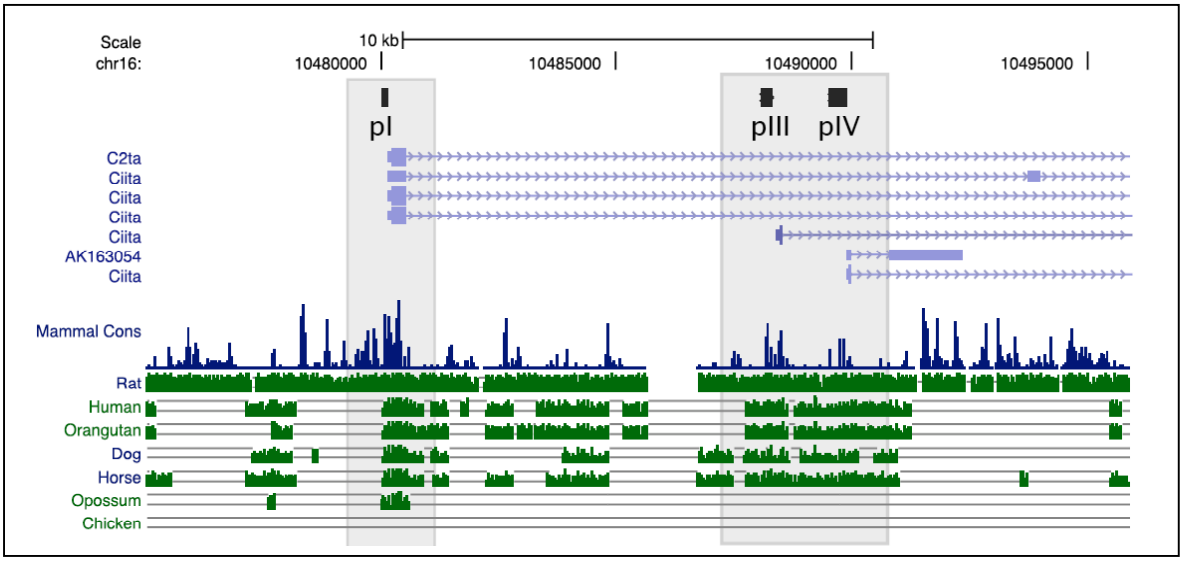


Figure 8. CIITA promoters that conserved through the human to mouse
The conservation of CIITA promoters, pI, pIII, and pIV was determined using the UCSC genome browser.

The DNA homology map shown in Figure 8 displays the species conservation on the different promoters. The gap points to the deletion of pII in mouse (Figure 8).

CIITA promoter I

CIITA promoter I is used in dendritic cells or macrophages for constitutive CIITA expression. Promoter I directed CIITA has an extra 94 amino acids in the N-terminus that encodes a caspase activation and recruitment domain (CARD); therefore, pI expresses the longest CIITA isoform and is132 kDa (Nickerson et al., 2001). The specific function of this CARD domain is unknown. Promoter I derived CIITA transcripts vary in their expression depending upon developmental stage or cytokine stimulation (LeibundGut-Landmann et al., 2004). For example, pI CIITA is expressed at high levels in immature dendritic cells, but is silenced in differentiated mature dendritic cells (Landmann et al., 2001; Pai et al., 2002). Histone deacetylases can reverse pI CIITA silencing during dendritic cell differentiation, indicating that the transcriptional regulation of CIITA pI uses epigenetic mechanisms (Sebastian et al., 2008). Additionally, CIITA pI specific transcripts are also detected in low levels from bone marrow-derived macrophages and increase expression in response to IFN-γ stimulation. CIITA pIV-dependent transcripts are also increased in IFN-γ treated macrophages; however, pI-directed CIITA transcripts are more stable than pIV transcripts (Pai et al., 2002), suggesting that the CARD domain may provide stability to the transcript. The mechanism by which this occurs has not been defined.

CIITA promoter II

CIITA promoter II is only identified in human cells and produces low level transcripts in B cells. In mice, there is no sequence homology of promoter II. The role of promoter II in CIITA expression is not clearly understood. In one report, transcripts initiating downstream of pII in response to IFN-γ stimulation were observed (van der Stoep et al., 2007). These transcripts are expressed at low levels and may not have an important biological role.

CIITA promoter III

CIITA promoter III is employed for constitutive CIITA expression in B-lymphocytes and activated T lymphocytes (Lennon et al., 1997; Lennon et al., 1998); however, CIITA is silenced during B cell to plasma cell differentiation that also leads to MHC class II loss in plasma cells (Green et al., 2006; P. Silacci, 1994). Under the control of CIITA pIII, 124 kDa isoform of CIITA is produced (Figure 7). Regulatory factors and proximal cis elements for CIITA pIII have been described (Figure 9). In vivo footprinting analysis identified five cis-regulatory elements between -380 to +120 from the transcriptional start site (Ghosh et al., 1999). These elements were termed ARE1 (TEF-like element), ARE2, Site A, B, and C. ARE1 and 2 elements are critically required for pIII-depedent CIITA transcription (Ghosh et al., 1999). Each element directly binds with specific transcription factors, such as PU.1, E47, SP1, and CREB (Green et al., 2006; van der Stoep et al., 2004) (Figure 9). PU.1 and E47/E2A cooperate to activate CIITA transcription in B cells through site C (Figure 9). PKC (protein kinase C) also impacts CIITA expression by controlling CREB phosphorylation and CBP binding at CIITA promoter III (Kwon et al., 2006). Besides B cells or activated T cells, CIITA promoter III encoded transcripts are also observed in immature dendritic cells, and are similarly silenced upon dendritic cell maturation as seen in B cell to plasma cell differentiation (Landmann et al., 2001). In addition to the core promoter region, a long distance promoter element, located 6kb upstream of CIITA promoter III is also required for monocytic cell and macrophage activation in response to IFN-γ stimulation in a STAT-1 dependent manner (Piskurich et al., 1998; van der Stoep et al., 2007). Recently, it was found that pIII is also induced by IFN-γ stimulation in human non-hematopoietic cells, but this required a region 2kb upstream of the pIII core promoter (Piskurich et al., 2006; Piskurich et al., 1998).

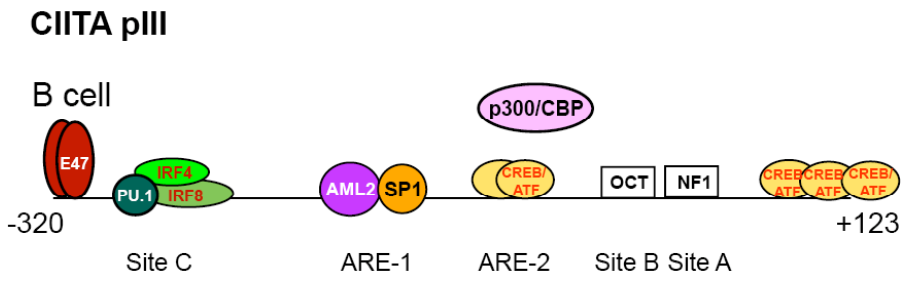


Figure 9. CIITA pIII binding transcription factors
The cis-regulatory elements of CIITA pIII is illustrated with transcription factors that are required for CIITA activation in B cells.

CIITA promoter IV

CIITA promoter IV is utilized in response to IFN-γ in non-antigen presenting cells and produces the shortest isoform of 121 kDa (Muhlethaler-Mottet et al., 1997; Steimle et al., 1994). In contrast with pI and pIII, pIV initiates translation from CIITA's common exon 2. Waldberger et al. showed that mice deleted in CIITA pIV fail to express MHC class II in non-hematopoietic cells (Waldburger et al., 2001). B-lymphocytes also express pIV directed CIITA mRNA in response to IFN-γ (Tooze et al., 2006). The signal pathway by which IFN-γ actives CIITA transcription, starts with the binding of IFN-γ to its cell surface receptor. IFN-γ activates receptor-associated Jaks (Janus kinases), which then lead to STAT-1 (signal transducer and activator of transcription) phosphorylation. The phosphorylated STAT-1 forms homodimers and migrates to the nucleus and activates transcription of target genes. IRF1 (interferon regulatory factor) and USF1 bind at CIITA promoter IV for transcription along with STAT1 to activate transcription (Morris et al., 2002; Piskurich et al., 1998). Moreover, BRG1, the Swi/Snf ATP-dependent nuclesome remodeling complex, is necessary to activate CIITA by binding before STAT-1 complexes at CIITA pIV (Mudhasani and Fontes, 2002).

Thus, CIITA has at least three or four distinct modes of regulation that are used by cells of immune system to precisely control its expression and ultimately MHC class II expression. As

described below, it is clear that the proximal promoter elements do not work alone and there is evidence for additional cis-elements and trans-acting factors that regulate this system.

B cell differentiation and CIITA expression

B cell differentiation into plasma cells:

During B cell development, immunoglobulin gene rearrangement in pro and pre B cells occurs in the bone marrow. Immunoglobulin expressing immature B cells migrates to the periphery and spleen, where they develop into mature B cells through several transitional stages. In the bone marrow or lymph nodes, follicular B cells are differentiated by T cell-dependent antigens and lead to the formation of germinal centers, where somatic hypermutation and selection to produce B cells expressing antibody with high affinity to antigens occurs. Then, antigen and T cell stimulated mature B cells leave the germinal center (GC) and differentiate to plasma cells or memory B cells (Figure 10). Memory B cells express immunoglobulin on the cell surface, and are required for rapid immune response to future invading antigens (McHeyzer-Williams and McHeyzer-Williams, 2005). Plasma cells produce secreted antibody for the humoral response to invading antigen (reviewed in (Shapiro-Shelef, 2005; Matthias, 2005)). During B cell to plasma cell differentiation, B cell specific surface proteins B220, CD19, MHC class II, CD21, and CD22 are down regulated (Schmidlin et al., 2009). Dysregulation of plasma cell differentiation leads to a number of autoimmune diseases, including Grave's disease, lupus erythematosus, and rheumatoid arthritis.

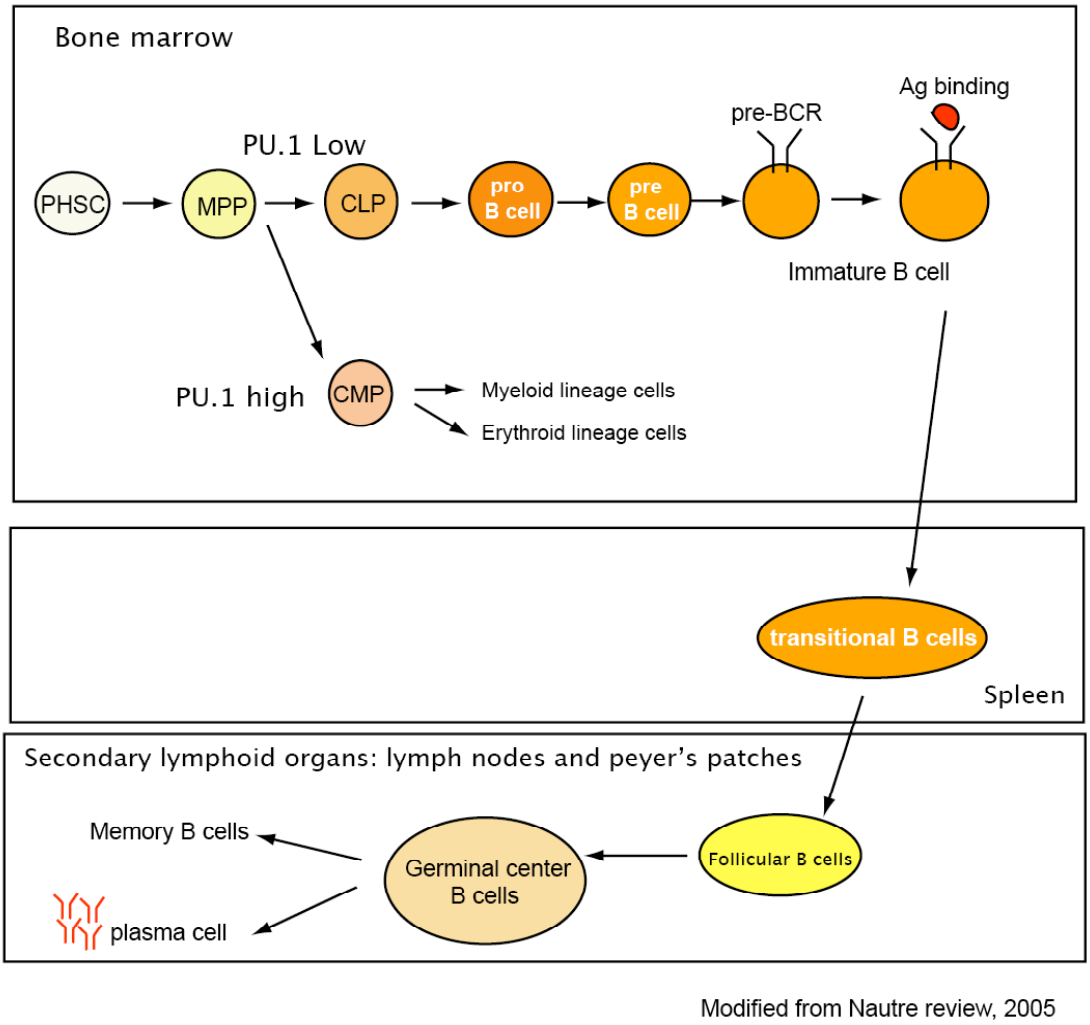


Figure 10. The process of B cell differentiation
The B cell differentiation process from bone marrow to secondary lymphoid organs is shown. Due to graded expression levels, PU.1 is the factor that determines either lymphoid and myeloid lineage cell development.

Transcription factor network during B cell differentiation

During the B cell to plasma cell transition, B cell specific surface protein and transcription factors are down regulated (Igarashi et al., 2007; O'Riordan and Grosschedl, 2000). As stated earlier, MHC class II, B220, and CD19 were lost from B cell surface during the B cell differentiation. However, syndecan-1, which recognizes extracellular matrix and growth factors, is induced. Thus, syndecan-1 can be used as cell surface marker to determine plasma cell differentiation state

(Seidel et al., 2000). In addition to cell surface proteins, B cell and plasma cell specific genes orchestrate B cell to plasma cell transition by reprogramming their expression during differentiation (Singh et al., 2005) (Matthias and Rolink, 2005; Shapiro-Shelef and Calame, 2005) (Figure 11).

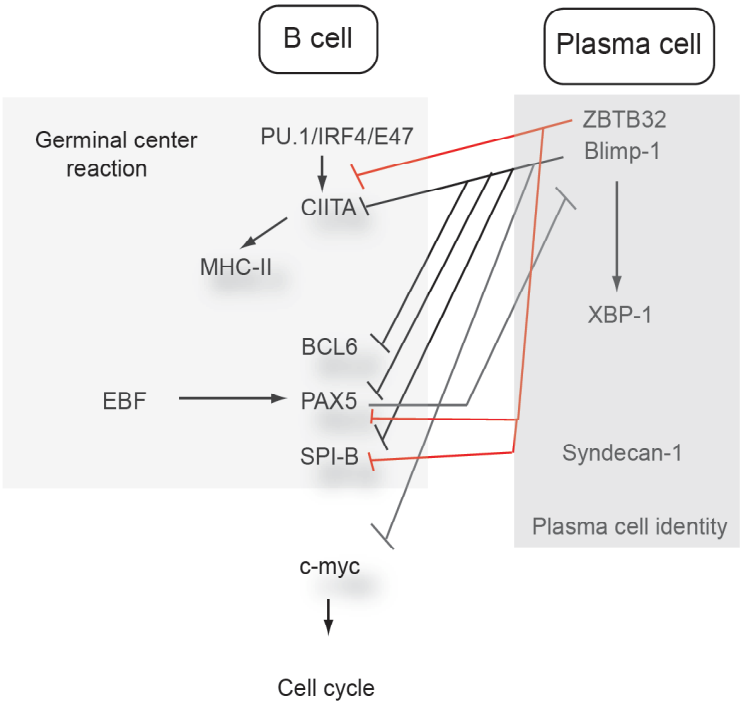


Figure 11. Transcription factors differentially regulated during B cell to plasma cell differentiation
The transcription factors that are expressed in B cells and plasma cells to maintain the cell fate are illustrated**.** Blimp-1 directly repress CIITA, Pax-5, BCL6, and Spi-B (Angelin-Duclos et al. 2000, Chen et al. 2007, Lin et al. 2002) .

This reprogramming is determined by a number of key transcription factors that are therefore induced or repressed during the process. The factors Pax-5, EBF1, PU.1, Spi-B, Spi-C, BCL6, Blimp-1, XBP-1, IRF-4, and ZBTB32 are key players in this process and are described below.

B cell specific transcription factor

Pax5 (paired homeodomain protein 5) and EBF1 (early B cell factor-1):

Pax5 is essential for B lymphoid lineage progression from the pro B cell to the mature B cell stage (Cozma et al., 2007; Decker et al., 2009; Hagman and Lukin, 2007). Pax5 represses genes that are not involved in B lineage development and activates B cell specific genes. Subsequently, Pax5 is silenced during the B cell to plasma cell transition due to direct repression by Blimp-1 (B lymphocyte induced maturation protein 1), which is expressed in plasma cells (Nera et al., 2006). Conditional Pax-5 knockout mice resulted in the conversion of mature B cells into T cells by dedifferentiation (Cobaleda et al., 2007a; Cobaleda et al., 2007b; Mikkola et al., 2002; Zandi et al., 2008). It has been reported that Pax5 is required for CIITA regulation (Horcher et al., 2001), but it is not clear how Pax-5 is involved. EBF1 induction leads to the B cell fate by increasing E47 activity (Hystad et al., 2007; Medina and Singh, 2005). While EBF1 can commit to B-cell fate independently of Pax5 (Hystad et al., 2007; Pongubala et al., 2008; Singh et al., 2005), it was thought that EBF1 activates Pax5 genes by binding to a Pax5 distal promoter region (Gao et al., 2009). Thus, these factors are more likely to function together.

ETS domain transcription factors

ETS transcription factors are evolutionally conserved, and are critical for lymphocyte development (Anderson et al., 2001). Spi-C, Spi-B, and PU. 1 are members of this transcription factors family and are described below.

PU.1 (Spleen focus forming virus proviral integration oncogene spi1):

PU.1 is encoded by the PU.1-Spi1-Sfpi1 proto-oncogene. Maki et al. isolated a cDNA that encoded PU.1 from macrophages and B cells (Goebl, 1990; Klemsz et al., 1990). The expression of PU.1 is required for the development of the common lymphoid and myeloid cell lineages that leads to B cells, T cells, and macrophages (Anderson et al., 2000; DeKoter

and Singh, 2000; Henkel et al., 1996; Scott et al., 1994). During common lymphoid lineage development, graded levels of PU.1 expression decide either B lymphocyte or macrophage fate (DeKoter and Singh, 2000) (Figures10). The disruption of PU.1 expression in mice resulted in neonatal lethality (Scott et al., 1994). PU.1 mutations also cause acute myeloid leukemia in mice (Cook et al., 2004; Houston et al., 2007). As a transcription factor, PU.1 binds to a purine rich sequence (GAGGAA) termed PU-box at promoter or enhancer elements to regulate gene expression. For example, PU.1 binds at the immunoglobulin light chain enhancer to regulate its transcription (Marecki et al., 2004). It has been reported that PU.1 interacts with other transcription factors, such as IRF8 to regulate transcription of its target genes (Rehli et al., 2000).

Spi-B (Spi-1/PU.1 related transcription factor):

Spi-B represses Blimp-1 and XBP-1 expression by binding directly to regulatory elements so that plasma cell differentiation is inhibited (Schmidlin et al., 2008). Spi-B knock out mice resulted in abnormal B cell receptor (BCR) signaling and poor antibody response after immunization (Su et al., 1997), indicating that the expression of Spi-B is important for B cell development. Dys-regulation of Spi-B is associated with B cell lymphomas, indicating its importance in maintaining normal B cell fate progression (Schmidlin et al., 2008).

Spi-C (Spi-1/PU.1 related transcription factor):

The expression of Spi-C is detected in macrophages, pre-B cells, and mature B cells, but not from plasma cell RNA preparations (Bemark et al., 1999; Carlsson et al., 2003). Spi-C and PU.1 cooperate reversely for pre-B cell development (Schweitzer et al., 2006). Spi-C and PU.1 recognize similar DNA sequence elements (Bemark et al., 1999), but do not appear to bind them at the same time. Some evidence suggests that they may work in a reciprocal manner during B cell development.

BCL6 (B-cell leukemia/lymphoma 6):

BCL6 gene encodes a POZ/zinc finger transcriptional repressor that is highly expressed in germinal center B cells, but not in plasma cells (Chang et al., 1996). BCL6 mRNA can be detected in many tissues; however, its protein expression is only detected in lymphocytes (Seyfert et al., 1996). BCL6 expression is required for germinal center (GC) formation and T-cell dependent antibody responses (Fujita et al., 2004; Greiner et al., 2000; Niu et al., 2003). To control germinal center formation, BCL6 represses genes that function in lymphocyte differentiation, such as Blimp-1 (B lymphocyte induced maturation protein 1) (Sciammas and Davis, 2005; Shaffer et al., 2002). However, when chromosomal translocation in the BCL6 genes occurs, dys-regulated BCL6 expression in germinal center B cells prevents the silencing of Blimp-1 and termination of the GC response for plasma cell differentiation (Baron et al., 2004; Camacho et al., 2004; Chaganti et al., 1998; Franco et al., 2004; Ohno, 2004). Thus, BCL6 is critical to B cell fate maintenance and a key repressor of a plasma cell progress.

Plasma cell specific transcription factors

Blimp-1 (B lymphocyte induced maturation protein 1):

Blimp-1 was identified from human cDNA encoding a zinc finger containing protein that bound to the positive regulatory domain 1 of the human IFN-β promoter and initially termed PRDM1 (Huang, 1994). A few years later, the M. Davis group isolated a murine cDNA for *prdm1* from BCL1 B cells differentiated with IL2, IL5 to plasma cells (Turner et al., 1994). They termed the gene Blimp-1 not knowing of the existence of the other clone. Blimp-1 is functionally conserved across species. Blimp-1 is the master transcriptional factor for plasma cell differentiation (Angelin-Duclos et al., 2002; Iwakoshi et al., 2003; Sciammas and Davis, 2005; Shaffer et al., 2002; Shapiro-Shelef et al., 2003). The *Blimp-1/Prdm1* gene is located on chromosome 6 in humans and chromosome 10 in mice. The Blimp-1 gene contains 8 exons composed of Set

domain in the N-terminus and a zinc finger domain in its C-terminus (exon 6, 7, 8). As a transcriptional repressor, Blimp-1 (PRD1-BF) recruits other factors to target genes. For example, G9a was recruited to the promoter of IFN-β promoter by Blimp-1 (Gyory et al., 2004). Blimp-1 is critical for T cell, B cell, or non-lymphoid cell development (Chang et al., 2000; Crotty et al.; Fink, 2006; Messika et al., 1998). In B cell development, Blimp-1 represses B-cell specific transcriptional factors, CIITA, Pax-5, Spi-B, and BCL6 to promote B-cell to plasma cell differentiation (Angelin-Duclos et al., 2000; Chen et al., 2007; Lin et al., 2002; Nera et al., 2006). Conditional Blimp-1 knock out mice in mature B cells resulted in reduced levels of immunoglobulin secretion even when class switch recombination and the peripheral B cell population were normal (Shapiro-Shelef et al., 2005). Microarray cDNA profile comparison between Blimp-1 expressing and non-expresssing Burkitt lymphoma cell lines identified a number of genes that are affected by Blimp-1(Sciammas and Davis, 2005). Among those, c-myc, Spi-B, Pax-5, Id3, and CIITA were found to be directly repressed by Blimp-1 (Chen et al., 2007; Lin et al., 1997; Tooze et al., 2006). Blimp-1 also represses c-myc expression, which is required for cell cycle progression (Messika et al., 1998). Thus, cell division is repressed in plasma cells due to the high level of Blimp-1 (Lin et al., 1997). For this reason, ectopic expression of Blimp-1 in B cells leads to cell death. XBP-1 is also found as a downstream gene of Blimp-1 (Shaffer et al., 2004). However, in this case XBP-1 is activated. The expression of Blimp-1 protein is also detected from human and mouse T cells (Kallies et al., 2009; Martins and Calame, 2008; Rutishauser et al., 2009; Savitsky et al., 2007). Mouse thymocytes increase Blimp-1 expression upon anti-CD3, acti-CD28, and IL2 stimulation (Shin et al., 2009). Blimp-1 is also observed in regulatory T cells (Martins et al., 2006). Chromatin immunoprecipitation analysis showed that IL2, T bet, and BCL6 are direct targets of Blimp-1 during T cell development (Cimmino et al., 2008). Together, these data place Blimp-1 at the center of several terminal cell fate differentiation processes.

XBP-1 (X-box binding protein 1):

XBP-1 was originally isolated as an MHC class II X box transcription factor (Liou et al., 1990; Ono et al., 1991). Subsequently, it was found to be expressed ubiquitously and not play a role in MHC class II transcription. In B cells, XBP-1 is induced upon B cell activation and increased in plasma cells (Reimold et al., 2001). XBP-1 deficient mice resulted in normal numbers of activated B-lymphocytes that proliferate and formed normal germinal centers but secreted little immunoglobulin of any isotype such that plasma cell function is disrupted (Reimold et al., 2001).

IRF4 (interferon regulatory factor 4):

IRF4, also known as MUM1 (multiple myeloma antigen 1), is expressed in plasma cells and activated T cells (Falini et al., 2000). IRF4 was found to collaborate with the ETS domain transcription protein PU.1, Spi-B, E47, as well as BCL6, STAT6, and Blimp-1 (van der Stoep et al., 2004). IRF4 is required for plasma cell differentiation and cytotoxic T cell development so that IRF4-depleted cells cannot produce antibody efficiently due to reduced number of plasma cells (Falini et al., 2000; Fillatreau and Radbruch, 2006; Klein et al., 2006; Lu, 2008; Teng et al., 2007).

ZBTB32 (zinc finger and BTB domain containing 32 protein):

The Zinc finger and BTB domain containing 32 protein also named PLZP (PLZF like zinc finger protein), FAZF (Fanconi anemia zinc finger), TZFP (testis zinc finger protein), and Rog (repressor of GATA), contains a BTB/POZ (bric a brac-tram track-broad complex/poxvirus zinc finger) domain that serves as a protein-protein interaction motif (Bilic and Ellmeier, 2007; Dai et al., 2002; Miaw et al., 2000). BTB/POZ domain containing proteins collaborate with other repressor complexes, such as N-Cor, Sin3a/SMRT and histone deacetylase to mediate gene silencing (Bilic and Ellmeier, 2007). A number of studies have shown that ZBTB32 is important for T lymphocyte proliferation, cytokine production (Piazza et al., 2004); however, the role of

ZBTB32 in B cell differentiation has not been studied. Chapter 2 of this dissertation identified ZBTB32 as a major silencer of CIITA during plasma cell differentiation.

microRNAs:

In addition to cell type specific transcriptional factors, microRNA, which influence the development of lymphocytes such as B cells or T cells by affecting their target mRNA expression, were identified as a post-transcriptional regulator during B cell development (Zhang et al., 2009). miRNAs are small non-coding RNAs that play a key role in cellular function (Bi et al., 2009). Recently, distinct profiles of microRNA expression were observed during lymphocyte differentiation. In the different B cell development stage, a number of microRNA species are differentially expressed (Carissimi et al., 2009; Xiao and Rajewsky, 2009). Comparing normal B cell and lymphoma cells, several miRNAs invovled in tumorigenesis were discovered (Lionetti et al., 2009). For instance, miRNA 155, which regulates AID (activation induced deaminase) and PU.1 expression, was increased in chronic lymphocytic leukemia cells when compared to normal cells from some patients (Faraoni et al., 2009; Tili et al., 2009; Worm et al., 2009). The full roles of microRNAs in lymphocyte development have yet to be descerned.

CIITA regulation during B cell differentiation

The expression of MHC class II is detected as early as the B cell lineage which is specified in normal bone marrow (Benacerraf, 1981). It was shown that early B cell IA expression is required for B cell maturation (Miki et al., 1992). Using transgenic mice harboring the antisense DNA to IAβ chain gene showed that mouse B cell development was perturbed in spleen from transgenic mice if IA expression is reduced (Miki et al., 1992). During B cell differentiation, CIITA expression is increased and constitutively expressed in mature B cells stage; however, when B cells transition into plasma cells, MHC class II expression is lost due to loss of CIITA expression in plasma cells (Dellabona et al., 1989; Green et al., 2006; P. Silacci, 1994). Previously, somatic

cell hybridization of human B cells with murine plasmacytoma cells resulted in MHC-II repression (Dellabona et al., 1989). It was hypothesized that the suppression is due to a trans acting suppressor of mouse origin pre-existing in the plasmaocytoma cells. We now interpreted that CIITA silencing during B cell differentiation is due to a dominant repressor expressed in plasma cells. Blimp-1, which is essential for the terminal differentiation of B-lymphocytes into plasma cells was later identified as a direct CIITA repressor (Angelin-Duclos et al., 2000; Chen et al., 2007; Shaffer et al., 2002; Tooze et al., 2006), suggesting that Blimp-1 may have been the suppressor discovered in the above cell fusion experiment. Blimp-1 binds to CIITA promoter III in B cells and promoter IV in myeloma cells (Chen et al., 2007; Tooze et al., 2006). Also LSD-1, a histone demethylase, binds at CIITA pIII by interacting with Blimp-1 (Su et al., 2009). Fujita et al. found that BCL6 and MTA3 expression in the human plasma cell line H929 is sufficient to repress plasma cell specific genes, Blimp-1 and syndecan-1 (Fujita et al., 2004). Ectopic BCL6 and MTA3 expression in H929 induced CIITA. Moreover, recently it was shown that EZH2, which is part of the Polycomb repressive complex involved in gene silencing, mediated CIITA promoter IV silencing in uvenal cancer cells (Holling et al., 2007). It is possible that EZH2 is also involved in CIITA pIII repression in B-lymphocytes. However, other factors that might regulate CIITA silencing during B cell to plasma cell transition are not known.

Chromatin structure during B cell differentiation in CIITA gene

Epigenetic mechanisms alter gene expression patterns without changing DNA sequences. The CIITA gene is epigenetically regulated during B cell differentiation and in fetal trophoblasts (Green et al., 2006; Morris et al., 2002). Epigenetic regulation is mediated by post-translational histone modifications, DNA methylation, nucleosome positioning, and higher order chromosomal organization of the genome.

Histone modifications

The nucleosome is composed of a histone octamer containing an H3-H4 tetramer and two H2A-H2B dimers, which are wrapped around 146 bp of DNA (Bradbury et al., 1976; Moutschen, 1976) (Wu and Grunstein, 2000). The N-terminal tails of histones can be post-translationally modified to regulate gene expression by acetylation, phosphorylation, methylation, and ubiquitylation (Imhof and Wolffe, 1998; Van Hooser et al., 1998; Wolffe, 1998; Wolffe and Guschin, 2000) (Kouzarides, 2007; Lachner et al., 2003). Many of the enzymes that mediate these modifications have been identified (Sterner and Berger, 2000). Histone modifications predict the status of gene expression (Heintzman et al., 2007; Hon et al., 2009). Histone acetylation has been suggested to regulate gene activation by resulting in neutralization of lysine residues in histone N-terminal tails leading to an open chromatin structures (Turner, 2000). Histone acetylation at H3K9, K18, and H4K8 at transcriptional regulatory regions indicates transcriptional activation. H3K4^{me2} and H3K4^{me3} appear to be enriched in activated promoters and reflect chromosomal accessibility by other trans regulatory factors (Hon et al., 2009; Kouskouti and Talianidis, 2005). H3K4^{me1} is enriched at enhancers. H3K4^{me2} is indicative of the basal transcription level by positioning nucleosome in a broader pattern while H3K4^{me3} is located at core promoter region (Barski et al., 2007). H3K4 methylation can be removed by histone demethylases, such as LSD1/BHC110 or the Jarid1 subfamily of jumanji domain containing proteins (Okada et al., 2007; Shi et al., 2004). On the other hand, H3K9 and H3K27 methylation are hallmarks of heterochromatin and stable repression (reviewed in (Li et al., 2007)). The H3K9^{me2} is catalyzed by the G9a histone methyltransferase, which was found to interact with Blimp-1 to suppress IFN-β gene expression (Gyory et al., 2004). H3K27^{me3} is a mark that is catalyzed by components of the polycomb repressor complex, such as Ezh2. Holling et al. showed that EZH2 is involved in CIITA promoter IV specific silencing in melanoma cells, which displayed H3K27^{me3} histone modification (Holling et al., 2007; Margueron et al., 2008).

H2A.Z is a histone variant of H2A that is found preferentially at active promoter regions and associates with CTCF binding sites (Raisner et al., 2005) (Barski et al., 2007). H2A.Z has a different amino terminal sequence than H2A; thus, this may influence the nuclesome stability by affecting interactions with other histones and factors (Santisteban et al., 2000; Thakar et al., 2009).

Green et al. showed that all human CIITA promoters have open chromatin marks in B cell lines. However, plasma cell lines lost all active histone marks from CIITA promoters and displayed at least one repressive histone mark, such as $H3K9^{me2}$ (Green et al., 2006). Thus, CIITA regulation during B cell to plasma cell transition involves histone modifications to change chromatin accessibility to the regulatory region.

Chapter 1 examines the change in key histone marks in B cell and plasma cells in the mouse.

CIITA regulation by distal regulatory element

In addition to proximal regulatory elements, there are additional cis regulatory elements, such as enhancers, insulators, and locus control regions that can regulate gene expression (Spilianakis and Flavell, 2004). These elements contribute to gene expression from either upstream or downstream positions with respect to gene promoters (Halfon, 2006). Some elements may function over large distances. Recently, Ni et al. found distal regulatory elements that are critical for CIITA promoter IV regulation in IFN-γ induced human Hela cells (Ni et al., 2008). These elements interacted with each other and were dependent on the activity of BRG-1. STAT1 and USF1, transcription factors that are essential for pIV specific CIITA expression, were found to bind these elements as well. However, in B cells, distal cis regulatory elements that are involved in CIITA regulation have not been identified. Identification of novel cis-regulatory elements, which are critical for cell type or developmental specific CIITA expression, will be important to fully understand CIITA regulation.

One way in which distal elements could function is through direct interaction with the promoters (Majumder et al., 2008; Spilianakis and Flavell, 2004). Boss and Reith proposed a model for possible loop formation between distal regulatory elements identified from human Hela cells (Reith and Boss, 2008). IFN-γ stimulation increases the long distance interactions between distal regulatory elements to activate CIITA promoter IV dependent transcription. Without IFN-γ stimulation, some of long distance interactions were generated but were not stable (Figure 12) (Ni et al., 2008).

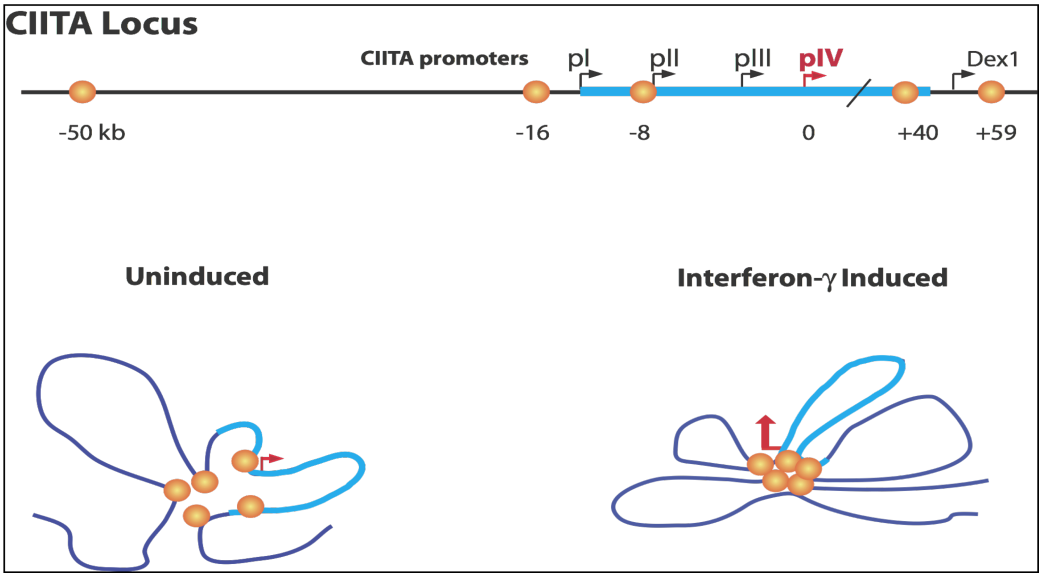


Figure 12. Model for long distance chromosomal looping between distal regulatory elements
A set of distal cis-regulatory elements is required for CIITA pIV-dependent transcription in response to IFN-γ stimulation by stabilizing the long distance interactions between distal regulatory elements (Boss and Reith, Nature immunology; Ni et al. 2008).

CTCF (CCCTC binding factor) is a 130kDa protein and highly conserved in eukaryotes (Filippova et al., 1996). CTCF, a chromatin insulator protein, is able to organize long distance chromosomal interactions (Dunn and Davie, 2003; Splinter et al., 2006). Corces et al. describe CTCF as a "master weaver of the genome", stressing the importance of CTCF in genome organization (Phillips and Corces, 2009). CTCF was first found as a c-myc gene binding protein

required for gene repression (Lobanenkov et al., 1990). Besides acting as a repressor, CTCF plays a role as an activator, enhancer blocker, and a heterochromatin barrier insulator (Filippova et al., 1996). CTCF is a key factor for cell development as well. CTCF knock out mice die during embryogenesis (Heath et al., 2008). In human and murine dendritic cells, CTCF is expressed, but down regulated during dendritic cell maturation (Koesters et al., 2007). CTCF interacts with a number of proteins including YY1, CHD8 (chromodomain helicase), and the cohesin complex (Rubio et al., 2008) (Donohoe et al., 2007; Ishihara et al., 2006). Chip-on- chip data revealed that CTCF and cohesin co-localized to more than 7813 out of 13, 894 CTCF sites in the human genomes (Wendt et al., 2008). Majumer et al. identified ten CTCF sites within the MHC class II locus and showed that CTCF is important for chromosomal organization for the expression of MHC class II molecules by interacting with CIITA bound to MHC class II genes (Majumder et al., 2008).

Summary

Understanding the process of B cell to plasma cell transition is critical to help design approaches to improve immune responses. As a major model of this event, we chose to investigate the mechanisms of CIITA and MHC class II silencing during B cell to plasma cell differentiation. In this thesis, we sought to study CIITA regulation in several ways: by identifying distal cis regulatory elements that regulate B cell expression; by determining the histone codes that process B cell to plasma cell transition at all CIITA promoters; and by identifying novel factors that silence CIITA in plasma cells. To this end, a distal cis regulatory element located 11kb upstream of the CIITA promoter III transcription start sites, was identified in B cells and termed HSS1. We found that PU.1 binds to HSS1, as well as CIITA promoter III. PU.1 was found to be required for long distance interactions between HSS1 and CIITA promoter III. Chromatin immunoprecipitations showed a reciprocal transition in H3K4 methylation when B cells were compared to plasma cells. Lastly, cDNA microarray experiments describe the gene expression

profiles that determined differentially or similarly expressed genes during B cell differentiation. ZBTB32 was identified as a major differentially expressed gene and found to function as a repressor of CIITA expression in differentiated plasma cells. ZBTB32 directly represses CIITA pIII dependent transcription by interacting at the promoter region and collaborates with other repressor factors including EZH2 and Blimp-1. ZBTB32 knock out mice supported our findings that ZBTB32 is required for CIITA silencing by delaying the repression of other B cell specific genes, such as BCL6. Therefore, we discovered that ZBTB32 is critical to initiate plasma cell differentiation.

In summary, this study will help to understand the genetic programming that is required for B cell to plasma cell differentiation and improve future immunotherapies that seek to control immune responses, such as those used for vaccination.

MATERIALS AND METHODS

Cells and culture

Cell line

The murine BCL1 3B3 B cell line (CRL-1669, American Tissue Type Culture (ATCC), Manassas, VA), P3X63Ag8.653 (referred to as P3X) plasma cell line (CRL-1580, ATCC), human B cell line Raji (CCL-86), and plasma cell line NCI-H929 (CRL-9068) cell lines were purchased from ATCC. The Burkitts lymphoma B cell line Raji was grown in RPMI 1640 medium supplemented with 5% FBS (HyClone Laboratories), 5% bovine calf serum (HyClone Laboratories). The plasmacytoma cell line H929 was cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol. Murine BCL1 cells and P3X63Ag8 plasma cells were grown in RPMI 1640 medium (Mediatech Inc., Manassas, VA) supplemented with 10% heat inactivated FBS (Hyclone Laboratory, Logan, VT), 10 mM HEPES (HyClone Laboratory), 1 mM sodium pyruvate (HyClone Laboratory), 1X non-essential amino acid (HyClone Laboratory), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Louis, MO). HEK293FT cells were cultured in DMEM with 10% heat inactivated FBS to produce lentivirus.

Primary B cells and plasmablast

C57BL/6 mice were purchased from the Jackson Laboratory and five-week old mice were used to obtain primary B cells from the spleen. Primary B cells were incubated with anti-CD43 and negatively selected using MACS paramagnetic beads and columns following the manufacturer's protocol (Miltenyi Biotech, Inc. Auburn, CA). To induce plasma cell differentiation, purified murine primary B cells were stimulated with culture media containing IL-2 (20 ng/ml, Sigma-Aldrich), IL-5 (10 ng/ml, Sigma-Aldrich) and LPS (20 μg/ml, Sigma-Aldrich) for the indicated time as previously described. Mice were housed in the Emory University School of Medicine

Facilities. All animal experiments were approved by the Emory University Institutional Animal Care and Use Committee.

To obtain primary plasmablasts from the spleen, Balb/c mice (6 per experiment) were infected by intraperitoneal injection with 2 X 10^5 plaque forming units of LCMV Armstrong strain. Eight days after infection, mice were euthanized and their spleens harvested. After homogenization and RBC lysis with ACK buffer (Gibco Inc.), splenocytes were suspended in DPBS with 2% FBS and 2 mM EDTA and stained with PE- or APC-labeled anti mouse CD138, PerCP-labeled anti mouse B220, and APC- or PE-labeled anti mouse CD44. All antibodies were purchased from BD Pharmingen. CD138-positive cells were enriched by magnetic bead positive selection using LS columns and anti-PE or anti APC-microbeads (Miltenyi Biotec). $CD138^{hi}$ $CD44^{hi}$ cells were then sorted by flow cytometry. Post sort purity was ~90-95% and approximately 5-10 million sorted cells were obtained per experiment.

Assessment of plasma cell purity by ELISPOT

ELISPOT analyses were performed essentially as described. Multiscreen HA plates (Millipore Corporation) were coated with goat anti-mouse IgG/IgA/IgM capture antibody (0.25 μg/well) (Caltag) and blocked with 10% FCS in PBS. Three fold serial dilutions of sorted plasma cells were seeded into each well in growth medium (RPMI 1640 with 10% FCS, 10 mM HEPES, 5.5 μM β-mercaptoethanol, and 100 U/ml penicillin-streptomycin). Plates were incubated 6-8 hours at 37°C in a 5% CO_2 atmosphere. After washing, foci of antibody secretion were detected with anti IgG-biotin or anti IgM-biotin (Southern Biotech) and Avidin-HRP (Vector Laboratories), using chromogen 3-amino-9-ethylcarbazole (AEC) substrate (Sigma Aldrich). Spots were counted manually and divided by the number of cells seeded to determine the proportion of antibody secreting cells in the sorted population.

DNase I hypersensitivity assay

DNase I hypersensitivity assay was performed as described by Lu et al. Here, 2 x 10^7 cells were used to prepare nuclei. Preparations of nuclei were treated with increasing concentrations of DNase I (Roche Ltd, Indianapolis, IN). The DNA was then purified and digested to completion with *Eco*RV and *Bam*HI (NEB, Inc. Beverly, MA). Nuclease sensitivity for the *CIITA* locus was determined by Southern blotting using radiolabeled probes and visualized by autoradiography. The probe for *CIITA* was derived from sequence positions 10,471,913 to 10,472,354 on chromosome 16 as described by the University California Santa Cruz Genome Browser database (http://genome.ucsc.edu/ (Kent et al., 2002)). A PCR-based DNase I hypersensitivity assay was based on that of Oestreich et al. (Oestreich et al., 2008). In this assay, nuclei from BCL1 and P3X cells were isolated and treated with 0, 40, 80, or 160 u/ml of DNase I for 3 minutes at 25 °C. The reaction was stopped and the DNA purified. DNA from the DNase I treated nuclei was PCR amplified using a set of primers that spanned HSS1, pI, pIII, and pIV (Y9, Y11, Y19, and Y22, respectively). Detection and quantitation of these amplicons was carried out by real-time PCR. A relative hypersensitivity was compared to the results obtained for an insensitive region (Y6). The data from at least three biological replicates were normalized to Y6 for each concentration of DNase I used and then plotted as fold over the untreated sample.

PCR Primer Sequences

All PCR primer sequences are provided in each chapter.

RNA isolation and RT-real time PCR

Total RNA was extracted from the indicated cells using RNeasy mini prep kits (Qiagen, Inc., Valencia, CA). Reverse transcriptase (Invitrogen, Inc., Carlsbad, CA) was used to produce cDNA according to the manufacturer's directions. SYBR green dye incorporation into the PCR product using ~1/100th of the cDNA as a template was measured by an iCycler with optical assembly (Biorad, Inc., Hercules, CA). Parallel RT-PCR reactions were performed with 18s

rRNA primers to normalize between samples. Following 18s rRNA normalization, the average of three biological replicates were plotted relative to the indicated control sample.

cDNA microarray analysis

RNA was prepared from purified murine primary B cells that were untreated and treated as described above with IL-2, IL-5, and LPS for 1 day. This RNA was submitted to the Emory Winship Cancer Institute DNA microarray core facility to conduct cDNA microarray analysis. The Applied Biosystems Mouse Genome Survey Microarray containing ~33,000 probes was used. Single color microarray technology was used to measure the absolute level of RNA. Two biological replicates were used. The data were analyzed using SAM (significant analysis of microarray) software to generate significant difference. Clustering algorithms by average linkage were performed to identify expression patterns within data. Genes with 2-fold change in expression are presented in Supplemental Table 1 (Chapter 2). The full dataset has been deposited into and will be available on the NCBI GEO database (Accession number GSE17999) following acceptance.

Chromatin immunoprecipitations (ChIP)

ChIP assays were conducted as previously described. 4×10^7 cells were crosslinked with formaldehyde (1%) for 15 min at room temperature. Cross-linked chromatin was purified and sonicated to generate an average DNA size of 600 bp. The crosslinked, sonicated chromatin (30 μg) was used for immunoprecipitations with the indicated antibody. The company and catalog number for each antibody used are listed in each chapter. DNA was extracted by phenol-chloroform and ethanol precipitated after reversing the cross-links. One tenth of immunoprecipitated sample was employed for quantitative real-time PCR using SYBR green incorporation for the *CIITA* sequence of interest. A standard curve using sonicated murine genomic DNA was used to determine the relative amount of DNA in each sample. The average

of at least three experiments was normalized to an irrelevant antibody control (TCR) for each amplicon and plotted as fold over the indicated control sample. Antibodies were purchased from a variety of sources as follows, and indicated in the each chapter.

BAC modification, transfection, and analysis

The protocol for BAC modification followed that described previously(Gong et al., 2002; Jeong and Epstein, 2005). The BAC encoding *CIITA* (RP23-240H17) was purchased from Children's Hospital Oakland Research Institute (CHORI, Oakland, CA). The shuttle vector (pLD53SCA-E-B), which was used for introduction of BAC modification, was obtained from Dr. N. Heniz (Rockefeller University). The shuttle vector has an R6Kγ origin, *RecA* gene, *SacB* gene, and an ampicillin resistant gene. For deleting HSS1, the *CIITA* sequences between chr 16: 10,475,871 - 10,476,371 (UCSC genome Bioinformatics) and chr16: 10,477,371 - 10,477,870 were cloned into the *Asc*I and *Pac*I restriction sites of the shuttle vector. Similarly, for deleting pIII, chr 16: 10,487,673 - 10,488,074 and 10,488,317 - 10,488,691 were cloned into the same sites. These sequences represent the homologous targeting arms surrounding the sequences to be deleted. To generate homologous recombination events, BAC containing host bacteria were made competent and transformed with the shuttle vector by electroporation using Bio-Rad Gene Pulser. The cells are selected and grown in 1 ml of LB medium containing ampicilin (50 μg/ml) and chloramphenicol (15 μg/ml). The culture was diluted 1:1000 and incubated for 16 h at 37°C. The culture was again diluted 1:5000 and incubated for an additional 8 h at 37°C. Cells were diluted and spread onto LB plates containing the above antibiotics. Colonies were analyzed for integration of the targeting shuttle vector into the BAC by PCR. To resolve the recombinants (i.e., remove the shuttle vector DNA), co-integrated BAC containing bacteria were inoculated into Luria broth cultures supplemented with chloramphenicol (15 μg/ml) for 1 h at 37°C and plated on plates containing chloramphenicol and 5% sucrose. Colonies were screened by PCR

for the resolved BAC and extensive restriction enzyme digestion was carried out to ensure that the recombination and resolution processes affected only the region mutated.

BAC DNAs were transfected into the human *CIITA* mutant cell line RJ2.2.5 by nucleofection using an Amaxa Inc. Nucleoportator with transfection kit V according to the manufacturer's protocols. In each transfection, approximately 2 X 10^6 cells and 8 μg of DNA were used. At 48 h post transfection, RNA was isolated using RNAeasy kits (Qiagen, Inc). Real-time RT-PCR was used to analyze the murine, BAC-encoded *CIITA* transcripts using primers specific for each CIITA isotype (Supplemental Table I). Transcripts were normalized to 18s rRNA levels. Transfection efficiency was monitored by examining the levels of the *NuBP1* mRNA transcript encoded within the *CIITA* region BAC used. As variation of the *NuBP1* transcript was ~1% between samples, no additional normalization was conducted. The data were averaged from three independent transfections and plotted with standard error. A student T test was used to determine whether the differences observed were significant.

Plasmids and transfection

Myc tagged *ZBTB32* and Flag tagged EZH2, RFX5 and Blimp-1 were cloned into the pcDNA 3.1(-) expression vector directly from PCR amplified cDNA from P3X63Ag8 or Raji (RFX5). Other expression plasmids, which were used in transient transfection, were purchased from OriGENE Technologies, Inc.: *Asfb* (SC113719), *CBX2* (MC204062), *HMGN3* (SC110044), *Rad54* (MC205395), *SATB1* (MC200989), *SglII* (MC 205210), *TfDP1* (SC127935). 2X10^6 of log phase growing Raji cells were transfected using an Amaxa Nucleofector following manufacturer's protocol.

Reporter assay

The pGL3 promoter (Promega) containing the firefly reporter gene was used in the reporter transcriptional assay. Chicken β-globin 5'HS4 region, CTCF binding sequence 1 , and 2 were

inserted upstream of the promoter and downstream of enhancer. Renilla luciferase vector, which is constitutively expressed, were co-transcfected for normalizaton. Raji cells were transfected using electrophoresion. After 24hour post transfection, promoter activity was tested following by manufacture's protocol.

RNAi

Fo ShRNA specific to PU.1, a series of lentivirus PU.1 shRNA constructs was purchased from Open Biosystem, Inc. (catalog number; RMM4534, Clone ID; TRCN0000009497, TRCN0000009498, TRCN0000009499, TRCN0000009500, TRCN0000009501). These lentivus constructs are based on the base vector pLKO.1.1, allowing for selection by puromycin. HEK 293FT packaging cells were used as the host for transfection of the lentivirus constructs with the pseudo envelope protein VSV-G using FuGENE 6 cationic liposomes (Roche Ltd.). After 48 h and 72 h post transfection, virus was harvested and used to infect murine B cell line BCL1 in the presence of polybrene (0.8 μg/ml). The efficiency of silencing of PU.1 was assessed by western blot as indicated.

For shRNA to ZBTB32 experiment, lentivirus constructs (pLKO.1) harboring shRNA to specific *ZBTB32* (cat. RMM4534-NM_021397; clone number, TRCN0000096484, TRCN0000096485, TRCN0000096486, TRCN0000096487, TRCN0000096488) were purchased from Open Biosystems. For primary B cell experiments, five different lentivirus that express shRNA specific to *ZBTB32* were infected into purified murine primary B cells, cells were then treated with IL-2, IL-5, and LPS to induce plasma cell differentiation for five days. To deplete *ZBTB32* in the plasma cell line P3X63Ag8, lentivirus expressing shRNA to *ZBTB32* was used, and cells were selected using puromycin for 6 days encoded in the pLKO.1 vector.

Flag tagged *ZBTB32* plasmid was cloned into YFP expressing lentivirus vector (pHR-UBQ-IRES-eYFP). These lentivirus constructs were co-transfected with the pseudo envelope protein VSV-G gene into HEK293FT packaging cells using FuGENE 6 transfection reagent (Roche). At

48 and 72 hours post transfection, virus was harvested, and infected into purified primary murine B cells in the presence of 8 µg/ml polybrene (Sigma Aldrich). Infection efficiency was determined by YFP expression and western blot using Flag antibody.

Flow cytometry

Flow cytometry was performed following standard protocols using a FACSCalibur. Briefly, $1X10^6$ control or IL-2, IL-5, and LPS stimulated primary B cells and lentivirus infected BCL1 cells or P3x cell after puromycine selection with 1mg/ml and 4 mg/ml for 5 day and 6 days, respectively washed with PBS containing 0.1% BSA, incubated with PE conjugated IA/E or CD138 (syndecan-1) antibodies (BD Pharmingen) for 30 min on ice and washed again in PBS containing 0.1% BSA. Flow cytometry data was analyzed using CellQuest. All experiments were performed at least three times from independently isolated and purified cells.

Chromatin conformation capture (3C) assays

The 3C assays were performed essentially as described (Majumder et al., 2008; Miele et al., 2006). Briefly, $1X10^7$ cells were crosslinked with 2% formaldehyde for 10 min. The reaction was quenched with glycine (0.125 M). Cells were lysed in 10 mM Tris pH8, 10 mM NaCl, and 0.2 % NP-40 followed by 15 strokes using a dounce homogenizer. The resulting nuclei were washed in NEB2 restriction enzyme buffer, resuspended in the same buffer containing SDS (0.3 %), and incubated for 1 h at 37°C. To sequester SDS, 2 % Triton X-100 was added, and incubated for 1 h at 37°C. 400 U *Hin*dIII was added and incubated overnight at 37°C. *Hin*dIII was inactivated with SDS (1.6 %) and incubating for 25 min at 68°C. The samples were diluted 40 fold in ligation buffer (30 mM Tris, 10 mM MgCl, 1% triton X-100, and 0.1 M ATP) and ligated with 200 U T4 DNA ligase overnight at 16°C. The crosslinks within 3C library products were reversed and the DNA purified by overnight treatment with proteinase K at 65°C as per assay protocol. Quantitative real-time PCR using a standard curve was conducted to measure the

frequency of the 3C products within each sample. Standard curves for 3C assays were generated using a *CIITA* encoding BAC (RP23-240H17) that was *Hin*dIII digested and then religated to generate all possible 3C products within the locus. A 3C frequency was determined by averaging the amount of 3C product produced for a given amplicon (from three independent experiments) and dividing that value by the amount of 3C product determined for an irrelevant restriction fragment that does not interact. In this case, the pIII-H1 3C product served as the background control.

Coimmunoprecipitation and western blot

Flag-or Myc-tagged ZBTB32, Blimp-1, EZH2, and RFX5 expression vectors were transiently transfected into HEK293T cells for 2 days. Whole cell lysates were prepared from each transfection. Following the manufacturer's protocol, antibodies were pre-incubated with magnetic beads (Invitrogen Corporation) overnight. Protein lysates were immunoprecipitated with antibody pre-bound beads for 3 hour at 4°C. After incubation, the beads were washed in 50 mM Tris (pH. 8), 150 mM NaCl, and 1% NP-40, and eluted with SDS-PAGE loading buffer. Immunoblots were performed to determine protein precipitation. Human plasma cell line H929 nuclear extracts were prepared as described previously (Fujita et al., 2004). Nuclear extract (250 µg) was incubated with specific antibodies for 1 hour on ice, and added 40 µl protein A sepharose following 3 hour incubation at 4°C. Precipitates were loaded in SDS-PAGE and immunoblotting was conducted with the indicated antibodies.

Immunofluorescence staining

Murine plasma cell line, P3X63Ag8 and ex vivo differentiated primary B cells were washed with PBS, and incubated with poly-L-lysine coated cover slips for 1 hour at room temperature. Cells then were fixed with 2% paraformaldehyde for 15 min at room temperature, and blocked with 1% BSA, 0.05% saponin (Sigma Aldrich) in PBS for 30 min. After blocking, cells were incubated

with goat anti-Blimp-1 and rabbit anti-ZBTB32 antisera in blocking solution overnight at 4°C. Cells were washed with 0.05% saponin/PBS four times, and incubated with Alexa fluor 488 for anti-goat IgG and Alexa fluor 594 for anti-rabbit IgG for 1 hour at room temperature. The cover slips were mounted with mounting medium (Vector Laboratory), which contains DAPI. The images were analyzed by a confocal microscope (Olympus inverted 1X81 microscope/Olympus Fluoview FV1000) and visualized using the FV10-ASW version 1.7b program.

II. STUDIES

The following chapters consist of manuscripts that have either been published or will be published. Chapter I described the identification of distal regulatory element that is required for CIITA activation in B cells. Chapter 2 explained the identification of regulatory factors that repress CIITA expression during B cell to plasma cell differentiation.

Chapter: 1

Hyesuk Yoon, Jeremy M. Boss. (2010)
PU.1 binds to a distal regulatory element that is necessary for B-cell specific expression on the class II transactivator.
In press

This paper was written by Hye Suk Yoon and edited by Dr. Jeremy Boss.

PU.1 binds to a distal regulatory element that is necessary for B-cell specific expression of the class II transactivator[1]

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Running title: HSS1 and PU.1 regulate *CIITA* expression in B cells

Abstract

The transcriptional coactivator *CIITA* regulates MHC class II genes. In the mouse, *CIITA* is expressed from three distinct promoters (pI, pIII, and pIV) in a developmental and cell type specific manner with pIII being responsible for B lymphocyte specific expression. Although the promoter proximal sequences that regulate *CIITA* in B cells have been described, nothing is known about additional distal elements that may regulate its expression in B cells. Sequence homology comparisons, DNase I hypersensitivity assays, and histone modification analysis revealed a potential regulatory element located 11 kb upstream of pIII. Deletion of this element, termed HSS1, in a bacterial artificial chromosome (BAC) encoding the entire *CIITA* locus and surrounding genes, resulted in a complete loss of *CIITA* expression from the BAC following transfection into B cells. HSS1 and pIII displayed open chromatin architecture features in B cell but not in plasma cell lines, which are silenced for *CIITA* expression. PU.1 was found to bind HSS1 and pIII in B cells but not in plasma cells. Depletion of PU.1 by shRNA reduced CIITA expression. Chromatin conformation capture assays showed that HSS1 interacted directly with pIII in B cells and that PU.1 was important for this interaction. These results provide evidence that HSS1 is required for B-cell specific expression of *CIITA*, and that HSS1 functions by interacting with pIII, forming a long distance chromatin loop that is part mediated through PU.1.

Introduction

Major histocompatibility complex class II (MHC-II) genes encode proteins that present peptide antigens to CD4+ T lymphocytes, a process that results in the initiation of adaptive immune responses (1, 2). The class II transactivator (CIITA) plays an essential role in this process by serving as the limiting transcription factor controlling MHC-II gene expression (3). Mutations in *CIITA* are a cause of bare lymphocyte syndrome, an inherited immunodeficiency in which MHC-II gene expression is absent (4, 5). *CIITA* is constitutively expressed in antigen presenting cells, such as B cells, macrophages, and dendritic cells (6). *CIITA* and therefore MHC-II expression can be induced by IFN-γ treatment in most cell types (7). In the mouse, three distinct promoters control *CIITA* expression: promoters I, III, and IV (8). Each promoter specifies the transcription of an unique exon 1, which is ultimately spliced into a downstream common exon, resulting in the production of distinct CIITA isoforms. The three *CIITA* promoters are activated in a cell type or cytokine dependent manner. Promoters I and III are utilized for the constitutive *CIITA* expression in myeloid cells and lymphoid cells, respectively (6, 8-10). Promoter IV directs *CIITA* expression in response to IFN-γ stimulation in non-lymphoid cells (9).

In B cells, *CIITA* is constitutively expressed and associates with all MHC-II promoters, recruiting histone acetyltransferases (HATs) and chromatin remodeling proteins to activate MHC-II transcription (3, 11, 12). CIITA also associates with the insulator protein CTCF to form long-range interactions with at least some human MHC-II promoter regions (13). However, when B cells terminally differentiate into plasma cells, *CIITA* expression is silenced, ultimately resulting in the loss of MHC-II expression (14, 15). We previously showed that during this transition histone modifications associated with active transcription were lost and replaced by at least one mark associated with gene silencing (15). Coupling these observations with those that show that the *CIITA* pIV DNA is methylated in cell types that are refractory to the induction of *CIITA* by IFN-γ (16-18), suggest that the locus has the potential for epigenetic regulation.

Five cis-regulatory elements located between -545 bp to -113 bp of the pIII transcription initiation site were identified in B cells (8, 19). These elements, termed ARE1, ARE2, site A, site B, and site C, are occupied by the transcription factors E47, PU.1/IRF1/IRF4, SP1, CREB/CBP, and Oct1 (15, 20, 21). Site C binds PU.1, and PU.1 was found to be essential for B cell specific *CIITA* expression (15, 21). Following differentiation to plasma cells, the occupancy of these sites by the above activators is lost and the master repressor Blimp-1 binds to this region (22, 23). The histone H3 lysine 4 demethylase LSD-1 also associates with this region and removes activation associated histone modifications (23). These latter events are likely critical to the silencing of *CIITA* pIII during the plasma cell differentiation process.

PU.1 (SFFV proviral integration 1) is a member of the ETS domain transcription factor family and is encoded by the PU.1-Spil-Sfpil proto-oncogene (24, 25). PU.1 expression is required for the development of common lymphoid and myeloid progenitors that give rise to B cells, T cells, natural killer cells, and macrophage (26-31). Targeted disruption of PU.1 in the mouse resulted in neonatal lethality (32). As a sequence-specific transcription factor, PU.1 binds at promoters and lymphoid specific enhancer regions either by itself or interacting with other factors, like IRF4, to regulate target genes (33-35). Although PU.1 is required for *CIITA* expression, it is not known how it may coordinate interactions and activate *CIITA* expression.

The regulation of each *CIITA* promoter (pI, pIII, and pIV) by their proximal promoter regulatory regions has been well studied (8, 16, 20, 21). In contrast, little is known about whether there are other distal cis-regulatory elements, which are required for *CIITA* regulation. Recently, Ni et al. described a series of distal elements within the *CIITA* gene that were responsive to IFN-γ and regulated expression through pIV (36). These results suggest the possibility that distal elements may be required for B-cell specific expression as well. To address this issue, experiments were designed to identify novel distal elements that could regulate *CIITA* in B cells. One element that was identified and termed HSS1 was required for B-cell specific *CIITA* expression through pIII. HSS1 was occupied by PU.1 in B cells but not in plasma cells. The

mechanism of action of both PU.1 and HSS1 was found to involve the ability of HSS1 to interact directly with the pIII promoter region through a long-distance chromatin loop. Intriguingly, this loop was in part dependent on PU.1 expression and was significantly reduced in plasma cells, suggesting that PU.1 plays a role in loop formation and that maximum loop formation is necessary for the high levels of *CIITA* found in B cells. Together, these results suggest that multiple elements are required to control *CIITA* expression in B cells and provide these cells with the ability to present antigens.

Materials and Methods

Cells and culture

The murine BCL1 3B3 B cell line (CRL-1669, American Tissue Type Culture (ATCC), Manassas, VA) and P3X63Ag8.653 (referred to as P3X) plasma cell line (CRL-1580, ATCC) were cultured in RPMI 1640 medium (Mediatech Inc., Manassas, VA) supplemented with 10% heat inactivated FBS (Hyclone Laboratory, Logan, VT), 10 mM HEPES (HyClone Laboratory), 1 mM sodium pyruvate (HyClone Laboratory), 1X non-essential amino acid (HyClone Laboratory), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Louis, MO). C57BL/6 mice were purchased from the Jackson Laboratory and five-week old mice were used to obtain primary B cells from the spleen. Primary B cells were incubated with anti-CD43 and negatively selected using MACS paramagnetic beads and columns following the manufacturer's protocol (Miltenyi Biotech, Inc. Auburn, CA). To induce plasma cell differentiation, purified murine primary B cells were stimulated with culture media containing IL-2 (20 ng/ml, Sigma-Aldrich), IL-5 (10 ng/ml, Sigma-Aldrich) and LPS (20 μg/ml, Sigma-Aldrich) for the indicated time as previously described (15).

DNase I hypersensitivity assay

DNase I hypersensitivity assay was performed as described by Lu et al. (37). Here, 2 x 10^7 cells were used to prepare nuclei. Preparations of nuclei were treated with increasing concentrations of DNase I (Roche Ltd, Indianapolis, IN). The DNA was then purified and digested to completion with *Eco*RV and *Bam*HI (NEB, Inc. Beverly, MA). Nuclease sensitivity for the *CIITA* locus was determined by Southern blotting using radiolabeled probes and visualized by autoradiography. The probe for *CIITA* was derived from sequence positions 10,471,913 to 10,472,354 on chromosome 16 as described by the University California Santa Cruz Genome Browser database (http://genome.ucsc.edu/ (38)). A PCR-based DNase I hypersensitivity assay was based on that of Oestreich et al. (39). In this assay, nuclei from BCL1 and P3X cells were isolated and treated

with 0, 40, 80, or 160 u/ml of DNase I for 3 minutes at 25 °C. The reaction was stopped and the DNA purified. DNA from the DNase I treated nuclei was PCR amplified using a set of primers that spanned HSS1, pI, pIII, and pIV (Y9, Y11, Y19, and Y22, respectively). Detection and quantitation of these amplicons was carried out by real-time PCR. A relative hypersensitivity was compared to the results obtained for an insensitive region (Y6). The data from at least three biological replicates were normalized to Y6 for each concentration of DNase I used and then plotted as fold over the untreated sample.

PCR Primer Sequences

All PCR primer sequences are provided in Supplemental Table 1.

RNA isolation and RT-real time PCR

Total RNA was extracted from the indicated cells using RNeasy mini prep kits (Qiagen, Inc., Valencia, CA). Reverse transcriptase (Invitrogen, Inc., Carlsbad, CA) was used to produce cDNA according to the manufacturer's directions. SYBR green dye incorporation into the PCR product using ~1/100th of the cDNA as a template was measured by an iCycler with optical assembly (Biorad, Inc., Hercules, CA). Parallel RT-PCR reactions were performed with 18s rRNA primers to normalize between samples. Following 18s rRNA normalization, the average of three biological replicates were plotted relative to the indicated control sample.

Chromatin immunoprecipitations (ChIP)

ChIP assays were conducted as previously described (11). 4 x 10^7 cells were crosslinked with formaldehyde (1%) for 15 min at room temperature. Cross-linked chromatin was purified and sonicated to generate an average DNA size of 600 bp. The crosslinked, sonicated chromatin (30 μg) was used for immunoprecipitations with the indicated antibody. The company and catalog number for each antibody used are listed in Supplemental Table 2. DNA was extracted by

phenol-chloroform and ethanol precipitated after reversing the crosslinks. One tenth of immunoprecipitated sample was employed for quantitative real-time PCR using SYBR green incorporation for the *CIITA* sequence of interest. A standard curve using sonicated murine genomic DNA was used to determine the relative amount of DNA in each sample. The average of at least three experiments was normalized to an irrelevant antibody control (TCR) for each amplicon and plotted as fold over the indicated control sample.

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with chloramphenicol (15 μg/ml) for 1 h at 37°C and plated on plates containing chloramphenicol and 5% sucrose. Colonies were screened by PCR for the resolved BAC and extensive restriction enzyme digestion was carried out to ensure that the recombination and resolution processes affected only the region mutated.

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RNAi and Flow Cytometry

A series of lentivirus PU.1 shRNA constructs was purchased from Open Biosystem, Inc. (catalog number; RMM4534, Clone ID; TRCN0000009497, TRCN0000009498, TRCN0000009499, TRCN0000009500, TRCN0000009501). These lentivus constructs are based on the base vector pLKO.1.1, allowing for selection by puromycin. HEK 293FT packaging cells were used as the host for transfection of the lentivirus constructs with the pseudo envelope protein VSV-G using FuGENE 6 cationic liposomes (Roche Ltd.). After 48 h and 72 h post transfection, virus was harvested and used to infect murine B cell line BCL1 in the presence of polybrene (0.8 μg/ml). The efficiency of silencing of PU.1 was assessed by western blot as indicated.

Lentivirus infected BCL1 B cells were grown in the presence of puromycin (1 mg/ml) for 5 days. $1x10^6$ cells were collected, washed with PBS containing 0.1% BSA and stained with phycoerytherin conjugated IA/E antibody for 30 min on ice. Samples were analyzed on a FACScalibur and the data analyzed by CellQuest and Flowjo 8.8.6 software.

Chromatin conformation capture (3C) assays

The 3C assays were performed essentially as described (13, 42). Briefly, $1X10^7$ cells were crosslinked with 2% formaldehyde for 10 min. The reaction was quenched with glycine (0.125 M). Cells were lysed in 10 mM Tris pH8, 10 mM NaCl, and 0.2 % NP-40 followed by 15 strokes using a dounce homogenizer. The resulting nuclei were washed in NEB2 restriction enzyme buffer, resuspended in the same buffer containing SDS (0.3 %), and incubated for 1 h at 37°C. To sequester SDS, 2 % Triton X-100 was added, and incubated for 1 h at 37°C. 400 U *Hin*dIII was added and incubated overnight at 37°C. *Hin*dIII was inactivated with SDS (1.6 %) and incubating for 25 min at 68°C. The samples were diluted 40 fold in ligation buffer (30 mM Tris, 10 mM MgCl, 1% triton X-100, and 0.1 M ATP) and ligated with 200 U T4 DNA ligase overnight at 16°C. The crosslinks within 3C library products were reversed and the DNA purified by overnight treatment with proteinase K at 65°C as per assay protocol (13, 42). Quantitative real-time PCR using a standard curve was conducted to measure the frequency of the 3C products within each sample. Standard curves for 3C assays were generated using a *CIITA* encoding BAC (RP23-240H17) that was *Hin*dIII digested and then religated to generate all possible 3C products within the locus. A 3C frequency was determined by averaging the amount of 3C product produced for a given amplicon (from three independent experiments) and dividing that value by the amount of 3C product determined for an irrelevant restriction fragment that does not interact. In this case, the pIII-H1 3C product served as the background control.

Results

A conserved sequence located -11 kb upstream of pIII is hypersensitive to DNase I

To date, only the promoter proximal sequences (within 350 bp) of *CIITA* pIII have been shown to regulate B-cell specific *CIITA* expression. To identify additional regions, a series of general assays were conducted on the sequences upstream of pIII. The sensitivity of chromatin to DNase I has been found to be associated with regulatory regions (43). Thus, as a first approach DNase I hypersensitivity assays were employed on the murine B cell line BCL1 and the plasma cell line P3X, which represent *CIITA* expressing and silenced cell lines, respectively. In BCL1 cells, a hypersensitive site appeared at ~ 11 kb upstream of pIII (Figure 1A). A strong DNase I hypersensitive site was also observed at -13 kb from pIII, but only in the *CIITA* negative P3X plasma cell line (Figure 1A). Thus, this region appears to be differentially accessible to DNase I, suggesting that it has some regulatory function. The -11 kb site specific to B cells was termed hypersensitive site 1 or HSS1 and the -13 kb site HSS2.

A PCR-based DNase I hypersensitivity assay was used to assess HSS1 in primary murine splenic B cells (Figure 1B). Here, HSS1 was sensitive to increasing concentrations of DNase I in both primary murine B cells and BCL1 cells (Figure 1B). Promoter III also displayed strong DNase I hypersensitivity in the primary cells. Promoters I and IV were not sensitive to DNase I in these cells, thereby providing specificity controls. A region located -14 kb from pIII (represented by primer set Y6) was used as a control and showed no changes in DNase I hypersensitivity in BCL1 or primary B cells (data not shown).

Cross species genomic DNA comparisons using the University of California Santa Cruz Genome Browser tools, found that HSS1 was highly conserved while HSS2 showed only low levels of sequence conservation (Figure 1C). No microRNA sequences or other coding/non-coding sequence features were found within these regions. In human HeLa cells, sequences within HSS1 region were recently shown to participate in IFN-γ induction through pIV (36),

suggesting that this region may function as a complex regulatory component and may contribute to B-cell specific expression of *CIITA*. Thus, its characterization and function were investigated further. Southern blot based DNase I hypersensitivity assays were also performed for the region between pI and pIII. A hypersensitive site was observed ~2 kb 3' to pI in both BCL1 and P3X cells. Because this region was not differentially sensitive to DNase I, it was not explored further.

Chromatin modifications at HSS1 reveal an accessible and active nucleosome structure

To further characterize this element and to determine if other potential regulatory regions were present within this upstream segment of the *CIITA* gene, a series of chromatin immunoprecipitations (ChIP) assays using antibodies to the acetylated forms of histones H3 and H4 were conducted. These modifications are typically associated with regulatory regions and expressed genes (44). Here, we sought to find regions that were differentially modified between B cells (BCL1) and plasma cells (P3X) as such regions could indicate a putative role in B-cell meditated expression. Twenty-three amplicons spaced at ~1 kb intervals, spanning the region between pIV and -18 kb upstream of pIII were used to analyze precipitated chromatin. The regions corresponding to pIII and pIV showed 47-107-fold levels of histone H3 acetylation over the control antibody in BCL1 B cells that was significantly less in the P3X plasma cell line (Figure 2). This result was similar to that observed for human B and plasma cell lines (15). Promoter I displayed H3 acetylation levels that were 9-fold over control but not differentially modulated between the two cell lines. HSS1 displayed levels of acetylated histone H3 that were 25- and 6-fold over the control when comparing the B cell and plasma cell lines, respectively, suggesting that histone H3 acetylation of this region was enhanced in B-cells. Similarly, pIII and pIV showed high levels of histone H4 acetylation in BCL1 cells but not in P3X cells. Compared with pIII and pIV, pI showed significantly lower levels of histone H4 acetylation that did not change between the B and plasma cell lines. In agreement with the histone H3 ChIP result, HSS1 also showed high levels of H4 acetylation in B cells (175-fold over control) and lower levels in

plasma cells (29-fold). The sequences extending upstream of HSS1 to -13 kb (HSS2), showed histone H4 acetylation levels between 54 and 98-fold over control; although the differences between B and plasma cells was not more than 2 fold.

Analysis of activation/silencing associated chromatin modifications for select regions can provide a fundamental assessment of the general chromatin architecture of a region (accessibility), as well as the general gene expression activity of a region (active/inactive) (45). Additional ChIP assays were conducted on HSS1 and comparisons were made between the three promoter regions and a control region, Y4 (Figure 3). Y4 (-16 kb from pIII) represents a region with no known regulatory or promoter function. Four histone acetylation modifications were assessed: H3K9, H3K18, H4K8, and H4K16 (Figure 3). Nucleosomes at pIII were highly acetylated at histone H3K9, H3K18, and H4K8 (218 to 1600-fold over control) in BCL1 cells and substantially less in the P3X cells. Each of these marks was therefore associated with active transcription of *CIITA*. Although not at the level of pIII, HSS1 showed differential levels (BCL1 vs. P3X cells) for these three modifications as well. Promoter I showed acetylation of H3K18 and H4K8; whereas pIV displayed H3K9, H3K18, and H4K8 acetylation modifications. However, pI and pIV modifications were not indicative of *CIITA* expression, suggesting that they may represent a general feature of the region in the lymphocyte lineage. None of the regions displayed histone H4 K16 acetylation levels that were greater than the control region Y4.

The degree of methylation of H3 K4 is associated with gene transcription or poised RNA polymerase binding (46). Histone H3 K4 monomethylation has been associated with enhancer activity and dimethylation with gene expression and accessibility; whereas trimethylation of this residue is associated with RNA polymerase binding and active transcription (46-48). All three configurations of histone H3 lysine methylation were observed at HSS1 in B cells and were reduced in plasma cells (Figure 3). Intriguingly, pI also displayed strong H3K4^{me1} at this residue in B cells; whereas this modification appeared at high levels at pIII and pIV in plasma cells. In contrast, H3K4^{me2} and K4^{me3} levels showed a direct relationship with *CIITA* expression.

Promoter III showed the highest levels of these marks as anticipated as this promoter is predominantly used in B cells. Promoter IV also showed these two active marks, as some transcription from pIV is observed in B cells (data not shown). Promoter I did not display H3K4^{me2} or K4^{me3} modifications, suggesting that this promoter region is not transcriptionally active in BCL1 cells. RT-PCR for pI in BCL1 cells showed no detectable transcripts (data not shown). The histone variant H2A.Z, which is often associated with active promoter regions (49), was observed at the highest levels in BCL1 cells at pIII and HSS1 and at lower levels at the other sites.

Repressive chromatin modifications H3K27^{me2} and K27^{me3}, which are associated with the Polycomb repressor complex, were examined (Figure 3). Low levels of H3K27^{me2} were associated with pI and possibly with HSS1 in BCL1 cells. By contrast, P3X plasma cells showed high levels of H3K27^{me3} associated with all three promoter regions but not HSS1. Thus, the three promoters display histone code patterns that are mostly associated with gene expression, and in general, the histone modification patterns of HSS1 also follow the transcriptional state of the locus in B cells.

HSS1 is required for CIITA expression

The above data suggest the possibility that HSS1 may function in the regulation of *CIITA* in B cells. To examine the regulatory potential of HSS1, we employed a bacterial artificial chromosome (BAC) modification system (40, 41) that would allow the introduction of a mutation into the *CIITA* gene and test its effect on controlling *CIITA* expression in the context of the locus. Thus, using the BAC system, the effect of deleting HSS1 on the expression of *CIITA* from each of the promoters can be assessed. The BAC (RP23-240H17) encodes the murine *CIITA* locus with 88,771 bp upstream of pIII and 51,386 bp downstream of *CIITA's* last exon (Figure 4A). This includes all proximal promoter regulatory regions and other regulatory regions (16, 21, 36, 50) known to regulate *CIITA* expression. To modify the BAC, two homologous recombination-

targeting shuttle vectors were created. These vectors were designed to generate a 1 kb and 500 bp deletion of HSS1 and pIII, and were termed ΔHSS1 and ΔpIII, respectively (Figure 4A). The pIII deletion includes both the transcription start site and all promoter proximal B-cell specific regulatory motifs that were characterized previously to control expression from pIII (8, 51). The targeting vectors were used to transform *E. coli* containing the *CIITA* BAC and co-integrations were screened and isolated, followed by resolution of the targeting vector sequences from the BAC DNA. The mutated BACs were assessed for integrity by restriction enzyme digestion (data not shown) and by PCR (Figure 4B). The modified BACs contained only the intended deletions.

To assess the expression of *CIITA* from the BAC DNA, wild-type and modified *CIITA* BACs were transfected into the human B-cell line RJ2.2.5. RJ2.2.5, originally derived from Raji cells contains heterozygous deletions for *CIITA* and is MHC-II negative (4, 52, 53). Following transfection of wild-type and mutant (ΔHSS1 and ΔpIII) *CIITA* BAC DNAs into RJ2.2.5 cells, RNA was purified and murine *CIITA* promoter I, III, and IV specific mRNA levels were assessed by real-time RT-PCR. Mock-transfected cells showed that the primers are specific for murine *CIITA* as they did not detect any of the mutant *CIITA* transcripts expressed in RJ2.2.5. The wild-type BAC expressed *CIITA* from pIII and pI at levels of ~370-fold and 27-fold over the mock transfected cells, respectively (Fig 4C). The ΔHSS1 mutation resulted in nearly a complete loss of pIII expression, suggesting that HSS1 element is critical to pIII-mediated expression. The ΔHSS1 mutation had a similar influence on pI. As anticipated, the DpIII mutation caused a complete loss of pIII directed *CIITA* expression. Surprisingly, the DpIII mutation also affected expression from pI. This result suggests that the B-cell specific elements contained within the DpIII deletion were responsible for expression from pI. In contrast, no expression was detected from pIV irrespective of the *CIITA* BAC constructs assayed. Low levels of both pI and pIV *CIITA* mRNA isoforms were detected in Raji cells but these levels were 43-64 fold lower than the

pIII isoform (data not shown). Thus, with the exception of pIV, the BAC mimics to a large extent the properties of the *CIITA* locus, and HSS1 plays a critical role in expression from pIII.

PU.1 binds to HSS1 and CIITA pIII

To predict the identity of factors that may bind HSS1 in B cells, the DNA sequence of HSS1 was analyzed using the Genomatix software program. The analysis found that HSS1 had two highly significant potential PU.1 binding sites (GGAA) with perfect matches, yielding core similarity scores of 1.0. These sequences were located at chr16: 10,476,423 and 10,476,550 bp (University of California Santa Cruz (UCSC) genome browser). Other putative sites, including those for CREB and PLZF were also identified but their similarity scores were lower (0.89 and 0.86, respectively). Because PU.1 is a transcription factor that binds to lymphocyte specific enhancer regions (33, 34) and is responsible for B cell and myeloid cell fate decisions (30, 54) its role was examined further. To determine the extent of PU.1 binding in vivo, ChIP assays were conducted across the locus as above, using chromatin prepared from BCL1 and P3X cells (Figure 5A). The result showed that PU.1 bound to HSS1 in BCL1 cells (Figure 5A). Robust PU.1 binding was also detected at pIII in BCL1 cells, which was in agreement with previous studies that showed PU.1 binding to pIII (15). Low levels of PU.1 binding were detected in BCL1 cells at pI and at a region located 2 kb upstream of pIII. No binding of PU.1 at any site was detected in P3X plasma cells. This result is consistent with the very low levels of PU.1 that are found in P3X cells (data not shown). Thus, recruitment of PU.1 to at least two and possibly four sites may be important for the B-cell specific expression of *CIITA*.

To examine the dynamics of PU.1 occupancy at HSS1 during the B cell to plasma cell transition, chromatin preparations from primary murine B cells *ex vivo* stimulated with IL-2, IL-5, and LPS to induce plasma cell differentiation (15) from day 0 through day 5 were examined. This treatment results in the silencing of *CIITA* expression (15). Similar, robust levels of PU.1 binding were observed at HSS1 and pIII in the untreated freshly isolated murine B cells. PU.1

occupancy was lost rapidly during the differentiation process (Figure 5B). This correlated with the loss of PU.1 protein that occurs during the differentiation process (Figure 5C).

PU.1 is required for CIITA expression in BCL1 B cells

The ChIP results indicate that PU.1 binds directly to HSS1 and pIII and may regulate *CIITA* expression. To determine the role of PU.1 for *CIITA* regulation in B cells, the expression of PU.1 was depleted from BCL1 B cells using RNAi. A set of five lentivirus vectors expressing different shRNAs specific to PU.1 and the selectable marker puromycin were produced and used to infect BCL1 cells. Infected cells were selected with puromycin for 5 days. PU.1 shRNA vectors 2, 3, 4, and 5 reduced PU.1 protein to varying degrees (Figure 6A). Although vector 3 had the most pronounced effect on PU.1 expression levels, in some experiments the cells died following infection with this vector, and therefore it was not used further. To assess *CIITA* levels after PU.1 knockdown from B cells, total RNA was purified and real time RT-PCR was carried out to determine the level of *CIITA* and other B cell specific gene mRNAs. PU.1 knockdown using shRNA vectors 2, 4, and 5 resulted in a significant reduction of *CIITA* and I-A expression (Figure 6B) despite the fact that none of the vectors were able to completely ablate PU.1 expression in the culture. Other B cell specific genes, including Pax5, Spi-C, Spi-B, and CD19 were not altered in their expression by any of the shRNAs (Figure 6B). A reduction in MHC-II surface expression was also observed following infection with the lentivirus containing shRNA5 (Figure 6C). These data suggest that PU.1 plays an essential role in *CIITA* expression in B cells.

Long distance interactions between HSS1 and CIITA pIII form in B cells

One way in which HSS1 may contribute to the regulation of *CIITA* in B cells is for the region to interact directly with the pIII regulatory region. To determine if HSS1 can interact with pIII, the chromatin conformation capture assay (3C) was employed (55). In 3C assays, chromatin is crosslinked with formaldehyde to fix interactions within the cells. Purified chromatin is then

digested to completion with a restriction enzyme, such as *Hin*dIII. The samples are diluted greatly such that non-crosslinked DNAs are no longer spatially associated, and the crosslinked DNAs are ligated. Following reversal of the crosslinks and DNA purification, the formation of these novel "3C" junctions are evaluated by PCR. A map of the *CIITA* locus with primer positions and *Hind*III sites used is shown (Figure 7A). In these experiments, the pIII encoding restriction fragment was used as the anchor to test for interactions between it and other sequences. A 3C product was observed between HSS1 (primer H2) and pIII (Figure 7B and 7C), indicating that an interaction between these DNAs occurs. This 3C product was reduced by 53% in its frequency in P3X cells (Figure 7B and C). No interactions between pIII and Primer H1 (Figure 7B) or H7 (Figure 7C), which represent regions upstream of HSS1 and downstream of pIII, respectively were observed. In BCL1 cells, a 3C product was also observed between pI and pIII (Figure 7B and C). The pI/pIII 3C product was reduced by 65% in P3X cells, suggesting that it is B-cell specific. All 3C interactions were found to require formaldehyde crosslinking (data not shown) and the presence of DNA ligase (Figure 7B). Equal loading of samples was controlled by the internal amplification of a single restriction fragment (Figure 7B, primers Y19F and Y19R). All restriction sites assessed were equally accessible to restriction enzymes irrespective of cell type or conditions of the assay (Supplemental Data Figure 1). Additionally, the ability of a digested fragment to self ligate and form a 3C product was also evaluated (Figure 7B, primers H4-H5) and demonstrated that these sites were equally accessible between B cells and plasma cells.

To determine the role of PU.1 in the ability of pIII to interact with HSS1 and pI, 3C assays were performed on BCL1 cells infected with the PU.1 shRNA lentivirus vector 5. Compared to pLKO.1 lentivirus infected cells, the shPU.1 lentivirus showed a 52% reduction in the 3C product formed between HSS1 and pIII. Intriguingly, interactions between pIII and pI were unchanged by PU.1 knockdown. No interaction was observed with the control region (primer H7-H6). Another way to deplete PU.1 from the system is to *ex vivo* differentiate primary

B cells to plasma cells with LPS, IL-2, and IL-5 as above. In concordance with the loss of PU.1 occupancy at HSS1 and pIII, *ex vivo* differentiation resulted in a 65% decrease in the frequency of 3C product formation between HSS1 and pIII and a 62% loss of the interaction between pI and pIII. These results suggest that PU.1 plays a role in HSS1 interacting with pIII but that interactions between pI and pIII are dependent on another set of factors.

Discussion

In the mouse, *CIITA* expression is regulated from three promoter regions, with each region initiating the transcription of a unique isoform of CIITA (8). The three promoters are regulated in a cell-type dependent manner, with pIII functioning in lymphoid cells. In B cells, pIII is expressed constitutively but is silenced as B cells transition to plasma cells. Several groups have shown that the proximal region 5' to the pIII transcription start site encodes a complex set of binding sites that is critical to B cell specific expression (20, 51). However, no other regions associated with pIII expression in B cells have been described. Using a number of tools, we identified a region located upstream of pIII and pI that is critical for B cell specific expression. This region, termed HSS1 is highly conserved, hypersensitive to DNase I treatment, and displayed chromatin marks that were associated with an active and accessible locus. A key parameter showing that HSS1 was critical to B-cell specific expression of *CIITA* was the deletion of this element from a BAC construct. Due to the multiple promoters that control *CIITA,* this experiment provided an "in context" evaluation of the role of HSS1 with respect to all the *CIITA* promoters. While pIII-dependent transcripts were robustly expressed in the BAC system, low levels of pI but no levels of pIV specific *CIITA* expression were observed. The pI transcripts were affected by the presence of HSS1 and pIII, suggesting that transcription from pI was likely controlled by factors bound at these elements. Analysis of *CIITA* transcript utilization from the human B cell line Raji, which is the parent of RJ2.2.5 showed that pI and pIV produced minimal levels of steady-state transcripts compared to pIII. In contrast, no pI or pIV transcripts could be detected from the murine BCL1 cells (data not shown). Thus, there is some variation between the human and murine systems or cell lines.

DNase I hypersensitivity Southern blots also identified a robust hypersensitive site that was present in the plasma cell line P3X but not in B cells. Cross species conservation of HSS2 was not high, and HSS2 displayed significant levels of histone H4 acetylation but oddly near background levels of histone H3 acetylation. Intriguingly, the region just upstream of HSS2

showed no histone modifications associated with an active locus. While such demarcations can sometimes suggest that the element functions as a boundary element, no binding of the insulator factor CTCF to HSS2 was found (data not shown). Thus, at this time the function of HSS2 is not known. DNase I hypersensitive sites were not detected at pI or at the PU.1 site located between pI and pIII (Figure 1B and data not shown). While this is inconsistent with the ChIP data for the low levels of binding of PU.1, it is consistent with the ChIP data for active histone modifications that were observed in these regions. Thus, the DNase I hypersensitivity assays correlate more closely with the histone modifications and chromatin conformation rather than the binding of a factor.

In a previous study (15), we found that chromatin histone modifications that were in general associated with active gene expression were lost at the human *CIITA* proximal promoter regions when human B cell lines were compared to plasma cell lines. This suggested that there were global changes that controlled chromatin accessibility and the overall architecture of the locus. Here similar findings were observed for the murine B cell line BCL1 and plasma cell line P3X. Intriguingly, in the plasma cell line P3X, chromatin marks associated with Polycomb gene silencing (45, 56) were observed at all three *CIITA* promoters, suggesting that there is an active mechanism that results in the recruitment of Polycomb factors to silence *CIITA* expression. The polycomb H3K27^{me3} methyltransferase EZH2 was found to play a role in regulating pIV in uveal melanoma cells (18). Preliminary data showed that EZH2 binds to pIII in plasma cells (unpublished data).

Genome wide studies have helped identify the global locations to various histone marks (47, 48). Histone H3 K4 methylation marks are mostly indicative of the gene expression state of a locus. H3K4^{me3} is most often associated with promoter regions of actively transcribed genes (46). For *CIITA* in B cells histone H3K4^{me3} was most associated with pIII, but was also found at lower levels at pIV and HSS1. Only background levels of histone H3K4^{me3} were observed in plasma cells, and thus, this mark is clearly associated with *CIITA* expression. Histone H3K4^{me2} is

more broadly associated with gene expression and can be found covering large segments of the promoter and upstream regulatory regions of genes (46) and perhaps is a mark of accessibility. H3K4^{me2} was lower in plasma cells, suggesting that the region may be less accessible; perhaps due to silencing marks associated with Polycomb. While histone H3K4^{me1} was reported to be associated with enhancer regions (48), the patterns for *CIITA* promoters and HSS1 correlated with transcription initiation in that pIII and pIV showed high levels of this modification in plasma cells; whereas the opposite was observed for HSS1 and pI in B cells. The finding of multiple modifications within a region may reflect the number of nucleosomes assessed in the ChIP assays, as well as the dynamic nature of the marks that is likely to occur.

For B cells, *CIITA* expression and the interaction between HSS1 and pIII was in part dependent on PU.1. Depletion of PU.1 by RNAi or through the use of plasma cells or differentiation of B cells to plasma cells, showed significant reduction in *CIITA* mRNA levels and in HSS1 interactions with pIII. The inability to completely deplete PU.1 levels in the RNAi and *ex vivo* differentiation systems are likely to underplay PU.1's role in transcription of *CIITA*. Conservatively, we conclude that PU.1 is necessary for full activity of HSS1 and B-cell specific expression of *CIITA*, but that other factors are likely to be involved. The finding of PU.1 at both HSS1 and pIII was intriguing and may suggest a role for PU.1 to help bridge these regions together as its reduction by shRNA resulted in a decrease in the 3C product. We suggest that the ChIP data reflect direct in vivo binding of PU.1 to both sites and that this is not due to 3C interactions between these sites. This conclusion is based on the equal and robust ChIP results for primary B cells (Figure 5B) and the fact that the conditions used to fix the cells have not shown indirect ChIP binding due to 3C interactions at other sites that are known to interact (13). Whether PU.1 interacts with other DNA binding proteins or whether it interacts with a set of coactivators to stabilize the interactions between pIII and HSS1 can only be speculation at this point.

Recently, Ni et al. identified a series of sequences within the *CIITA* locus of human HeLa cells that bound STAT1 and IRF1 in response to IFN-γ signaling. Some of these elements were required for the IFN-γ expression through pIV, the *CIITA* promoter responsible for IFN-γ expression in non-hematopoietic cells (36). A role for the IFN-g elements in B cells was not determined. We found that HSS1 is the murine homolog to the human region termed -16 (36) as referenced from the pIV transcriptional start site. In IFN-γ treated HeLa cells, -16 interacted with pIV in a BRG1-dependent manner. BRG1 is the ATPase of the Swi/Snf chromatin-remodeling complex that is associated with the expression of many genes and likely facilitates nucleosome movement to allow the interactions to occur (57).

With the discovery that HSS1 functions in *CIITA* regulation through pIII in B cells and that there were multiple elements found to fully orchestrate pIV regulation by IFN-γ in HeLa cells (36), two questions arise. How many total elements will be required to regulate *CIITA* in a tissue specific manner and how will promoter usage be derived? Five elements were required for IFN-g signaling through pIV in Hela cells (36). Our current findings imply that at least HSS1 responds to multiple signals, controlling both B cell and IFN-g-inducible expression. It is possible that like HSS1, other elements that were discovered in the IFN- g system will specify expression from the different *CIITA* promoters. However, whereas pI was found to interact with pIII in B cells it was not found to interact with pIV in the IFN-g system (36). Thus, it may be that only a limited set of these elements may function as master regulators of *CIITA* and that a set of promoter specific elements will also exist. HSS1 has the potential to be one such master regulatory element for *CIITA*. For such elements, the transcription factors that bind to the master element could determine which promoter is used. For IFN-γ treatment of HeLa cells STAT1/IRF1 are the key factors that likely target pIV transcription; whereas, in B cells, PU.1 is at the very least, a key component of HSS1 and pIII B cell regulatory mechanism. While little is known about even the proximal promoter elements for pI, it is possible that PU.1, which is also

expressed in myeloid derived dentritic cells (30), also plays a role in this cell lineage for *CIITA* expression. Considering that HSS1 has the potential to bind PU.1, it is possible that it will also function in the regulation of pI. Nonetheless, the current data suggest that the HSS1/-16 element may function in multiple cell type contexts with its use dependent on the specific set of transcription factors that are expressed or induced.

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[2] Abbreviations used in this paper: 3C, chromatin conformation capture; ChIP, chromatin immunoprecipitaiton; CTCF, CCCTC transcription factor; HSS1, hypersensitive site 1; HSS2, hypersensitive site 2; LSD-1, lysine-specific demethylase 1; promoter I, III, IV, pI, pIII, pIV

Disclosures

The authors have no financial conflicts of interest.

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Figure Legends

Figure 1. B cell and plasma cell specific DNase I hypersensitive sites are located in conserved regions upstream of *CIITA* promoter I. A. Southern blot DNase I hypersensitivity assay performed on the murine B-cell line BCL1 and plasma cell line, P3X63ag8.653 (referred to as P3X). Triangles denote increasing DNase I concentration. A schematic diagram of the region analyzed and the probe location is shown. B. PCR based DNase I hypersensitivity assay on purified murine splenic B cells and BCL1 cells. DNase I treated and control samples were subjected to real-time PCR amplification over the designated regions, represented by the primer amplicon sets, which are described in Supplemental Table 1. The amount of PCR product for each sample was divided into the undigested sample and the average of three reactions was plotted. C. The regions corresponding to HSS1 and HSS2 were analyzed for sequence conservation using the University of California Santa Cruz Genome Browser (38). The screen shot from that analysis is shown with an annotated schematic.

Figure 2. HSS1 and *CIITA* promoters display reduced histone acetylation in plasma cells compared to B cells.

Chromatin prepared from the murine B-cell line BCL1 and plasma-cell line P3X were subjected to ChIP analysis using antibodies to the general acetylated (ac) forms of histone H3 and H4. A non-specific control antibody to the TCR was used to normalize the values for each amplicon. The position of the twenty-three amplicons used are schematically represented over the *CIITA* locus encompassing pIV through -18 kb upstream of pIII, which was designated as the origin (0 kb). All ChIP assays were performed three times from independent chromatin preparations. Real-time PCR was used with a standard curve generated for each amplicon to determine values for each of the ChIP reactions. These values were averaged for the three experiments,

normalized to the amount of chromatin in each set of reactions prior to ChIP, and plotted with respect to the values obtained with the TCR antibody as fold over control. The shaded sections highlight ChIP amplicons for HSS2, HSS1, pI, pIII, and pIV. The // indicates a region that could not be amplified due to repetitive DNA.

Figure 3. HSS1 and the *CIITA* promoters display differential histone modifications. ChIP for the indicated histone modifications were performed on BCL1 and P3X cells as above. The relative location of the five amplicons used in this series is shown in the schematic. Y4 represents an amplicon located at -16 kb from pIII and serves as a non-regulatory region control for comparison across the locus. ChIP assays were performed three times from independent chromatin preparations as above. Asterisks indicate B and plasma differences that were significant ($p<0.05$) as determined by student T tests. ac, acetylation; me, methylation (mono, di, tri).

Figure 4. HSS1 is required for B cell expression of *CIITA* from pIII. A. A schematic representation of the murine *CIITA* encoding BAC and the surrounding loci is shown with the indicated deletion mutations that were created in HSS1 (DHSS1) and pIII (DpIII). Primers used to assess the integrity of the BAC constructs are shown. B. PCR across the regions that were deleted in each of the mutant *CIITA* BAC constructs using the indicated primer pairs showed specificity of the intended deletions. C. Wild-type and mutant BACs were transfected into the *CIITA*-deficient human B-cell line RJ2.2.5 by nucleoporation. Forty-eight h post transfection, the cells were harvested, and the RNA analyzed by real-time RT-PCR using primers specific for each of the *CIITA* promoter-dependent transcripts as shown in A. Three independent transfections were performed and the results were averaged with respect to the levels of 18s rRNA and plotted as relative expression with standard error. Student T tests showed that the differences between wt

and DHSS1 and DpIII were highly significant (*) for promoters I and III ($p<0.04$). No differences were observed at pIV.

Figure 5. PU.1 binds to HSS1 and *CIITA* pIII.

A. ChIP assays were conducted to assess the binding of PU.1 to HSS1 and other sites across the locus using chromatin prepared from BCL1 and P3X cells. The binding of PU.1 was analyzed on the *CIITA* gene using primers that represent sequences spaced at intervals of ~1 kb between -11 kb through +4 kb relative to *CIITA* promoter III. B. Purified splenic B cells were used for PU.1 ChIP assays either immediately (black bars) or *ex vivo* differentiated in LPS, IL-2, and IL-5 for 1, 3, or 5 days (gray bars) as indicated. The regions analyzed by ChIP are stated. In all of these assays three independent replicates were carried out, averaged and plotted vs the non-specific control with standard error. All real-time PCR values were determined as above by comparison to a standard curve for the amplicon generated from genomic DNA. Student T tests were used to determine the significance of the differences from undifferentiated cells. HSS1, pI, and pIII showed statistical significance of $p<0.05$ between untreated and all days post treatment. C. Western blot of murine splenic B cells (day 0) *ex vivo* differentiated to plasma cells (days 1, 2, and 3) that was stained for PU.1 and actin.

Figure 6. PU.1 is required for *CIITA* expression in BCL1 cells.

A. Five different shRNA lentiviral expression vectors were used to knockdown the expression of PU.1 in BCL1 B cells. Infected cells were selected using puromycin for five days. Lysates prepared from the resistant cells were assayed by immunoblotting for PU.1 and actin protein. B. RNA from PU.1-shRNA-depleted BCL1 cells was purified and analyzed by real-time RT-PCR for the levels of the indicated transcripts. These experiments were performed three times from independent shRNA infections. The results of each experiment were averaged and plotted with respect to the parent lentiviral construct (pLKO.1) infected cells. Significance was determined

using a student T test and comparisons between control and PU.1 shRNA with p values of <0.05 are indicated with an asterisk. C. Flow cytometry was used to assess the level of MHC-II surface expression on pLKO.1 and shPU.1 vector 5 infected BCL1 B cells.

Figure 7. A long distance interaction occurs between HSS1 and *CIITA* pIII.

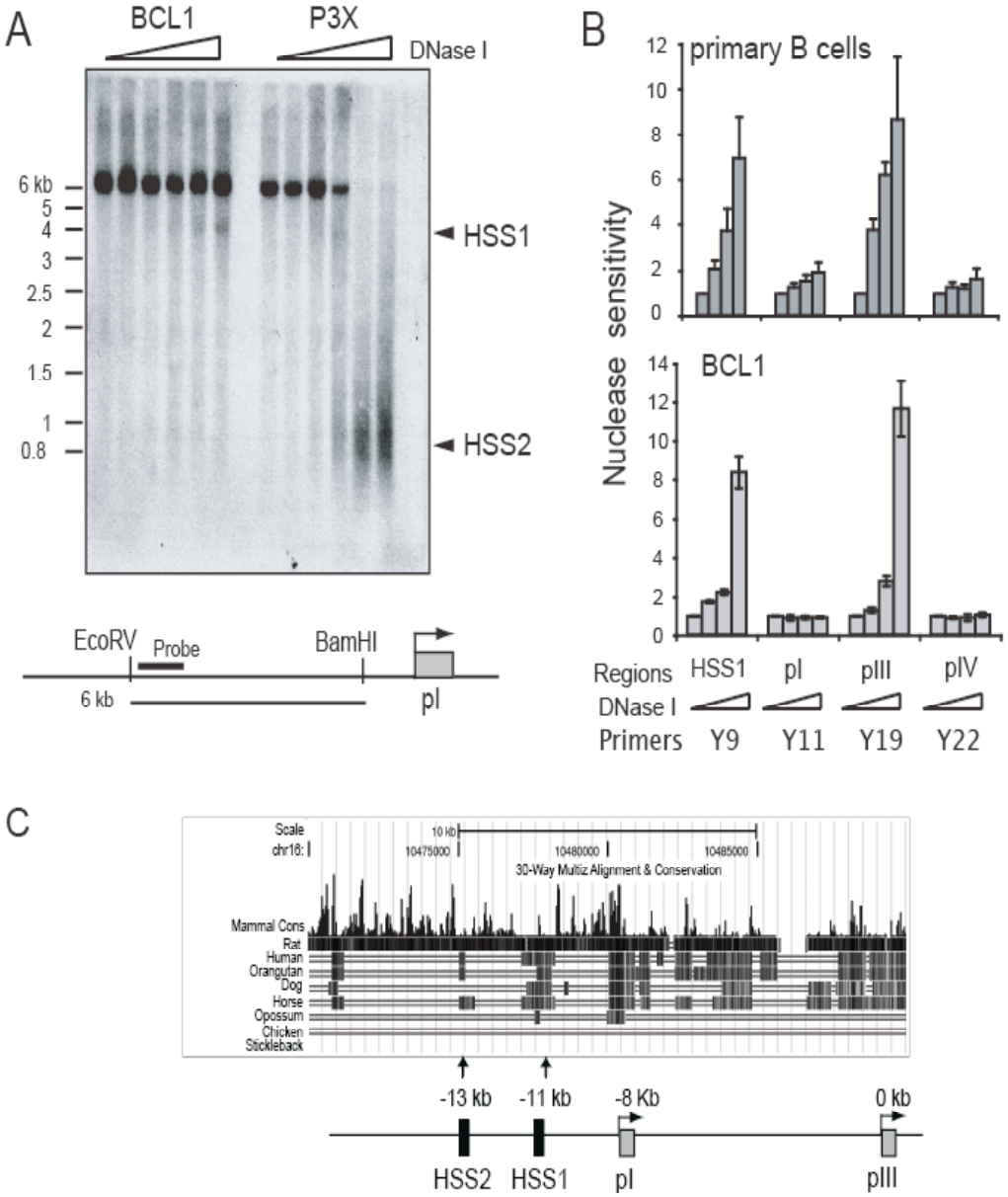
A. A schematic of *CIITA* region analyzed by 3C assays is shown with *Hin*dIII sites, 3C primers, and relative position of HSS1 and the three *CIITA* promoters is shown. Note that one of the pIII region specific primers has been termed PIII. B. Schematic representation of 3C products for pIII interacting with HSS1 (primer H2) and pI (primer H3) are shown. These 3C products were cloned and sequenced to verify their location. Agarose gel electrophoresis of 3C reactions was carried out with the indicated primer pairs using BCL1 and P3X cells. C. Real-time PCR analysis of 3C products formed between pIII and the H7 downstream region, HSS1, and pI in BCL1 and P3X cells. A relative crosslink frequency was determined by calculating the amount of 3C product between pIII and the experimental restriction fragment and dividing it by the amount of 3C product formed for pIII and H1 (an irrelevant upstream restriction fragment (see Fig 7B)). The average of three independent experiments is shown with standard error and a student T test evaluation of the significance. No significant interactions were observed between pIII and 3C primer H7 were observed. Relative 3C crosslink frequency for pIII interactions in (D) BCL1 cells infected with pLKO.1 and PU.1 shRNA lentiviral vector 5; and (E) undifferentiated and day 3 e*x vivo* differentiated murine splenic B cells are presented as in C.

Supplemental Figure 1. Restriction site accessibility

All restriction sites assessed for 3C were equally accessible to digestion by *Hind*III irrespective of the cell line or the condition used. A. A schematic of *CIITA* region analyzed by 3C assays is shown with *Hind* III sites, and primers that were used to assess *Hin*dIII accessibility. Nuclei from crosslinked cells prepared for 3C was digested with *Hind* III or left undigested. 30 ng of the

purified DNA from the above reactions was used to perform PCR with indicated primers. Agarose gel electrophoresis used to assess each of the PCR products with (B) comparing BCL1 and P3X cells; and (C) undifferentiated and 3 day ex vivo differentiated primary splenic B cells.

Figure 1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5
4
3
2.5
2
1.5
1
0.8
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HSS2
EcoRV
Probe
BamHI
6 kb
pI
B
primary B cells
Nuclease sensitivity
BCL1
Regions
HSS1
pI
pIII
pIV
DNase I
Primers
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Y19
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C
Scale
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30-Way Multiz Alignment & Conservation
Mammal Cons
Rat
Human
Orangutan
Dog
Horse
Opossum
Chicken
Stickleback
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-11 kb
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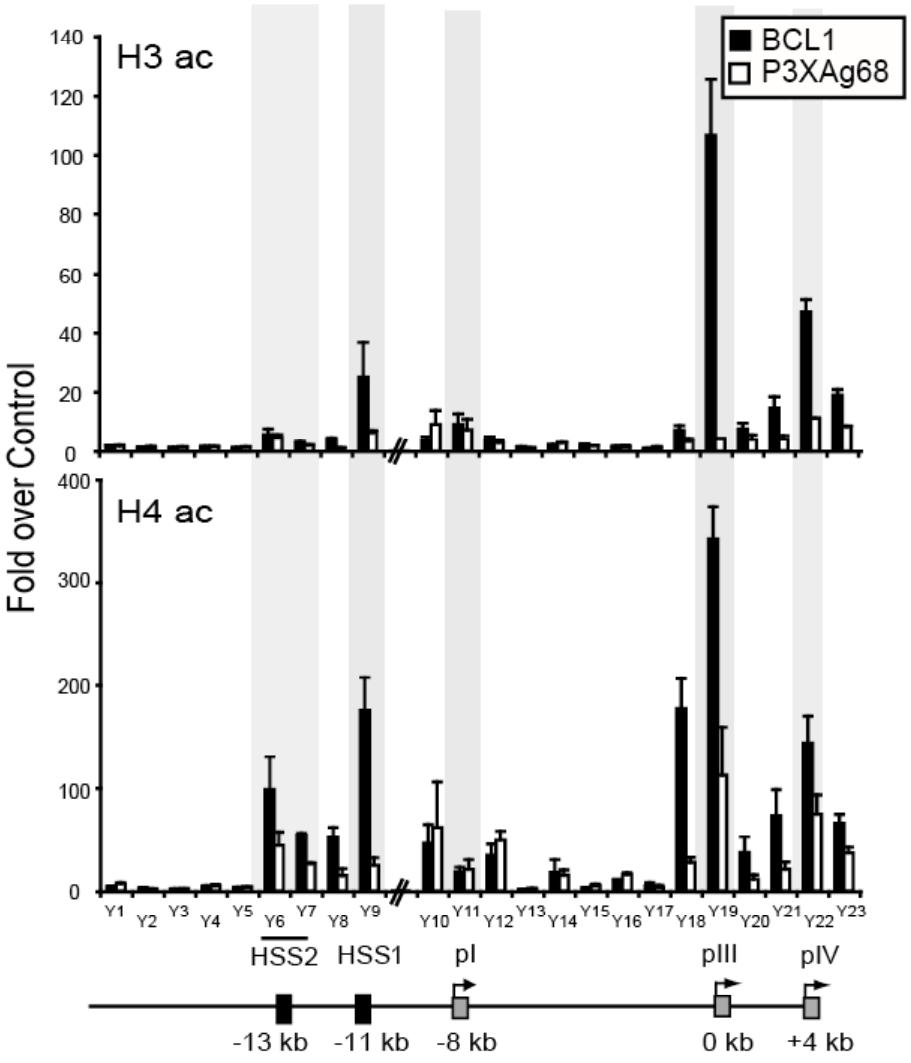
0 kb
HSS2
HSS1
pI
pIII

Figure 2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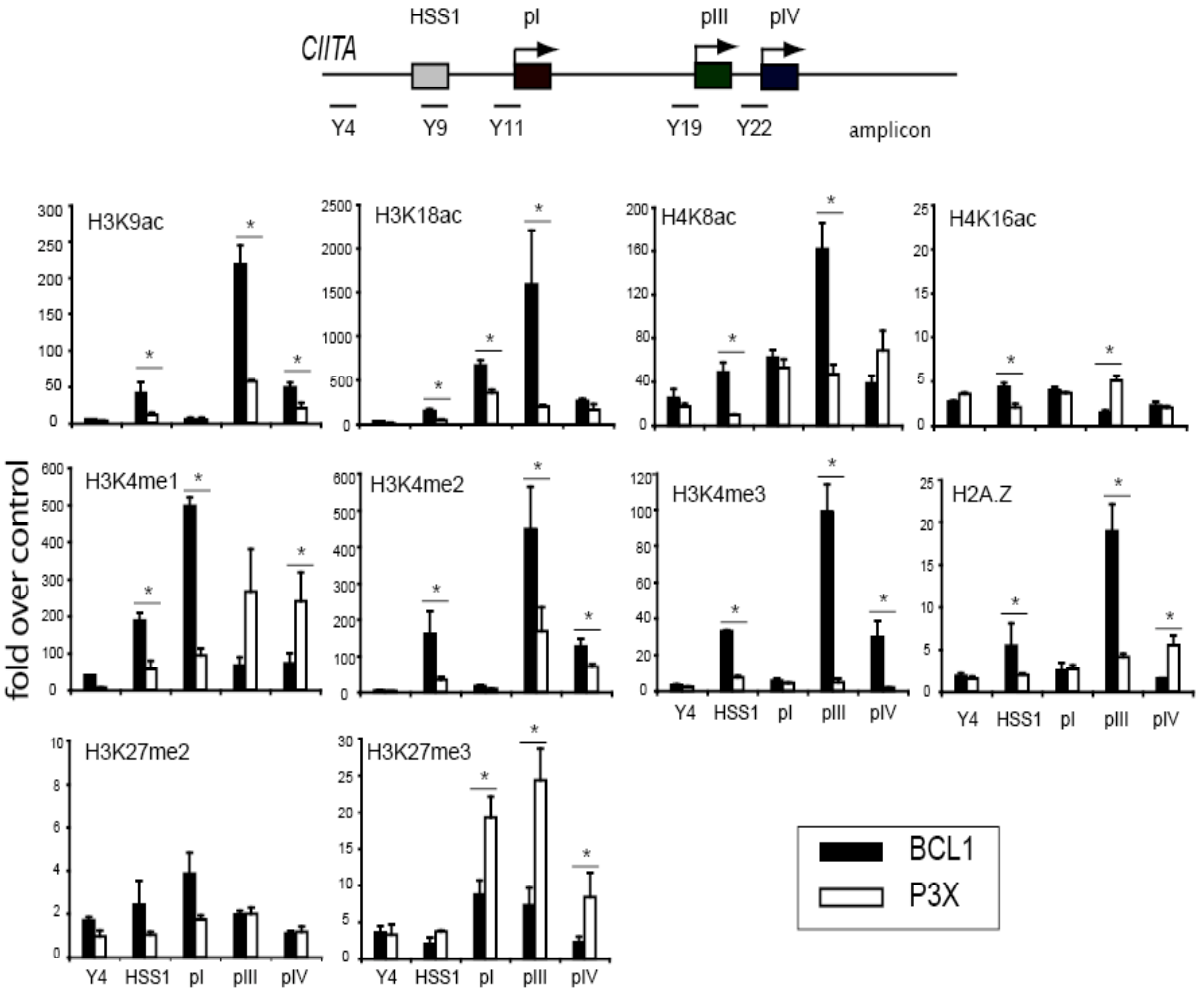
H4 ac
BCL1
P3XAg68
Fold over Control
140
120
100
80
60
40
20
0
400
300
200
100
0
Y1 Y2 Y3 Y4 Y5 Y6 Y7 Y8 Y9 Y10 Y11 Y12 Y13 Y14 Y15 Y16 Y17 Y18 Y19 Y20 Y21 Y22 Y23
HSS2
HSS1
pI
pIII
pIV
-13 kb
-11 kb
-8 kb
0 kb
+4 kb

Figure 3


CIITA
HSS1
pI
pIII
pIV
Y4
Y9
Y11
Y19
Y22
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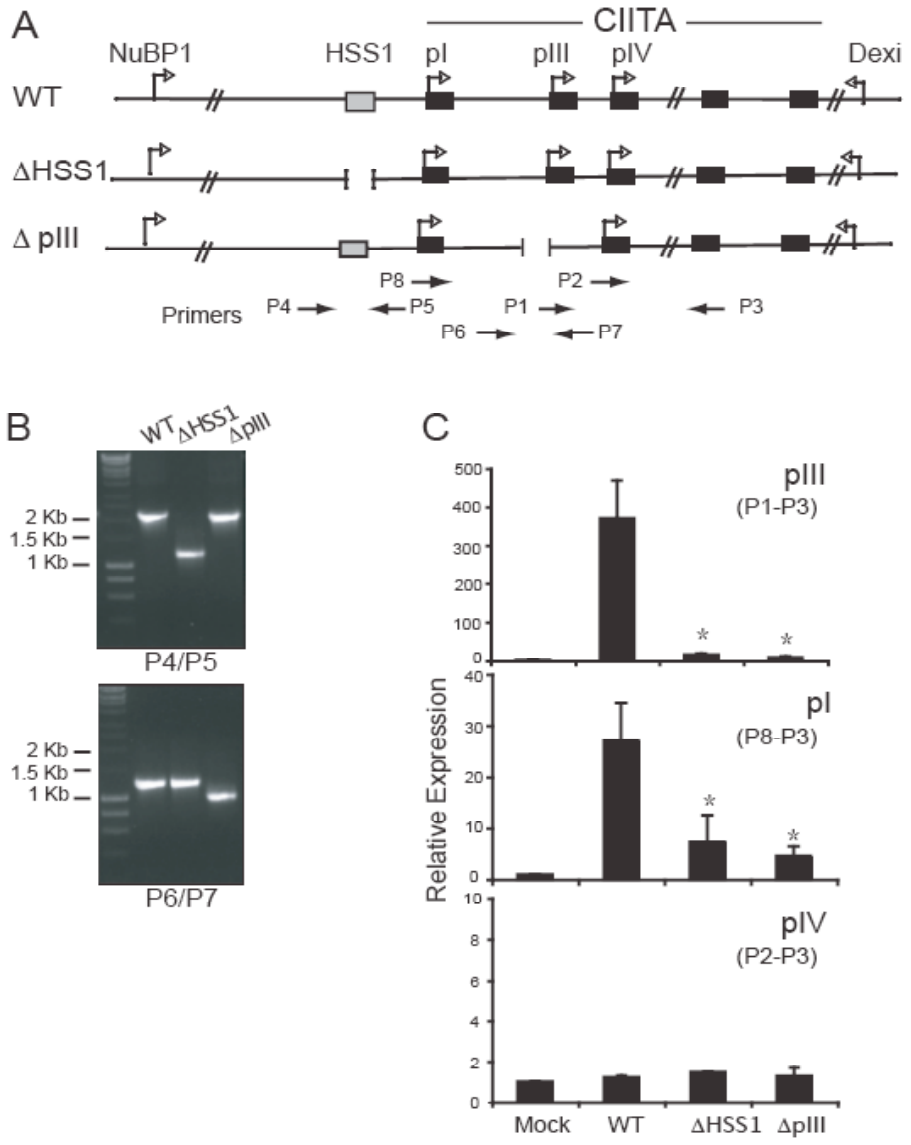
H4K16ac
H3K4me1
H3K4me2
H3K4me3
H2A.Z
H3K27me2
H3K27me3
fold over control
Y4 HSS1 pI pIII pIV
BCL1
P3X

Figure 4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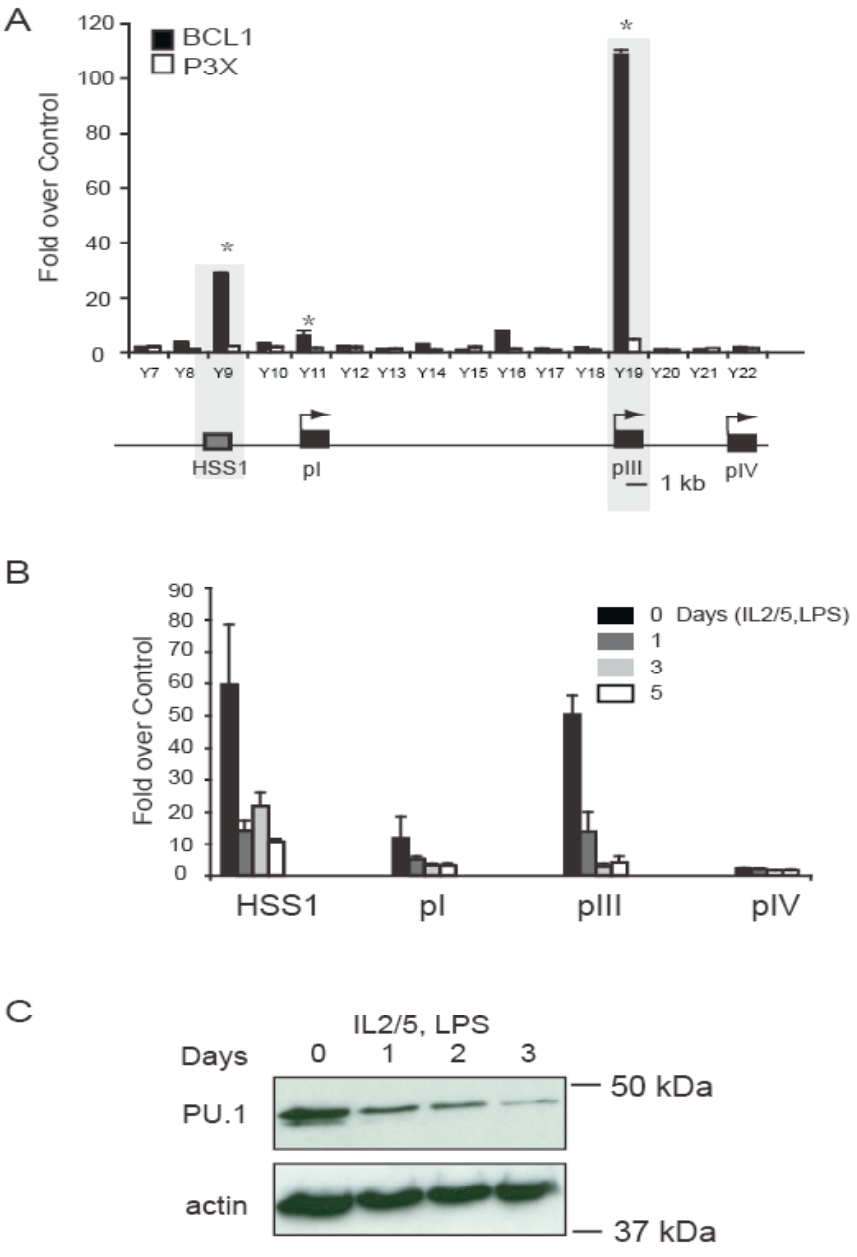
pIII
pIV
Dexi
WT
ΔHSS1
Δ pIII
P8
P2
Primers
P4
P5
P1
P3
P6
P7
B
WT
ΔHSS1
ΔpIII
2 Kb
1.5 Kb
1 Kb
P4/P5
2 Kb
1.5 Kb
1 Kb
P6/P7
C
pIII
(P1-P3)
pI
(P8-P3)
pIV
(P2-P3)
Relative Expression
Mock
WT
ΔHSS1
ΔpIII

Figure 5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P3X
Fold over Control
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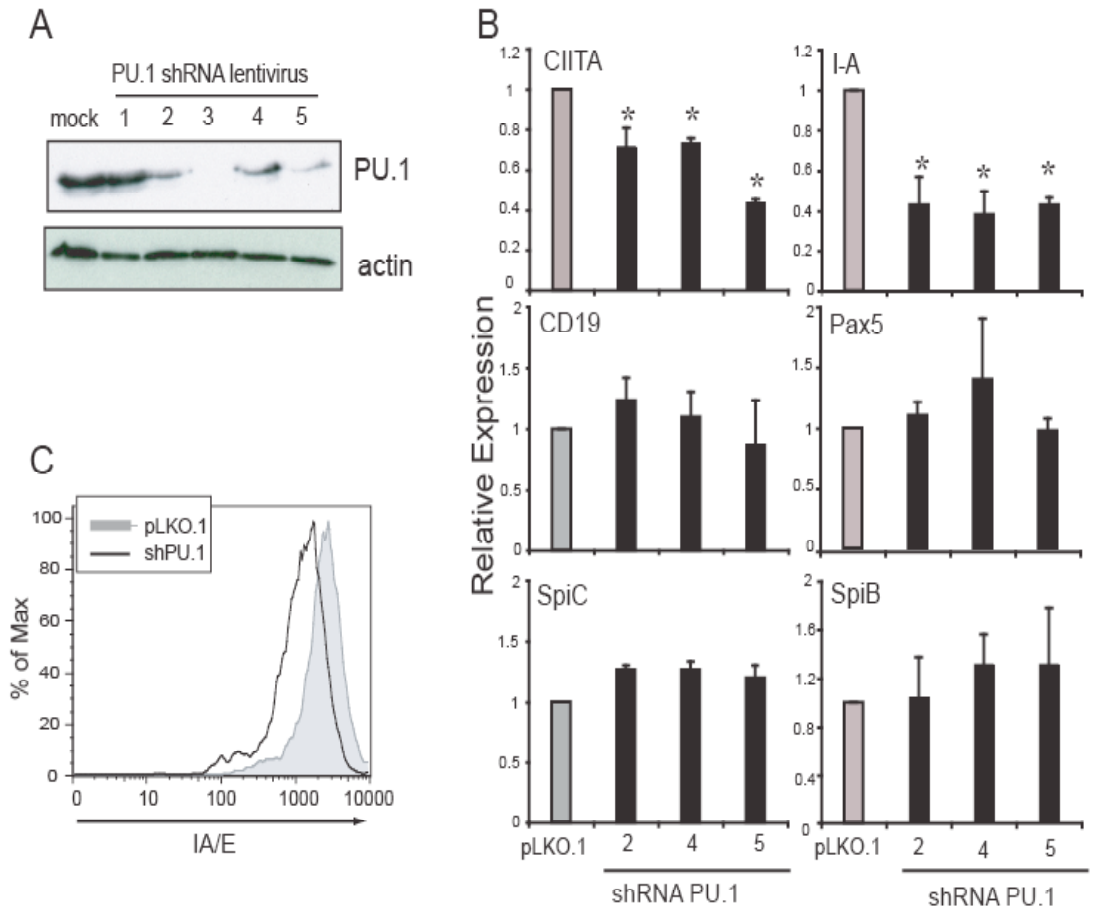
100
80
60
40
20
0
Y7 Y8 Y9 Y10 Y11 Y12 Y13 Y14 Y15 Y16 Y17 Y18 Y19 Y20 Y21 Y22
HSS1
pI
pIII
pIV
1 kb
B
Fold over Control
0 Days (IL2/5,LPS)
1
3
5
HSS1
pI
pIII
pIV
C
IL2/5, LPS
Days 0 1 2 3
PU.1
50 kDa
actin
37 kDa

Figure 6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PU.1
actin
B
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I-A
CD19
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SpiB
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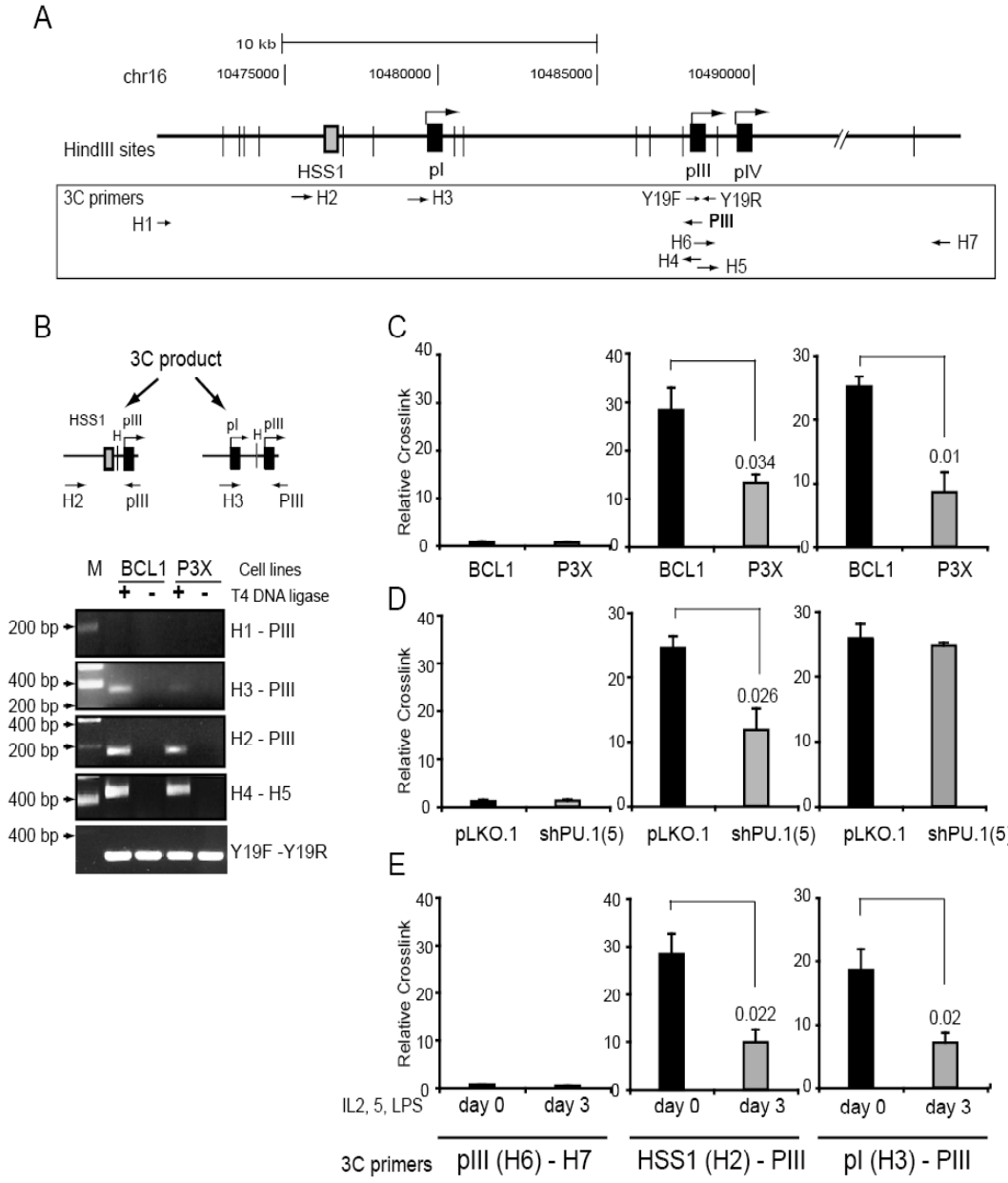
shRNA PU.1
C
pLKO.1
shPU.1
% of Max
IA/E

Figure 7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H7
H4
H5
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H
pI
H2
pIII
H3
PIII
M
BCL1
P3X
Cell lines
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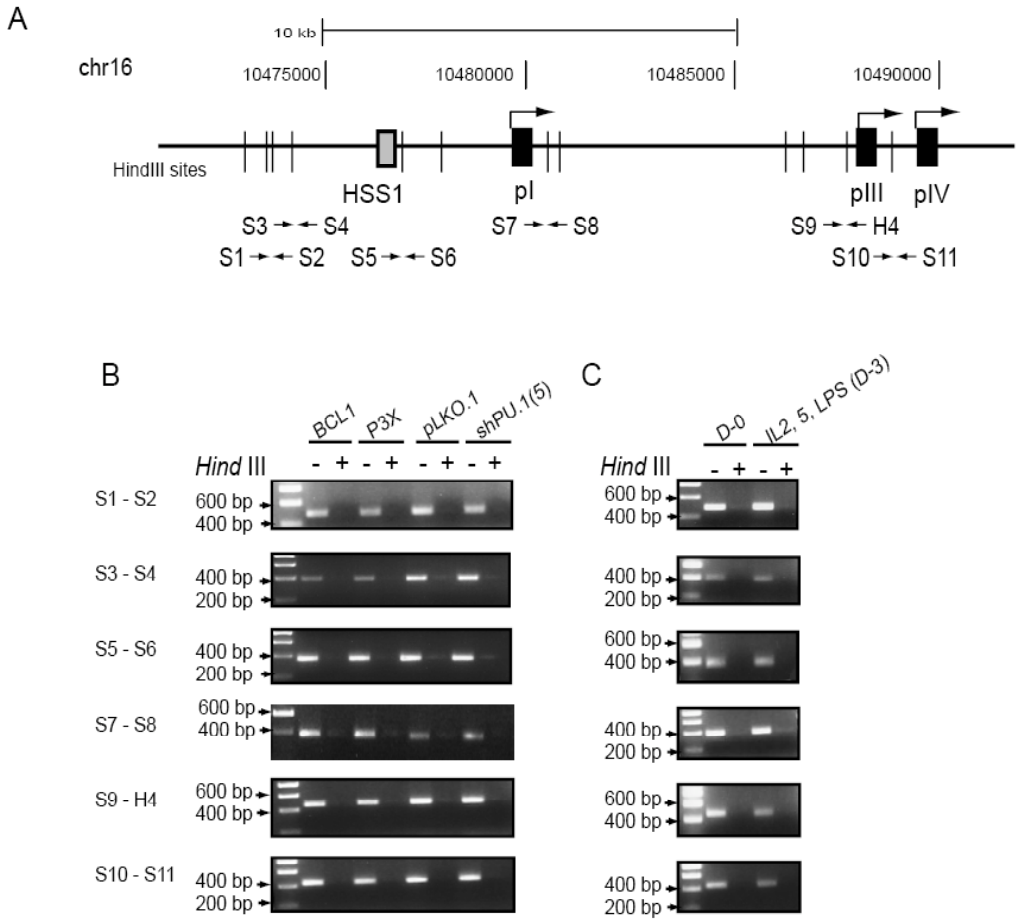
200 bp
H1 - PIII
400 bp
200 bp
H3 - PIII
400 bp
200 bp
H2 - PIII
400 bp
H4 - H5
400 bp
Y19F -Y19R
C
Relative Crosslink
0.034
0.01
BCL1
P3X
D
Relative Crosslink
0.026
pLKO.1
shPU.1(5)
E
Relative Crosslink
0.022
0.02
IL2, 5, LPS
day 0
day 3
3C primers
pIII (H6) - H7
HSS1 (H2) - PIII
pI (H3) - PIII

Supplement Figure 1



Supplement Table 1

ChIP Primers

Location on Ch16*	primer		Sequence
10,468,811	Y1	Forward	5-CAAGTCAGCGTGAGGCATGG
10,468,902		Reverse	5-GGCAATGGCGGCACTAAGG
10,469,536	Y2	Forward	5-GGCAGCAGGCAGCAAAGC
10,469,696		Reverse	5-TCCAGCAGGTGAGGCAAGG
10,470,554	Y3	Forward	5-GGACACTGGCTGCTGATGC
10,470,660		Reverse	5-GCTTGCTTGCTTGATTGATTGATG
10,471,497	Y4	Forward	5-TAAAGGCAAAGAGAGGGACAAAGG
10,471,616		Reverse	5-GGCAAGCAACAACAGGAGAAATC
10,472,687	Y5	Forward	5-CCTGTCTGTCTGTCACCTACC
10,472,841		Reverse	5-GCTATGCTGGCGGCTGTC
10,473,738	Y6	Forward	5-TAGCACATAACAGCGGTAGATTAC
10,473,835		Reverse	5-CCCAAGTTTGAGACAAGCAGAC
10,474,409	Y7	Forward	5-CTCTGATGTCTTGATGGTGCTAAC
10,474,579		Reverse	5-TCCTCCTGCCACACTCTCC
10,475,654	Y8	Forward	5-ACTCTGGTGTCCTACTCTGTTG
10,475,807		Reverse	5-AAGGGTGGTGTGGTTGCG
10,476,378	Y9 (HSS1)	Forward	5-CCCGAAGCCAGTGAGAACC
10,476,481		Reverse	5-TGTGTCTGTGTGTTTGTATGTAGG
10,479,684	Y10	Forward	5-TTGTGAGGCAGTCTTGTGTAGC
10,479,795		Reverse	5-AAGTGGGAATGGCGATATGAGC
10,480,035	Y11 (pI)	Forward	5-CTTATTGCTGTCCAAGTCACCCC
10,480,174		Reverse	5-TCCAGCCTTGCAGCATCCAAA
10,480,374	Y12	Forward	5-TGAGCCTGATGGTGATGC
10,480,542		Reverse	5-TGGTCATACATTGTTGCCTTG
10,481,600	Y13	Forward	5-TTGGGTGCTGAGTATGGGAGTC
10,481,745		Reverse	5-GCTGGTGGAGTGAGGGATGG
10,482,464	Y14	Forward	5-GTGCTTCTCCTGGGACAATGG
10,482,613		Reverse	5-AAGGAACCAGCGTACTCAGAAC
10,483,370	Y15	Forward	5-GTTGTCGCACGGCTCCAG
10,483,501		Reverse	5-CCCTTCCTAGCTCTCCCTTCC
10,484,196	Y16	Forward	5-CAGCCTGCTATGTATCCTCTTG
10,484,373		Reverse	5-GCAATAGCTTGGAGTAGGTAGG
10,485,287	Y14	Forward	5-TCTACATCTCCAGCGTCATCC
10,485,455		Reverse	5-TGTGCTGTTACCTGGTCTCC
10,486,867	Y18	Forward	5-CACAGTGATGCCAGTCTAGG
10,487,003		Reverse	5-CACACGATGCCCAAGTCC
10,488,157	Y19 (pIII)	Forward	5-GCCCACAAGAAGGAACTGAAATTAAC
10,488,305		Reverse	5-AGGAGCCCAGAACCTACCAAGC
10,488,576	Y20	Forward	5-CTTGGGTGGTTTCAGCATTCC
10,488,693		Reverse	5-TTAAGGAAGAGGAGAAGGAAGAGG
10,489,114	Y21	Forward	5-ATCTGGCACCGTGAAGTAACC
10,489,265		Reverse	5-CCTTCTCTTCCACCCTGAACC
10,489,917	Y22 (pIV)	Forward	5-GCAGGCAGCACTCAGAAGC
10,489,988		Reverse	5-TCGGATTCCCAACCTAACTTTAGC
10,490,100	Y23	Forward	5-GGATCTTGGACGGACTGTATGC
10,490,176		Reverse	5-CGCCACTTGCCTTCACTACC

BAC modification and real time RT-PCR primers for Figure 4

Chromosome 16	Primer	Sequence
10,488,421	P1	5-GCAGCTGGACTACAGACGTTAC
10,489,907	P2	5-GAGACTGCATGCAGGCAGCA
10,501,917	P3	5-GGTCGGCATCACTGTTAAGGA
10,475,771	P4	5-ACAATTGCTGTAGATATGCGCAA
10,477,949	P5	5-ATTTCCCTCTGTGGACCTGTCAT
10,487,571	P6	5-GGTATGCATGACCACATCACA
10,488,770	P7	5-TACTCAAAGACAGAAATAGGC
10,480,334	P8	5-AAGAGCTGCTCTCACGGGAAT

RT PCR primers for Figure 5

Target		Sequence
I-A	Forward	5-GACGCAGCGCATACGGCTC
	Reverse	5-CCGCCGCAGGGAGGTGCT
CD19	Forward	5-AGGACTGGAAGAAGAAGG
	Reverse	5-TCATAAGACTCAGCATTGG
Pax5	Forward	5-CTCCCAGATGTAGTCCGCCAAAG
	Reverse	5-GCTTGATGCTTCCTGTCTCATAATACC
SpiC	Forward	5-AACACTCGCTGAACTCTGG
	Reverse	5-GCCTCGCTGAACTGGTAG
SpiB	Forward	5-AGGACTTCACCAGCCAGACC
	Reverse	5-TCGGAGCCAGCCAAGAGG

Chromatin Conformation Capture (3C) assay primers

Chromosome 16	Primer	Sequence
10,452,430	H1	5-ACTGCTCTGGAAGAATGAATGTAAC
10,476,826	H2	5-GGAGAGCAGGTAGGTCTGATATAG
10,480,375	H3	5-TGAGCCTGATGGTGATGC
10,487,991	H4	5-AGCTCCAGCTTCTGCTCGCAG
10,488,572	H5	5-AGGTCTTGGGTGGTTTCAG
10,487,855	pIII	5-TTCAGGGTTGTGGTGGTAGG
10,488,692	H6	5-TCCTCTTCCTTCTCCTCTTC
10,518,117	H7	5-TCTGCCCTTTGTGGTTTCG

HindIII accessibility

Chromosome 16	Primer	Sequence
10,473,410	S1	5-CTCAGGCTTCCTTCCAAGTCTATTTCTAACTTG
10,473,666	S2	5-GTATATTCCACACTGGCCTTGAATCC
10,474,012	S3	5-GCAATGCTAGGCTCTCTAAGGCAGACCGGA
10,474,383	S4	5-GAGAGTAGATGACATCACTTCAGTCGACT
10,476,645	S5	5-AGTGTCTGACAGAAGTCCATAATGG
10,476,987	S6	5-CTATACATGCAAGTATAGTAGACAC
10,480,432	S7	5-GACTTAGACTTGACTTTCTTGAGCTGGGTCT
10,480,787	S8	5-TAGATGGTGTGTCTGTTGGAAGCAG
10,487,548	S9	5-ACATTCTACACTGTGGTATTACAGGTATGCATG
10,488,610	S10	5-AATCTGCACCATGACTTCCAGGGGCCTCT
10,489,016	S11	5-AACTGAGACTAAGAACGGTAATT

*Position relative to the University California Santa Cruz Genome Browser Build July 2007

Supplement Table 2

Company	Catalog #	Abtibody
Millipore	06-599	Acetyl H3
Millipore	06-866	Acetyl H4
Millipore	07-352	Acetyl H3 Lys9
Millipore	07-328	Acetyl H4 Lys8
Millipore	07-329	Acetyl H4 Lys16
Millipore	07-030	Dimethyl H3 Lys4
Millipore	07-473	Trimethyl H3 Lys4
Millipore	07-452	Dimethyl H3 K27
SantaCruz	sc-352x	PU.1
Abcam	ab1191	Acetyl H3 Lys18
Abcam	ab6002	Trimethyl H3 Lys27
Abcam	ab8895	Monomethyl H3 Lys4
Abcam	ab4174	H2A.Z
BD Phamingen	557000	anti mouse IA/IE

Chapter: 2

Hyesuk Yoon, Carl W. Davis, Rafi Ahmed, Jeremy M. Boss.
ZBTB32/Repressor of GATA3 is required for silencing the class II transactivator and MHC class II gene expression during differentiation to plasma cells.
In preparation

Hyesuk Yoon contributed all experiments. Carl W. Davis, Ph.D carried out the primary plasmablast purification.

This paper was written by Hye Suk Yoon and edited by Dr. Jeremy Boss.

ZBTB32 / Repressor of GATA3 is required for silencing the class II transactivator and regulating part of the B cell program during differentiation to plasma cells

Hyesuk Yoon, Carl W. Davis, Rafi Ahmed, and Jeremy M. Boss*

Summary

ZBTB32/repressor of GATA3 was identified as a novel repressor of class II transactivator (*CIITA)* and MHC-II gene expression during the early phase of plasma cell differentiation, occurring at a time when *Blimp-1*, the regulator of plasma cell fate and suppressor of *CIITA,* had not reached peak levels. Knockdown of ZBTB32 in plasma cells restored *CIITA* expression, but also that of the B cell fate regulators *Pax5 and SpiB*. In plasma cells, ZBTB32 and the polycomb repressor complex bound directly to the *CIITA* gene prior to Blimp-1 binding. At later stages of plasma cell differentiation, ZBTB32 and Blimp-1 coimmunoprecipitated and colocalized within the nucleus. These results suggest that ZBTB32 initiates *CIITA* silencing during B cell differentiation and may play a greater role in plasma cell formation.

Introduction

To initiate and regulate the adaptive and humoral immune responses, major histocompatibility complex class II (MHC-II) genes encode heterodimeric proteins that present antigenic peptides to CD4 T lymphocytes [1-3]. MHC-II genes are transcriptionally regulated by the MHC class II transactivator (CIITA), with expression occurring on a limited number of cell types, including dendritic cells, macrophages, and B lymphocytes [4-6]. CIITA is a transcriptional coactivator that connects the DNA binding transcription factors at MHC-II promoters with chromatin modifying complexes and the RNA polymerase machinery [7-10]. Depending on the species, the expression of CIITA is controlled by three-four tissue specific promoters[11, 12]. Following immune stimulation, activated B lymphocytes differentiate/mature into antibody secreting plasma cells [13]. As B cells transition to plasma cells, the expression of CIITA is silenced, resulting in the loss of MHC-II expression[14, 6].

Plasma cell differentiation requires an extensive epigenetic reprogramming and physical changes that allow them to secrete high levels of serum antibodies[15, 16]. This process represents a critical event in the development and control of the humoral immune response. The B lymphocyte induced maturation protein-1 (Blimp-1) encoded by the *pdrm1* gene has been shown to be an important master regulator of this transition as ectopic expression of Blimp-1 in some B cell lines induces plasma cell differentiation[17, 18], including the silencing of CIITA and MHC-II expression[19-21]. Moreover, conditional Blimp-1 deficient B cells showed an absence of plasma cells, leading to a decrease of serum immunoglobulin even though B cell development to this point was normal[22, 23]. Blimp-1 functions as a transcriptional repressor of gene activity[23, 24]. However, Blimp-1 is only part of this cell fate decision. A complex network of activators and repressors controls B cell fate and development. For example, while B cell lymphoma-6 (BCL-6) maintains the B cell program and represses *Blimp-1* expression, Blimp-1 represses the B cell program by silencing *BCL-6*, *Pax5*, and *SpiB*, critical modulators of B cell fate [24, 25]. Blimp-1

also serves to induce the plasma cell program by contributing to the regulation of *XBP-1* and *syndecan*-1[26, 27]. With respect to *CIITA* silencing, Blimp-1 was found to bind directly to sequences upstream of two of the tissue specific promoters of the *CIITA* gene (promoters III and IV), suggesting that it directly repressed *CIITA* expression[20, 28]. The mechanism of Blimp-1 action is still not well defined. In one report Blimp-1 was found to interact with histone H3 lysine dimethyltransferase G9a in regulating the expression of the interferon β gene[29, 30]. Additional participating factors and mechanism of Blimp-1 action are largely unknown.

The gene *ZBTB32* encodes a transcriptional repressor and member of the zinc finger broad complex, tramtrack, bric-a-brac protein family of proteins. ZBTB32, which is also known as repressor of GATA3 (ROG), PLZF like zinc finger protein (PLZP), and Fanconi anemia zinc finger (FAZF), was identified in several screens involving either immune cell tumorigenesis or development[30-33]. In T cells, ROG activity inhibits the development of Th2 cells by interfering with the activity of GATA3, a factor required for Th2 development and *IL-4* expression[34, 35]. ZBTB32/ROG also plays a role in lymphocyte homeostasis[31, 35]. However, a role for ZBTB32 in terminal differentiation events, such as B to plasma cell has not been described.

While Blimp-1 is highly expressed in plasma cells, it is not fully expressed at early time points when *CIITA* is repressed and the *CIITA* locus has lost the binding of positive regulators of transcription and its active chromatin marks[36]. This suggests that other factors may initiate or work with Blimp-1 to silence *CIITA*. Using cDNA microarray analysis a set of transcriptional/chromatin regulators of *MHC-II* and *CIITA* silencing during the B-plasma cell transition were identified. Of these, *ZBTB32* stood out as being highly induced early in an *ex vivo* B cell-plasma cell differentiation protocol. Further analysis showed that ZBTB32 could actively suppress *CIITA* gene expression and its removal by shRNA knockdown led to derepression of *CIITA* silencing. The mechanism was explored and found to be through the direct binding of ZBTB32 to the promoter region of *CIITA* and interaction with the polycomb repressive complex protein EZH2, which was also identified during the screen. Moreover, suppression of ZBTB32 in

plasma cells resulted in derepression of the B-cell specific regulator *Pax5*, suggesting that ZBTB32 may function to regulate additional aspects of the B cell developmental program.

Results

A large number of transcription factors and chromatin remodeling proteins are differentially regulated during *ex vivo* B cell differentiation

To investigate the breadth of factors that may be required for silencing *CIITA* expression during differentiation from B cells to plasma cells an *ex vivo* differentiation model was used[36]. Freshly isolated and purified $CD43^-$ splenic B cells were cultured with IL-2, IL-5, and LPS, which induces their differentiation. To assess differentiation, MHC-II and CD138 (syndecan-1), which represent B cell and plasma cell markers respectively, were examined by flow cytometry (Figure 1a). After 5 days of culture, MHC-II levels were reduced and CD138 levels were increased. Similarly, *I-Aα* and *CIITA* mRNA levels were greatly reduced under these conditions. Time course analysis of *CIITA* and *Blimp-1* showed that *CIITA* levels were significantly repressed within a 24 hour time period, but that *Blimp-1* levels were still not at their peak (Figure 1b), suggesting that other factors that were induced early may play an important role in this process. RNA transcripts from control and splenic B cells treated with IL-2, IL-5, and LPS for 24 hours were compared using cDNA microarray to identify potential candidate genes that were differentially expressed between B cells and this 24 h time point. Of the 33,000 murine targets queried, 2,370 genes were differentially expressed by more than 2 fold ($p<0.05$) with 1,169 were up regulated and 1,201 were down modulated (Figure 1c, Supplemental data table 1). In addition to the *MHC-II* genes, some of the many highly regulated genes were transcription factors or modifiers of chromatin structure (Table 1). For example, key B cell transcription factors, such as *Pax-5, BCL6, EBF1, Bach1, CD19, SpiB,* and *SpiC* were significantly repressed during the differentiation process. Enhancer of zest 2 (EZH2) and Chromobox homology 2 (CBX2), which

are members of the chromatin remodeling polycomb group (PcG) repressor complexes PRC2 and PRC1, respectively, were induced, whereas EZH1, a component of PRC2 complex was repressed. The expression of other chromatin assembly/remodeling factor genes, *Chalf, TfDP1, Asf1, HMGN3,* and *Smyd2* were also increased. Additionally, *ZBTB32*, which encodes a zinc finger repressor protein previously characterized in the regulation of T helper cell differentiation as a repressor of GATA3, was increased ~100 fold during the process. These results suggest that some of these genes may function to regulate *CIITA* expression and/or B cell differentiation.

B cell specific genes show diverse kinetics of regulation

To verify the cDNA microarray data and to provide a kinetic view of the expression of some of the differentially regulated genes, real-time RT-PCR was carried out on 12 repressed genes and 12 induced genes over a 5 day period following treatment with IL-2, IL-5, and LPS. For the repressed genes, two overall kinetic profiles were observed. One profile showed a near continual decline in gene expression over the time course (Figures 1b and 2). *CIITA, BCL6, BTLA, PDCD4, SpiB,* and *SpiC* fell into this group. In the second group, there was either a delay or a transient induction of the gene expression that peaked between 24 and 72 hours before returning to the baseline or lower at 5 days (*EBF1, MLL1, MTA3*). For the induced genes, there were two-three profiles. The profile exhibited by *Blimp-1* and *XBP-1* showed a steady increase in expression over the time course (Figures 1b and 2). Most of the other induced genes, including *ZBTB32* showed a transient, high burst of induction over the first 2 days with the final levels still significantly above the starting state in resting B cells (e.g., *Chalf2, EZH2, PRMT1, TfDP1, Asf1b, and IgG*). *Smyd2, G9a,* and *Stat6* showed a transient induction with a return to the B cell level by the end of the time course.

To determine whether a similar set of genes were differentially regulated in primary plasma cells, primary plasmablasts (CD138+CD44+) were purified from the spleens of mice at eight days following LCMV infection (Figure 3a), and the RNA profiles of 24 genes was

analyzed. Enrichment led to a population of cells that were 94% CD138+CD44+ (Figure 3a). As expected [37], *CD19* and *c-Myc* genes were repressed in the plasmablasts (Figure 3c). *c-Myc* was not repressed in the *ex vivo* system, mostly likely due to the stimulation provided by LPS in the differentiation cocktail. Although there were differences in magnitude, for the most part, the induction/repression of genes was similar to that observed in the *ex vivo* system (Figures 2 and 3c). Induction of *Blimp-1*, *XBP-1*, and *syndecan-1* was more pronounced and the levels of *ZBTB32* were ~13 fold higher than those in B cells. *Asf1b* and *TfDP1* were also induced but not at the same level as in the *ex vivo* system. Overall, these results verify the cDNA microarray data and provide a series of candidates that may regulate MHC-II genes and potentially other genes involved in B cell differentiation.

ZBTB32 regulates *CIITA* and *MHC-II* expression

To determine if some of the induced plasma cell transcription factors could suppress *CIITA* expression, cDNA expression vectors encoding nine of the genes were obtained and transiently transfected into the MHC-II and CIITA positive B cell line Raji (Figure 4a). Selection of this set of candidates was based on the overall induction strength and known function such that transcription repressors and chromatin assembly/remodeling proteins were chosen. The transient transfection efficiency by nucleoporation of Raji cells ranged between 35 and 60% (unpublished data). Of the genes transfected only three had a statistically significant effect on the repression of *CIITA*. These included *CBX2* and *EZH2*, which are members of the polycomb PRC1 and PRC2 complexes, respectively, and *ZBTB32*. Because ZBTB32 was the most efficient at repression and represented a novel candidate for *CIITA* silencing, its specificity of silencing was assessed by examining *HLA-DRA, MTA3, and BCL6* expression (Figure 4b). As expected due to a reduction in *CIITA*, *HLA-DRA* expression was reduced. *MTA3* and *BCL6* expression was not affected.

To determine if ZBTB32 could repress *CIITA* in primary murine B cells, YFP lentiviral expression vector co-expressing ZBTB32 was used to infect freshly purified murine B cells. The

levels of *CIITA* mRNA were assessed after 4 days. In these cultures a low level of LPS (5 µg/ml) was provided to maintain cell viability over the course of the experiment. The level of *CIITA* mRNA in the YFP-lentiviral cultures was similar to uninfected; whereas, the ectopic expression of ZBTB32 in primary murine B cells repressed *CIITA* expression (Figure 4c).

While the above results suggest that *ZBTB32* regulates *CIITA* when over expressed, a key question is what happens during development to *CIITA* expression if ZBTB32 is depleted from the system. To address this question, *ZBTB32* shRNA expressing lentiviral vectors were used to infect the plasmacytoma cell line P3X63Ag8 and determine if *CIITA* and other B cell specific genes would be derepressed in its absence. P3X63Ag8 cells represent fully differentiated plasma cells and express *ZBTB32* (Figure 5a). Three of the five lentiviral shRNA vectors were able to reduce ZBTB32 protein to varying degrees (Figure 5a). RNA from cells infected with these three vectors was further analyzed for expression of B and plasma cell genes. While each of the three shRNA vectors used had an effect on *CIITA* expression, *ZBTB32* shRNA vector 3 showed the greatest effect on *CIITA* derepression, resulting in a ~2,700 fold increase in its expression (Figure 5b). The other vectors (2 and 4) showed a 100 and 200 fold increase, respectively (Figure 5b). Consistent with *CIITA* expression, *I-Aα* expression was derepressed (70-300 fold) using the same vectors. Surprisingly, all three vectors substantially derepressed the mRNA levels of the B cell transcription factors *Pax5* and *SpiB*. The levels of *Pax5* mRNA increased between 200-800 fold increase. The levels of *BCL6* mRNA were slightly induced at levels between 2 and 3 fold depending on the shRNA. While no significant difference was observed for *syndecan-1* or *XBP-1* mRNAs, a 20-50% reduction in *Blimp-1* mRNA levels was observed.

To determine if ZBTB32 silencing could derepress *CIITA* expression during the *ex vivo* differentiation process, freshly isolated primary murine B cells that were treated to undergo *ex vivo* differentiation to plasma cells were infected with the lentiviral shRNA *ZBTB32* vectors (Figures 5a and c). Using *ZBTB32* shRNA vectors 2, 3, and 4, the levels of *CIITA* mRNA were significantly derepressed when compared to the empty viral vector control over the five day time

course of the experiment. The relative level of *BCL-6* mRNA remained the same. Together, these data demonstrate that *ZBTB32* is a component of the genetic pathway that represses *CIITA* transcription during the transition from activated B cell to plasma cell.

There are two zbtb32 knock out mice models that were studied in previous. Using zbtb32 knock out mice, we performed the ex vivo B cell differentiation. Primary B cell from zbtb32 knock out mice failed to repress CIITA expression at day 1 during ex vivo differentiation with IL2, IL5, and LPS, but ultimately repressed at day 5. The level of BCL6 mRNA repression was also delayed in zbtb32 knock out mice during differentiation (Figure 9). Interestingly, Blimp-1 knock out mice repressed CIITA expression at day 1 as wild type mice while the similar level of zbtb32 expression compared to wild type mice was detected.

ZBTB32 binds to the CIITA's B cell specific promoter region

To elucidate the mechanism and provide direct evidence that *ZBTB32* regulates *CIITA* expression, chromatin immunoprecipitation (ChIP) assays were conducted on the three different promoters associated with tissue specific expression of *CIITA*. Promoters I, III, and IV are responsible for *CIITA* expression in myeloid cell, lymphoid cell, and IFNγ-induced expression in non-hematopoietic cells, respectively [4, 6, 11]. ChIP amplicons were designed for each promoter region (Figure 6a). A three-day *ex vivo* differentiation time course on primary murine B cells was carried out and ChIP was conducted on days 0, 2, and 3. ZBTB32 was found to bind specifically to the 5' side of promoter III in the differentiated cells at day 2 with an increase in binding at day 3. No binding was detected in B cells. Blimp-1 binding was also tested and found to bind to pIII in agreement with previous reports[20, 38], but the relative increase in Blimp-1 occupancy was not significant until day 3. EZH2 and the histone H3 lysine 4 demethylase LSD1 also bound to this region in a time course that was more consistent with the binding of ZBTB32 than Blimp-1's. The binding of all of these factors to pI and pIV was low to background and did not change during the time course, indicating that silencing of *CIITA* was initiating at the upstream region of

pIII. Whereas ZBTB32, Blimp-1, EZH2, and LSD1 bound in the plasma cell state, PU.1 was bound only in the B cell stage. PU.1 binding to pIII in B cells is also in agreement with previous reports [36, 39, 40]. PU.1 also bound to a region just upstream of pI, but does not bind to pIV (Figure 6b). The decrease in PU.1 binding as cells transition between B and plasma cell stages was associated with a loss of protein (Figure 6c). In contrast the level of EZH2 increased. Thus, these data support a direct association of ZBTB32 with regulating *CIITA* gene expression in B cells. Moreover, the data demonstrate a direct role for EZH2 and LSD1 at promoter III. Because ZBTB32 binding to CIITA pIII precedes that of Blimp-1, we hypothesized that ZBTB32 binding is necessary for the binding of Blimp-1. To test this, ChIP assays for Blimp-1 were performed on chromatin isolated from P3X63Ag8 plasmacytoma cells after ZBTB32 protein depletion by shRNA. In comparison to untreated and control vector infected P3X63Ag8 cells, ZBTB32 depleted P3X63Ag8 cells displayed a remarkable reduction in Blimp-1 binding to pIII of CIITA (Figure 8). This suggests that ZBTB32 binding is necessary for Blimp-1 binding to the CIITA pIII promoter region.

ZBTB32 associates with Blimp-1 and the polycomb protein EZH2

The ChIP data suggest the hypothesis that ZBTB32 may participate in silencing *CIITA* by interacting in a complex with Blimp-1 and EZH2. To determine if that may be the case, immunoprecipitations using epitope tagged molecules were carried out following the transfection of expression plasmids encoding these genes into cells. Flag-tagged versions of EZH2, Blimp-1, and RFX5 were each co-expressed with myc-tagged ZBTB32 in HEK293T cells. Both Flag-EZH2 and Flag-Blimp-1 were found to coimmunoprecipitate with myc-ZBTB32 (Figure 7a), suggesting that each of these proteins interacts with ZBTB32. As a negative control, RFX5, a transcription factor that binds MHC-II gene promoters did not coprecipitate with ZBTB32. A similar set of assays was carried out on the endogenous proteins using human H929 plasma cells (Figure 7b). The result showed that ZBTB32 interacts with Blimp-1.

The above experiments suggest that ZBTB32 and Blimp-1 may colocalize in the nucleus of cells. To determine if this is the case immunohistochemical staining of P3X63Ag8 cells was performed. The confocal image showed considerable overlap in spatial positioning, suggesting colocalization (Figure7 c. d). To determine if colocalization also occurred during *ex vivo* differentiation, primary B cells were stimulated with IL-2, IL-5, and LPS to initiate B cell differentiation. Immunohistochemical staining over a three-day time course showed that Blimp-1 expression occurred after ZBTB32 was expressed, and co-localized with ZBTB32 in the nucleus of the 3-day differentiated cells (Figure7e).

Discussion

The silencing of *CIITA* and *MHC-II* gene expression represents a terminal event for the ability of a plasma cell to receive further TCR-MHC-II help from T lymphocytes, effectively removing that pathway and signaling system from the plasma cell. The transition from B cell to plasma cell represents a critical fate decision leading to effective humoral immune responses. During this transition, positive regulators of *CIITA* expression are evicted from its promoters, followed by the removal of all transcriptionally active chromatin marks and the deposition of repressive modifications to the chromatin structure [36]. Blimp-1 was identified as a key regulator of this transition [41] and as a repressor of *CIITA* expression [42]. However, silencing of *CIITA* begins at a time before Blimp-1 reaches a high level of expression. While it could be that the *CIITA* gene is sensitive to low levels of Blimp-1 expression, this inconsistency prompted us to search for factors expressed early in this transition that could mediate either fully or in part, *CIITA* silencing. In addition to identifying ZBTB32 as a mediator of early *CIITA* repression, the up regulation and recruitment of the polycomb repressor complex and histone demethylase LSD1 to the *CIITA* gene begin to define the steps for repression.

An *ex vivo* system to globally stimulate and differentiate naïve B cells was chosen such that sufficient material for analysis could be generated from early time points in the process. Intriguingly, the cDNA microarrays identified ~2,370 genes whose expression had already changed at 24 hours, with several genes showing log differences in their expression patterns. Verification of the microarray results using primary plasmablasts isolated 8 days after virus infection, provided proof that the *ex vivo* system mimicked to a large extent a plasma cell fate differentiation process. Previously, a number of gene expression profile comparisons between B cell and plasma cell lines or tumors representing these cell states were performed to identify genes that were associated with these genetic programs [43]. Consistent with our studies, a number of hallmark genes were clearly differentially regulated, including the up regulation of cell cycle/proliferation associated genes and *IL6, IRF4, IRF6, TfDP1*. Likewise, as in this study *Pax5, Bach1, CD19, BCL6, SpiB* and others were among some of the key down modulated genes observed previously. Many of these genes were found previously to be direct targets of Blimp1, including *Pax5, Id3, CIITA, c-Myc,* and *SpiB*[44]. Although ZBTB32 was not identified in any of the B cell/plasma cell microarray screens reported, it was identified in a number of T cell related functional screens, including as a suppressor of GATA3 transactivation for Th2 cell differentiation[34]. In these studies it was termed ROG for repressor of GATA3. In other studies of T cell differentiation and cancer, ZBTB32 was identified and termed FAXF, FAZF, TZFD, and ZNF538 [30]. Along with the study described here, ZBTB32 appears to have an extended function in lymphocyte differentiation.

Using the *ex vivo* differentiation system, a kinetic analysis of expression of select genes revealed an intriguing set of profiles. While most of the repressed genes tested displayed a steady decline in their steady state mRNA levels, the induced genes examined, showed at least two profiles. One profile that was pronounced by *Blimp-1* and *XBP-1* showed steady increases over time. The other showed a transient increase in expression peaking at day 2-3 and then a drop in steady state levels. In most cases the final steady state level after 5 days of treatment was still

higher than the B cell level. The transient expression of these transcription factors and chromatin related genes, such as *ZBTB32, Chalf2, EZH2, PRMT1, TfDP1*, could be necessary for initiating the plasma cell program, whereas the final expression level could be necessary for its maintenance.

Differential expression of the polycomb complex genes *EZH1, EZH2*, and *CBX2* was previously observed, and recent studies have shown that B cells express more *EZH1* than plasma cells, which express more *EZH2, CBX1,* and *CBX2* [43]. The change in PcG protein expression profiles suggests a unique role for EZH2 as opposed to EZH1 during B lymphocyte differentiation. Consistent with these findings was the observation that histone H3 lysine 27 di and trimethylation epigenetic marks, which are encountered with gene silencing, were observed in plasma cells at the CIITA promoter region III (data not shown). Such marks are placed by the polycomb group complex [45, 46].

ZBTB32 is a member of the BTB containing zinc finger protein family that recruits co-repressors, such as N-CoR and HDACs to its target genes[35]. ZBTB32 over expression and shRNA analysis revealed that ZBTB32 expression was a key component of the early steps in the *CIITA* repression. Sequence analysis of promoter III of the *CIITA* gene revealed a potential BTB-ZF family member binding site within 150 bp upstream of transcription start site where the reported Blimp-1 binding site is present [20]. Indeed, ChIP for ZBTB32 at pIII but not pI or pIV provided direct evidence for ZBTB32 functioning as a repressor of *CIITA* transcription. The ChIP analysis revealed additional information with respect to the mechanism. Because EZH2 was induced early in the differentiation process and previous reports have shown that EZH2 could repress pIV-mediated transcription in uveal melanoma cells[47], ChIP data were collected for EZH2 binding to *CIITA*. Intriguingly, EZH2 was found only at pIII and not at pI and pIV, suggesting a targeting of the polycomb complex to the B cell specific promoter. Because H3 K4 methylation is lost at pIII, it was not surprising that LSD-1, a H3 lysine 4 histone demethylase was found associated with pIII during the differentiation process. Others have reported that Blimp-1 can

interact with LSD1 in the human plasma cell line H929[38]. However, while LSD1 was found at the CIITA pIII in plasma cells, during the differentiation phase its occupancy was detected prior in Blimp-1's. Thus, the data are currently consistent with a model for the regulation of *CIITA* during plasma cell differentiation in which ZBTB32 is induced and binds to pIII. The binding of ZBTB32 initiates the recruitment of LSD-1, and the polycomb complex containing EZH2. During this transition, *CIITA* activators like PU.1 are evicted from the DNA. As Blimp-1 is induced and expressed, it occupies the *CIITA* promoter to aid in the commitment to silenced chromatin state and the plasma cell program. These repressive chromatin modifiers alter the histone code at the locus by removing activation marks and replacing them with the polycomb H3 lysine27 methylation signature for silenced genes.

Additional support for this model was provided by the analyses showing that ZBTB32, Blimp-1, and EZH2 could be coprecipitated in plasma cells or when over expressed in an *in vitro* system. Moreover, immunohistochemical staining of plasma cells and *ex vivo* differentiated plasma cells showed that ZBTB32 not only appears earlier in the nucleus than Blimp-1 but intriguingly that there is significant colocalization of the factors. This raises the issue of the breadth that ZBTB32 plays in plasma cell differentiation. One potential candidate for ZBTB32 regulation is *Pax5*. ShRNA knockdown of ZBTB32 in the plasma cell line resulted in a strong derepression of *Pax5*, indicating that its control is in the pathway mediated by ZBTB32 and that it may be a direct target of ZBTB32. Previously, Kallies et al. [48] provided evidence that the plasma cell program was initiated through the loss of *Pax5*, and that this occurred at a time prior to Blimp-1 expression. They suggested that an unknown factor could be the mediator of this process. Although significant experimentation is required to show that ZBTB32 is this factor, the current data are supportive of such an hypothesis.

While there are two mouse models that have introduced target deletions of the ZBTB32 gene [33], neither has investigated the role of ZBTB32 in plasma cell differentiation. In these mice peripheral and splenic B cells appear normal although they are reduced in overall numbers.

Reduction in lymphocytes, specifically the T cell pool was reported to be associated with a defect in lymphocyte homeostasis[33]. Thus, the role of ZBTB32 likely extends earlier than the formation of B and T cells. In summary, the data reported here introduce ZBTB32 as a major regulator of antigen processing and presentation and implicate it as a repressor in terminal B cell differentiation.

Materials and Methods

Cells, cell culture, and flow cytometry

CW13.20-3B3 (CRL-1669) referred to a BCL1 cells, Raji (CCL-86), P3X63Ag8.653 (CRL-1580), and NCI-H929 (CRL-9068) cell lines were purchased from ATCC. Murine BCL1 cells and P3X63Ag8 plasma cells were grown in RPMI 1640 medium (Mediatech) supplemented with 10% heat inactivated FBS (Hyclone Laboratories), 10 mM HEPES (HyClone Laboratories), 1 mM sodium pyruvate (HyClone Laboratories), 1X non-essential amino acid (HyClone Laboratories), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich). The Burkitts lymphoma B cell line Raji was grown in RPMI 1640 medium supplemented with 5% FBS (HyClone Laboratories), 5% bovine calf serum (HyClone Laboratories). The plasmacytoma cell line H929 was cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol. C57BL/6 and Balb/c mice were purchased from the Jackson Laboratory. Primary murine splenic B cells were purified from five week old C57BL/6 mice using MACS columns as previously described [36]. Purified murine primary B cells were incubated in culture media supplemented with IL-2 (20 ng/ml, Sigma-Aldrich), IL-5 (10 ng/ml, Sigma-Aldrich), and LPS (20 μg/ml, Sigma-Aldrich) for indicated time. HEK293FT cells were cultured in DMEM with 10% heat inactivated FBS to produce lentivirus.

Flow cytometry was performed following standard protocols using a FACSCalibur. Briefly, $1X10^6$ control or IL-2, IL-5, and LPS stimulated primary B cells were washed with PBS containing 0.1% BSA, incubated with PE conjugated IA/E or CD138 (syndecan-1) antibodies (BD Pharmingen) for 30 min on ice and washed again in PBS containing 0.1% BSA. Flow cytometry data was analyzed using CellQuest. All experiments were performed at least three times from independently isolated and purified cells.

To obtain primary plasmablasts from the spleen, Balb/c mice (6 per experiment) were infected by intraperitoneal injection with $2 X 10^5$ plaque forming units of LCMV Armstrong strain [49]. Eight days after infection, mice were euthanized and their spleens harvested. After homogenization and RBC lysis with ACK buffer (Gibco Inc.), splenocytes were suspended in DPBS with 2% FBS and 2 mM EDTA and stained with PE- or APC-labeled anti mouse CD138, PerCP-labeled anti mouse B220, and APC- or PE-labeled anti mouse CD44. All antibodies were purchased from BD Pharmingen. CD138-positive cells were enriched by magnetic bead positive selection using LS columns and anti-PE or anti APC-microbeads (Miltenyi Biotec). $CD138^{hi}$ $CD44^{hi}$ cells were then sorted by flow cytometry. Post sort purity was ~90-95% and approximately 5-10 million sorted cells were obtained per experiment.

Assessment of plasma cell purity by ELISPOT

ELISPOT analyses were performed essentially as described. Multiscreen HA plates (Millipore Corporation) were coated with goat anti-mouse IgG/IgA/IgM capture antibody (0.25 μg/well) (Caltag) and blocked with 10% FCS in PBS. Three fold serial dilutions of sorted plasma cells were seeded into each well in growth medium (RPMI 1640 with 10% FCS, 10 mM HEPES, 5.5 μM β-mercaptoethanol, and 100 U/ml penicillin-streptomycin). Plates were incubated 6-8 hours at 37°C in a 5% CO_2 atmosphere. After washing, foci of antibody secretion were detected with anti IgG-biotin or anti IgM-biotin (Southern Biotech) and Avidin-HRP (Vector Laboratories), using chromogen 3-amino-9-ethylcarbazole (AEC) substrate (Sigma Aldrich). Spots were

counted manually and divided by the number of cells seeded to determine the proportion of antibody secreting cells in the sorted population.

RNA isolation and RT-real time PCR

Total RNA was isolated using RNeasy mini prep kit (Qiagen, Inc.). 2 μg RNA was used to generate cDNA with reverse transcriptase (Invitrogen, Inc.) and random hexamer (Applied Biosystem, Inc.). Approximately $1/100^{th}$ of the reverse transcription generated cDNA was analyzed by real time PCR using an iCycler (Biorad, Inc.) to measure SYBR green incorporation of the PCR product. Primers that used in qRT-real time PCR are provided in Supplemental Table 2. 18s rRNA measurements by real-time PCR were used to normalize between samples. Normalized data from at least three independent cell preparations were averaged and expressed as fold over control treated samples. Student T tests were used to determine if significant differences between control and experimental samples were observed.

cDNA microarray analysis

RNA was prepared from purified murine primary B cells that were untreated and treated as described above with IL-2, IL-5, and LPS for 1 day. This RNA was submitted to the Emory Winship Cancer Institute DNA microarray core facility to conduct cDNA microarray analysis. The Applied Biosystems Mouse Genome Survey Microarray containing ~33,000 probes was used. Single color microarray technology was used to measure the absolute level of RNA. Two biological replicates were used. The data were analyzed using SAM (significant analysis of microarray) software to generate significant difference. Clustering algorithms by average linkage were performed to identify expression patterns within data [50].

Plasmids and transfection

Myc tagged ZBTB32 and Flag tagged EZH2, RFX5 and Blimp-1 were cloned into the pcDNA 3.1(-) expression vector directly from PCR amplified cDNA from P3X63Ag8 or Raji (RFX5). Other expression plasmids, which were used in transient transfection, were purchased from OriGENE Technologies, Inc.: *Asfb* (SC113719), *CBX2* (MC204062), *HMGN3* (SC110044), *Rad54* (MC205395), *SATB1* (MC200989), *SglII* (MC 205210), *TfDP1* (SC127935). $2X10^6$ of log phase growing Raji cells were transfected using an Amaxa Nucleofector following manufacturer's protocol.

Lentivirus generation and infection

Flag tagged ZBTB32 plasmid was cloned into YFP expressing lentivirus vector (pHR-UBQ-IRES-eYFP). These lentivirus constructs were co-transfected with the pseudo envelope protein VSV-G gene into HEK293FT packaging cells using FuGENE 6 transfection reagent (Roche). At 48 and 72 hours post transfection, virus was harvested, and infected into purified primary murine B cells in the presence of 8 μg/ml polybrene (Sigma Aldrich). Infection efficiency was determined by YFP expression and western blot using Flag antibody. For shRNA experiment, lentivirus constructs (pLKO.1) harboring shRNA to specific ZBTB32 (cat. RMM4534-NM_021397; clone number, TRCN0000096484, TRCN0000096485, TRCN0000096486, TRCN0000096487, TRCN0000096488) were purchased from Open Biosystems. For primary B cell experiments, five different lentivirus that express shRNA specific to ZBTB32 were infected into purified murine primary B cells, cells were then treated with IL-2, IL-5, and LPS to induce plasma cell differentiation for five days. To deplete ZBTB32 in the plasma cell line P3X63Ag8, lentivirus expressing shRNA to ZBTB32 was used, and cells were selected using puromycin for 6 days encoded in the pLKO.1 vector.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously [36, 51]. $4x10^7$ cells from murine primary B cell, which was untreated or treated with IL-2, IL-5, and LPS, were crosslinked with 1% formaldehyde for 15 min in culture media. Chromatin was isolated and sonicated to generate average 600 bp size of DNA. Chromatin (30 µg) was pre-cleared, and immunoprecipitated with specific antibodies overnight at 4°C. DNA was purified from the reverse crosslinked chromatin and 1/10 of each sample was analyzed by quantitative real time PCR using SYBR green incorporation for the CIITA DNA sequence of interest. Primers for CIITA promoter I, III, and IV are provided in Supplemental Table 2. Quantitation for each real-time PCR assay was carried out by comparison to a standard curve generated to genomic DNA. The average of at least three experiments was normalized to an irrelevant antibody control for the locus and plotted with respect to the amount of input chromatin.

Antibodies were purchased from a variety of sources as follows: LSD-1 (cat.ab17721) from Abcam, Ltd.; ZBTB32 (sc-25358), PU.1 (sc-352), and Blimp-1 (sc-13206) from Santa Cruz Biotechnology, Inc.; EZH2 (07-689) and monoclonal Myc (16-213) from Millipore, Inc.; Flag (F4042) and secondary anti-rabbit antibodies (A0545) from Sigma-Aldrich. For immunoprecipitation, the rabbit polyclonal Blimp-1 antibody was generated in collaboration with Rockland Farms, Inc.

Coimmunoprecipitation and western blot

Flag-or Myc-tagged ZBTB32, Blimp-1, EZH2, and RFX5 expression vectors were transiently transfected into HEK293T cells for 2 days. Whole cell lysates were prepared from each transfection. Following the manufacturer's protocol, antibodies were pre-incubated with magnetic beads (Invitrogen Corporation) overnight. Protein lysates were immunoprecipitated with antibody pre-bound beads for 3 hour at 4°C. After incubation, the beads were washed in 50 mM Tris (pH. 8), 150 mM NaCl, and 1% NP-40, and eluted with SDS-PAGE loading buffer. Immunoblots were performed to determine protein precipitation. Human plasma cell line H929

nuclear extracts were prepared as described previously [52]. Nuclear extract (250 μg) was incubated with specific antibodies for 1 hour on ice, and added 40 μl protein A sepharose following 3 hour incubation at 4°C. Precipitates were loaded in SDS-PAGE and immunoblotting was conducted with the indicated antibodies.

Immunofluorescence staining

Murine plasma cell line, P3X63Ag8 and *ex vivo* differentiated primary B cells were washed with PBS, and incubated with poly-L-lysine coated cover slips for 1 hour at room temperature. Cells then were fixed with 2% paraformaldehyde for 15 min at room temperature, and blocked with 1% BSA, 0.05% saponin (Sigma Aldrich) in PBS for 30 min. After blocking, cells were incubated with goat anti-Blimp-1 and rabbit anti-ZBTB32 antisera in blocking solution overnight at 4°C. Cells were washed with 0.05% saponin/PBS four times, and incubated with Alexa fluor 488 for anti-goat IgG and Alexa fluor 594 for anti-rabbit IgG for 1 hour at room temperature. The cover slips were mounted with mounting medium (Vector Laboratory), which contains DAPI. The images were analyzed by a confocal microscope (Olympus inverted 1X81 microscope/Olympus Fluoview FV1000) and visualized using the FV10-ASW version 1.7b program. .

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Figure Legends

Figure 1. *Ex vivo* treatment with IL-2, IL-5, and LPS differentiates primary murine B cells to plasma cells. Treatment of primary mouse B cells purified from splenocytes of five week old C57BL/B6 mice with IL-2, IL-5, and LPS for five days resulted in the loss of B cell markers and acquisition of a plasma cell expression. (A) $1X10^6$ cells collected from control and IL-2, IL-5, and LPS stimulated cells for five days were stained with PE conjugated I-A/E and CD138 (syndecan-1) antibodies and analyzed by flow cytometry. (B) Representing three independent experiments, the steady state RNA levels of *CIITA* and *Blimp-1* from IL-2, IL-5. LPS treated

cultures were measured by quantitative RT-PCR. The results were expressed relative to the day 0 time point. (C) *Ex vivo* differentiation leads to approximately 2,300 changes in gene expression. A cDNA profile from the expressed RNA from two independent experiments of control and *ex vivo* differentiated primary B cells with IL-2, IL-5, and LPS for 1 day was generated as described in the materials and methods. The data were normalized and the differences were analyzed using SAM (significant analysis of microarray) software to measure the fold change between controls and experiments. Genes with >2-fold change in expression were clustered using average linkage and Tree View was used to visualize the result of microarray and generate the presented heat map.

Figure 2. Kinetic profiles of selected transcription factors during *ex vivo* B cell differentiation. Quantitative RT-PCR was used to determine the steady state RNA levels of representative genes encoding transcription factors and chromatin remodeling proteins identified from the cDNA microarrays over a five-day time course of *ex vivo* differentiation of murine B cells with IL-2, IL-5, LPS. At least four different kinetic profiles were observed. The data were analyzed and normalized to the expression level of 18s rRNA and plotted with respect to the day 0 time point from three independent experiments.

Figure 3. Eight day old primary murine plasmablasts repress B cell genes while inducing plasma cell genes. Primary murine splenic plasmablasts were collected and analyzed for expression of select transcription factors. (A) Eight days following infection with LCMV, splenocytes were isolated and plasmablasts were sorted based on their expression of high levels of CD138 (syndecan-1) and CD44. (B) Purified cells from A were tested for antibody secretion by ELISPOT. Between 70% and 100% of the sorted cells were secreting antibody. (C) Steady state RNA levels of selected genes representing B cell and plasma cell genetic program were analyzed by qRT-PCR. Samples were analyzed as above using three independent RNA samples.

Figure 4. ZBTB32 and the polycomb complex proteins EZH2, and CBX2 repress CIITA expression. (A) cDNA expression vectors encoding nine transcription factors/chromatin assembly proteins identified from the cDNA microarrays were transfected into the human CIITA positive B cell line Raji by nucleoporation. This experiment has a transfection efficiency between 30-65% (data not shown). At 3 days post transfection, RNA was extracted from three individual experiments, and the level of *CIITA* mRNA level was evaluated by qRT-PCR. (B) The expression of *HLA-DRA, MTA3*, and *BCL6* mRNA steady state levels was also evaluated after ZBTB32 transfection into Raji cells. (C) Purified primary murine B cells were infected with lentivirus expressing either YFP or ZBTB32. Cultures were treated with 5 μg/ml LPS and 8 μg/ml polybrene for 4 days, and RNA was prepared and analyzed for *CIITA* mRNA levels as above. All experiments were conducted three times. The data were averaged, normalized to the expression of 18s rRNA and presented with respect to the levels of the control gene's expression, which was arbitrarily set a 1. In A and C the control gene was *CIITA*. In B, the control genes represent the target genes. Asterisks indicate differences between control and experiments that show significant differences with $p < 0.05$.

Figure 5. ZBTB32 is required for CIITA silencing during B cell development. ShRNAs were used to deplete cells of ZBTB32 and observe the effect on *CIITA* gene expression. (A) Lentivirus containing five different shRNAs specific to ZBTB32 were produced, and infected into murine plasma cell line, P3X63Ag8 cells or purified primary B cells together with IL-2, IL-5, and LPS to induce B cell differentiation, respectively. The efficiency of knock down to ZBTB32 was evaluated by western blot; left, P3X63Ag8; right, primary B cells. (B) Quantitative RT-PCR was performed on RNA isolated 6 days after infection of P3X63Ag8 cells with lentivirus expressing three different shRNAs as indicated. The RNA levels of *CIITA* and other B cell specific genes, *Pax-5, BCL6,* and *Spi-B,* as well as plasma cell specific genes, *Blimp-1, XBP-1,* and *syndecan-1* from the shRNA treated cells were examined as indicated and compared to the

control samples. Here, control samples represent a lentiviral infection that does not express an shRNA. The results are plotted with respect to the level from the control infection. (C) Primary B cells were infected with lentivirus harboring shRNAs to ZBTB32 or empty vector (control). After 5 days with IL-2, IL-5, and LPS treatment the levels of *CIITA* and *BCL6* mRNA expression were determined by qRT-PCR. RNA levels were expressed with respect to the control samples. The level of RNA from undifferentiated primary B cells is shown (gray bars). All experiments were performed three times and for B and C the data are averaged.

Figure 6. ZBTB32 binds at CIITA promoter III, but not pI or pIV in differentiated plasma cells. (A) schematic representation of the three CIITA promoters (pI, pIII, and pIV) is shown. (B) Chromatin prepared from primary B cells that was untreated and treated with IL-2, IL-5, and LPS for indicated time points was used in a ChIP assay. Using antibodies to specific ZBTB32, Blimp-1, EZH2, LSD-1, and PU.1, the occupancy of these factors was tested. The amount of input chromatin was used to normalize results from these experiments. The averaged data from three independent experiments were plotted with respect to the amount of chromatin precipitated with an irrelevant antibody. (C) During *ex vivo* B cell differentiation, the level of indicated protein expression was determined by immunoblot. Actin immunoblots demonstrate even loading of samples.

Figure 7. ZBTB32 associates with Blimp-1 and the polycomb protein EZH2.

(A) Flag-tagged Blimp-1, EZH2, and RFX5 were transfected with Myc-tagged ZBTB32 in HEK293T cells as indicated. Whole cell lysates were prepared and immunoprecipitated with anti-Flag antibody. Precipitates were blotted with anti-Myc antibody. 10 % input of the lysates and the precipitate were separated by SDS-PAGE and determined by the indicated antibody. (B) Coimmunoprecipitation of Blimp-1 and ZBTB32 were performed with nuclear extracts of H929 plasma cells. Immunoprecipitation and western blot were conducted. The input represents 16 %

of lysate used. Blots were representative of most 2 experiments. (C), (D) Immunohistochemical staining of P3X63Ag8 using Blimp-1 and ZBTB32 antibodies were performed and evaluated by confocal microscope (C). Enlargement of cells described by arrow in C (D). Nuclei were co-stained with DAPI. (E) Immunohistochemical staining of Blimp-1 and ZBTB32 during *ex vivo* differentiation was detected.

Figure 8. ZBTB32 is required for Blimp-1 recruitment to CIITA promoter III.

P3X63Ag8 cells were infected with ZBTB32 shRNA lentiviral vectors 3 and 4. After 6 days of selection in puromycin, ChIP assays for Blimp-1 binding at CIITA pIII were performed. These experiments were performed with three independent chromatin preparations as above.

Figure 9. zbtb32 knock out mice fail to repress CIITA at early phase (Day 1) during B cell differentiation.

Primary B cells were purified from zbtb32 KO, Blimp-1 KO, and wild type mice and then, ex vivo differentiated into plasma cell with IL2, IL5, and LPS. During indicated time points, RNA was prepared and quantitative RT-real time PCR was conducted. At least three experiments were performed, and data were analyzed over undifferentiated primary B cells.

Supplemental Table 1. cDNA microarray table

Genes that were changed more than two fold in gene expression are listed with annotation.

Supplemental Table 2. Primers for RT-real time PCR and Chromatin IP-coupled real time PCR

Figure 1

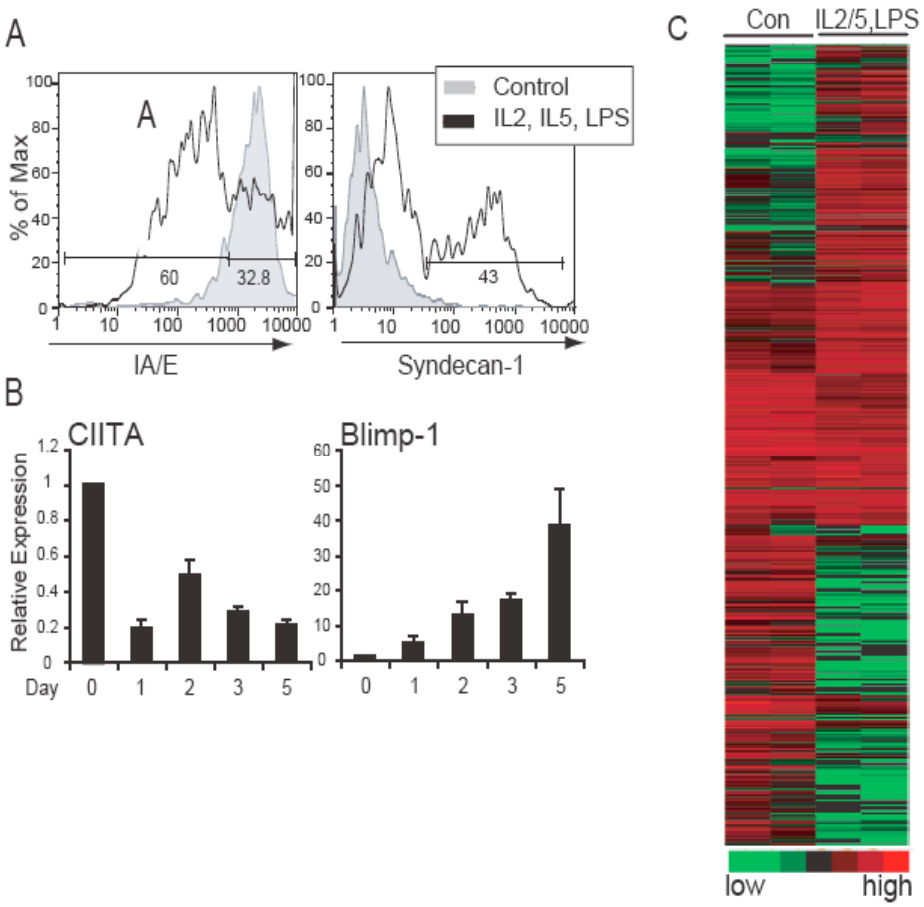

A
% of Max
A
Control
IL2, IL5, LPS
60
32.8
43
IA/E
Syndecan-1
B
CIITA
Blimp-1
Relative Expression
Day
0
1
2
3
5
C
Con
IL2/5,LPS
low
high

Table 1

Table 1

Differential expression of selected genes during ex vivo plasma cell differentiation

Induced genes				Repressed genes					
Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
ZBTB32	102.2	Tcf19	8.2	ATF7	0.50	Pdcd4	0.33	Bank1	0.18
Msfd2	92.3	HMGN3	7.8	Pax5	0.48	KLF13	0.31	Satb1	0.18
Ptgir	68.1	TfDP1	7.5	ELF2	0.48	SpiB	0.31	EZH1	0.17
Hist1h2ag	64.3	PRDM16	6.9	Setd2	0.46	Prkar1b	0.3	Myst3	0.12
cdc6	30.0	HMGB2	6.6	Chmp1b	0.45	CD19	0.3	SpiC	0.01
IL2R	29.0	EZH2	6.5	Bach1	0.42	Foxo1	0.29	MHC related Genes	
IL6	27.2	CBX2	6.0	KLF7	0.42	Mef2d	0.27	Gene	Fold Change
Pbk	19.3	Mxd3	5.6	Jarid2	0.40	Hhex	0.25	H2-Eb1	0.48
Batf	15.9	Nudcd2	5.2	MLL5	0.39	KLF3	0.24	H2-Q7	0.43
Chalf	15.7	Timeless	5.1	Arid1a	0.38	Btla	0.22	H2-T23	0.32
Solt	11.1	Bard1	3.9	JMJD1c	0.38	BCL6	0.22	H2-DMb1	0.18
Luzp5	9.8	IRF6	3.8	EBF1	0.37	Pink1	0.21	H2-DMa	0.16
Smyd2	9.3	PRMT1	3.4	MLL1	0.36	MLL3	0.21	H2-Oa	0.14
Asf1	8.3	HDAC2	2.9	Zhx2	0.35	Cabc1	0.21	CIITA	0.14
		ZFP265	2.9	JMJD1a	0.33	p450	0.2	H2-Ob	0.07

Figure 2

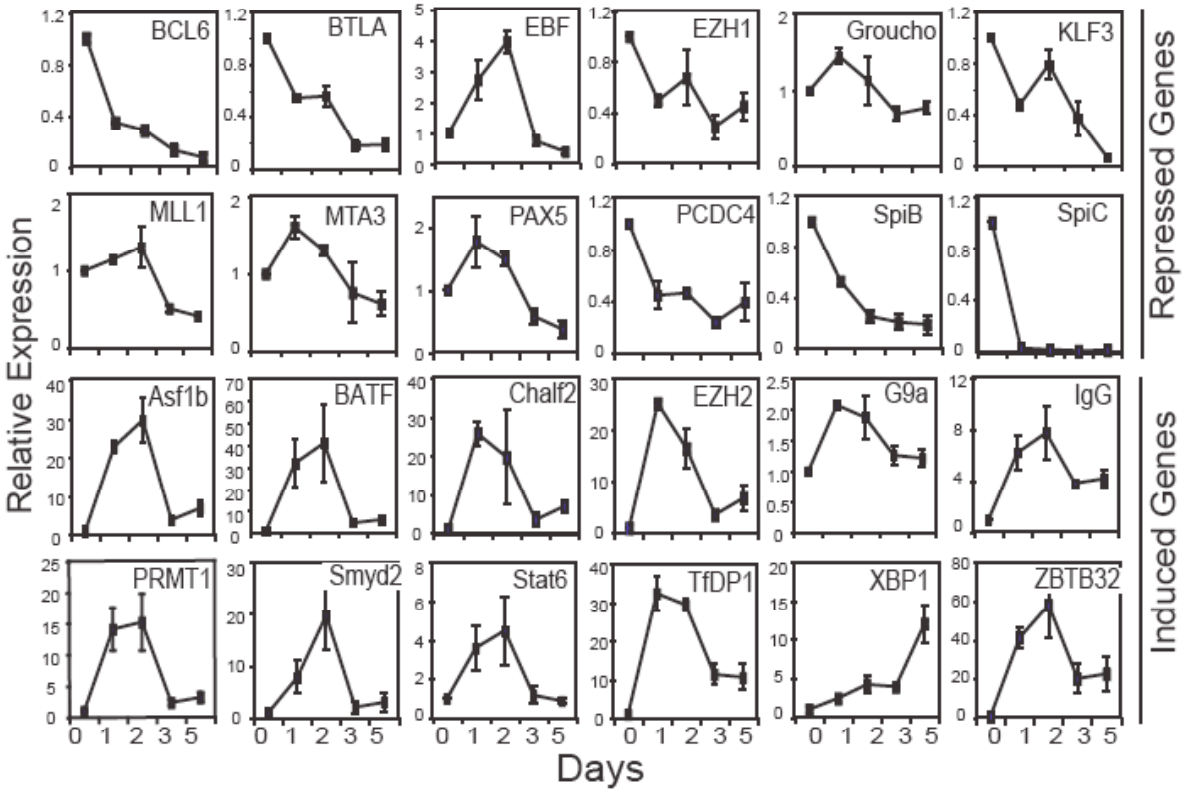

BCL6
BTLA
EBF
EZH1
Groucho
KLF3
MLL1
MTA3
PAX5
PCDC4
SpiB
SpiC
Asf1b
BATF
Chalf2
EZH2
G9a
IgG
PRMT1
Smyd2
Stat6
TfDP1
XBP1
ZBTB32
Relative Expression
Days
Repressed Genes
Induced Genes

Figure 3

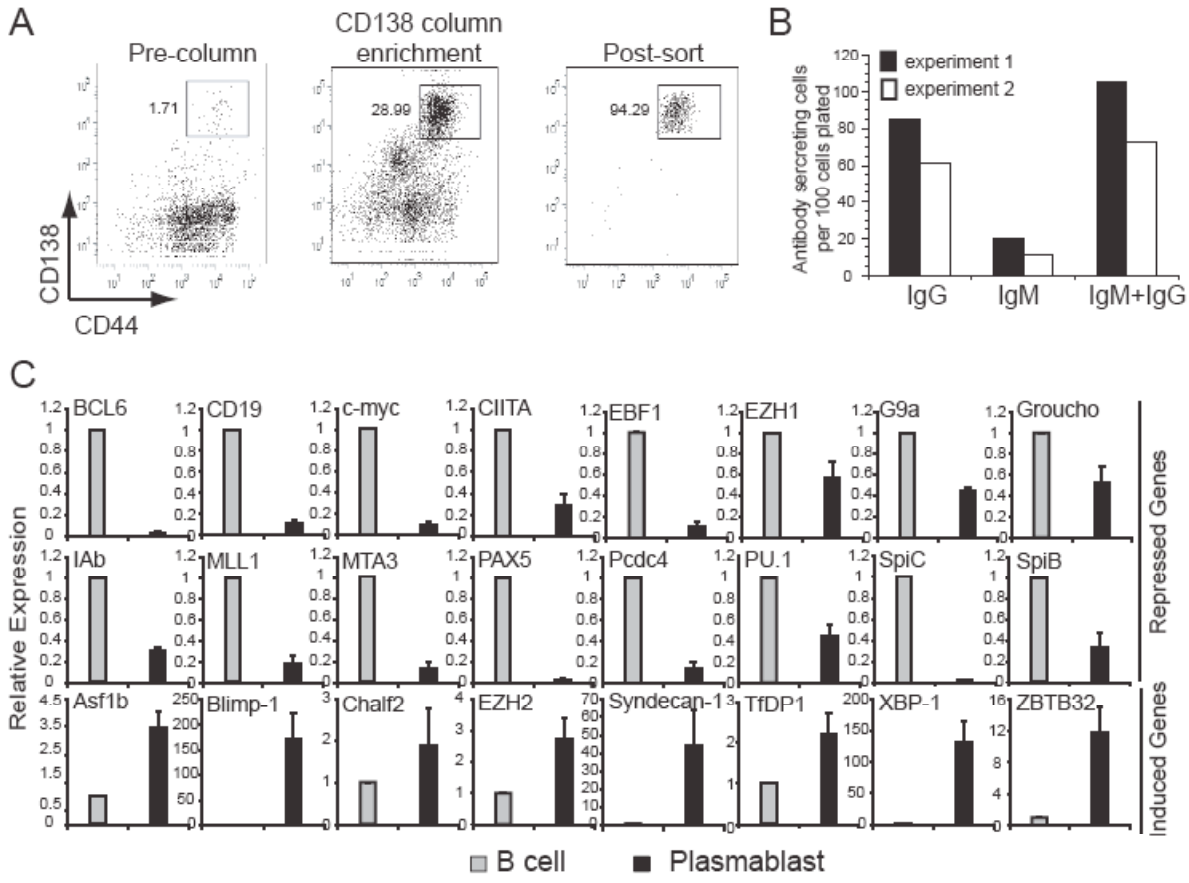

A
Pre-column
CD138 column enrichment
Post-sort
1.71
28.99
94.29
CD138
CD44
B
experiment 1
experiment 2
Antibody sercreting cells per 100 cells plated
IgG
IgM
IgM+IgG
C
Relative Expression
BCL6
CD19
c-myc
CIITA
EBF1
EZH1
G9a
Groucho
IAb
MLL1
MTA3
PAX5
Pcdc4
PU.1
SpiC
SpiB
Asf1b
Blimp-1
Chalf2
EZH2
Syndecan-1
TfDP1
XBP-1
ZBTB32
Repressed Genes
Induced Genes
B cell
Plasmablast

Figure 4

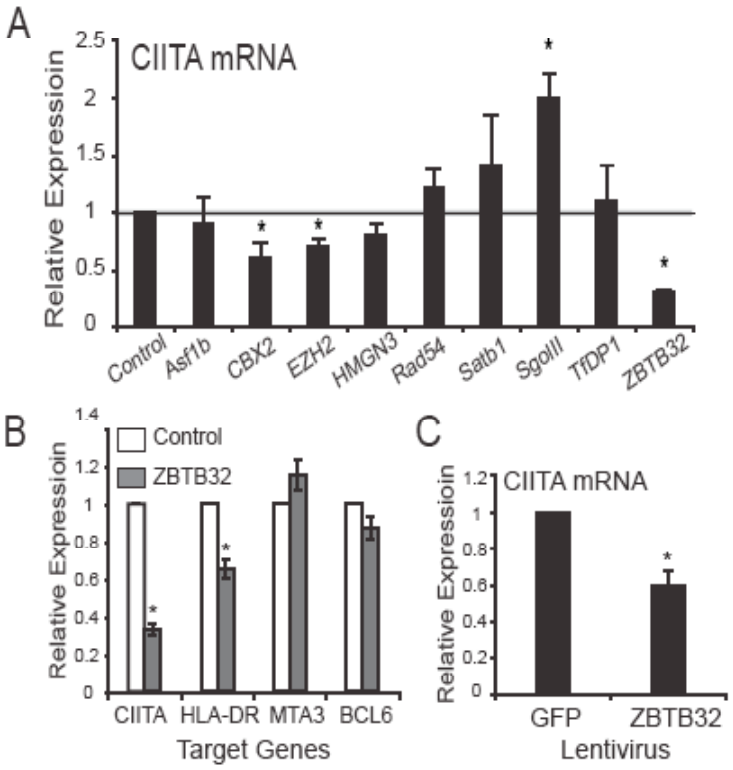

A
CIITA mRNA
Relative Expressioin
2.5
2
1.5
1
0.5
0
Control
Asf1b
CBX2
EZH2
HMGN3
Rad54
Satb1
SgolII
TfDP1
ZBTB32
B
Control
ZBTB32
Relative Expressioin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CIITA
HLA-DR
MTA3
BCL6
Target Genes
C
CIITA mRNA
Relative Expressioin
1.2
1
0.8
0.6
0.4
0.2
0
GFP
ZBTB32
Lentivirus

Figure 5

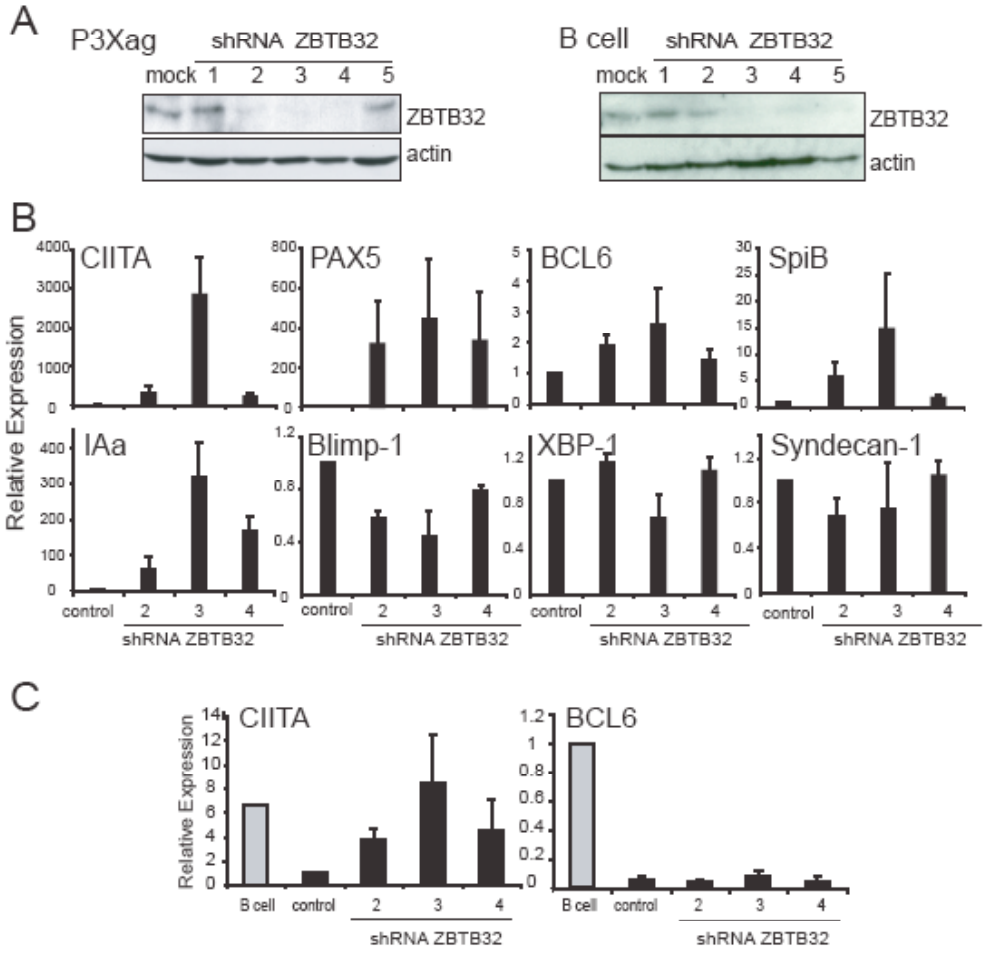

A
P3Xag
shRNA ZBTB32
mock 1 2 3 4 5
ZBTB32
actin
B cell
shRNA ZBTB32
mock 1 2 3 4 5
ZBTB32
actin
B
Relative Expression
CIITA
PAX5
BCL6
SpiB
IAa
Blimp-1
XBP-1
Syndecan-1
control 2 3 4
shRNA ZBTB32
C
Relative Expression
CIITA
BCL6
B cell control 2 3 4
shRNA ZBTB32

Figure 6

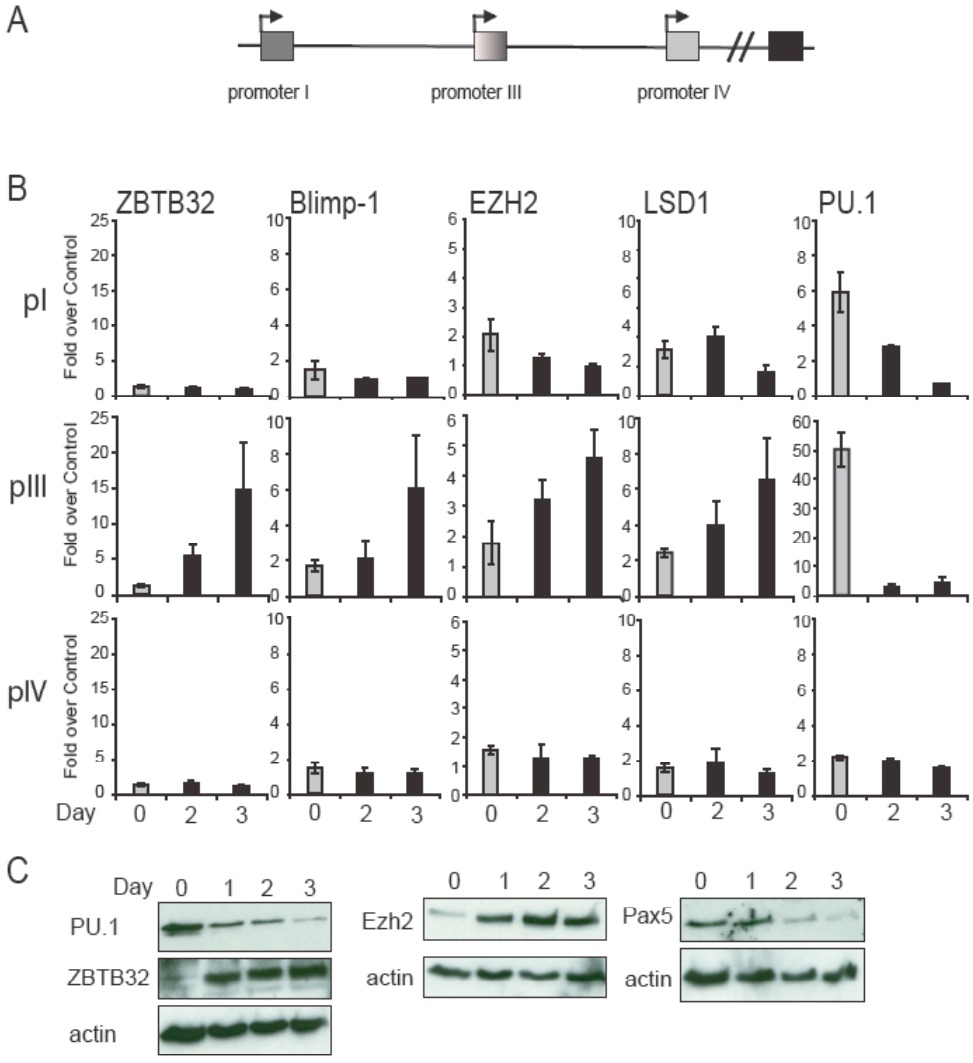

A
promoter I
promoter III
promoter IV
B
ZBTB32
Blimp-1
EZH2
LSD1
PU.1
pI
pIII
pIV
Fold over Control
Day
0 2 3
C
Day 0 1 2 3
PU.1
ZBTB32
actin
0 1 2 3
Ezh2
actin
0 1 2 3
Pax5
actin

Figure 7

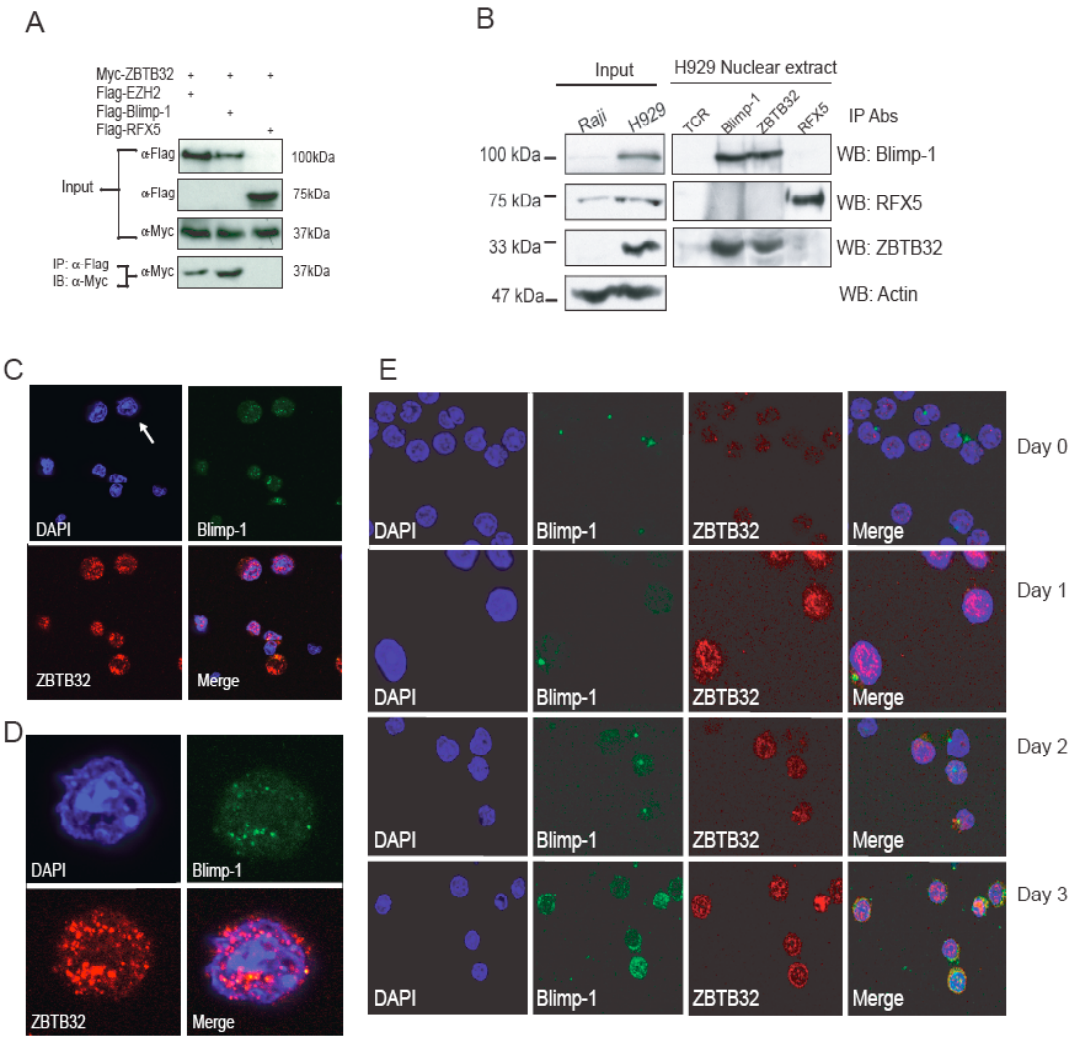

A
Myc-ZBTB32 + + +
Flag-EZH2 +
Flag-Blimp-1 +
Flag-RFX5 +
Input
α-Flag
α-Flag
α-Myc
100kDa
75kDa
37kDa
IP: α-Flag
IB: α-Myc
α-Myc
37kDa
B
Input
H929 Nuclear extract
Raji
H929
TCR
Blimp-1
ZBTB32
RFX5
IP Abs
100 kDa
75 kDa
33 kDa
47 kDa
WB: Blimp-1
WB: RFX5
WB: ZBTB32
WB: Actin
C
DAPI
Blimp-1
ZBTB32
Merge
D
DAPI
Blimp-1
ZBTB32
Merge
E
DAPI
Blimp-1
ZBTB32
Merge
Day 0
Day 1
Day 2
Day 3

Figure 8

pIII

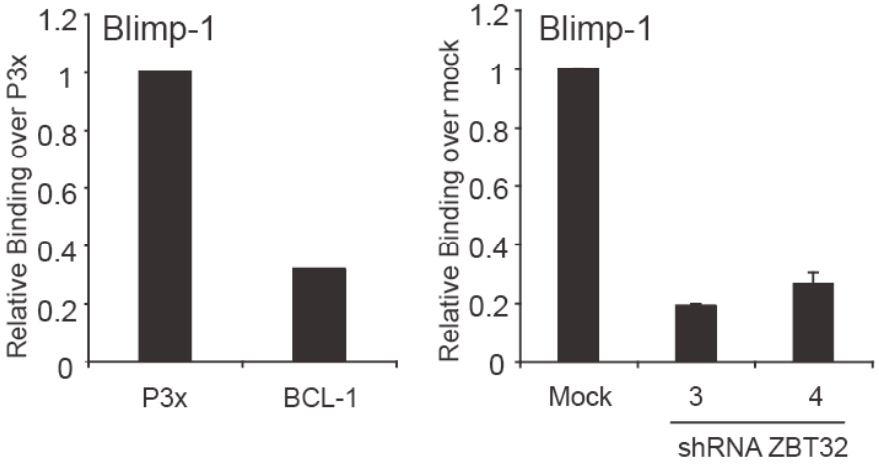


Table 2

Genes		Sequence
18srRNA	FF	5-GTAACCCGTTGAACCCCATT
	RR	5-CCATCCAATCGGTAGTAGCG
Asf1b	FF	5-CTGACGACCTGGAGTGGAAG
	RR	5-AGGAATGAGGGATGGGTTTGG
BCL6	FF	5-CACACTCGAATTCACTCTG
	RR	5-TATTGCACCTTGGTGTTGG
Blimp-1	FF	5-ATGGAGGACGCTGATATGAC
	RR	5-CCTTACTTACCACGCCAATAAC
CIITA	FF	5-GCAGCTGGACTACAGACGTTAC
	RR	5-GGAGTTCCAGGTAGCTGCCCTCTG
Chaf2	FF	5-ACAAGAAGACAGCCATCATCC
	RR	5-CCACATCGTCATCCTCATCC
c-myc	FF	5-GGGCCAGCCCTGAGCCCCTAGTGC
	RR	5-ATGGAGATGAGCCCGACTCCGACC
EBF	FF	5-TCCCAGCCCTTGCTAACAC
	RR	5-CTTGCAGGCTGTTCCCGTT
EZH1	FF	5-CGCCGCCGCCGAAGG
	RR	5-CCAGTCATTGCCAGTGTCTCTATC
EZH2	FF	5-CAGTTCGTGCCCTTGTGTGATAGC
	RR	5-CACTCTCGGACAGCCAGGTAGC
Groucho	FF	5-CCTGCCTGCTATGCTCTG
	RR	5-TTGTCCAAACCACCTGTCC
G9a	FF	5-GGTGGTGGGCGATGACTTC
	RR	5-TTCTTCCTCCTCCTCCTCTTCC
IAb	FF	5-GACGCAGCGCATACGGCTC
	RR	5-CCGCCGCAGGGAGGTGCT
IgG	FF	5-GCTAAATCTTGCTGCTGGGACTC
	RR	5-TGTCAGTGTCACCGTGTCTTCC
MLL1	FF	5-TGAGGACAAGAAGCGGAAGG
	RR	5-AGCGGCAAGAGGACTAACG
MTA3	FF	5-CCGAGAGCAAATTGAAACAAG
	RR	5-AGGAATACCACTGATGAGACTG
Pax5	FF	5-CTCCCAGATGTAGTCCGCCAAAG
	RR	5-GCTTGATGCTTCCTGTCTCATAATACC
Pcdc4	FF	5-AGCGGTTAGAAGTGGAGTTGC
	RR	5-TCCTGGTCGTCATCATAGTTTGG
Prmt1	FF	5-GTTACTGCCTCTTCTACG
	RR	5-TGTCGGTCCTCAATGG
PU.1	FF	5-TCTCGTCCAAGCACAAGG
	RR	5-GCCGCTGAACTGGTAGG
Smyd2	FF	5-ACCAAGGATAAGGACAAAGCCAAGG
	RR	5-TGACATTGCGTGCGTATCTGACC
SpiB	FF	5-AGGACTTCACCAGCCAGACC
	RR	5-TCGGAGCCAGCCAAGAGG
SpiC	FF	5-AACACTCGCTGAACTCTGG
	RR	5-GCCTCGCTGAACTGGTAG
Stat6	FF	5-CACTATAAGCCCGAACAGAT
	RR	5-CTACCATAGTCACATCTGA
Syndecan-1	FF	5-GCCACAGGTGCTTCTCAGAG
	RR	5-TGCCTTCGTCCTTCTTCTTCATG
TfDP1	FF	5-AGAGGAGGCTGGAGAGG
	RR	5-GGTGTTGACAATGATGAAGG
XBP1	FF	5-GGGCATTCTGGACAAGTTGGAC
	RR	5-AGAGAAAGGGAGGCTGGTAAGG
ZBTB32	FF	5-CGTCTGGGCAAGGGTTCAC
	RR	5-CCAGAGCGAGCAGGAGAGG

Supplement Figure 1

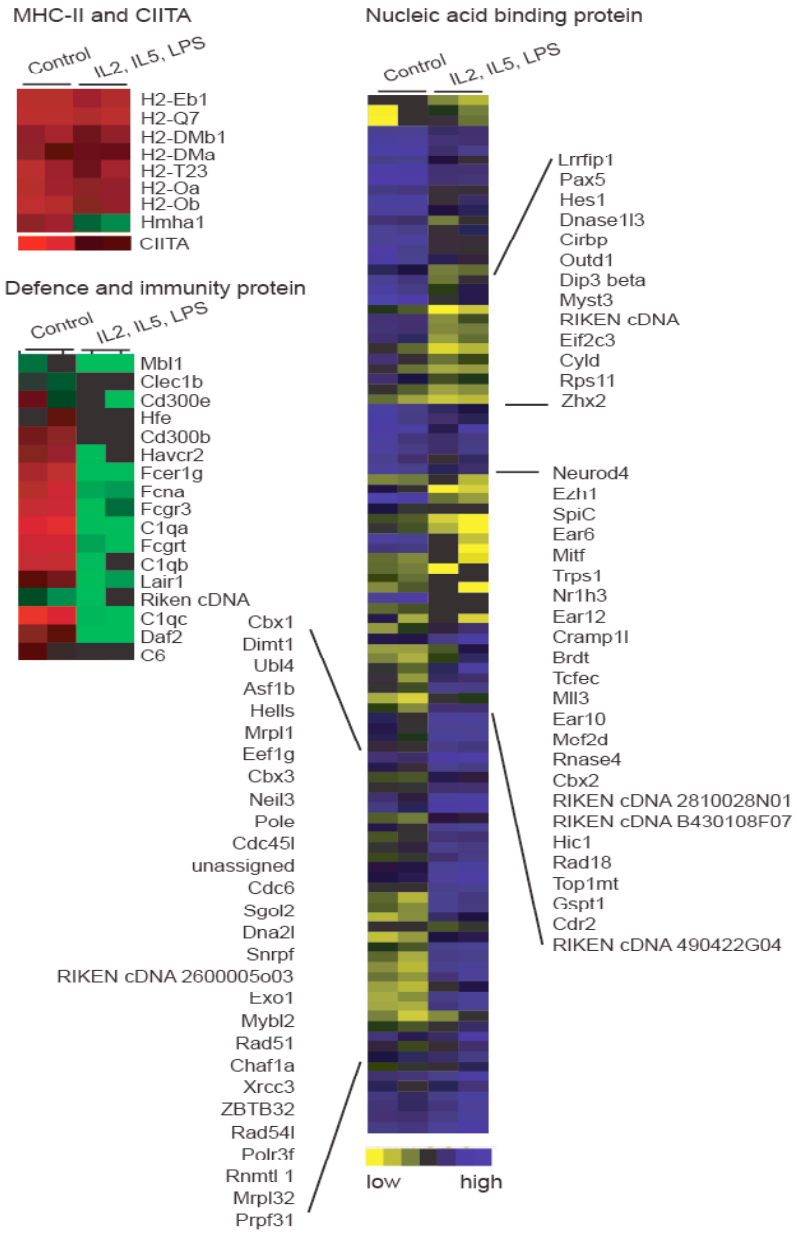
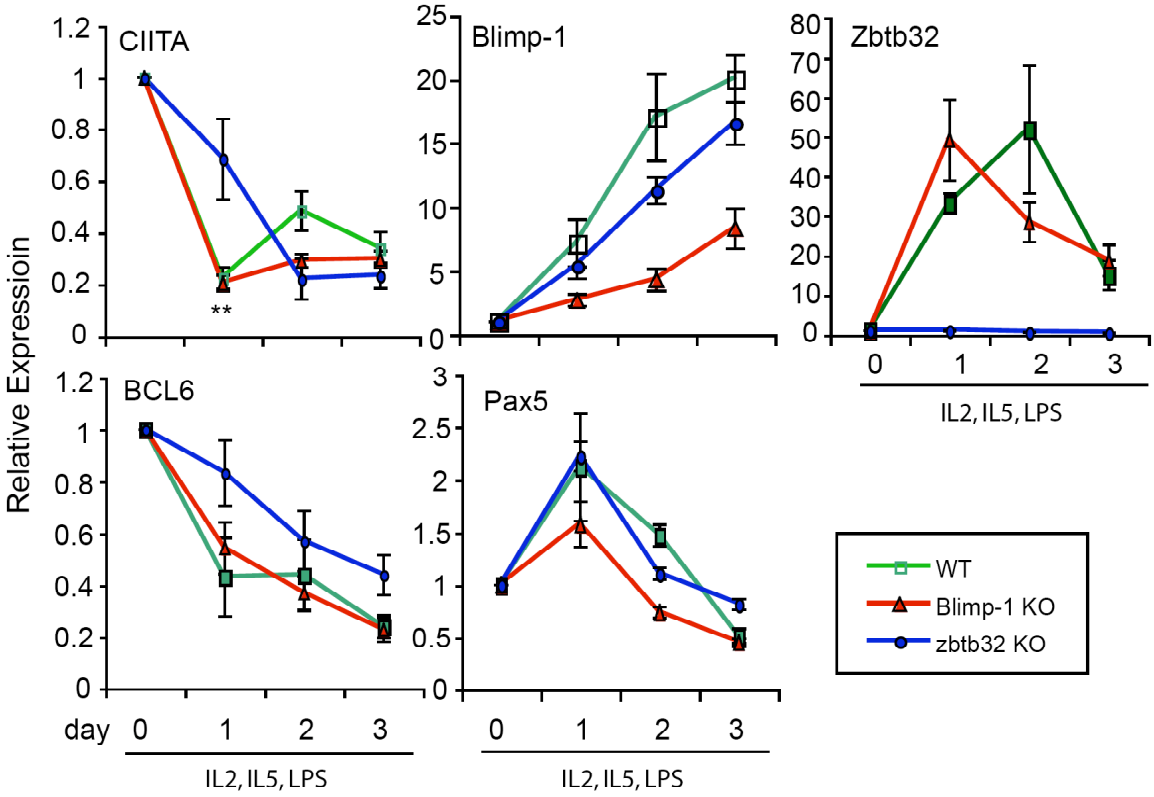


Figure 9


Relative Expressioin
CIITA
Blimp-1
Zbtb32
BCL6
Pax5
**
day
0
1
2
3
IL2, IL5, LPS
WT
Blimp-1 KO
zbtb32 KO

APPENDIX A:

The regulation of CIITA by CTCF (CCCTC binding factor)

This work is in progress.

siRNA, knockdown of CTCF was conducted by Parimal Majumder, Ph.D. Hyesuk Yoon carried out all other experiments including RT-real time PCR to determine gene expression in CTCF knock down cells.

This was written by Hye Suk Yoon and edited by Dr. Jeremy Boss.

CTCF (CCCTC binding factor) is a zinc finger protein that is involved in gene regulation by transcription repression, activation, or insulation (Dunn, 2003). CTCF also plays a role in chromosomal organization by long-range chromosome interaction (Majumder et al., 2008; Splinter et al., 2006). Kim et al. and Barski et al. found that the human genome has 13,804 binding sites in fibroblast and 20, 282 sites in resting human T cells, respectively, using ChIP-on-chip or ChIP-seq approaches (Kim et al., 2007; Barski et al., 2007). While most of CTCF binding sites are located in intergenic regions (~46%), other sites are found in intronic, exonic, or within genes (Kim et al., 2007). Several genes, such as immunoglobulin and MHC class II, were regulated by chromosomal organization through CTCF interactions in their regulatory regions on the genes (Splinter et al., 2006).

We identified CTCF sites on CIITA genes using genome comparison followed by chromatin Immunoprecipitation (ChIP). These CTCF binding sites termed CTCF1 and CTCF2 are located in the intronbetween exon 16 and exon 17 of the human CIITA gene (Figure 1, 2). The CTCF1 site shows a greater level of species conservation (Figure 1).

We examined the role of these CTCF sites in CIITA regulation. We used the human B cell line Raji and the human plasma cell line H929 that are CIITA positive and negative cells, respectively, to test if CTCF influences CIITA expression. Human B cell line Raji and plasma cell line H929 expressed similar levels of CTCF protein (Figure 2A). Chromatin immunoprecipitation was carried out to determine CTCF binding at two potential CTCF binding sites CTCF1 and CTCF2, as well as a irrelevant control locus located between CIITA promoter I and II (Figure 2B). Interestingly, CTCF bound to both CTCF1 and CTCF2 sites in B cell and plasma cell. However, B cells showed more CTCF binding than plasma cells, but the difference was not dramatic (Figure 2B). CTCF binding was not observed in the control region. In B cells, CIITA is constitutively expressed under the control of CIITA promoter III; therefore, it was

tested whether CTCF binding on CTCF1 and CTCF2 sites are also detected in other cells besides B cells. To determine CTCF binding in different cells, A431 cell lines that induce CIITA promoter IV-dependent transcripts in response to IFN-γ were used. CTCF binding was detected from A431 cells with and without IFN-γ stimulation at both CTCF1 and CTCF2 (Figure 2B). The human T cell line Jurkat cell was also examined for CTCF binding. The data showed that CTCF bound both CTCF 1 and CTCF2 (data not shown). Therefore, CTCF binding on CTCF1 and CTCF2 is not dependent on cell types or cytokine stimulation.

CTCF binding also plays a role in enhancer blocking insulator. Gomez et al. showed that XL9 sequence located between HLA-DR and HLA-DQ interacts with CTCF and displayed enhancer-blocking activity (Gomez, 2005). Therefore, we determined if CTCF1 and CTCF2 have enhancer blocking insulator activity. CTCF1 and CTCF2 sequences were introduced into the pGL3 enhancer and promoter plasmid containing the firefly reporter genes, and the promoter activity was measured by production of lucifease. The data revealed that both CTCF1 and CTCF2 displayed enhancer- blocking activity in B cells and plasma cells (Figure 3). Thus, CTCF1 and CTCF2 appear to be insulators and CTCF binding at these sites may be essential to prevent the transcription regulatory effects from other genes or regulatory elements. Therefore, we examined the role of CTCF in CIITA expression along with CIITA neighboring genes, NuBP-1 and Dexi. If CTCF binding on the CIITA gene functions to regulate gene expression of CIITA and neighboring genes, different gene expression profiles might be observed when comparing B cells with CTCF depleted B cells. RNAi was used to deplete CTCF from Raji B cells. After 72 hour post transfection, RNA was prepared, and gene expression of CIITA and its neighboring genes were assessed. The level of HLA-DR mRNA that was repressed by CTCF depletion from Raji cells in previous study was also assessed. The expression of BCL6, which is not located on chromosome 16, was measured as well. HLA-DR expression was diminished after CTCF knock down as shown previously, while BCL6 expression was not changed after CTCF knock down. Interestingly, CIITA and NuBP-1 whose genes are transcribed in same orientation were repressed

in CTCF depleted cells; on the other hand, the Dexi gene that is transcribed in reverse orientation to CIITA was not affected by CTCF depletion (Figure 5), indicating the possibility that the presence of CTCF binding around CIITA genes is critical for prohibiting the spread of gene silencing or activation from surrounding genes. This depletion assay needs to be conducted in plasma cells to determine the role of CTCF in CIITA silencing. However, the role of CTCF binding at CTCF1 and CTCF2 is not understood. Further, studies using BAC modification, which deletes CTCF1 and CTCF2 region from BAC encoding CIITA and neighboring genes, are necessary to determine the function of CTCF1 and CTCF2 region.

Additionally, other CTCF binding sites were identified in the mouse CIITA by Chip analysis. These sites surround pI (Figure 5). Interestingly, one site is located upstream of pIII, where DNA methylation was detected using immunoprecipitation for methylated DNA (MeDIP) in plasma cells (Figure 6), but not in B cells. This result suggests that DNA methylation at the CIITA promoter III in plasma cells prevents CTCF binding. The possible model proposed by this finding is described in the discussion.

In summary, several CTCF binding sites on the CIITA gene were identified in humans. CTCF1 and CTCF2 elements located between exon 15 and exon 16 of CIITA bind to CTCF independent of cell type. Both sites are function as enhancer-blocking insulators, but it is not clear how these elements insulate CIITA from neighboring its genes. Additionally, CTCF binding sites were also found in mice. One is located 1 kb upstream of promoter I, and binds CTCF in both B cells and plasma cells. Another site is located 500 bp upstream from the CIITA promoter III region, and only binds to CTCF in B cells, but not in plasma cells where DNA is methylated.

For further study, these elements need to be mutated to determine how they affect CIITA expression in B cells and plasma cells.

Figure 1

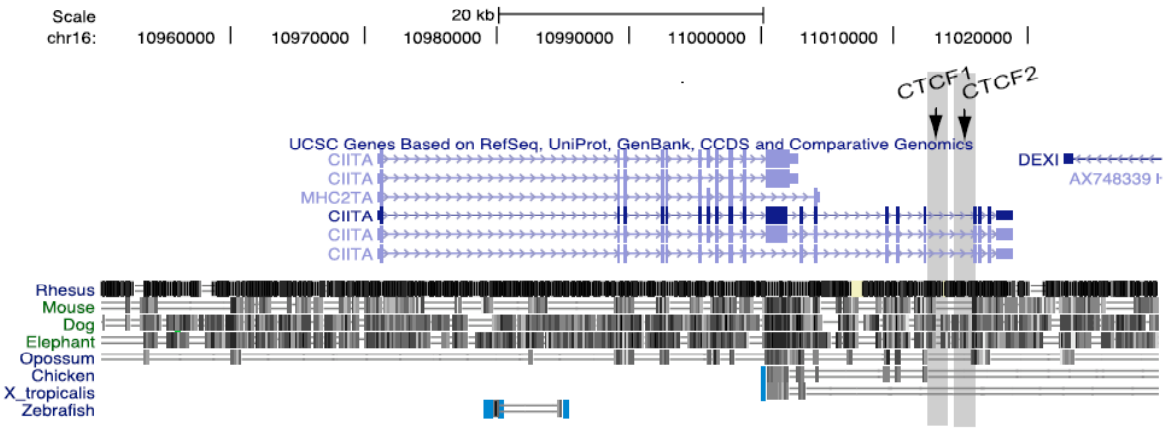

Scale
chr16:
20 kb
10960000
10970000
10980000
10990000
11000000
11010000
11020000
CTCF1
CTCF2
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
CIITA
CIITA
MHC2TA
CIITA
CIITA
CIITA
DEXI
AX748339
Rhesus
Mouse
Dog
Elephant
Opossum
Chicken
X_tropicalis
Zebrafish

Figure 2

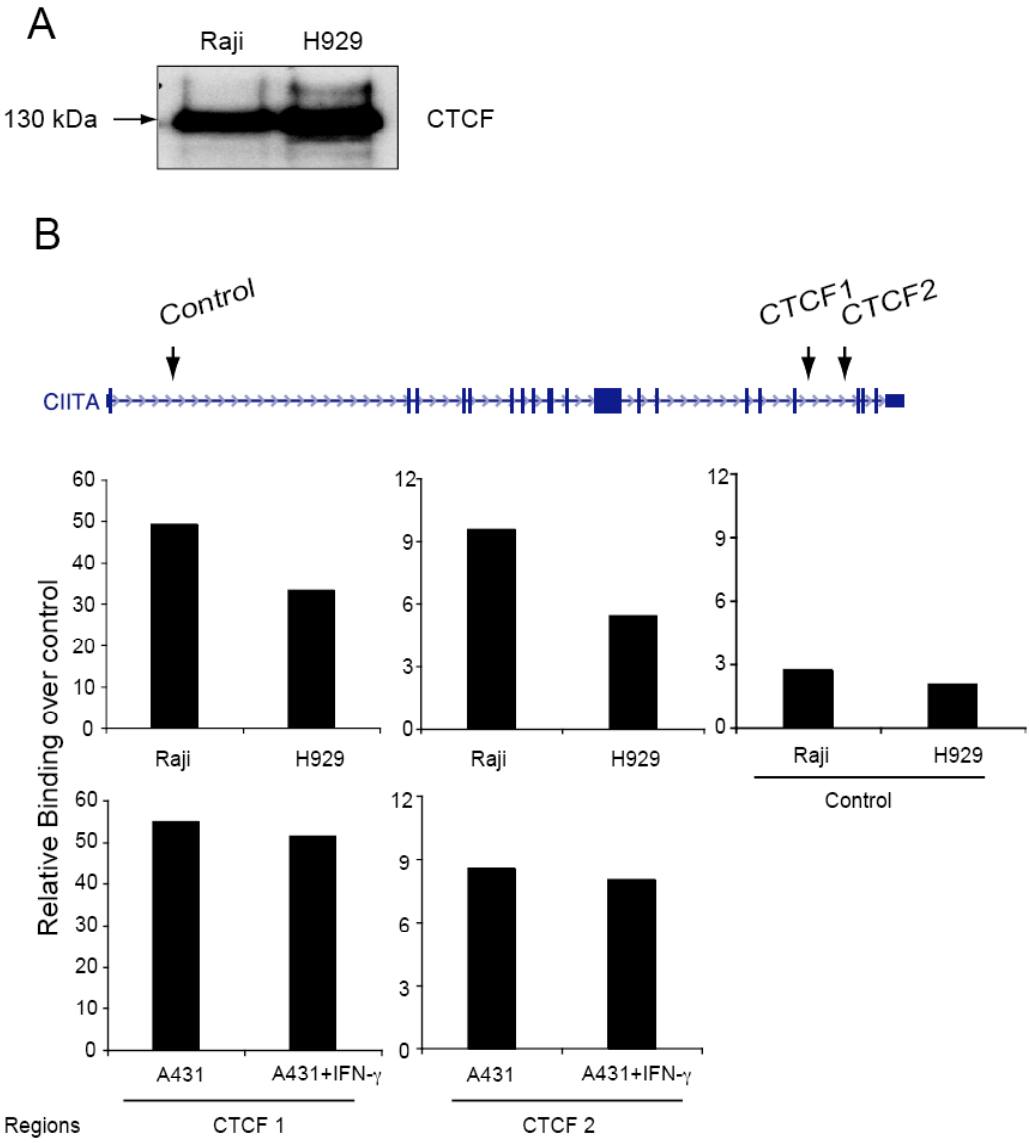

A
Raji
H929
130 kDa
CTCF
B
Control
CTCF1
CTCF2
CIITA
Relative Binding over control
Raji
H929
Control
A431
A431+IFN-γ
Regions
CTCF 1
CTCF 2

Figure 3

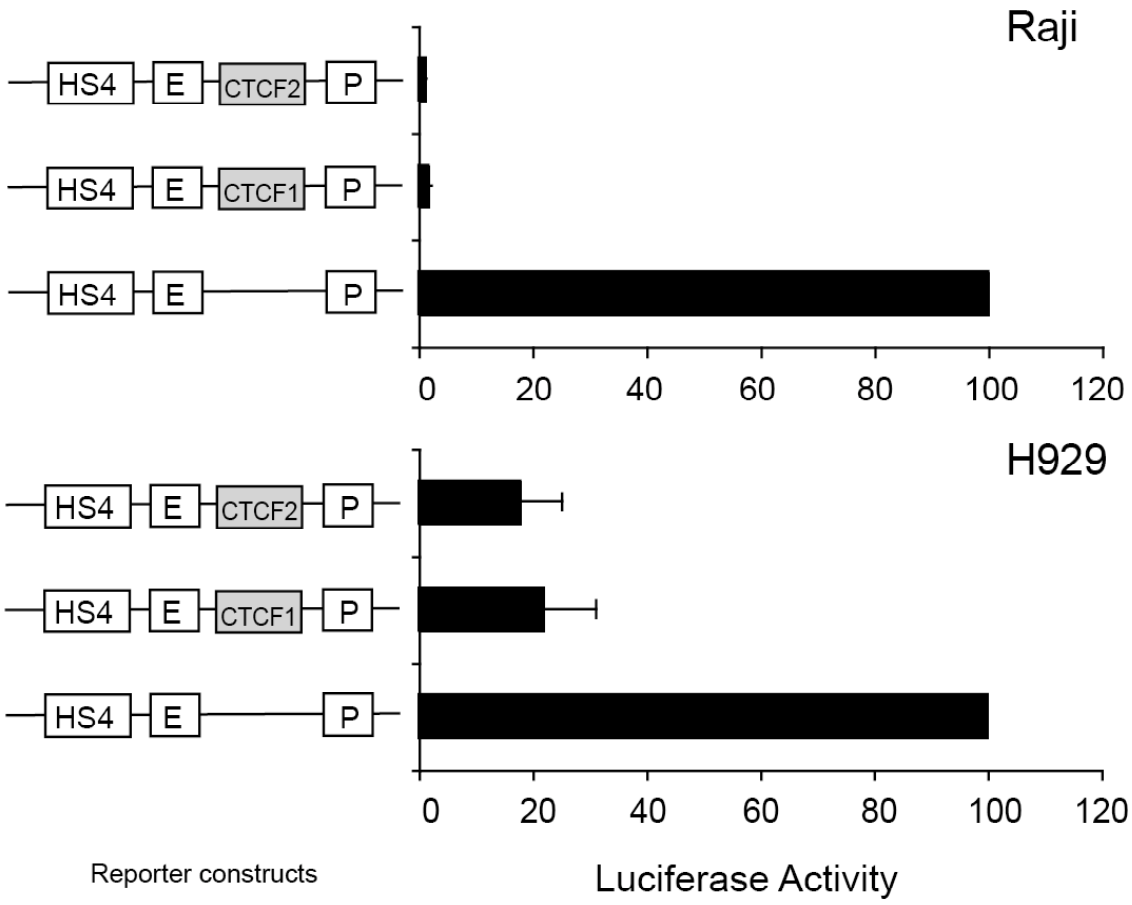

Raji
HS4
E
CTCF2
P
HS4
E
CTCF1
P
HS4
E
P
0
20
40
60
80
100
120
H929
HS4
E
CTCF2
P
HS4
E
CTCF1
P
HS4
E
P
0
20
40
60
80
100
120
Reporter constructs
Luciferase Activity

Figure 4

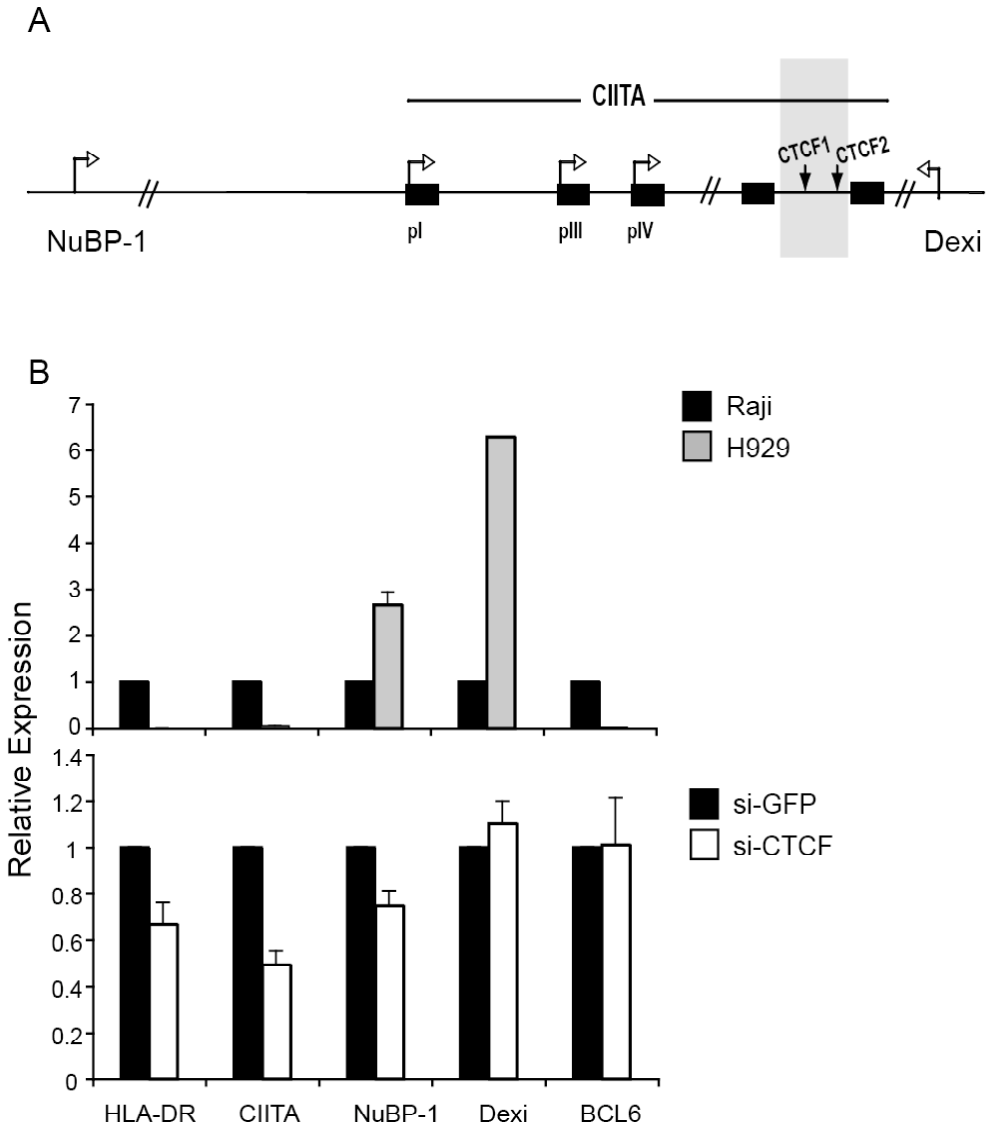

A
CIITA
CTCF1
CTCF2
NuBP-1
pI
pIII
pIV
Dexi
B
Raji
H929
si-GFP
si-CTCF
Relative Expression
HLA-DR
CIITA
NuBP-1
Dexi
BCL6

Figure 5

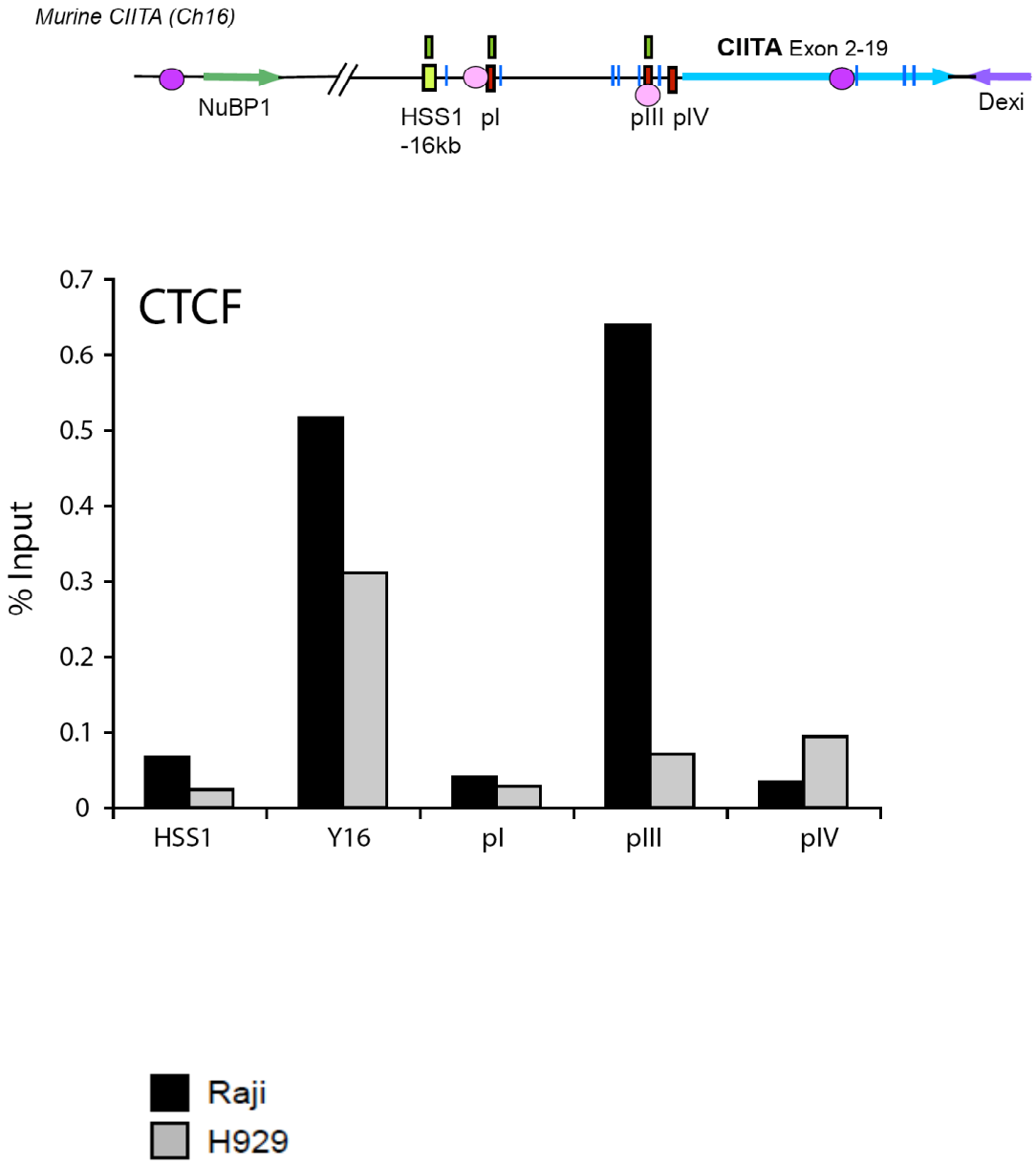

Murine CIITA (Ch16)
CIITA Exon 2-19
NuBP1
HSS1
-16kb
pI
pIII
pIV
Dexi
CTCF
% Input
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
HSS1
Y16
pI
pIII
pIV
Raji
H929

Figure 6

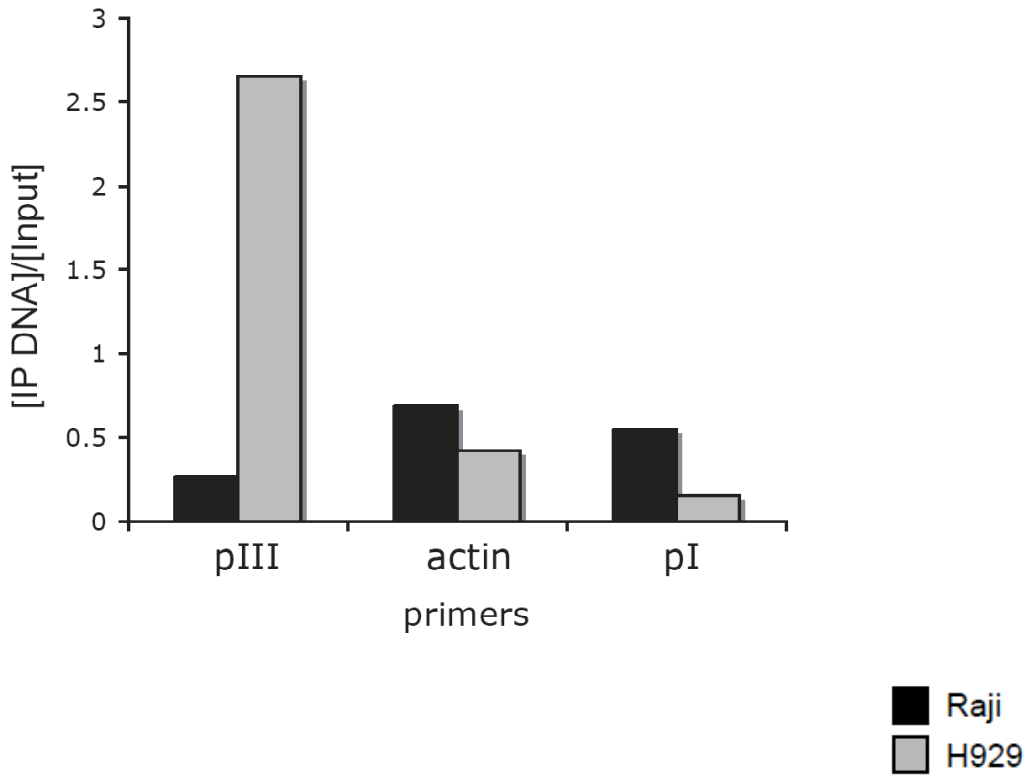


Figure legend

Figure 1. The schematic diagram for CTCF binding sites in the human CIITA gene.

The regions represented to CTCF1 and CTCF2, which were identified by ChIP-in-chip, were analyzed for sequence conservation using the Unversity of California Santa Cruz Genome Browser.

Figure2. CTCF binds to introgenic regions of CIITA gene.

A. Western blot to determine CTCF expression was conducted using nuclear extract of human B cell line Raji and plasma cell line H929. B. A ChIP assay was conducted on Raji and H929 cell chromatin using CTCF antibody. The results were analyzed as described in materials and methods. The TCR antibody was used as an irrelevant antibody, and the amplicon located between CIITA promoter I and III was used as an irrelevant locus control. The chromatin of A431 cells that was stimulated with IFN-γ for 24 hour was also used to measure CTCF binding at indicated locus.

Figure 3. CTCF1 and CTCF2 functions as an enhancer-blocking activity.

Two CTCF binding sequences were introduced into the pGL3 enhancer and promoter plasmid containing the firefly reporter genes. After 24 h post transfection, the promoter activity was measured.

Figure 4. CTCF is required for the regulation in CIITA and neighboring gene's expression.

Quantitative RT-real time PCR was conducted to measure the mRNA level of CIITA, HLA-DR, NuBP1, Dexi, and BCL6 from Raji cells, H929 cells (A); siGFP or siCTCF tranfected Raji cells for 3 day (B).

Figure 5. CTCF binds to upstream of CIITA promoter I and promoter III. ChIP assays were conducted on the chromatin of murine B cell line BCL1 and plasma cell line P3X to determine the binding of CTCF to indicated locus. CTCF binding sites were illustrated as a pink, and the green bar indicates PU.1 binding sites.

Figure 6. CIITA promoter III DNA is methylated in plasma cells, but not in B cells. Methylated DNA immunoprecipitation (MeDIP) was conducted to examine the DNA methylation at CIITA promoter I and CIITA promoter III. As an irrelevant locus control, primer for actin promoter was used.

APPENDIX B:

The plasma cell de-differentiation by re-programming of gene expression

This is preliminary data. Further studies are required.

This was written by Hye Suk Yoon and edited by Dr. Jeremy Boss.

CIITA is silenced in plasma cells so that MHC class II expression is lost during B cell to plasma cell differentiation (Figure 1A, B). Reprogramming the expression of transcriptional factor during development is a key regulatory mechanism to define the cell fate (Matthias, 2005; Medina, 2005). During B cell to plasma cell differentiation, B cells lose the cell surface protein B220, MHC class II, and CD19 while differentiated plasma cell obtained syndecan-1 on the surface. Syndecan-1 is generally used as a plasma cell fate marker. In addition to cell surface protein, the expression of transcriptional factors also changes. Blimp-1, which is identified as a plasma cell specific transcriptional factor and is known to repress CIITA expression, increased (Figure 1A) while BCL1, Pax5, and other B cell specific genes are repressed (data is not shown). Fujita et al. showed that ectopically introduced BCL6 in human plasma cell line H929 initiates CIITA expression; in contrast, BCL6 represses Blimp-1 and syndecan-1 expression from plasma cells (Fujita, 2004). To determine if this occurred in murine plasma cells, we introduced BCL6 and MTA3 into murine plasma cell line P3X using an adenovirus or retrovirus expression system (Figure 2). Consistent with the human cell line, the murine plasma cell line can initiate CIITA expression after BCL6 expression, but not MTA3 expression. MTA3 co-expression with BCL6 induced CIITA expression higher than BCL6 infected P3X, indicating that BCL6 collaborates with MTA3 to regulate gene expression. It has been shown that BCL6 co-immunoprecipitated with MTA3 as well as other repressor complexes (Fujita, 2004). The plasma cell specific genes, Blimp-1 and syndecan-1 were repressed after BCL6 expression (Figure 2C).

In this part of thesis, we would like to determine how chromatin accessibility at the promoter regions of CIITA is changed by BCL6 or MTA3 for CIITA regulation. To carry out this study, the presence of general histone modifications, such as $H3^{ac}$, $H4^{ac}$ and $H3K4^{me2}$ at CIITA promoters were determined after ectopic BCL6 expression in the plasma cell line P3X. Interestingly, CIITA promoter III, which B cells use for constitutive CIITA expression showed the highest level of all active histone modifications while promoter I and IV appeared to be not affected (Figure 3). These data indicate that CIITA regulation by BCL6 and MTA3 involves the

change of chromatin architecture to allow histone modification factors to bind at the regulatory regions. However, how BCL6 re-initiates CIITA expression is not clearly understood. BCL6 binding at CIITA promoter III was not found (data is not shown), indicating BCL6 may regulate CIITA indirectly. Because BCL6 is known to repress Blimp-1, which represses CIITA in plasma cell, it is possible Blimp-1 repression by BCL6 prevents Blimp-1 binding at CIITA promoter so that CIITA and MHC class II can be expressed. In this study, we showed that BCL6 and MTA3 transcriptional factor were sufficient to program gene expression from plasma cell to B cells. In this process, histone modifications likely play a role to change chromatin accessibility for gene activation.

In summary, transcription factors that are expressed differentially during B cell development determine cell fate. BCL6 and MTA3 were identified as B cell specific transcription factors, and collaborate to repress plasma cell specific genes, such as Blimp-1. In plasma cells Blimp-1 represses B cell specific genes, like CIITA, Pax-5, and BCL6. In this study, BCL6 and MTA3 were expressed in plasma cell using an adenovirus or retrovirus expression system. This resulted in the re-expression of CIITA in the plasma cells and the repression of Blimp-1 and syndecan-1 expression. It was found that this process involves the gain of active histone modifications at CIITA promoter III. However, BCL6 or MTA3 did not directly bind to CIITA promoter III to mediate this process, suggesting that this is an indirect mechanism.

Figure 1

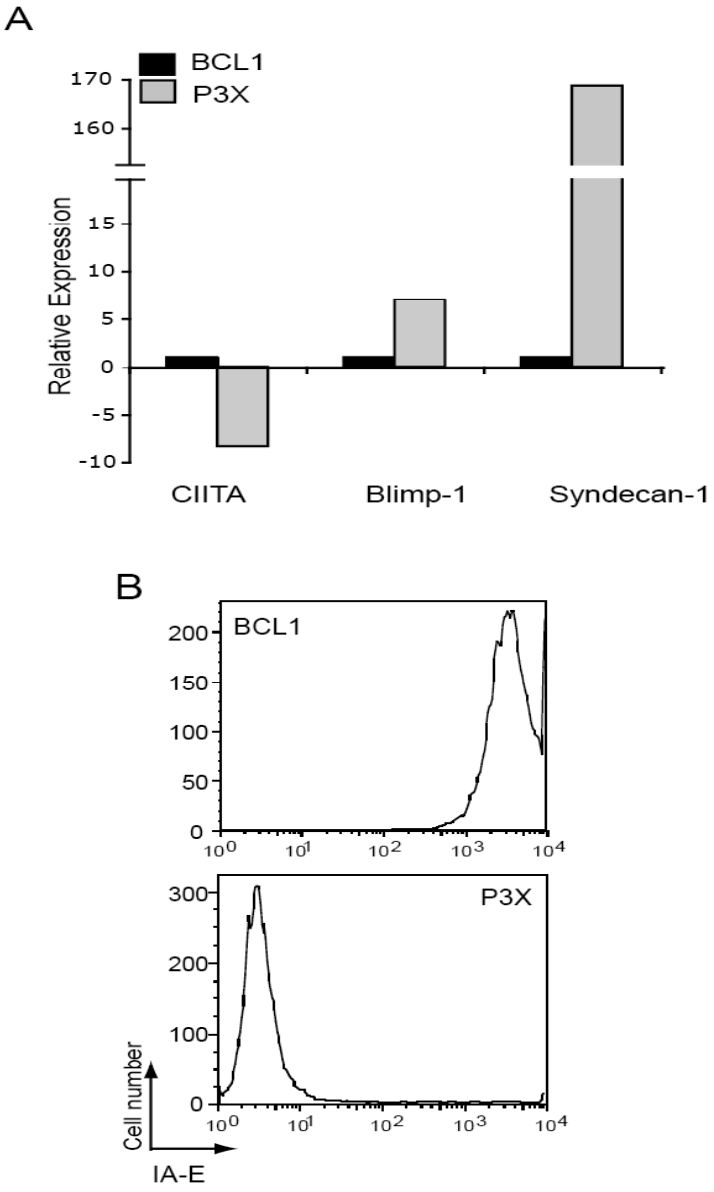

A
BCL1
P3X
170
160
15
10
5
0
-5
-10
Relative Expression
CIITA
Blimp-1
Syndecan-1
B
BCL1
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4
P3X
300
200
100
0
Cell number
IA-E

Figure 2

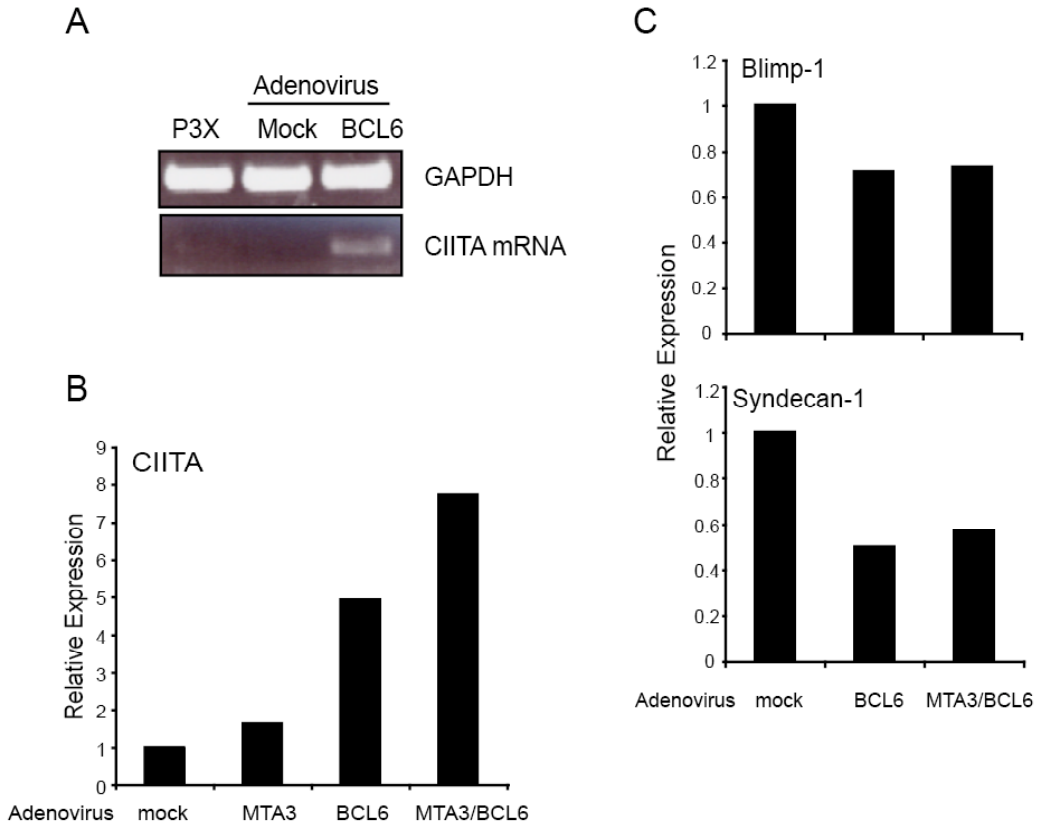

A
Adenovirus
P3X
Mock
BCL6
GAPDH
CIITA mRNA
B
CIITA
Relative Expression
Adenovirus
mock
MTA3
BCL6
MTA3/BCL6
C
Blimp-1
Syndecan-1
Relative Expression
Adenovirus
mock
BCL6
MTA3/BCL6

Figure 3

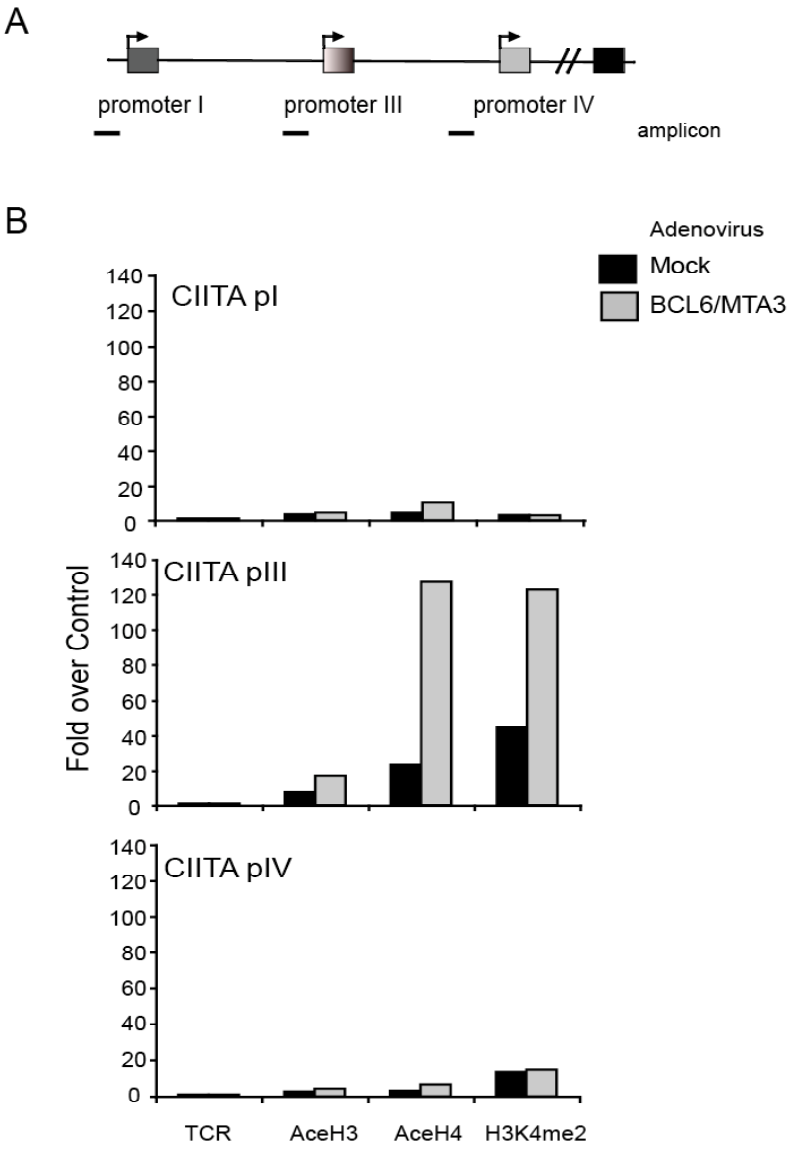

A
promoter I
promoter III
promoter IV
amplicon
B
Adenovirus
Mock
BCL6/MTA3
CIITA pI
CIITA pIII
CIITA pIV
Fold over Control
140
120
100
80
60
40
20
0
TCR
AceH3
AceH4
H3K4me2

Figure legend

Figure1. The gene expression patterns and MHC class II expression of murine B cell line BCL1 and plasma cell line P3X.

(A). qRT-real time PCR was performed to determine the level of CIITA, Blimp-1, and syndecan-1 mRNA expression from BCL1 and P3X cells. (B) IA/E cell surface expression was determined by flow cytometry.

Figure 2. Ectopic BCL6 and MTA3 expression in plasma cell line P3X initiates CIITA expression, but suppress plasma cell specific genes, Blimp-1 and syndecan-1.

BCL6 and MTA3 expressing adenovirus was infected into murine plasma cell line P3X for 5 days. RNA was prepared for qRT-real time PCR to assess the mRNA level of CIITA, Blimp-1, and syndcan-1 (A, B, C).

Figure 3. The initiation of CIITA expression in plasma cells by B cell specific repressor BCL6 and MTA3 is mediated by introducing activation marks to CIITA promoters.

ChIP assays were conducted on the chromatin of plasma cell line P3X expressing BCL6 and MTA3 to determine the binding of indicated antibodies at CIITA promoter I, III, and IV.

Discussion

Mature B cells constitutively express CIITA, which is the transcriptional coactivator of MHC class II expression. MHC class II molecules present antigen on B cell surfaces, which allows CD4+ T cells to recognize the presented antigen (Benacerraf, 1981; Long, 1992). This process directs B cell differentiation into plasma cells, which will produce high levels of secreted antibody. In this process, CIITA is silenced so that MHC class II expression is off in plasma cells. The loss of MHC class II proteins from plasma cells prevents further stimulation from activated CD4+ T cells. This in effect fixes the idiotypic and isotypic nature of the antibody that plasma cells produce. Thus, the regulation of CIITA expression in B cell to plasma cell differentiation is critical to direct precise control of humoral immune responses. The work described in this thesis is important to understand the regulation of MHC class II gene and their master regulator CIITA, and will be useful for immune-based therapies to enhance or reduce immune responses.

CIITA transcriptional regulation during B cell differentiation

CIITA transcription is controlled by at least three independent promoters (pI, pIII, and pIV) whose regulation is directed in a cell type specific or developmental-specific manner (Muhlethaler-Mottet et al., 1997). In B cells, CIITA promoter III, which is located within -350bp to +150bp from the transcription start site, controls constitutive CIITA expression. However, little is known about other distal regulatory elements that are required for CIITA expression. Two sets of data suggest that CIITA is controlled by additional elements rather than by just the local promoter regions. First, Green et al. described that all CIITA promoters in human B cells have open chromatin structure by showing active histone modifications, $H3^{ac}$, $H4^{ac}$, H3 $K4^{me2}$, $H3K4^{me3}$, even though CIITA promoter III is only employed in B cells for constitutive CIITA expression (Green et al., 2006). This suggested that a locus control region may be present in the CIITA locus. Second, Ni et al. recently identified that human Hela cells portrayed a series of

distal regulatory regions on CIITA gene that are important for induction of CIITA in response to IFN-γ stimulation (Figure 1). STAT1, IRF1, and BRG-1, which are also key transcriptional activators that bind at CIITA pIV, were found to bind these distal elements. For CIITA pIV, several of these elements interact through long distance chromosomal interaction upon IFN-γ stimulation, while the interaction of other elements is constitutively present without IFN-γ stimulation (Ni et al., 2008).

Figure 1. Distal regulatory elements on CIITA genes identified in Hela cell
The distal regulatory elements that are required for CIITA pIV-dependent transcription as illustrated from Ni et al. (*Nature Immunology*, 2008) is shown.

Therefore, a number of approaches were employed to investigate potential distal regulatory elements in B cells to study CIITA regulation. DNase I hypersensitivity assays, which generally identifies regulatory elements, or the status of gene activation were employed. Two hypersensitivity sites were discovered. The HSS1 site was hypersentive in a murine B cell line BCL1 while HSS2 was hypersensitive in the plasma cell line P3X. HSS1 is located 11kb upstream of CIITA promoter III. HSS1 is the murine homolog to the -16kb site, which was one of the distal regulatory elements that Ni et al. identified. The nomenclature of distal elements reflects the distance from pIV (Figure 13). Freshly isolated primary B cells also showed that HSS1 is hypersensitive to DNase I, indicating that HSS1 was not an artifact of a transformed cell line. HSS1 is highly conserved between mouse and human, but HSS2 has less conservation. Because of this high conservation, we chose to focus on HSS1 and examine its role in CIITA regulation during B cell to plasma cell differentiation.

In addition to the DNase I hypersensitivity assay and genome comparison, histone modifications also can identify active regions of the genome. In a previous study, we found that active histone modifications associated with gene activation were lost at the human and mouse CIITA proximal promoter region during the B cell to plasma cell transition (Green et al., 2006). This result suggested that the chromosomal accessibility and architecture of the locus may regulate CIITA expression.

Hon et al. identified some histone modification patterns of cis-regulatory elements that distinguish promoters and enhancers (Hon et al., 2009). General active histone modifications, such as H3K18^{ac}, K9^{ac}, or H4K8 ^{ac}, indicate gene activation or an open chromatin features and allow transcription factor binding at a locus (Jenuwein and Allis, 2001; Turner, 2000). H3K4 methylation also reflects gene activation state. H3K4^{me2} or H3K4^{me3} were generally enriched in promoter regions while H3K4^{me1} reflected an enhancer signature in Hela cells (Barski et al., 2007; Hon et al., 2009). Interestingly, HSS1 displayed significant levels of H3K4^{me1}, suggesting that HSS1 possibly functions as an enhancer. HSS1 and CIITA pIII showed H3K4^{me2} and H3K4^{me3} in B cells. All H3K4 methylation modifications were higher in B cells than in plasma cells, indicating both a regulatory status as well as transcriptional status for CIITA pIII. General active histone modifications, H3K9^{ac}, H3K18^{ac}, and H4K8^{ac} were also found at HSS1 and pIIII regions in B cells, but low level in plasma cells as illustrated in Figure 2 (Figure 2). Thus, HSS1 displays an active signature in B cells. Differential levels of these above histone marks were also observed in the pIV region, but these were lower levels than the level at pIII. In B cells pIII transcripts are 10 fold higher than pIV. The fact that pIV also has an open chromatin structure in B cells implies that distal regulatory elements may be important to direct the transcription machinery to pIII as opposed to one of the other promoters.

Intriguingly, while B cells displayed active histone marks at CIITA promoters, plasma cells showed repressive histone marks, H3K9^{me2} and H3K27^{me3} at CIITA promoter III. H3K9 ^{me2} leads

to gene silencing and is mediated by the histone methyltransferase G9a (Tachibana et al., 2001). G9a is known to interact with transcription factors Blimp-1 and Groucho to repress target genes; however, G9a was not found at CIITA pIII in plasma cells while Blimp-1 binding was detected (Gyory et al., 2004; Ren et al., 1999) (data not shown). Therefore, other repressor complexes may modify H3K9^{me2} at CIITA pIII. Alternatively, placement of that modification may only regulate transient association of G9a at a time point that was not assayed. In the mouse, H3K27^{me3}, which is a marker placed by the Polycomb repressive complex (PRC), was observed at all CIITA promoters in the plasma cell line P3X, indicating that Polycomb factor binding is involved in CIITA silencing. Using *ex vivo* differentiated primary B cell, it was found that EZH2 binding increased at CIITA pIII during CIITA silencing. EZH2, a component of PRC 2, is a histone methyltransferase specific to H3K27^{me3}. These results suggest that EZH2 and PRC2 bind at CIITA promoters with Blimp-1 to repress CIITA transcription. A previous study showed that EZH2 is involved in CIITA promoter IV silencing in uveal melanoma cells; therefore, Polycomb mediated gene silencing is one of the regulatory mechanisms for CIITA repression (Holling et al., 2007).

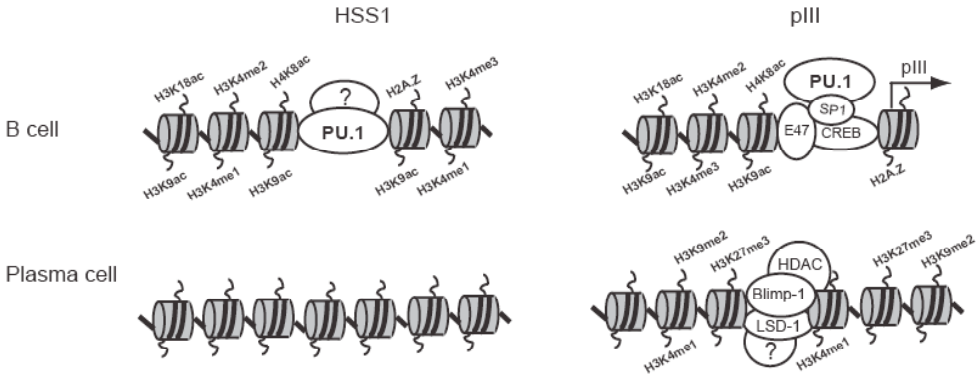


Figure 2. Histone modifications at HSS1 and CIITA pIII in B cells and plasma cells
B cells showed more active histone modifications at both HSS1 and pIII than plasma cells. Additionally, a set of transcription factors is associated with both regions to express CIITA expression in B cells. However, active histone modifications are replaced by repressive histone modifications, such as H3K9^{me2} and H3K27^{me3} when B cells transition to plasma cells.

Characterizing HSS1 as a novel regulatory element by a number of tools provides a clue that HSS1 may play a critical role for CIITA regulation in B cells. The deletion of HSS1 sequence from a bacterial artificial chromosome (BAC) construct encoding 180kb of the CIITA locus and its neighboring genes, Dexi and NuBP1, provided compelling data, indicating that HSS1 is critical for B-cell specific CIITA expression. CIITA promoter III-dependent transcripts were robustly reduced in HSS1-deleted BAC constructs compared to the wild type. Interestingly, pI specific transcripts were also affected by presence of HSS1 and pIII. This finding provokes the possibility that pI is also controlled by HSS1 or pIII binding factors. In contrast, pIV dependent transcription from the BAC construct was not observed in B cells. Ni et al. showed that -16kb/HSS1 element interacts with pIV in response to IFN- γ stimulation (Ni et al., 2008). Therefore, pIV regulation by HSS1 may be only involved in IFN-γ stimulated cells while HSS1 may contribute to both pI and pIII regulation.

Proximal regulatory promoter regions generally control transcription; however, the distal regulatory regions are also required to enhance or repress gene expression. Chromatin organization of distal regulatory elements is likely to be critical for gene expression. One of the ways to organize chromatin is through long-range chromosomal interactions that bring distal regulatory and elements into close proximity to facilitate gene expression or insulate a genes from other regulatory elements (reviewed in (Phillips and Corces, 2009)). In IFN-γ stimulated Hela cells, it was found that the -16kb region/HSS1 interacts with promoter IV in a BRG-1 dependent manner to regulate CIITA pIV transcription (Ni et al., 2008). In B cells, HSS1 also interacted with promoter III. These data imply that B cells express a factor that mediates long-distance interactions between HSS1 and pIII. Chromatin immunoprecipitation data coupled with Genome Matrix analysis showed that PU.1 binds at both HSS1 and pIII in the murine B cell line BCL1 and primary murine B cells, but not in plasma cells or differentiated primary plasma cells. ShRNA to PU.1 results in reduced frequency of long distance interactions between HSS1 and

pIII. shRNA to PU.1 also repressed CIITA transcription levels, indicating that CIITA transcription and the long chromosomal interaction between HSS1 and pIII are in part dependent on PU.1. However, PU.1 depletion was not sufficient to reduce 100% of the interaction, suggesting that PU.1 may cooperate with other trans-regulatory factors. It has been reported that PU.1 interacts with IRF4, IRF8, and E47, which were also found at pIII in B cells and thought to act CIITA expression synergistically (van der Stoep et al., 2004). Additionally, the binding of CREB was also found at HSS1 and pIII (data not shown). Thus, these factors may be involved in HSS1 mediated CIITA regulation and may be required for stabilization of long distance interactions between HSS1 and pIII. Intriguingly, pI and pIII also interacted in B cells, but this interaction was independent of PU.1 binding at pI or pIII, as shRNA to PU.1 failed to reduce the long- distance interactions between pI and pIII. This finding arise several questions.

The distal elements identified by Ni et al. from human Hela cells showed conservation with the murine CIITA gene except at the -30kb and -50kb regions, which are inverted in the mouse. Considering the fact that human CIITA and mouse CIITA genes have more than 90% homology and -16kb and HSS1 has been used in IFN-γ stimulated cell and B cell, respectively, it is possible that the other distal regulatory elements identified by Ni et al. may play a role in B cells and macrophage expression of CIITA.

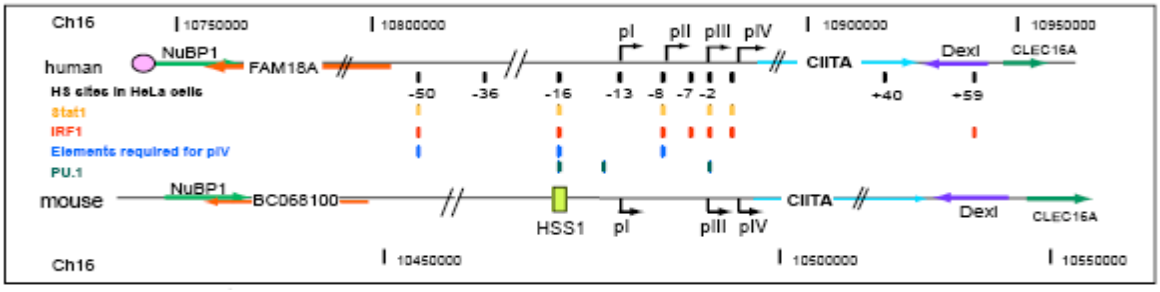


Figure 3. HSS1 and human distal regulatory elements on CIITA genes
The distal regulatory elements that were found in both humans and mice identified by Ni et al. (Nature immunology, 2008) and this study are illustrated with the factors binding at those sites.

The key questions now are how many elements are required for CIITA regulation in B cells or in other cell types and how are the promoter chosen in a cell type specific manner? Because we found that HSS1 is involved in controlling both constitutive B cell systems and IFN-γ inducible systems, it is possible that it is also required for pI transcription. The cell type specific transcription factors that bind these distal elements may be critical to decide chromosomal loop formation and promoter usage. STAT1/IRF1 are the factors that direct pIV transcription while PU.1 binding in B cells coordinates pIII usage (Figure 3). Importantly, macrophages and dendritic cells display a higher level of PU.1 binding at pI than in B cells (data not shown). In splenic DC the level of PU.1 binding at pIII is present but substantially lower than that of B cells. This may suggest that another mechanism may distinguish pI and pII promoter selection.

In B cells, the interaction between pI and pIII was not dependent upon PU.1 binding; thus it is proposed that other factor(s) are involved in this interaction. Using Genome Matrix analysis and Chip-on-chip data from Kim et al, several CTCF site were found on the human CIITA gene (Kim et al., 2007) and homologous sites were identified in the mouse (data not shown). CTCF generally binds at insulator elements and blocks enhancer activity to a promoter (Dunn and Davie, 2003). In mice, using ChIP analysis, we found CTCF sites upstream of both CIITA promoter I and promoter III (Figure 4). These CTCF sites were found by conventional ChIP to bind CTCF. One is at +40 kb from the transcriptioin start site of pIV (Appendix A). The other sites surrounded pI. If these CTCF sites interact in B cells, this could effectively place pI in a loop and insulates it, allowing HSS1 to interact with pIII. Interestingly, in plasma cells, the pIII proximal CTCF DNA is methylated and displays 4-8 fold reduced CTCF binding. This methylation and reduced CTCF binding could disrupt chromatin structure and facilitate CIITA silencing. Future work in this mode is needed to test the above model based on my data.

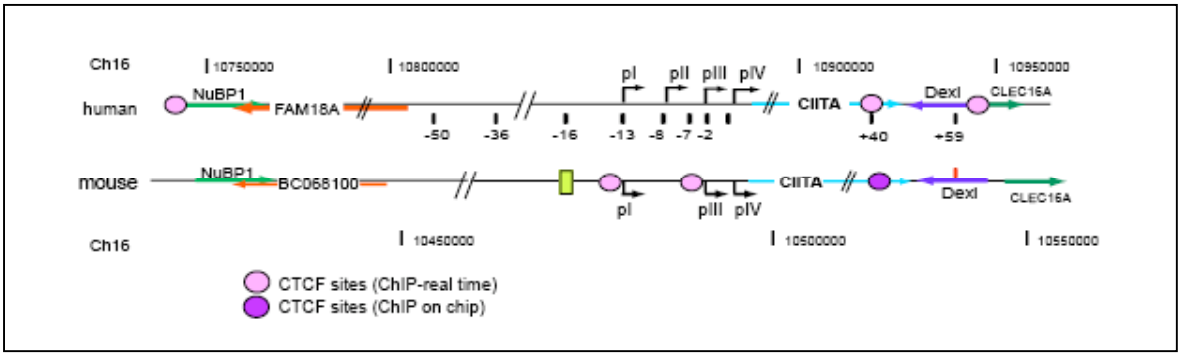


Figure 4. CTCF sites on CIITA genes
CTCF binding sites that were identified using chromatin immunoprecipitation in both humans and mice are shown.

A role for the CTCF site at +40 kb is not clear. However, siRNA to CTCF transcfection in the human B cell line Raji resulted in a reciprocal gene profile between CIITA and its surrounding genes, Dexi, NuBP-1, suggesting that CTCF may be involved in blocking the spreading of gene expression to its neighbors (Appendix A).

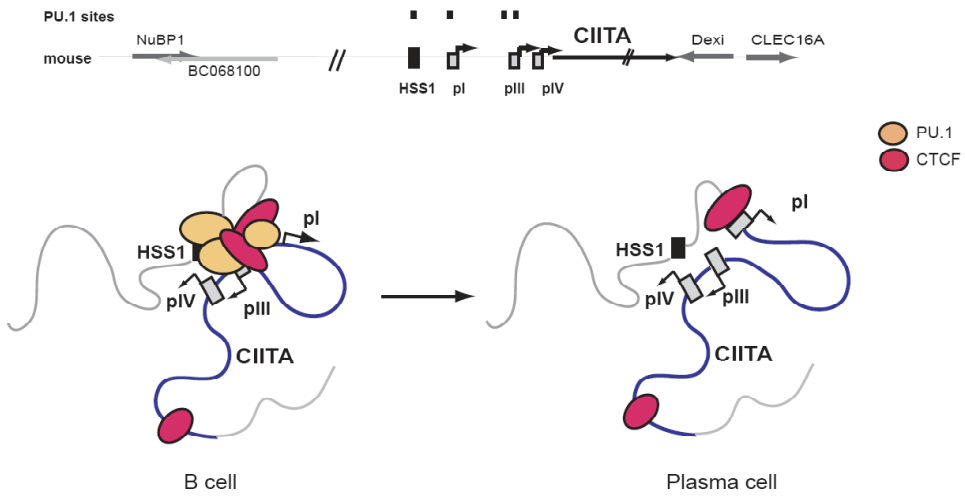


Figure 5. Final proposed model of CIITA regulation by HSS1 distal regulatory element
The locus of CIITA promoters and binding sites of PU.1 and CTCF are illustrated. Two proposed structure can be hypothesized from the data generated in this. Thus, these reflect the presence or absence of CTCF-CTCF interaction, as well as promoter regulatory site interactions.

CIITA regulation by transcription factors that are required for B cell differentiation to plasma cells

The reprogramming of gene expression is a key mechanism to control the cell development and to decide cell fate. A number of transcription factors, which are expressed in a B cell or plasma cell specific manner, orchestrate B cell to plasma cell differentiation. It was found that Blimp-1 and XBP1 are induced while BCL6, Pax5, MTA3, and other B cell factors are repressed during B cell differentiation to plasma cell. Fujita et al. showed that ectopic BCL6 and MTA3 expression in the human plasma cell line H929 repressed plasma cell specific factors, Blimp-1 and syndecan-1, while inducing B cell factor CIITA expression (Fujita et al., 2004), suggesting that reprogramming of transcription factor expression during cell development plays a key role in cell fate determination. Additionally, inhibiting the expression of cell specific factors could have a severe effect. For example, Blimp-1 conditional knock-out mice show reduced levels of antibody secretion in plasma cells even though B lymphocyte development was normal (Shapiro-Shelef et al., 2003). B cell specific transcription factor BCL6 knock out mice failed to form germinal centers to mediate B cell differentiation (Fukuda et al., 1997). These findings indicate that transcription factor reprogramming is critical to direct precise cell development.

In this thesis, we sought to investigate how known cell type specific factors are important for CIITA regulation during B cell differentiation. While a number of transcriptional activators were found at proximal CIITA promoters, it was not clear how CIITA is silenced during the B cell to plasma cell transition and what regulator direct this phenomenon. Blimp-1 was identified previously as a key factor for plasma cell differentiation and as a repressor of CIITA expression (Doody et al., 2007). However, *ex vivo* differentiation system using primary murine B cells showed that CIITA silencing is observed before Blimp-1 reaches its highest level. Moreover, Blimp-1 over expressed in B cell lines failed to suppress CIITA expression efficiently. This

suggests two possibilities. First, low levels of Blimp-1 expression are sufficient for CIITA silencing. Second, other factors that are expressed early in *ex vivo* differentiation are required.

cDNA microarray approach is a great tool to elucidate gene expression profiles. We employed cDNA microarrays to identify the factors involved in CIITA regulation during B cell differentiation by comparing gene expression patterns. Greory et al. reported gene expression profiles between IgM+ B cells and plasma cells (Underhill et al., 2003). In this study, a large number of genes showed altered expression when two cell types were compared. In our system, we found that 1169 genes are reduced while 1201 genes are induced during B cell differentiation. Interestingly, we found that EZH2 was significantly induced with CBX2, another Polycomb complex component. However, EZH1, which is also a PcG component, was down regulated as B cells transitioned to plasma cells. Recent studies have revealed that B cells express EZH1 while plasma cells express more EZH2, CBX1, and CBX2. This consistency of data indicates that our assay using *ex vivo* B cell differentiation is a viable model to study B cell differentiation. The PcG protein expression profile suggests that a Polycomb complex is required for B cell maturation to reprogram gene expression for lymphocyte differentiation. These data were consistent with our observation of $H3K27^{me3}$ at CIITA promoter regions in plasma cells. We failed to observe Blimp-1 expression at 1 day following *ex vivo* differentiated B cells even though CIITA expression was diminished. Therefore, it was thought that repression of CIITA by Blimp-1 in plasma cells required other regulatory factor(s). Such factors would be induced before Blimp-1 expression during the B cell differentiation process. Using cDNA micorarrays, Shaffer et al. identified several target genes for Blimp-1. These include Pax5, Id3, CIITA, c-Myc, and SpiB. ZBTB32 was not found. It is important to point out that the Shaffer et al. experiments involved fully differentiated cell lines and did not monitor the transition (Shaffer et al., 2002). The failure to detect ZBTB32 may have to do with the chip used.

Following cDNA microarray data, our gene expression profiles during *ex vivo* B cell differentiation to determine the kinetics of gene expression at the single time point showed that the pattern of gene expression could be classified into three groups. First, CIITA silencing occurred rapidly with other B cell specific factors, such as BCL-6, SpiB, and SpiC. Other B cell factors, including MTA3, PU.1, EBF, EZH1, and MLL1 were slowly repressed. These profiles indicate that CIITA silencing is an early event of B cell to plasma cell differentiation with BCL6, Spi-C and Spi-B. Second, Blimp-1 and XBP-1, which are known as a plasma cell specific factors, were slowly up regulated during B cell differentiation and reached high levels of expression at the end of the process (5 day). Third, a number of chromatin remodeling and transcription factors, ZBTB32, Chalf2, EZH2, PRMT1, TfDP1 appeared to be induced transiently at early stages of the process. It should be noted that cell viability was not an issue at the 5 days time point. This transient expression explains similar patterns were observed in plasmablast, which were purified 8 days post LCMV infection. This data reveals that transiently up regulated chromatin remodeling or transcription factors are engaged in early events of B cell differentiation, and when B cells terminally differentiated to plasma cells, terminally differentiated plasma cell fate was formed by master plasma cell transcriptional factors, such as Blimp-1, by completely suppressing B cell markers.

ZBTB32 was found as one of the most significantly differentiated genes after 1 day of B cell differentiation. ZBTB32 is a member of BTB containing zinc finger proteins that recruit other co-repressor to the target genes. While ZBTB32 appears to have a role in T lymphocytes development, its role in B cells and plasma cell differentiation was not investigated.

With ZBTB32, several other candidates from the cDNA microarray analysis were used to evaluate their function as novel repressors of CIITA during the B cell differentiation. Of the 10 analyzed only ZBTB32, EZH2, and CBX2 could repress CIITA when over expressed in B cells.

Although Raji cells expressed very low levels of ZBTB32 mRNA, high levels of ZBTB32 protein expression was detected only in human and murine plasma cell lines or *ex vivo* differentiated B cells. Consistent with this data, the experiment using primary B cells in which lentivirus expressing ZBTB32 was able to repress CIITA transcription.

ShRNA approaches to knock down primary B cell and plasma cell with lentivirus harboring shRNA specific to ZBTB32 indicated that ZBTB32 is a factor that represses CIITA during B cell to plasma cell differentiation. *Knock down* of ZBTB32 during *ex vivo* differentiation re-stored CIITA expression from plasma cells, validating that ZBTB32 is required for CIITA suppression in plasma cells. Intriguingly, the experiment that knock down ZBTB32 in plasma cell line, P3X, resulted in dramatic up regulation of CIITA, as well as MHC class II expression. Pax5 was significantly deregulated following zbtb32 knock down. Other B cell markers, such as BCL6 and SpiB were also slightly de-repressed from plasma cells. Pax5, BCL6, and SpiB are identified as the target genes of Blimp-1, suggesting that ZBTB32 might cooperate with Blimp-1 to repress B cell specific genes for plasma cell formation. However, knockdown of ZBTB32 from plasma cells did not significantly influence the expression of plasma cell markers, XBP-1, syndecan-1. Blimp-1 mRNA levels were reduced 10% after ZBTB32 knockdown. Therefore, ZBTB32 significantly influences the repression of B cell specific genes, but not the activation of plasma cell specific genes.

There are two mouse models that have introduced targeted deletions into the ZBTB32 gene (Piazza et al., 2004). In these mice peripheral and splenic B cells appear normal although they are reduced in overall number. Reduction in lymphocytes, specifically the T cell pool was reported. Thus, ZBTB32 is important to maintain the lymphocyte homeostasis. Using B cells from ZBTB32 KO mice, we found that CIITA was not repressed at day 1, but ultimately was repressed at day 5, time when Blimp-1 was fully induced. Calame et al. produced conditional Blimp-1 KO

mice (Shapiro-Shelef et al., 2003). Blimp-1 knock out from this system was only 90% efficient in depleting Blimp-1. Using our *ex vivo* system, CIITA was silenced in Blimp-1 KO mice at day 1. This finding suggests that ZBTB32 acts first to silence CIITA and then Blimp-1 may be required for the maintenace of silencing. Alternatively, the low level of expressed Blimp-1 (approximately 10%) may be sufficient to mediate B cell differentiation and CIITA silencing. Another possibility is that our *ex vivo* differentiation system using IL2, IL5, and LPS induces B cell differentiation independent of Blimp-1 expression. Thus, ZBTB32 was sufficient to initiate B cell differentiation and CIITA silencing without Blimp-1. This may be supported by the fact that Blimp-1 knock out mice express high levels of ZBTB32 mRNA during *ex vivo* differentiation. Others have argued that Blimp-1 is not required for the initiation of plasma cell differentiation to produce antibodies (Kallies et al., 2007) and another factor is required to start differentiation by the repression of Pax-5 expression (Lin et al., 2002; Messika et al., 1998). It is formally possible that ZBTB32 is that factor that is required to start.

Fujita et al. showed that BCL6 and MTA3 expression in plasma cells led to de-differentiation of plasma cells by repressing plasma cell specific genes, Blimp-1, and by activating B cell specific genes, CIITA and MHC class II (Fujita et al., 2004). Using a murine system, BCL6 expression in plasma cells was sufficient to reinitiate CIITA expression and MTA3 and BCL6 expression in plasma cells induced CIITA dramatically. However, there is no evidence that BCL6 interacts with any of the CIITA promoters directly (Appendix B). Interestingly, ZBTB32 knock out B cells showed delayed BCL6 repression during B cell differentiation compared to wild-type mice. During T cell development, BCL6 and ZBTB32 orchestrate differential T cell development (reviewed in (Bilic and Ellmeier, 2007)). Thus, it is possible that differential expression of BCL6 and ZBTB32 during B cell lymphocyte development is also a mechanism for CIITA regulation.

ZBTB32 protein is composed of two domains: a zinc finger domain to bind target DNA sequences and BTB domain for protein - protein interaction. In plasma cells, ZBTB32 directly

binds at CIITA promoter III, but not pI or pIV. Other repressors EZH2, LSD1, and Blimp-1 also bind to pIII in plasma cells. Lin et al showed that histone lysine demethylase LSD1 was found to interact with Blimp-1 at CIITA promoter III and other Blimp-1 target gene promoters, such as Pax5 and SpiB in the human plasma cell line H929 (Su, 2009).

Chromatin IP results imply that ZBTB32, Blimp-1, EZH2, and LSD-1 bind to CIITA promoter III during B cell differentiation even though Blimp-1 binding at promoter III was delayed by at least a day. This fact gives us a clue that these proteins may interact as a protein complex to bind at CIITA promoter III. Because ZBTB32 is a BTB domain containing protein, which has been reported to interact with other repressors such as HDAC, SMRT, NcoR (reviewed in (Bilic, 2007)), it may recruit Blimp-1 and EZH2 at CIITA pIII to mediate CIITA silencing. The result from zbtb32 knock out plasma cell line, P3X showed reduced levels of Blimp-1 mRNA. Thus, it is possible that ZBTB32 is required for Blimp-1 recruitment to CIITA promoter III. However, it is not clear whether this result is due to reduced expression of Blimp-1 in zbtb32 knock out plasma cells or ZBTB32 is really required for Blimp-1 association at CIITA promoter III. Further studies need to be performed to understand this process. This proposes the possibility that ZBTB32 and Blimp-1 might have similar profiles for their target genes by binding at similar regulatory region on the genes. It is also not known how many other factors are in the ZBTB32 repressor complex with Blimp-1 and how many genes are possibly regulated by ZBTB32. Future, ChIP-seq or ChIP-on-chip studies will help to elucidate the answer to this question. Protein purification studies will also help to identify the ZBTB32 repressor complex.

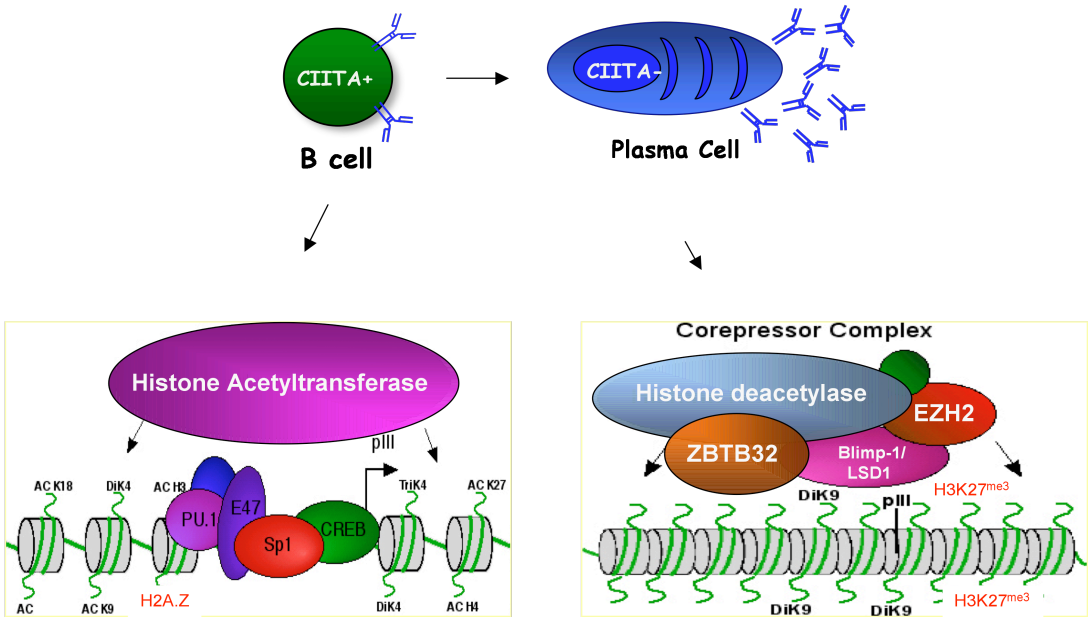


Figure 6. Final model for CIITA regulation during B cell differentiation

In summary

CIITA regulation is controlled by multiple mechanisms. Distal cis-regulatory elements organize CIITA genes to activate or repress expression in a cell type and developmental stage specific manner. Additionally, trans-regulators are also necessary for CIITA activation or repression by binding at cis-regulatory elements. We introduce here HSS1 as a novel cis-regulatory element for CIITA expression in B cells and PU.1 as a trans factor for HSS1-mediated CIITA promoter III regulation. Additionally, ZBTB32 is introduced as a key regulator of antigen processing by regulating CIITA expression and a repressor in terminal B cell differentiation. ZBTB32 plays an essential role to repress CIITA in plasma cells. ZBTB32 directly represses CIITA by binding at promoter III. Finally, ZBTB32 collaborates with Blimp-1 to repress CIITA in plasma cells by recruiting the Polycomb complex protein silencing machinery. This epigenetic modification may be stably maintained. Thus, my studies have identified several novel components of the antigen processing pathways that function to initiate and/or maintain adaptive immune responses.

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