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Epigenetic Regulation of the Major Histocompatibility Complex Class II Genes

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Biochemistry, Cell, and Developmental Biology

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B.Sc., Yonsei University, 2003

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Abstract

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The major histocompatibility class II genes (MHC-II) are a fundamental part of our adaptive immune system. Without proper expression of these genes, our bodies fail to mount an effective response against bacterial and viral infections, or may also lead to cancer and autoimmune diseases. The class-II transactivator (CIITA) is regarded the master regulator of MHC-II genes. CIITA can recruit various transcriptional coactivators to the promoter of MHC-II genes to activate transcription, such as CBP/p300 and PCAF. These transcription factors can modify histones positioned at cis-regulatory sites to alter the transcription level of genes. At an MHC-II gene, *HLA-DRA*, various histone acetylation and methylation modifications accumulated with the activation of transcription in both B cells and epithelial cells treated with interferon-γ, but not in mutant B cells that were lacking the expression of essential transcription factors. Using a dual-crosslinking chromatin immunoprecipitation approach, multiple subunits of the histone methyltransferase complex MLL and histone acetyltransferase complexes STAGA and ATAC were determined to bind at various regions across the gene and regulatory regions. In an unbiased approach to discover novel CIITA interacting proteins, a biotin-ligase-recognition-peptide fusion CIITA expression construct was cloned and expressed in B cells. By tandem mass spectrometry, the interacting proteins were determined. Recently, a genome-wide association study determined that a single nucleotide polymorphism within the first intron of *HLA-DRA* was highly correlated with late-onset Parkinson's disease (PD). It was hypothesized that a novel cis-regulatory element may be within its close proximity. PD and control whole blood samples were collected, and MHC-II expression levels were assessed in B cells and monocytes. Through these and further studies, a greater understanding is gained of the factors involved in MHC-II gene expression and how the expression of these genes influence various humans diseases.

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Chapter 1. Introduction

Regulation of Major Histocompatibility Complex Class II Genes

Nancy M. Choi

Introduction – Part 1 –

MHC-II Complex Function And Its Role In The Immune System

Higher eukaryotes contain a complex system of cellular and molecular signaling that distinguishes self from non-self entities termed the immune system. The immune system's objective is to detect foreign substances that could potentially harm the organism and respond accordingly by neutralizing or clearing them. Microorganisms, helminth parasites, environmental particulates, etc., may all potentially lead to hazardous health consequences for an exposed organism, or the host. The host cells will sense the presence of these entities through molecular interactions between the foreign particles eliciting the response, the antigens, and its own cell surface proteins. These recognition signals are transmitted internally to the transcriptional network of the host cells to express necessary genes to activate their defense functions.

The mammalian immune system consists of innate and adaptive components. Innate immunity is an immediate and rapid response that occurs non-discriminately towards invasive agents that leaves no lasting effects once it is cleared. In contrast, the adaptive immune system is a delayed and gradual response that recognizes molecular characteristics of an antigen and responds specifically towards the source of the antigen. This specific recognition is carried out through cell surface receptors unique to each immune cell. For example, the B-cell and T-cell receptors are sculpted through genetic recombination of the gene segments, allowing for their tremendous diversity (Tonegawa, 1983). Cells that express the specific receptor that can interact with the antigen become activated (Waldron et al., 1973). The signaling molecules that interact with the cell surface receptor will then relay

signals, commanding the cells to proliferate and differentiate and carry out further immune functions (Smith-Garvin et al., 2009). When the antigen is cleared, a small number of immune cells are retained within the organism called 'memory cells' that can respond rapidly and in greater magnitude against the antigen in future encounters (Sallusto et al., 2010). A crucial element in the interaction between antigen and immune cells, and hence our overall adaptive immunity, are molecules called the major histocompatibility complexes (MHC).

T cells are immune cells that undertake the role of directly eliminating infected cells, or activating other immune cells, macrophages, B cells, etc., to mount antigen specific immune responses. In order to do this, they must be able to recognize antigens. However, the structure of free antigen is imperceptible to T cells. Antigen needs to be presented in a visible context, and this is the role of the MHC molecules (Rosenthal and Shevach, 1973). T-cell receptors will interact with the peptides presented on an MHC molecule with the help of co-receptors. All of these interactions need to occur cooperatively to elicit a full T-cell response (Smith-Garvin et al., 2009). There are two groups of MHC molecules, class-I and II, with our focus being on the latter. Both of these MHC groups will be discussed in later sections.

When antigen presentation to T cells is disrupted, the consequences are a variety of human maladies. The bare lymphocyte syndrome (BLS) is a heritable severe combined immunodeficiency caused by a loss of transcription and surface expression of MHC-II (Durandy et al., 1983; Lisowska-Grospierre et al., 1985). People that carry certain MHC-II gene alleles and single nucleotide polymorphisms have been shown to have a higher susceptibility to autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, etc. (Graham et al., 2002; Gregersen et al., 1987; Modin et al., 2004). Fibrosarcoma, mammary, colon, and renal adenocarcinoma cells are more capable of

establishing tumors by tuning down their MHC-II expression (Frangione et al., 2010; Meazza et al., 2003). Various parasites, human immunodeficiency virus, human cytomegalovirus, *Chlamydia trachomatis, Mycobacterium tuberculosis*, also down regulate MHC-II expression to evade immune surveillance (Kanazawa et al., 2000; Miller et al., 1998; Pai et al., 2003; Zhong et al., 1999).

MHC-II mediated antigen presentation to T cells is a fundamental step in acquiring adaptive immunity. Learning how the MHC-II genes are expressed and how the complexes function is imperative to our understanding of how our bodies defend themselves against foreign agents.

Antigen Presentation By Major Histocompatibility Complexes

The major histocompatibility complexes consist of two groups of molecules, class I (MHC-I) and class II (MHC-II). In the human genome, the MHC genes are all present on chromosome 6 (location: 6p21.3). The MHC locus is one of the most gene dense regions of the human genome. The gene names all start with the common nomenclature human leukocyte antigen (HLA). The heavy chains of MHC-I are named HLA-A, B, and C. There are three isotypes of MHC-II molecules, HLA-DR, DQ, and DP, which consist of two polypeptide chains each, alpha and beta; the genes encoding for the alpha and beta chains are named *HLA-DRA* (alpha chain), *HLA-DRB* (beta chain), *HLA-DQA*, etc.

The MHC-I genes are expressed in most all cell types and classically function in antigen specific immunity against intracellular pathogens. The MHC-I molecules consist of a α heavy chain as mentioned, and a smaller β-2 microglobulin light chain that non-covalently associate with each other. The α chain alone forms the groove where an antigenic peptide of 8-10 amino acids can bind (Castellino et al., 1997).

When a cell is invaded by an intracellular pathogen, such as a virus, the proteosome complex within the cytosol of the cell digests the virus proteins into small peptides that are actively transported into the endoplasmic reticulum (ER) by the TAP1/TAP2 transporter complex. MHC-I is stably maintained in the ER membrane by the peptide loading complex, which consists of TAP1/TAP2, tapasin, ERp57, and calreticulin, until ready to be loaded with a peptide (Hansen and Bouvier, 2009). The viral peptides are loaded onto the MHC-I within the ER and are then transported to the extracellular surface of the invaded cell. Here, the MHC-I::peptide complex can be recognized by a specific cognate T-cell receptor (TCR) expressed on the surface of a CD8 (cluster of differentiation 8) T-cell. CD8 is a co-receptor molecule that assists in the recognition and binding of MHC-I and the TCR (Luescher et al., 1995). The peptide specific CD8 T-cell then becomes activated to express and secrete cytotoxic proteins, such as perforin and multiple granzyme proteins. This causes the cell invaded by virus to undergo apoptosis, hence limiting further propagation of the pathogen (Anthony et al., 2010). While the MHC-I molecules have a crucial function in immunity, the discussion of the MHC-I family of complexes will be limited to above as this thesis focuses on our understanding of the MHC-II complex.

MHC-II genes, in contrast to the MHC-I genes, are expressed in a restricted number of cell types or under certain stimulating conditions, and have a greater role in the bodies' defense against extracellular pathogens. MHC-II molecules are formed of two polypeptide chains of about equal size called α and β. The two chains also non-covalently interact, but unlike MHC-I, they cooperate to form a groove together where a peptide can bind (Kaufman et al., 1984). In the case of MHC-II molecules, the groove is non-restricting in size, therefore it can accommodate larger peptides than MHC-I molecules, theoretically of unlimited size, but usually 15-20 amino acids long (Castellino et al., 1997).

To be displayed by an MHC-II, extracellular pathogens and particles are taken up through phagocytosis or other endocytic pathways by professional antigen presenting cells (pAPC). These foreign particles are digested into smaller peptides by endocytic proteases, such as the various cathepsin proteases, CatD, B, F, H, L, S, and Z (Lennon-Duménil et al., 2002). The MHC-II, is expressed and incorporated into the ER membrane where it interacts with invariant chain (Ii), a molecule that stably maintains the structure of MHC-II (Viville et al., 1993). Within the endosomal compartments of the cell, the proteases digest away all of Ii in a stepwise manner except for a domain called CLIP that is left bound to the peptide-binding groove of the MHC-II molecule. CLIP is exchanged for an antigenic peptide in a late endosomal compartment of low pH with the assistance of MHC-II homologs, HLA-DM and HLA-DO (Rocha and Neefjes, 2008). HLA-DM is a chaperone for MHC-II complexes that facilitates the release of CLIP and holds the peptide-less complex together until a strong interacting peptide binds to the groove (Sloan et al., 1995). The final MHC-II:peptide interacting complex is then transported to the extracellular plasma membrane. At the surface of the cell, the MHC-II:peptide complex is specifically recognized by a compatible TCR with the help of a co-receptor molecule, CD4. This interaction activates the CD4-expressing T cell, which leads to the expression of cytokines that further activate the function of other immune cells.

As mentioned, MHC-II is expressed only in a selected number of cell types. They are expressed in macrophages, dendritic cells, and B-lymphocytes collectively called professional antigen presenting cells, and in thymic epithelial cells. Macrophages express greater levels of MHC-II when stimulated with interferon-γ (IFN-γ) (Steeg et al., 1982), and due to their high phagocytic activity, macrophages are very efficient at presenting antigens to T cells. This interaction will in turn activate the T cell to secrete IFN-γ, forming a positive

feedback loop, further enhancing the antigen presentation function in macrophages. Unlike macrophages, dendritic cells are not efficient in phagocytosis but very efficient in receptor-mediated endocytosis and macropinocytosis, making them also very effective antigen presenting cells (Sallusto et al., 1995). Antigen uptake is active in immature dendritic cells compared to mature dendritic cells, whereas, MHC-II expression is higher at the mature stages of development (Cella et al., 1997). Because of this separation of antigen acquisition and presentation, dendritic cells are thought to uptake antigens at the site of infection and deliver them to lymph nodes where they interact with T cells. In B cells, MHC-II genes are expressed early during development and silenced as the cells mature and become plasma cells (Latron et al., 1988). B cells have low phagocytosis and macropinocytosis activity and mainly acquire antigen through internalization of immunoglobulin receptors on the cell surface (Lanzavecchia, 1990). B cells are the least efficient of the pAPCs in antigen presentation (Vidard et al., 1992).

Thymic epithelial cells, though not considered pAPCs, also express MHC-II genes (Lorenz and Alien, 1989). In these cells, both MHC-I and MHC-II molecules play an essential role in T-cell positive and negative selection. Through this T cell maturation process, the immune system acquires a T cell repertoire that recognizes a diverse array of non-self peptides that are not highly reactive towards self-antigens. Expression of MHC-II genes in thymic epithelial cells is limited or absent until exposed to IFN-γ (Rigaud et al., 1996). Other cell types such as endothelial and fibroblast cells also express MHC-II genes after encountering IFN-γ. While these cells may be able to express comparable levels of MHC-II to pAPCs after IFN-γ stimulation, they are not good T cells activators as they lack co-stimulatory molecules or the ability to express stimulatory cytokines (Geppert and Lipsky, 1985).

A CD4+ T cell that interacts with an MHC-II::peptide complex can differentiate into various subtypes depending on the cytokines in its milieu (Murphy and Reiner, 2002). The Th1 subtype secretes IFN-γ to elicit a positive feedback loop, enforcing T cell development further down the Th1 cell lineage. This will launch a cellular defense response towards intracellular pathogens through activating the phagocytic activity of macrophages. Th2 cells produce interleukin-4 (IL-4), IL-5, and IL-13 that enforces the development of the Th2 lineage cells. These cytokines activate a humoral response against extracellular pathogens. Th2 cells will activate B cells to develop into plasma cells, specialized to produce large quantities of antigen specific antibodies, and produce antibodies that can neutralize the pathogen. Both are essential cellular developmental pathways for T cell mediated immunity and unachievable without the MHC-II complex.

Essential Regulatory Elements of MHC-II Transcription

Antigen presentation to T cells is a major function of pAPCs and when this process is disrupted, T cells are unable to recognize the pathogens, hence leading to a loss of any pathogen specific activation of the adaptive immune system. A dramatic realization of this are the symptoms of a severe immunodeficiency called the bare lymphocyte syndrome (BLS) as previously mentioned. Patients that inherit this recessive genetic disorder suffer from reoccurring viral and bacterial infections and in most cases do not survive beyond early childhood (Reith and Mach, 2001). BLS is a genetically heterogeneous disease that can be characterized by the lack of mRNA expression of all MHC-II genes that may be accompanied by reduction of MHC-I in some cases. Early on, it was thought that a shared trans-activating element would be responsible as the expression of all the MHC-II genes was simultaneously lost.

Through cell fusion studies, it was determined that there were four complementation groups of BLS (Reith and Mach, 2001). Rigorous biochemical studies further elucidated which proteins were absent in each complementation group. Three were determined to form a complex constituted of three regulatory factor X (RFX) proteins named RFX5, RFXAP, and RFXB (Durand et al., 1997; Nagarajan et al., 1999; Steimle et al., 1995). The fourth complementation group was later determined to be null for the master regulator of MHC-II genes, the class II transactivator (CIITA) (Steimle et al., 1993). All four trans-acting factors are essential for the expression of MHC-II genes and mounting an antigen specific immune response as seen by their null phenotypes, a total loss of adaptive immunity.

These essential transcription factors all bind to a region upstream of the transcription start site (TSS) called the *WXY* box. The *WXY* box is highly conserved at the proximal promoter of all the MHC-II genes, HLA-DM, HLA-DO, and Ii (Masternak and Reith, 2002). For the *HLA-DRA* gene promoter, it spans between -59 to -139 bps (Benoist and Mathis, 1990). Due to this conservation of proximal promoter sequence, these genes are transcribed concordantly in general. The *WXY* box consists of the W, X1, X2, and Y boxes. Not much is known of what transcription factor binds to the W box. The X1 box has been shown to recruit the RFX complex, and the X2 box to bind the CREB dimer complex (Moreno et al., 1999). The Y box has been established to bind the trimeric NF-Y complex that consists of NF-Ya, b, and c. These DNA binding transcription factors are found at the promoter regardless of transcription, ubiquitously. CIITA, on the other hand, does not bind DNA directly, nor is it expressed ubiquitously.

CIITA binds to the scaffold formed by the RFX, CREB, and NF-Y proteins at the promoter proximal regulatory region (Kara and Glimcher, 1991). The promoter is not protected in an in vivo DNase protection assay in BLS cell lines, such as Ramia, Nacera,

6.1.6, all of which are missing a component of the RFX complex. Therefore, without any one of the RFX proteins, the transcriptional machinery fails to assemble at the promoter. In contrast, RJ2.2.5, a CIITA null B cell line, is fully protected at the promoter despite the loss of CIITA (Kara and Glimcher, 1991), meaning the other DNA binding factors are still present. Similarly, the NF-Y proteins are also necessary for the complex to form on the *WXY* box, also as determined by in vivo genomic footprinting assays (Linhoff et al., 1997).

Between the TSS and *WXY* are also other promoter elements that should be mentioned. Some MHC-II genes, certain alleles of HLA-DP and HLA-DO for example, have been shown to contain a CAAT box in their promoters, but there is no evidence that they are functionally required (Benoist and Mathis, 1990; Servenius et al., 1987). MHC-II gene promoters also contain a TATA box that binds the TATA box binding protein (TBP). TBP interacts with general transcription factors to initiate transcription, and has also been shown to interact with CIITA to regulate MHC-II genes (Mahanta et al., 1997). *HLA-DRA* promoter has an additional octamer binding site that could bind Oct-2 and Bob-1 that may together enhance the expression of *HLA-DRA* in B cells (Fontes et al., 1996). There is, however, no *in vivo* evidence of these two proteins binding at these regions, therefore the function of Oct-2 and Bob-1 is still unknown. HLA-DM and Ii contain active *WXY* box elements, but also NF-kB and Sp1 sites, which have been shown to be necessary for full activity of the promoters (Brown et al., 1994; Radley et al., 1994; Wright et al., 1995).

Interestingly, the conserved *WXY* motif in the MHC-II promoters is also repeated throughout the MHC-II locus in the intergenic regions. Through computational alignment searching, 19 additional homology sites were found within the MHC-II region (Gomez et al., 2005). These sites were termed X-like (XL), as they were not associated with specific genes. Some of these sites are bound by RFX and CIITA, and are capable of activating reporter

genes in vitro. One particular XL site, *XL9*, positioned between *HLA-DRB1* and *HLA-DQA1*, binds a protein called CCCTC-binding factor (CTCF), and through the interaction between CTCF and transcription factors at the promoters, regulates the expression of its neighboring genes via long-range chromosomal interactions (Majumder et al., 2008). Further description of this transcriptional regulatory process can be found in Chapter 1. CTCF is known as an insulator binding protein that functions as an organizer of the genome (Phillips and Corces, 2009). Through immunoprecipitation followed by high-throughput sequencing, other CTCF binding sites were identified within the MHC-II region, making a total of ten sites (Majumder and Boss, 2010a). These sites have also been determined to interact with the promoters of nearby genes and possibly regulate their expression. Currently, other than the cis-regulatory elements mentioned above, there are no other identified DNA elements with regulatory activity.

Of the essential trans-regulatory elements, CIITA is regarded as the master regulator of MHC-II expression. It is not only a necessary factor, but its expression is highly controlled. Also, CIITA is the mediating factor that interacts with general transcription factors and recruits them to the promoter to initiate transcription; TBP, TFIID, $TAF_{II}250$, CREB binding protein (CBP), PCAF to name a few (Harton et al., 2001; Masternak and Reith, 2002; Spilianakis et al., 2000). The protein domains of CIITA had been characterized initially according to their sequence homology to known functional protein domains, and their functions were subsequently determined experimentally. Starting from the N-terminus, there is an activation (or acidic) domain, a proline-serine-threonine-rich (PST) domain, a GTP-binding domain (GBD), and leucine-rich repeat (LRR) domain at the C-terminus (Riley et al., 1995). The activation domain has been shown to interact with the coactivator proteins to activate MHC-II transcription (Fontes et al., 1999a). The PST domain may assist with

activation but no other specific functions are known. The GBD can bind GTP but the exact function of this is unclear (Harton et al., 1999). The LRR domains normally mediate protein-protein interactions, and not surprisingly, the LRR together with the GBD have been described to be necessary for the homodimerization of CIITA (Linhoff et al., 2001). The reason for CIITA dimerization is also unclear and needs further examination.

The regions of CIITA that interact with the other DNA binding *WXY* box proteins have also been determined. RFXB binds to the activation and PST domains, while RFX5 interacts with the GBD. RFXAP does not directly interact with CIITA (Masternak et al., 2000). Recently, it was determined that the RFX complex is actually a tetramer formed of a dimer of RFX5 and one molecule each of RFXAP and RFXB. Not any one of these factors alone can bind DNA efficiently and all three factors must be present for maximum stability (Garvie and Boss, 2008a). CREB binds to the larger region of CIITA spanning from the N terminus to the GBD, and the NF-Y proteins bind to the PST and GBD domains.

The expression of CIITA is highly regulated in contrast to the other MHC-II transcription factors. *CIITA* expression is regulated by three promoters in human cells, which convey when and where the gene is to be expressed. Promoter I allows the expression of *CIITA* in macrophages and conventional dendritic cells (Muhlethaler-Mottet et al., 1997). In macrophages, promoter I is also the dominant promoter during IFN-γ treatment induced transcription (Zinzow-Kramer et al., 2012). While initially transcripts had been cloned from a supposed promoter II in human, when human cell lines and tissue were examined for *CIITA* expression, no transcripts have been detected to be expressed from this promoter to date (Muhlethaler-Mottet et al., 1997). Promoter III determines *CIITA* expression in B-lymphocytes and plasmacytoid dendritic cells (LeibundGut-Landmann et al., 2004). Lastly, promoter IV is an inducible promoter that becomes activated when the cells

are exposed to the cytokine IFN-γ. Promoter IV can be activated in normally non-CIITA expressing cell types, and can also further activate the expression of CIITA in macrophages. As MHC-II expression is highly dependent on the activity of CIITA, the limited spatial and temporal expression of CIITA determines where MHC-II is expressed.

Histone Modifications And Transcriptional Regulation

Some of the mentioned transcriptional coactivators that CIITA interacts with and recruits to the MHC-II promoters have histone modifying catalytic activities. Histone modification as a gene expression regulatory mechanism has gained much interest in the recent years and is now recognized as a major mechanism of epigenetic transcriptional regulation. Currently, epigenetics is most widely accepted and defined as, "The study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression, (or) nuclear inheritance which is not based on differences in DNA sequence" (Holliday 94).

Heritable genetic information transmitted from parent to progeny is encoded in the form of deoxyribonucleic acid (DNA) (Avery et al., 1944) from various species of viruses to singular and multicellular organisms. DNA is formed of a phosphate-deoxyribose backbone and four bases, adenine, cytosine, thymine, and guanine constructed into a double helical structure (Watson and Crick, 1953). These four bases form a unique combination of sequences that encode for ribonucleic acid (RNA) and consequently the protein molecules that carry out the structural and mechanical functions of the cell. The human genome consists of approximately 3.2 gigabase pairs of DNA (McPherson et al., 2001). To package this material into the nucleus of a cell, the DNA must be highly compacted. Histones are

chemically basic proteins that have a high affinity for the acidic DNA; they foremost undertake the task of DNA compaction and organization.

In humans, there are four core histone proteins, also called the canonical histones, H2A, H2B, H3, and H4, as well as a linker histone H1. These proteins are expressed ubiquitously in all cells. There are also histone variants that are cell type, developmental stage, or species specifically expressed (Talbert and Henikoff, 2010). The canonical histones assemble as an octamer, two of each core histone protein, to form a unit called the nucleosome. DNA wraps around the nucleosomes, forming a beads-on-a-string structure (Oudet et al., 1975; Sollner-Webb and Felsenfeld, 1975). This is regarded as the first order of DNA compaction, or organization, and the linker histones are thought to provide a further degree of compaction (Noll and Kornberg, 1977).

Researchers initially made observations that histones inhibited RNA synthesis and thought of histones as repressive proteins. However, in a seminal article by V. G. Allfrey, R. Faulkner, and A. E. Mirsky, it was first reported that post-translational modifications of histone proteins could enhance RNA synthesis (Allfrey et al., 1964). Histone acetylation and methylation were found by this group to decorate the histone proteins, but only acetylation was described to be a gene activating modification. Histone methylation was regarded as a neutral or negative regulator until a protein methyltransferase, coactivator associated arginine methyltransferase 1 (CARM1), was determined to methylate histone H3 and activate transcription (Chen et al., 1999). Also, not much later, it was shown that histone H3 lysine4 methylation is associated with the transcriptionally active nuclei in tetrahymena (Strahl et al., 1999), the first to show that methylation of a specific residue correlated with active transcription.

The histone octamers form disc-like structures in each nucleosome; this consists of two central H3-H4 dimers with two peripherally associated H2A-H2B dimers (Finch et al., 1977; Luger et al., 1997). Approximately 146 base pairs of DNA are wrapped around the disc-like structures formed by the histone octamers in 1.65 left-handed helical turns (McGhee and Felsenfeld, 1980). The N-terminal tails of the histone proteins are unstructured; especially, the H3, H4, and H2B tails will protrude out from the DNA gyres, which facilitates the recognition by other proteins (Luger et al., 1997). These N-terminal tails can become extensively post-translationally modified, though modifications are not limited to the tails and may also occur within the interior of the nucleosomes. It is thought that the chemical properties of the modifications may alter the interaction dynamic between the DNA and histone octamer to render the DNA sequences more or less accessible; this is also referred to as 'open' and 'closed' chromatin. Some characterized post-translational histone modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation. These modifications are thought to have roles in transcriptional regulation of genes, chromatin organization, DNA damage repair, and during DNA replication (Kouzarides, 2007). Depending on the modification or combination of modifications, it was also proposed that this would form a 'histone code', which could be recognized by 'readers' to undertake specific functions (Strahl and Allis, 2000).

Most modifications mentioned occur on lysine residues; except methylation can be applied to lysine and arginine residues, while phosphorylation can only be applied to serine or threonine residues (Kouzarides, 2007). Methylation of lysine or arginine can occur once or multiple times on a single residue to produce mono-, di-, or trimethylated forms. With regards to transcriptional regulation, acetylation of lysine residues in general has been associated with activation of genes. A few other examples of marks of active transcription

include H3 serine10 phosphorylation (H3S10ph) and H2B lysine120 ubiquitylation (H2BK120ub). Methylation of histones can signify a range of functions depending on the specific residue and degree of modification. For example, H3K4 methylation deposition at the promoter region of genes is a well-characterized activation mark, while H3K27 methylation at these regions associated with transcriptional repression (Li et al., 2007). The degree of methylation adds an extra layer of control as in the example of H3K4 methylation. A monomethylated H3K4 (H3K4me1) is not associated with promoters of active genes but with regulatory elements of the intergenic regions of the genome. H3K4 dimethylation (H3K4me2) is positioned in accessible 'open' regions of the genome, while H3K4 trimethylation (H3K4me3) at promoter regions is regarded as the mark of active transcription. Our understanding of how histone modifications affect gene transcription advances with the discovery and identification of factors that apply and recognize these marks. Acetylation and methylation of histones are the most advanced in regards to our knowledge of the molecular machinery.

Histone modifying complexes and their role in transcriptional regulation

Histone modifications are applied, removed, and interpreted into a function by the so-called 'writers', 'erasers', and 'readers' of histone modifications. The lysine acetyltransferases (KAT) and histone deacetylases (HDAC) counteract each other, one being the 'writer' and the other the 'eraser', respectively. The acetylation marks can be 'read' by proteins that contain a bromodomain, such as the chromatin remodeling SWI/SNF family of proteins, but it can also be recognized by the plant homeodomain (PHD) finger structure (Glatt et al., 2011; Yap and Zhou, 2010).

The PHD domain is better known for its ability to bind histones with varying degrees of H3K4 methylation, but may also recognize the symmetrical methylation of H3R2 modification (Yap and Zhou, 2010). The royal family of domains, Tudor, PWWP, MBT, and chromodomains, will bind to various histone methylation marks of H3K4, H3K9, H3K27, H3K79, H4K20, etc.; different factors recognize specific modifications. The 'writers' for histone methylation are called lysine methyltransferases (KMT), and the 'erasers', lysine demethylases (KDM).

All of these players must interact and cooperate to achieve the appropriate level of histone modification and gene expression. The role of KATs, KMTs, HDACs, and chromatin remodeling factors in MHC-II transcriptional regulation is discussed in further detail in Chapters 2 and 3. Our understanding of the mechanism of how these factors function is continuously evolving and new factors are continuously being discovered. This is reflected in this thesis as it focuses on the recently determined histone acetyl- and methyltransferase multiprotein complexes and their role in MHC-II transcription.

The histone lysine acetyltransferases were the first proteins identified to modify histones and have a role in transcriptional regulation. Those of the CBP/p300 family (Ogryzko et al., 1996), GNAT family (GCN5 and PCAF) (Brownell et al., 1996; Yang et al., 1996), MYST family (dMOF) (Smith et al., 2000), as well as certain general transcription factors ($TAF_{II}250$) (Mizzen et al., 1996), and nuclear receptor cofactors (SRC-1) (Spencer et al., 1997) contain acetyltransferase activity. They share the catalytic activity of transferring an acetyl group from the acetyl coenzyme A (acetyl-CoA) to a lysine residue on the histone molecules (Allfrey et al., 1964). However, the KATs differ in their specificity for lysine residues. For example, when tested with nucleosomes as the substrate, CBP/p300 has a broad range of specificity with a stronger preference for H4 lysine residues, while PCAF has

a preference for H3K14 (Ogryzko et al., 1996; Schiltz et al., 1999), and MOF has been determined to prefer the H4K16 residue as a substrate (Smith et al., 2000). However, within a complex of proteins, the specificity may change, as in the case of GCN5. GCN5 alone prefers the acetylation of H3K14, while within the SAGA complex it will also acetylate H3K9 and H3K18 (Grant et al., 1999). Of these KATs, relatively little was known of how GCN5 regulates the MHC-II genes. Also, the subunits of different types of human GCN5/PCAF multiprotein complexes were recently determined by complex purification and mass spectrometry studies (Martinez et al., 2001; Wang et al., 2008). Therefore, little is known of how these different complexes may have a role in MHC-II gene regulation.

The homolog of GCN5 (general control nonrepressed) from Tetrahymena thermophila was the first cloned histone acetyltransferase (Brownell et al., 1996). Human GCN5 shares a 75% homology with another KAT, PCAF (p300/CREB-binding protein-associated factor) (Xu et al., 1998). These proteins are the catalytic subunits of large protein complexes that were initially characterized in *Saccharomyces cerevisiae* (Grant et al., 1997). From this study it was determined that there are at least two types of GCN5/PCAF containing complexes in yeast, which they termed SAGA and ADA. Later, the conserved complexes were purified in drosophila and human (Lee and Workman, 2007). In human, the two types of GCN5/PCAF containing complexes are called STAGA (SPT3-TAF9-GCN5/PCAF Acetylase) and ATAC (Ada Two-A-containing). These complexes share common factors that form a core that are homologous to the yeast Gcn5, Ada2, Ada3, and Sgf29 proteins. As the complexes became more complex in the higher order metazoans, the yeast Gcn5 protein diverged into two homologous proteins, GCN5 and PCAF, in humans. Similarly, Ada2 separated into two homologous proteins ADA2a and ADA2b in drosophila and human cells. STAGA and ATAC can contain either GCN5 or PCAF interchangeably, but

only the ADA2b is found in the STAGA complex and the ADA2a in ATAC (Nagy et al., 2010). Not much is known to date on how functionally different the STAGA and ATAC complexes are, and similarly little is known whether containing a GCN5 vs PCAF enables the complex to have certain functions or specificity for certain genes.

The histone modification specificity of these complexes is still debatable. As mentioned above, the SAGA type complexes have a preference for H3K14, H3K9, and H3K18 residues. The knockdown of ATAC2 in mice led to a global decrease of H3K9, H4K5, H4K12, and H4K16 acetylations (Guelman et al., 2009), while when ADA2a was knocked down in HeLa cells, H3K9ac and H3K14ac were reduced (Nagy et al., 2010). Whether, the two different types of complexes respond to different stimuli is also a debated topic. ATAC was observed to be responsive to activation of the phosphokinase C pathway (Nagy et al., 2010), while STAGA was recruited to p53-activated genes in response to UV induced DNA damage (Gamper et al., 2009). Depending on the stimulus for the MAPK pathway, it seems that either complex may be responsible for activating the response genes (Spedale et al., 2012). A recent ChIP-seq study showed that the two complexes are in most cases bound mutually exclusive to each other at gene promoters or enhancers (Krebs et al., 2011). The researchers also suggested that the two complexes bind not only to stress-responsive genes, but also housekeeping genes through the guidance of gene specific transcription factors.

Histone lysine methyltransferases, except for a few exceptions, contain a SET domain, and are included in one of the six SET-containing KMT subfamilies: SET1, SET2, SUV39, EZH, SMYD, and PRDM (Upadhyay and Cheng, 2011). Dot1 is the one exceptional KMT that does not contain a SET domain (Min et al., 2003). SET8, SET7/9, SUV4-20H1, SUV4-20H2, MLL5, SETD5 and SETD6, while containing a SET domain, are

not part of the six families due to lack of homology (Upadhyay and Cheng, 2011). A member of the Su(var) group of proteins, initially identified in a mutant screen for position effect variegation in drosophila, mammalian SUV39-H1 was the first of SET domain containing KMT proteins to be shown to methylate H3K9 and affect chromatin organization (Rea et al., 2000).

As the methylation modifications have more defined functions depending on the modified residue and the degree of methylation, the KMTs also are more specialized than the acetyltransferases to our current knowledge. For example, Set8/PR-Set7 methylates the unmethylated H4K20 residue (Oda et al., 2009) while SUV4-20H1 and SUV4-20H2 will di- and trimethylate the H4K20 residue, respectively (Schotta et al., 2008). Currently, of these modifications, we know in a Set8 knockout mouse, partial embryonic lethality is observed, and cells have difficulty progressing into S phase during cell divisions, possibly due to the involvement of Set8 in DNA damage repair (Jørgensen et al., 2007). The H4K20me2/me3 modifications have been associated with constitutively repressed regions of the chromatin and may have a role in maintaining the silenced state of these regions (Schotta et al., 2004).

Unlike H4K20 methylation, H3K4 methylation is a well-characterized modification for open regions of chromatin and actively transcribed genes. The enzyme responsible for this modification was first determined in Saccaromyces cerevisiae as the SET domain containing protein Set1 (Briggs et al., 2001). Set1 was identified for its homology to the Drosophila protein trithorax, which was already known for its role in regulating the expression of HOX genes during development (Kennison and Tamkun, 1988). Trithorax and polycomb, bind to HOX and other genes and counteract each other, activating and repressing gene expression, respectively, to maintain a balanced expression level (Chinwalla et al., 1995). This activity correlates with H3K4 methylation for gene activation and H3K27

methylation for silenced genes (Cao et al., 2002; Milne et al., 2002). The mammalian homolog of trithorax and Set1, MLL (mixed lineage leukemia), was the first to be shown to methylate H3K4 at the HOX genes to activate transcription in mice (Milne et al., 2002). The gene acquired its name by its association with acute lymphoblastic and myeloid leukemia when genetic alterations occur to the gene in myeloid cell types (Ennas et al., 1997).

Set1 in yeast cells was determined to be a part of a multisubunit complex called COMPASS (Complex Proteins Associated with Set1) (Miller et al., 2001). This is also the case for its mammalian counterpart. There are six trithorax homologous proteins in human cells, Set1A, Set1B, MLL, MLL2, MLL3, and MLL4, with an additional MLL5 that contains a SET domain but has limited homology to the other MLL proteins (Eissenberg and Shilatifard, 2009). They form a COMPASS-like complex that consist of a MLL protein along with the other core proteins WDR5, ASH2L, RbBP5, and DPY-30 (Dou et al., 2005; Hughes et al., 2004). Depending on the complex, they may contain unique subunits, such as menin for the MLL1 and MLL2 containing complexes, whereas MLL3 and MLL4 complexes may interact with the nuclear receptor proteins and coactivator proteins (Goo et al., 2003).

When assessed with modification specific antibodies, it was considered that the MLL complex was capable of applying all three states of H3K4 methylation and the subunits of the MLL complex regulated the degree of methylation (Dou et al., 2006). The modification specific antibodies however may recognize more than one type of modification. More recently, with mass spectrometry analysis, using in vitro reconstructed MLL complexes, it was determined that when MLL protein is in a reaction alone with substrate, it was a weak H3K4 monomethyltransferase. With the addition of WDR5, RbBP5, and ASH2L, the complex was an efficient H3K4 mono and dimethyltransferase, but an inefficient

trimethylating enzyme complex (Patel et al., 2009). The WDR5, ASH2L, RbBP5, and DPY-30 alone (WRAD) without MLL, which was previously considered as the catalytic component, was also shown to be capable of mono and dimethylation of H3K4 (Patel et al., 2009; Patel et al., 2011).

The histone acetyl- and methyltransferase proteins and complexes have been shown to interact and cooperate to activate transcription. For example, the MLL protein has a CBP binding domain, which was initially thought as the mechanism of transcriptional activation (Ernst et al., 2001). Also, the different modifying complexes in some cases share subunit proteins. WDR5 is not only a core component of the MLL histone methylating complexes, it has also been shown to be included in the GCN5/PCAF containing ATAC complex (Dou et al., 2005; Wang et al., 2008). Similarly, HCF-1 is also a shared component between the MLL and ATAC complexes complex (Dou et al., 2005; Wang et al., 2008). These factors interact with mediator, the general transcription factors and RNA polymerase II to initiate transcription (Natarajan et al., 1999), as well as interact with P-TEFb to start elongation of a paused RNA polymerase (Boehm et al., 2003). All of these factors, in turn must also interact with gene specific transcription factors to be able to respond to specific developmental and environmental cues.

The goal of the projects described herein attempt to address the following questions: For the essential immune system genes of the MHC-II family to be expressed cell type specifically and in response to environmental signals (i.e. interferon-γ), what are the transcriptional coactivators that interact with the essential transcription factors to activate transcription? These transcriptional coactivators in many cases contain histone-modifying activity. Therefore, what histone modifications occur at the MHC-II genes during

transcriptional activation? What is the role of these histone modifications in transcription of MHC-II? Is there a possible epigenetic memory mechanism that marks the MHC-II genes for future exposure to a previously encountered stimulus? Are there any yet unknown cis-regulatory elements within the MHC-II locus, which may be associated with human diseases such as Parkinson's disease? Determining the histone modifying factors that assemble at the MHC-II genes and identifying novel cis-regulatory elements will allow us to further understand the role of MHC-II genes as a fundamental element of our immune system and its relationship to human disease.

Introduction – Part 2 –

Regulation of Major Histocompatibility Complex Class II Genes

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Summary

The major histocompatibility complex class II (MHC-II) genes are regulated at the level of transcription. Recent studies have shown that chromatin modification is critical for efficient transcription of these genes, and a number of chromatin modifying complexes recruited to MHC-II genes have been described. The MHC-II genes are segregated from each other by a series of chromatin elements, termed MHC-II insulators. Interactions between MHC-insulators and the promoters of MHC-II genes are mediated by the insulator factor CCCTC-binding protein and are critical for efficient expression. This regulatory mechanism provides a novel view of how the entire MHC-II locus is assembled architecturally and can be coordinately controlled.

Introduction

The major histocompatibility complex class II (MHC-II) genes function to present antigenic peptides to CD4 T cells. As a result of this action adaptive immune responses are initiated, maintained, and regulated. There are three isotypes of classical MHC-II genes in human (*HLA-DR, DP, DQ*), which are each formed of two polypeptides, α and β chains (A and B genes) (Figure 1-1). The MHC-II-linked antigen presentation accessory genes (*HLA-DM* and *HLA-DO*), as well as the unlinked invariant chain (*Ii*) gene, which encode proteins that function in translocation and peptide selection/loading of MHC-II molecules, are coordinately regulated with the classical MHC-II genes. MHC-II genes are constitutively expressed by professional antigen presenting cells (macrophages, dendritic cells, and B cells) and in thymic epithelial cells. These genes can be induced in non-immune cells (e.g., fibroblasts, epithelial cells, and endothelial cells, etc.) following exposure to cytokines, of which interferon-γ (IFN-γ) is the most prominent (Collins et al., 1984). One molecular

genetic regulatory process controls the expression of this system and therefore the ability of cells to present antigens. This review will focus on advancements of the past decade that have determined the complex interplay between the promoter proximal cis- and trans-regulatory elements and the chromatin machinery that serves to activate gene expression, and the newly described chromatin architecture and dynamic organization of the locus.

MHC-II promoter proximal elements and factors

Until recently, control of this system was thought to occur primarily through the action of a highly conserved, promoter proximal, combinatorial cis-regulatory sequence located approximately 100-200 bp upstream of the transcription start site of each MHC-II gene. The sequence consists of the W/S/Z, X1, X2, and Y box elements (Reith and Mach, 2001). NF-Y (nuclear factor-Y) complex, comprised of NF-Ya, NF-Yb, and NF-Yc, binds to the Y box. Due to the histone fold like structure of NF-Yb and NF-Yc (Baxevanis et al., 1995), the NF-Y complex is thought to distort and compact the structure of DNA, thereby providing an additional level of specificity for combinatorial transcription factor binding (Guerra et al., 2007; Ronchi et al., 1995). NF-Y regulates many genes as the Y box contains the CCAAT sequence described many years ago as a canonical regulatory element. The X2 box is bound by CREB (cAMP response element binding protein) (Moreno et al., 1999), and as its name implies, cAMP-dependent activation pathways modulate CREB's activity in other gene systems. However, although CREB and its phosphorylated form were found at the *HLA-DRA* X2 box, CREB phosphorylation was not essential and only mildly enhanced class II transcription (Lochamy et al., 2007). CREB's role as a stabilizing partner of the X1 box proteins at the promoter is likely a more important function and a mechanism unique to the MHC-II system (Lochamy et al., 2007).

Studies of bare lymphocyte syndrome (BLS) patient-derived cell lines, which are defective for MHC-II gene expression, identified four complementation groups that were essential for MHC-II gene expression. The genetic bases for three of these were due to mutations in transcription factors that formed the X1 box binding factor RFX: RFX5 (regulatory factor X5), RFXAP, and RFX-B/RFXANK (Reith and Mach, 2001). Solution structure studies of RFX protein domains suggest that the RFX complex likely exists as a heterotetramer with two RFX5 molecules and monomers of RFXAP and RFX-B (Garvie et al., 2007; Laird et al., 2010). Irrespective of MHC-II gene expression, RFX interacts directly with CREB and NF-Y, forming a combinatorial DNA-protein complex on the X-Y box region. By itself this ternary complex is transcriptionally inactive, but is specifically recognized by CIITA (class II transactivator), which is required to activate transcription. CIITA mutations represent the genetic lesion of the fourth BLS complementation group, and of the above factors, is the only one that is rate limiting and highly regulated. As such, the presence/absence of CIITA determines if a cell can express MHC-II genes. Thus, CIITA is the master regulator of this system.

Despite being discovered more than 20 years ago, the role of the W box in this system is still not clear. Early reports suggested that RFX also bound to the W box (Jabrane-Ferrat et al., 1996). In contrast, more recent data suggest that this is not the case as purified RFX complexes did not interact with the W box sequences (Garvie and Boss, 2008b) and mutations in the W box did not affect binding of RFX to the X1 box (Muhlethaler-Mottet et al., 2004). Instead, W box mutations led to decreases in CIITA binding (Muhlethaler-Mottet et al., 2004). The distance between the W and X box elements was also crucial for CIITA binding, which suggests the possibility of a yet unidentified W-box binding factor at this locus (Muhlethaler-Mottet et al., 2004).

CIITA is expressed from three major promoters in a cell type and cytokine dependent manner. Each promoter produces a slightly different N-terminal isoform, but the need if any for distinct isoforms is still not clear. The domain features of CIITA were used to define and discover the NLR/NOD/NACHT family of molecules that play multiple roles in innate immune responses (Harton et al., 2002; Ting et al., 2008). CIITA was also shown to upregulate human MHC-I genes in response to IFN-γ, although the immunological relevance of this function was not fully established. Most recently, the NLR family member NLRC5 (nucleotide binding domain and leucine rich repeat containing 5) was shown to be the likely factor that is responsible for MHC-I induction by IFN-γ in vivo (Meissner et al., 2010). NLRC5 does not regulate MHC-II genes. Thus, NLR family members regulate both MHC-I and MHC-II genes. It will be important to ultimately determine how these transcriptional activators are able to function with specificity on the different MHC genes.

Posttranslational modifications of CIITA are important to modulate its function (Wu et al., 2009). CIITA phosphorylation is involved in regulating CIITA nuclear localization and self-interaction. Acetylation of CIITA by acetyltransferases PCAF (p300/CBP associated factor) and CBP (CREB binding protein) may be important for its nuclear localization and activity (Wu et al., 2009). By contrast, class I histone deacetylase HDAC1 (histone deacetylase 1) had a negative regulatory role by interacting with CIITA to restrict its interaction with the enhanceosome complex (Zika et al., 2003), and HDAC2 was shown to directly deacetylate CIITA and target it towards proteasomal degradation (Wu et al., 2009). Monoubiquitylation of CIITA stabilizes the protein at the MHC-II promoters, and phosphorylation of a nearby serine residue was required for this modification, which suggests an intricate crosstalk between posttranslational modifications that are necessary for optimal activity of CIITA (Bhat et al., 2010).

MHC-II epigenetic regulation through histones

Modification or the remodeling of nucleosomes has been shown to be associated with gene expression and silencing (Clapier and Cairns, 2009; Jenuwein and Allis, 2001). The *HLA-DRA* gene promoter was one of the first immune system genes in which this connection was demonstrated (Beresford and Boss, 2001). At least four multisubunit complexes are recruited to MHC-II promoters to mediate these effects: CBP/p300, STAGA/ATAC (SPT3–TAF9–GCN5/PCAF/Ada Two-A-containing), COMPASS/MLL (complex associated with Set1/mixed lineage leukemia), and SWI/SNF (mating type switching/sucrose non-fermenting).

Histone acetylation is important for MHC-II gene expression. Following IFN-γ treatment histone H4 acetylation occurred in parallel with RNA polymerase II recruitment preceding transcriptional initiation, while histone H3 acetylation levels increased with productive mRNA transcription and were dependent on RNA pol II elongation (Rybtsova et al., 2007). CBP/p300, PCAF, and GCN5 (general control nonderepressible 5) are histone acetyltransferases (HAT) that are recruited by CIITA to MHC-II promoters (Fontes et al., 1999b; Kretsovali et al., 1998; Spilianakis et al., 2000). Of these HATs, GCN5 and PCAF function as components of STAGA and ATAC complexes, and with CBP/p300 are likely responsible for all of the activation associated acetylation marks at the MHC-II promoters (Nagy and Tora, 2007). CIITA itself has been shown to possess HAT activity as well (Raval et al., 2001). The ATPase Sug1, a component of the 19S proteasome complex present in the nucleus, associates with acetylated histone H3 and appears to be required for increased acetylation or stability of this mark at MHC-II promoters. Intriguingly, Sug1 also associates with CBP and this association is important for the recruitment of CBP to MHC-II genes

(Koues et al., 2008). Histone hyperacetylation through the inhibition of HDACs induced recruitment of enhanceosome components, but also led to the activation of MHC-II gene expression in the absence of CIITA. This suggests that there are multiple roles of histone acetylation at the MHC-II promoters: at least one modifying CIITA and another modifying the chromatin structure such that transcription can be initiated more efficiently (Gialitakis et al., 2006; Zika et al., 2003).

Several histone methylation marks associated with gene activation are also found at MHC-II promoters. The COMPASS/MLL type complexes are responsible for histone H3 lysine 4 methylation (H3K4me) from yeast to humans and also function at MHC-II genes. These complexes have a core histone methyltransferase (HMT) component accompanied by WDR5 (WD repeat domain 5), ASH2L (ash2-like), and RBBP5 (retinoblastoma binding protein 5) (Eissenberg and Shilatifard, 2009), which were bound at the *HLA-DRA* promoter (Koues et al., 2010). BRE1 (brefeldin A sensitivity 1) and UTX1 (ubiquitously transcribed tetratricopeptide repeat gene on X chromosome 1), which are also involved in COMPASS/MLL related activation of genes, were also recruited to the MHC-II promoters with IFN-γ treatment (Bhat et al., 2010; Koues et al., 2009). Coupled with these events following IFN-γ treatment, was the induced interaction between MLL and PML (promyelocytic leukemia) and relocation of the MHC-II promoters to PML nuclear bodies, which was important for sustaining H3K4me2 at the MHC-II promoters (Gialitakis et al., 2010). H3K4me2 histone modification is associated with transcriptional competency of a region and together with relocation to PML bodies, suggests a mechanism of transcriptional memory through chromosomal restructuring that may have a lasting effect on gene expression after the stimulus is removed.

Remodeling of MHC-II promoter nucleosomes by the SWI/SNF complex (Mudhasani and Fontes, 2005) and nucleosome eviction at the W-X-Y box was also important for proper expression and transcription start site selection of MHC-II genes (Leimgruber et al., 2009). The mechanism of establishing the nucleosome free promoter region was unique as it was not mediated by strong nucleosome positioning signals but through binding of the W-X-Y box factors (Leimgruber et al., 2009).

With all of these complexes and perhaps other factors functioning to modify the chromatin structure and activate transcription, two important issues can be raised. The first question is whether any of these events are unique to MHC-II genes. Because the chromatin remodeling complexes share components with one another, it is likely that some complex components or their isoforms are specific to this system while others are ubiquitous to all genes. Even if these are ubiquitous events, the second issue is how are these activities coordinated in both time and space at MHC-II promoters. CIITA is clearly the pivotal factor that facilitates the chromatin modification machinery and the activation of the system. As described below, these events are not sufficient to fully express these genes.

Long-range chromosomal regulation of MHC-II

In the late 1980s a distal X-Y box element was identified in the murine *I-Ea* locus that was associated with its correct tissue specific regulation when introduced as a transgene (van Ewijk et al., 1988). In the *HLA-DRA* system, a functional distal X-Y element was also found (Masternak et al., 2003). A screen for additional X-Y motifs found 32 non-promoter X box-like (XL) sequences within the MHC-II locus. Like the above sequences, some were functional, having properties similar to the promoter proximal W-X-Y boxes (Gomez et al., 2005). In contrast to the above elements, the *XL9* site, located in the intergenic region

between *HLA-DRB1* and *-DQA1*, did not bind RFX or CIITA, had no enhancer activity, but was in chromatin that was highly acetylated (Majumder et al., 2006). Because *XL9* was between the *HLA-DR* and *-DQ* subregions, there was the possibility that the region functioned as a chromatin boundary/insulator element.

Chromatin boundary/insulator elements function to organize chromatin into independent regulatory domains (West and Fraser, 2005). Insulators have the property of being able to block the activity of an enhancer from activating a promoter when placed between them. Some insulators also serve as barriers or boundary elements by preventing the encroachment of heterochromatin into active genes (Gaszner and Felsenfeld, 2006). These activities have been ascribed to CTCF (CCCTC binding factor), although it is likely that CTCF interacts with several other factors to mediate these functions (Bell et al., 1999). CTCF, a zinc finger DNA-binding protein that can homodimerize, is associated with the formation of long range chromatin loops between adjacent CTCF sites. Depending on the cell type and genetic context, and likely through the chromatin loops that it organizes, CTCF is ascribed transcriptional repression or activation functions (Filippova et al., 1996; Hark et al., 2000; Vostrov et al., 2002). Because its binding to DNA is methylation dependent, CTCF has been shown to play a major role in genomic imprinting (Hark et al., 2000) and X-chromosome inactivation in mammals (Chao et al., 2002). But precisely how these regulatory events are mediated is not known.

Indeed, CTCF was found to bind to sequences close to *XL9,* and this region mediated strong enhancer blocking activity (Majumder et al., 2008). For simplicity, the initial name of the region was retained. Further analyses showed that CTCF interacted with CIITA and was required for a chromatin-looping event between the *XL9* and proximal promoter regions of *HLA-DRB1* and *HLA-DQA1* genes (Majumder et al., 2008). CTCF depletion by

RNAi resulted in a substantial reduction in *HLA-DRB1* and *HLA-DQA1* gene expression. These data suggested that CTCF and potentially *XL9* were critical for expression of these and perhaps all MHC-II genes.

The question of whether CTCF was required for all MHC-II genes was addressed by using RNAi depletion. All CIITA-regulated genes within the MHC-II locus required CTCF for maximal expression (Majumder and Boss, 2010b). The five non-CIITA genes within the locus (*TAP1, TAP2, PSMB8, PSMB9,* and *BRD2*) were not affected by CTCF depletion. The question as to whether *XL9* was the only CTCF site that interacted with MHC-II gene promoters was also addressed. Genome wide studies identified 17 CTCF sites within the human MHC-II, including *XL9* (Barski et al., 2007; Kim et al., 2007); however, traditional chromatin immunoprecipitation studies showed that only ten of those sites truly bound CTCF in B cells and several other cell types (Figure 1-1) (Majumder and Boss, 2010b). Intriguingly, the validated sites resided between MHC-II subregions, such that they surrounded the subregions and for the most part isolated the CIITA-regulated genes from the others (Figure 1-1). For example, *C1* and *XL9* surround the *HLA-DR* subregion, whereas *XL9* and *C2* surround *HLA-DQ*. This may suggest that part of the duplication process that created multiple MHC-II genes may have required a CTCF site to be present during the event as it is part of the regulatory machinery.

Using chromatin conformation capture (3C) assays, procedures that allow the detection of long-range chromatin interactions (Dekker, 2006), two sets of interactions involving these CTCF sites were uncovered (Majumder and Boss, 2010b). A basal state of interactions between each of the CTCF sites occurred irrespective of MHC-II gene expression and was similar in B cells and fibroblasts (Figure 1-3). For example, *C1* was found to interact with its closest neighbor *XL9* and to a lesser degree with *C2*, which is 250

kb away. Similarly *XL9* could also interact with *C2*. The interactions appear to decrease substantially as the distances exceed 200 kb. These chromatin loops have the potential therefore to isolate the *HLA-DR* and *HLA-DQ* subregions from each other and from the non-CIITA regulated genes. As such we have termed these sites "MHC-II insulators." While the data do not distinguish between single and multiple interactions, the fact that CTCF can form homodimers suggests that only a single set of basal interactions for MHC-II insulators exists for any chromosome. However, each chromosome within a cell may have a different set of interactions. This latter concept would suggest that a population of MHC-II expressing cells would have multiple MHC-II chromatin structures.

The second set of interactions overlays the first and occurs in the transcriptionally active state (Figure 1-3). These interactions are dependent on CIITA binding at MHC-II promoters and likely include the aid of all of the factors described in the first part of this review. In this set, CIITA bound W-X-Y box DNA regions interact directly with CTCF bound MHC-II insulators. For example, CTCF bound at the *C1* site interacts with the *HLA-DRA* proximal promoter forming a second long-range chromatin loop (Figure 1-3). Depletion of CTCF by RNAi disrupts MHC-II insulator interactions and transcription of MHC-II genes. Each CIITA bound proximal promoter region can interact with different CTCF bound MHC-II insulator regions. Again, the data do not distinguish between promoters interacting with one or more MHC-II insulators simultaneously. However, to do so would likely require that multiple binding sites are available for each interaction. Because these interactions are CIITA dependent, they are induced by IFN-γ exposure of cells, which induces the expression of CIITA. Aside from overall distance, it is not clear at this point if there are preferred combinations for MHC-II promoter regions and MHC-II insulators. While the overall functions of the MHC-II insulators appear to be similar, it is not known if

some MHC-II insulators are more efficient at driving MHC-II transcription than others. If this were the case, then some MHC-II insulator-promoter interactions could result in different levels of mRNA transcribed.

Recently, cohesins have been found to be associated with 75% of genome wide CTCF sites in humans (Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008). Cohesin is a multisubunit-ringed complex that was initially thought to only function in the pairing of sister chromatids during cell division. With the finding of cohesin subunits in all stages of the cell cycle, and its association with CTCF, it has now been shown that cohesin is necessary for the enhancer blocking activity of CTCF (Wendt et al., 2008). Thus, it was not surprising to find that cohesin was associated with each of the MHC-II insulators. It will be interesting to determine if cohesin is also required for MHC-II transcription and/or to form the architecture that is now attributed to CTCF binding at the MHC-II insulator regions. In considering the ringed structure of cohesin, it is tempting to speculate that it will provide stability to either or both sets of interactions observed in this system.

These newly described sets of interactions and dependence on CTCF for MHC-II transcription was not an expected mechanism. This raises the important question of how these structures contribute to the regulation of this system. Because CTCF bound sequences are in highly acetylated chromatin, MHC-II insulators organize the chromatin into a steady-state architecture that is readily accessible to transcriptional regulatory machinery. This is consistent with the fact that active histone marks were reduced following CTCF depletion (Majumder et al., 2008). Alternatively, the CTCF-related interactions may stabilize the association of the histone modifying complexes. Another possibility is that the MHC-II insulators may cause the formation of a transcriptional hub/factory, a location that might be associated with high concentrations of transcription components and machinery (Sutherland

and Bickmore, 2009). The presence of MHC-II gene promoters in transcription factories would also have the advantage of allowing these genes to be expressed efficiently.

Conclusion

The proper regulation of MHC-II genes is fundamental to achieving adaptive immunity. The system is complex with numerous pathways that not only target the modification of the transcription factors specific to the system but also the chromatin modification machinery that is required for expression. Once elucidated the components and processes in these pathways represent targets to manipulate the expression of this system in a therapeutic setting. The newly introduced role of chromatin insulators and the potential dynamic movement of MHC-II loci to structures within the nucleus also provide new insights into how genes are regulated and what the necessary steps are to coordinate the expression of a multi-gene family and biological mechanism such as antigen presentation.

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Figure legends

Figure 1-1. MHC-II locus

Schematic of classical and non-classical MHC-II genes (black), MHC-II pseudo-genes (grey), and non-MHC-II genes (blue) together form a very gene dense locus on the short arm of human chromosome 6. The locus is punctuated with 10 CTCF binding sites (*C1-C16*; pink) including *XL9*.

Figure 1-2. Promoter proximal MHC-II regulation

Highly conserved W-X-Y box is bound by RFX, CREB, and NF-Y. They form a unique structure that is recognized by CIITA. CIITA recruits indicated transcriptional co-activators and their associated complexes that modulate the activity of the enhanceosome

proteins and modify nucleosomes (ac; acetylation, me; methylation) surrounding the nucleosome free region (NFR) to regulate transcription. 19S P: 19S proteasome regulatory complex.

Figure 1-3. MHC-II insulator long-range looping model

In the 'OFF state', MHC-insulators bound by CTCF (orange), *C1* and *XL9*, interact to form a chromatin loop and interaction focus. Cohesin (blue) likely encircles the interacting CTCF foci maintaining the integrity/stability of the structure or its function. When MHC-II genes are induced (ON), CIITA present at the promoters interacts with CTCF bound insulators forming a second series of interactions and sub loops. While this cartoon represents the HLA-DR subregion, additional interactions are possible with these elements (Majumder and Boss, 2010b).

Figure 1-1

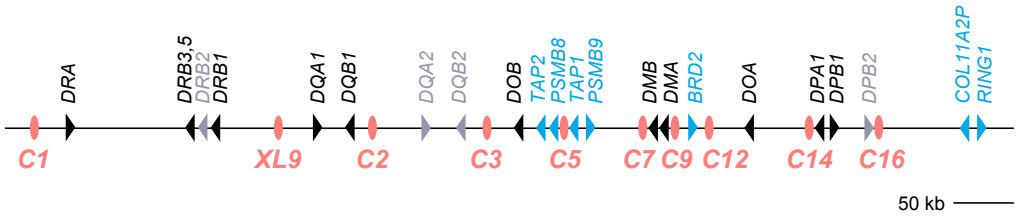


Figure 1-2

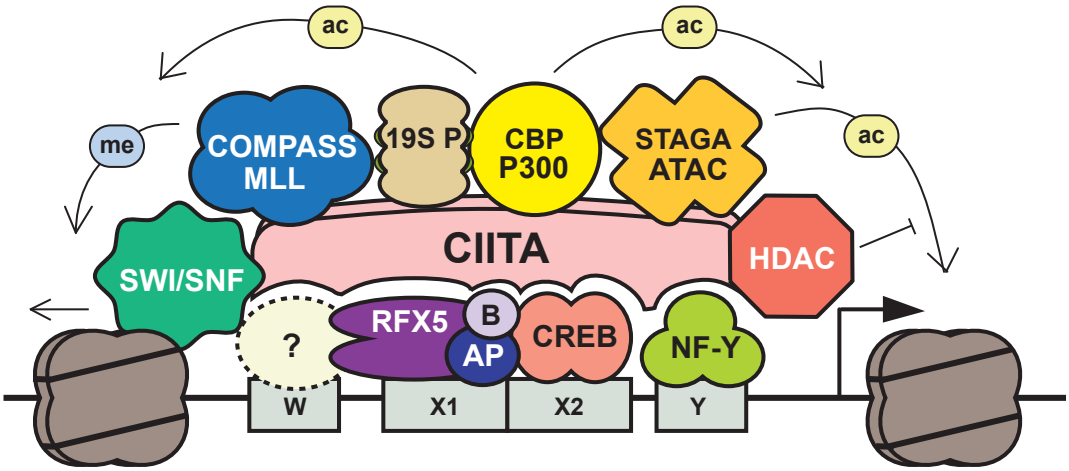
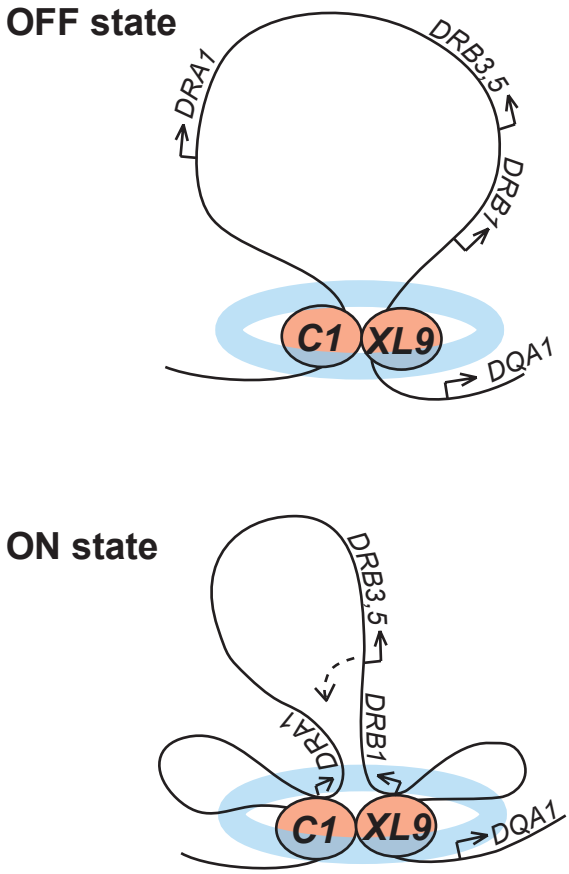


Figure 1-3


OFF state
DRA1
DRB3,5
DRB1
C1
XL9
DQA1
ON state
DRB3,5
DRB1
DRA1
C1
XL9
DQA1

Chapter 2. Multiple Histone Methyl and Acetyltransferase Complex Components Bind the HLA-DRA Gene

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Conceived and designed the experiments: JMB NMC. Performed the experiments: NMC. Analyzed the data: NMC JMB. Wrote the paper: NMC JMB.

Abstract

Major histocompatibility complex class II (MHC-II) genes are fundamental components that contribute to adaptive immune responses. While characterization of the chromatin features at the core promoter region of these genes has been studied, the scope of histone modifications and the modifying factors responsible for activation of these genes are less well defined. Using the MHC-II gene *HLA-DRA* as a model, the extent and distribution of major histone modifications associated with active expression were defined in interferon-γ induced epithelial cells, B cells, and B-cell mutants for *MHC-II* expression. With active transcription, nucleosome density around the proximal regulatory region was diminished and histone acetylation and methylation modifications were distributed throughout the gene in distinct patterns that were dependent on the modification examined. Irrespective of the location, the majority of these modifications were dependent on the binding of either the X-box binding factor RFX or the class II transactivator (CIITA) to the proximal regulatory region. Importantly, once established, the modifications were stable through multiple cell divisions after the activating stimulus was removed, suggesting that activation of this system resulted in an epigenetic state. A dual crosslinking chromatin immunoprecipitation method was used to detect histone modifying protein components that interacted across the gene. Components of the MLL methyltransferase and GCN5 acetyltransferase complexes were identified. Some MLL complex components were found to be CIITA independent, including MLL1, ASH2L and RbBP5. Likewise, GCN5 containing acetyltransferase complex components belonging to the ATAC and STAGA complexes were also identified. These results suggest that multiple complexes are either used or are assembled as the gene is activated for expression. Together the results define

and illustrate a complex network of histone modifying proteins and multisubunit complexes participating in *MHC-II* transcription.

Introduction

Antigen presentation is a paramount step in achieving adaptive immunity, where the major histocompatibility class II complex (MHC-II) proteins play a central role. The significance of MHC-II complexes is best illustrated in cases of bare lymphocyte syndrome (BLS) as patients that are unable to express MHC-II suffer from various bacterial and viral infections and usually do not survive beyond childhood (Reith and Mach, 2001). MHC-II proteins display antigenic peptides sampled from the endocytic compartments of the cell onto the cell surface; these peptides typically originate from extracellular pathogens but can include self, viral, or cancer-cell derived peptides. Recognition of MHC-II-peptide complexes by CD4 T cells triggers the expansion and differentiation of these T cells, leading to a host of antigen-specific immune responses (Rosenthal and Shevach, 1973). Proper expression of MHC-II proteins both spatially and temporally is critical, as aberrant expression can lead to an insufficient immune response or autoimmunity (Reith et al., 2005).

MHC-II genes are expressed constitutively in professional antigen presenting cells and thymic epithelial cells, and can also be induced in most other cell types following treatment with interferon-γ (IFN-γ) (Collins et al., 1984; Muhlethaler-Mottet et al., 1997). Cell-type dependent expression is largely controlled by regulation of a limiting transcription factor, the class II transactivator (CIITA) (Steimle et al., 1993). *MHC-II* genes share a highly conserved proximal upstream promoter region called the WXY box, where the factors RFXAP/B/5, CREB, and NF-Y bind directly, forming a scaffold that is recognized by

CIITA (Masternak et al., 2000; Moreno et al., 1999). This unique DNA-protein structure is collectively called the MHC-II enhanceosome (Gobin et al., 1998). The RFX proteins and CIITA are essential for *MHC-II* expression, as genetic deficiencies in these proteins leads to a MHC-II null phenotype and BLS (Reith and Mach, 2001). Located approximately 2.4 kb upstream of the transcription start site resides another WXY element that is fully functional. Initially described as a locus control element (Masternak et al., 2003) and later termed *XL4* for conserved homology with the WXY sequence (Gomez et al., 2005), *XL4* binds RFX and CIITA. Although the exact mechanism is unknown, it was proposed that *XL4* regulates *HLA-DRA* through a looping mechanism (Gomez et al., 2005). No other distal regulatory elements were reported to regulate *HLA-DRA*. CIITA also interacts with a multitude of coactivating factors and general transcription factors, which are recruited to the promoter to fine-tune the expression of *MHC-II* genes (reviewed in (Choi et al., 2010)). It has been previously shown by chromatin immunoprecipitation (ChIP) that multiple histone acetylation modifications and active methylation marks increased with constitutive and induced *MHC-II* expression at the proximal conserved promoter regions of some *MHC-II* genes, suggesting a role for these marks in regulation of this system.

Lysine acetylation modifications were the first histone modifications to be assigned an activating role in gene transcription, and were initially described to be localized at promoter regions, as well as enhancer and insulator elements of most genes (Allfrey et al., 1964; Heintzman et al., 2007). A well characterized histone lysine acetyltransferase (KAT) CREB-binding protein (CBP) is recruited to *MHC-II* genes through interactions with the N terminus of CIITA and possibly with phosphorylated CREB that is bound at the promoter (Fontes et al., 1999; Kretsovali et al., 1998; Lochamy et al., 2007). CBP and its homologue p300 are capable of acetylating all core histones but have a preference for histone H4K5 and

K8 when tested with mononucleosomes in vitro but can also acetylate (ac) histone H3K14 and K18 (Ogryzko et al., 1996; Schiltz et al., 1999). Another group of well-studied histone acetyltransferases of the GNAT family, PCAF (p300/CBP-associated factor) and GCN5 (general control nonderepressible 5), have a preference for histone H3 with specificity for H3K14 and H4K8 in vitro. PCAF and GCN5 have an expanded specificity for histone H3K9, K14, K18, K23, and K27 in vivo, depending on the complex of proteins with which they are incorporated (Grant et al., 1999; Kuo et al., 1996; Schiltz et al., 1999). GCN5 and PCAF are also recruited to the promoter of *MHC-II* genes (Spilianakis et al., 2003). PCAF has been shown to acetylate CIITA, altering its nuclear localization as a mechanism to regulate *MHC-II* transcription (Spilianakis et al., 2000). It has been shown previously that there is an accumulation of multiple acetylation marks on histone H3K9, K18, K27, and K14, as well as H4K5 and K8 in wild-type B cells and in non-myeloid cells with IFN-γ induction (Beresford and Boss, 2001; Rybtsova et al., 2007). The level of H3K18ac was affected when recruitment of CBP was reduced at the DRA promoter due to knockdown of the proteasome component Sug1 (Koues et al., 2008). However, the same treatments did not affect histone H3K9ac, suggesting a requirement for an independent (or redundant) KAT complex to catalyze this modification (Koues et al., 2008). While KATs may have the capability to acetylate diverse residues, they have a very restricted specificity in vivo, and by determining the various modifications that occur, it may be possible to identify the factors responsible for the modifications and their regulatory roles in transcription. Additionally, a number of complexes containing the same KATs have been described, with each complex likely having specificity for individual genes regulating distinct pathways. In the case of *MHC-II* genes, it is not known which complexes are actually bound.

Methylation of histones can cause varied outcomes depending on the modified residue and number of methyl groups (Kouzarides, 2007). The most well studied active transcription methylation marks are those of histone H3K4, where the three different levels of H3K4 methylation (me) have been shown to be associated with different regulatory functions and areas of the genome. In reference to the transcriptional start site (TSS), histone H3K4me3 (trimethylation) is associated with actively transcribed genes and focused to a narrow region immediately around the TSS. Histone H3K4me2 (dimethylation) has a broader deposition over the TSS that trails into the coding region and has been regarded as the 'open' chromatin mark, suggesting that the region is accessible but not necessarily transcribed. H3K4me1 (monomethylation) has attracted attention as the mark of enhancers and other regulatory elements, but is also found within the coding region of transcribed genes (Wang et al., 2009). Several groups have shown H3K4me2/3 (di and tri), H3R17me2, K36me2/3, and K79me2, all marks associated with transcriptionally active genes, to be induced with *HLA-DRA* expression, as well as loss of silencing marks H3K9me2/3 and H3K27me3 (Chou and Tomasi, 2008; Gialitakis et al., 2006; Gomez et al., 2005; Rybtsova et al., 2007; Zika et al., 2005).

The first identified H3K4 methylating enzyme was Set1 (SET domain containing 1) in yeast as a subunit of a multipartite complex called COMPASS (Complex Proteins Associated with Set1) (Briggs et al., 2001; Miller et al., 2001). Set1 and its human counterpart MLL1 (mixed myeloid leukemia) are both homologs of *Drosophila* trithorax, well known for its histone H3K4 methylating activity and role in the positive regulation of homeobox genes during development (Eissenberg and Shilatifard, 2010). Proteins of the MLL core complex have been shown to bind and promote H3K4 methylation at the *HLA-DRA* promoter (Koues et al., 2010), and intriguingly, interaction between MLL and PML

(promyelocytic leukemia) has been shown to prolong the dimethylation state of H3K4 and maintain transcriptional memory by localization to PML nuclear bodies (Gialitakis et al., 2010). Collectively, these data support the possibility of an MLL/COMPASS type histone lysine methyltransferase (KMT) complex having a role in *MHC-II* regulation.

While we have a basic understanding of some of the key promoter proximal histone modifications and some of the factors involved, we do not know the overall distribution of these modifications and factors, their stability or complexity, nor do we fully understand their dependence on CIITA. To further define the role of the major histone modifications associated with transcriptional activation to this gene, we assessed the levels of various modifications across the *HLA-DRA* MHC-II gene in constitutive and inducible cell systems. Using a dual crosslinking ChIP protocol to increase the radius of crosslinking and interaction capture, the observed histone modifications were correlated with the binding of specific coactivator/histone modifying complexes that are constitutively present or recruited to the locus upon induction with IFN-γ. The results showed that a multitude of factors, including those that make up the MLL complex and complexes containing GCN5 and PCAF, are involved at the *HLA-DRA* gene and are likely associated with regulating its expression.

Materials and Methods

Cell lines

The human Burkitt's lymphoma cell line, Raji, was purchased from the American Type Culture Collection (Manassas, VA) (Epstein and Barr, 1965), RJ2.2.5 cells are a CIITA-deficient cell line derived from Raji by γ-irradiation mutagenesis (Accolla, 1983; Steimle et al., 1993), and SJO cells are an RFX5-deficient cell line isolated from a BLS patient (Baxter-Lowe et al., 1989; Casper et al., 1990; Nocera et al., 1993). RJ2.2.5 and SJO are MHC-II

negative. SJO cells were generously provided by Dr. J. Gorski (Wisconsin Blood Center), and RJ2.2.5 cells were provided by Dr. R. Acolla (University of Insurbia, Italy). The above cell lines were cultured in RPMI supplemented with 5% fetal bovine serum, 5% bovine calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. A431, a human epithelial cell line, was obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. A431 cells were treated with 500 U/ml IFN-γ (PeproTech, Inc., Rocky Hill, NJ) for 24 hrs when indicated to induce the expression of *CIITA* and *HLA-DRA*. In some experiments, IFN-γ treated cells were washed with growth media and cultured in IFN-γ-free media for the indicated time.

qPCR and primers

For all real-time quantitative PCR reactions, Bio-Rad iCycler instruments (Bio-Rad Laboratories, Inc., Hercules, CA) with an iQ optical module were used to measure the amount of SYBR incorporated amplicons. DNA oligonucleotides used for primers listed in Table 1 were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and diluted to a final concentration of 100 nM for PCR reactions. All primers were tested by agarose gel electrophoresis to ensure that they formed single amplicon products of the correct size and optimized for Tm by temperature gradient real-time PCR followed by a melt curve analysis. Standard curves of sonicated genomic DNA were used to quantify the amount of starting material for every PCR reaction.

mRNA extraction and RT-PCR

IFN-γ treated and untreated A431 cells were grown until they were 80% confluent and RNA was prepared from cell pellets using the RNeasy kit (Qiagen, Venlo, Netherlands) according

to manufacturer's recommendations. 2 μg of total mRNA was used in reverse transcription reactions using Superscript II (Life Technologies Corp., Carlsbad, CA) with oligo dT and random hexamer primers (Life Technologies Corp., Carlsbad CA). Transcript specific primers (Table 1) were tested to produce single amplicon products prior to the qPCR reaction. Real-time PCR data were normalized to *GAPDH* mRNA expression using the ΔΔCt method (Livak and Schmittgen, 2001). All experiments were conducted at least three times from independent cell cultures and statistical significance was determined by Student's t-test.

Chromatin immunoprecipitation (ChIP)

For ChIP of histones, a conventional procedure was used as described (Beresford and Boss, 2001). For suspension cells, $4x10^7$ cells were crosslinked by 1% formaldehyde for 10 min. Adherent cells were plated the previous day onto two 15 cm plates such that they would be 80% confluent 16-24 hrs later when formaldehyde is applied. Following crosslinking, chromatin was sonicated until the majority of it was reduced to 200-600 bp fragments using the Bioruptor water bath sonicator (Diagenode, Denville, NJ) and the amount of chromatin DNA was measured using the Bio-Rad VersaFluor Fluorometer (Bio-Rad Laboratories, Inc., Hercules, CA). 10 μg of chromatin and 2.5 μg of antibodies were used per ChIP assay. 10% of each IP was used for each subsequent PCR reaction. Antisera were purchased from various manufactures as follows: anti-H3, H3K18ac, H3K4me1 (Abcam plc., Cambridge, United Kingdom), H3K9ac, H3K27ac, H4K16ac, H3K4me2, H3K4me3 (Millipore Corporation, Billerica, MA). A polyclonal anti-T cell receptor (TCR) antibody was used as a non-specific negative control antibody in the histone ChIP experiments. All data were presented as the percent of the input chromatin. Figures 2-1 and 2-2 were presented as

unnormalized data, whereas the same data presented in Supplemental Figures 2-S1 and 2-S2 were normalized to the levels of unmodified histone H3 at each amplicon.

For non-histone proteins, a secondary crosslinker was used in addition to formaldehyde. Disuccinimidyl glutarate (Nowak et al., 2005) (DSG, Proteochem, Denver, CO) was applied to cells at 2 mM in Sodium phosphate buffer (pH 8.0) for indicated times at room temperature. 1% formaldehyde was added to cells for the last 10 min of crosslinking with the DSG. All successive steps were identical to conventional ChIP. Except for the time course experiment in Figure 2-3A, all DSG treatments were carried out for 20 minutes. 30 μg of chromatin was immunoprecipitated with 5 μg of anti-CBP, p300, GCN5, PCAF, MLL1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ASH2L, RbBP5 (Bethyl Laboratories, Inc., Montgomery, TX), ATAC2, YEATS2, NC2-β, TRRAP, TADA1L, WDR5 (Abcam plc., Cambridge, United Kingdom), and DPY-30 (Abnova, Taipei, Taiwan). Anti-CIITA antibodies were prepared as previously described (Brown et al., 1998). ADA2a and ADA2b antibodies were a generous gift from Dr. R.G. Roeder at The Rockefeller University (Gamper et al., 2009). Unimmunized control rabbit IgG (Millipore Corporation, Billerica, MA) was used as a non-specific negative control antibody for all transcription factor/complex ChIP assays. The mean and standard error from these assays were provided as percent input of the chromatin added. For all ChIP assays, the Student's t-test was applied to determine statistical significance and all experiments were repeated at least three times with separate cell culture preparations.

siRNA treatment and immunoblotting

Raji cells were split the previous day to allow the cells to grow in log phase and collected the next day for nucleofection. ON-TARGET plus SMARTpool siRNA for non-targeting

control, GCN5, MLL1, and WDR5 were purchased from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA) and resuspended in nuclease free water. Using the Amaxa Nucleofection kit V (Lonza, Basel, Switzerland) $1x10^6$ cells were transfected with each indicated pool of siRNA [100 nM] and cultured for 72 hrs. These siRNA transfected cells were either collected for immunoblot, RNA analysis, or ChIP. For immunoblotting, cells were washed with PBS and lysed with RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) to generate whole cell lysates. Protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA) and equal amounts of lysate protein were resolved on a polyacrylamide gel and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk dissolved in TBS-Tween20 (0.01%) solution then probed with each indicated antiserum. Antibodies to beta-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to portray sample loading. HRP conjugated anti-mouse and anti-goat were from Sigma-Aldrich Co. (St. Louis, MO), and anti-rabbit was from Rockland Immunochemicals Inc. (Gilbertsville, PA), respectively. Chemiluminescence signal was recorded on BioMax XAR film (Kodak, Rochester, NY) and band intensities were measured using ImageQuant TL program (GE healthcare, Piscataway, NJ).

RESULTS

IFN-γ treatment leads to increased active histone marks at the *HLA-DRA* locus

Histone modifications and the factors that place these marks are responsible for the regulation of the chromatin state and may influence the expression of a gene. Identification of potential factor candidates can be obtained from an understanding of the presence and

distribution of the local chromatin modifications across the locus. To generate this information, a series of ChIP assays were conducted on cells that were induced for *MHC-II* gene expression, as well as those that constitutively expressed the genes. The *HLA-DRA* gene was used as the model *MHC-II* gene because it is monomorphic and expressed at high levels. The A431 epithelial cell line is negative for both *CIITA* and *MHC-II* gene expression. Treatment with IFN-γ induced expression of both *CIITA* and *HLA-DRA* genes ~300 and ~5,000 fold, respectively (Figure 2-1A).

To assess the distribution of histone modifications across the *HLA-DRA* locus, a set of amplicons were designed along the length of the *HLA-DRA* gene stretching from -2,500 to +3,500 bp (Figure 2-1B), which included the upstream regulatory element (here termed *XLA*) (Gomez et al., 2005; Masternak et al., 2003), to a region far downstream of the TSS. Amplicons for -300 and the major regulatory region, WXY box, are immediately adjacent to each other (Figure 2-1B). The -300 amplicon is where the first nucleosome 5' of the nucleosome free region has been reported to be positioned (Leimgruber et al., 2009). ChIP analyses of A431 cells (-/+ IFN-γ) were performed using anti-CIITA and various histone posttranslational modification specific antibodies that are associated with active transcription (Figure 2-1C). CIITA occupancy appeared only after IFN-γ induction and showed a sharp peak at the WXY and -300 regions, which confirms that it was indeed recruited to the *HLA-DRA* gene (Beresford and Boss, 2001; Masternak et al., 2000) in our assay, and also that the resolution of the experiment was ~300 bp. Analysis of unmodified histone H3 across the region showed minor differences across the gene in the uninduced state. However, following IFN-γ induction the distribution of nucleosomes was reduced at the WXY region compared to an upstream region (-600) as previously described (Leimgruber et al., 2009), suggesting that this region is more accessible in the active state.

Histone lysine acetylation marks were examined for H3K9, K18, K27, and H4 acetylation marks K8 and K16 (Figure 2-1C). In the uninduced state, very low levels of these modifications were observed, irrespective of the position across the gene. Following IFN-γ induction, a broad distribution of the acetylation marks appeared. Histone H3K18 and K27 acetylation showed relatively even distributions around the TSS and WXY box regions; whereas H3K9 and H4K8 displayed different patterns with H4K8 acetylation levels being higher upstream and H3K9 acetylation levels peaking at regions downstream from the TSS. Histone acetylation levels of H3K14, K23, and H4K5, K12, and K16 were also measured following IFN-γ treatment. Very low to no levels of these modifications were detected at the control or induced time point (data not shown) as represented by H4K16ac in Figure 2-1C. Thus these data point to H3K9 and H3K18 as the dominant H3, and H4K8 as the dominant H4 acetylation modifications associated with IFN-γ induced *HLA-DRA* gene expression. The levels of histone modifications in Figure 2-1C are plotted irrespective of histone H3 concentrations, whereas Supplemental Figure 2-S1 shows the same data normalized to the levels of histone H3 ChIP at each amplicon. Only slight differences in distribution over the WXY and -300 amplicons can be observed in comparing the two figures; which reflect the change in nucleosome density following IFN-γ treatment.

As H3K4 methylation is associated with active transcription, the three degrees of methylation were observed following IFN-γ treatment. H3K4me1 levels were significantly induced at all regions except the WXY box and +300. Surprisingly, the -600 amplicon showed high levels of the H3K4me1 modification. There is no reported regulatory activity for this region at this time. The finding of this modification may suggest a novel regulatory element at this location. The mark for histone H3K4me2 showed a broad distribution, but was clearly shifted downstream from the TSS. H3K4me3, a mark of active transcription was

more sharply positioned around the TSS with a peak downstream of the TSS. Significant levels of H3K4me1 and me2 modifications were also found at the upstream *XL4* element with IFN-γ induction. It is important to point out that the baseline levels of all the above modifications are typically very low, suggesting that CIITA occupancy is required for these modifications.

Active histone marks are CIITA and RFX5 dependent in B cells at *HLA-DRA*

To determine if a constitutively expressing system would have a similar histone modification distribution, a commonly used Burkitt's lymphoma B cell line, Raji, was examined. Raji cells express high levels of *CIITA* and all *MHC-II* genes, including *HLA-DRA*. To determine whether the above histone modifications were dependent on the presence of CIITA or RFX5, ChIP assays using CIITA- (RJ2.2.5) and RFX5- (SJO) deficient cell lines were compared to Raji. RJ2.2.5 cells were derived from Raji cells by γ-irradiation and SJO cells were B cells established from a bare lymphocyte syndrome patient (Accolla, 1983; Steimle et al., 1993). The region of analysis was expanded to +5800 with an additional amplicon at -2000. This analysis encompasses the entire open reading frame of *HLA-DRA* (Figure 2-2A).

In Raji cells, a high and tight peak of CIITA binding was observed at the WXY region as expected (Figure 2-2B, (Beresford and Boss, 2001)). Nucleosome density measured by histone H3 presence was variable between the three cells lines. In Raji cells, there is a clear reduction in nucleosomes at the -300/WXY region compared to its surrounding areas; whereas the nucleosome density in RJ2.2.5 or SJO cells did not show this preferential depletion at the proximal promoter region (Figure 2-2B). This agrees with the

IFN-γ induction data presented above, suggesting that only in the active state does the chromatin structure of the proximal promoter region become accessible.

The presence of the activation marks observed above was examined in the three cell lines. Universally, all four acetylation modifications were lower or absent in RJ2.2.5 and SJO cells compared to Raji. As RJ2.2.5 cells do not make a functional CIITA, and SJO cells do not bind CIITA due to a lack of RFX binding, these data demonstrate that CIITA is required for these modifications. In Raji cells, histone H3K9 and K18 acetylation values were extremely high and were broadly distributed extending upstream of the gene and only slightly into the open reading frame. H3K27 and H4K8 levels were also high and showed a similar distribution. In contrast to the IFN-γ pattern, *XL4* showed higher levels of the histone H3 acetylation marks in B cells, with the exception of H4K8ac, which had shown a relatively high level following induction by IFN-γ. As above, Figure 2-2 is plotted irrespective of the nucleosome density; whereas Supplemental Figure 2-S2 is plotted with respect to the histone H3 levels for each amplicon. While there are no major differences, histone modifications associated with the WXY and -300 regions are increased in Raji cells when normalized to the lower levels of nucleosomes over those sequences.

Histone H3K4 methylation showed distinct distributions among the wild-type and mutant B-cell lines. Monomethylation was strongest at the upstream *XL4* site and the surrounding region in all cell lines, which is indicative of this region functioning as an enhancer-like element (Heintzman et al., 2007). However, in Raji cells, monomethylation was markedly decreased at regions approaching the TSS, disappearing at +300, and reappearing at +1500 bp downstream of the TSS. In contrast monomethylation was higher at the +300 region in RJ2.2.5 and SJO cells and positioned evenly throughout the -600 to +600 regions. No pronounced peak at -600 was observed in any of the cells. Histone

H3K4me2 was broadly distributed in Raji cells and lower at all regions in RJ2.2.5 and SJO cells. The levels of H3K4me2 in SJO cells were significantly lower than RJ2.2.5 at the WXY box, suggesting that the lack of RFX binding to the region may be responsible for this additional loss of this histone modification. Histone H3K4me3, as expected showed sharp and high levels at regions close to the TSS in Raji cells, with its highest level at +600. In RJ2.2.5, low levels of H3K4me3 were observed close to the TSS. In contrast, no H3K4me3 was observed in SJO cells, again pointing to the role of RFX5 in assembly of the factors at the WXY box region (Dou et al., 2005).

Dual crosslinking ChIP increases pulldown efficiency

The spacer length of formaldehyde is limited to 2 Å, a distance that allows for the ChIP assay to be optimal for DNA binding proteins or their tightly interacting protein partners (Jackson, 1978). However, detection of coactivators, chromatin modifiers, or factors that interact at a greater distance from the DNA may not be as efficient under the standard conditions. To enhance the pulldown efficiency of our ChIP assays for indirect DNA-protein interactions, a dual crosslinking ChIP procedure was optimized using disuccinimidyl glutarate (DSG), which has a crosslinking arm of 7.7 Å (Nowak et al., 2005). Using this dual crosslinking assay, detection of CBP, a known coactivator that interacts with CIITA and binds to the promoter (Kretsovali et al., 1998; Zika et al., 2005), was significantly increased at the WXY box (Figure 2-3A). The increase in CBP detection was time dependent, and importantly, CBP binding was absent at a negative control region (-2,000) even at the longest time treatment of DSG. This demonstrates that there is no increase in non-specific binding due to the additional crosslinking step, while increasing the pulldown efficiency more than 9 and 11-fold when cells were DSG crosslinked for 20 or 30 min, respectively, compared to

cells only crosslinked with formaldehyde. Therefore, the DSG dual crosslinking procedure was employed in subsequent ChIP assays to examine coactivator proteins interactions with the *HLA-DRA* gene.

Additional controls were carried out across the region to assure that backgrounds levels were not increased at each amplicon, and previously reported coactivators could be captured (Figure 2-3B). Using all three cell lines, CIITA, RFX5, CBP, p300, and control IgG antisera were used in the dual crosslinking ChIP assays. The results showed a slight broadening of CIITA and RFX5 binding at the WXY/-300 regions. RJ2.2.5 showed RFX5 binding as before (Masternak et al., 2003) and SJO did not bind either factor. The dual crosslinking procedure did however produce higher levels of these factors at *XL4*. Control IgG levels were low at each amplicon. CBP and p300 binding was also captured at the WXY through -600 amplicons in Raji cells but not in RJ2.2.5 or SJO suggesting that their recruitment was dependent on CIITA. This role of CIITA recruiting these factors to the promoter region was reported previously (Fontes et al., 1999; Kretsovali et al., 1998; Spilianakis et al., 2003; Zika et al., 2005). However, significant recruitment of CBP and p300 were found at *XL4* in all three cell lines, suggesting that their recruitment to this region is independent of RFX5 and CIITA.

The MLL histone methyltransferase complex components are enriched at surrounding regions

The MLL histone methyltransferase core complex consists of MLL, WDR5, ASH2L, RbBP5, and DPY-30 (Dou et al., 2005). Together these proteins catalyze the addition of a methyl group to H3K4, producing mono-, di-, and trimethylated H3K4 in vitro and in vivo (Dou et al., 2005; Patel et al., 2009). As there were high levels of all three methylation states

observed at different regions across the *HLA-DRA* gene, ChIP assays were conducted on Raji, RJ2.2.5, and SJO chromatin preparations to determine if these MLL complex core proteins were present (Figure 2-4). It was expected that, because these factors are part of a core complex that binding to the *HLA-DRA* locus, would simply reflect the transcriptional activation state of the locus. This was not the case. Whereas all five proteins were bound across the locus in Raji cells, MLL1, ASH2L, and RbBP5 were mostly dependent on the presence of RFX5, while showing slight dependency on CIITA. DPY-30 was unique in that its binding was completely RFX5 and CIITA dependent as binding was not detected in either SJO or RJ2.2.5. WDR5 recruitment was completely independent of RFX5 or CIITA due to the fact that its level of occupancy was unchanged between the three cell types. RFX and CIITA independent binding of some of these subunits may explain why lower but significant levels of H3K4me2 were observed in RJ2.2.5 cells when compared to Raji cells.

IFN-γ induced A431 cells were also tested to determine the binding of the above factors. CIITA and RFX5 ChIPs were conducted initially to test the efficiency of dual crosslinking pulldown in the IFN-γ induced cells. CIITA binding was present only in the IFN-γ treated cells. The levels of pulldown achieved in the inducible system was ~4 fold lower than in B cells at the WXY box (Figure 2-5A). While there are low levels of RFX5 binding in the resting A431 cells, they are induced more than 2 fold with IFN-γ treatment. This has been observed previously, and is due to stabilization of the DNA binding components by CIITA (Reith et al., 1994a; Reith et al., 1994b). Therefore, an increased level of coactivator binding can be due to both the increased recruitment of RFX or CIITA. In uninduced cells, only WDR5 displayed significant binding above the background IgG control (Figure 2-5B). Following IFN-γ treatment, MLL1, WDR5, ASH2L and RbBP5 were associated with sequences surrounding the TSS and at *XL4*. Surprisingly, DPY-30 was not

observed at the locus under any of the conditions tested. Together, these results suggest that the MLL histone methyltransferase components are being recruited to the *HLA-DRA* locus most significantly at the promoter, but also within the body of the gene and regulatory regions.

Histone acetyltransferase complexes containing GCN5 and PCAF are present throughout the coding region and at upstream regulatory elements

The lysine acetyltransferases (KAT) CBP and GCN5 have been shown previously to bind the *HLA-DRA* promoter (Koues et al., 2008; Spilianakis et al., 2003; Zika et al., 2005). CBP, p300, and PCAF have been shown to interact directly with CIITA (Fontes et al., 1999; Kretsovali et al., 1998; Sisk et al., 2000; Spilianakis et al., 2000). To examine the breadth at which these KATs interact across the locus and to correlate that with the histone acetylation data, ChIP assays for these factors were carried out in the three B cell lines described above. Thus, to determine whether CIITA or RFX5 was indeed important in recruiting each of these factors to the *HLA-DRA* locus the binding of the above KATs in Raji, RJ2.2.5, and SJO cells were compared. There were no major differences in the protein expression levels of these KATs between the three cell lines as determined by immunoblot (Supplemental Figure 2-S3). At the WXY box region, the presence of CIITA was critical for CBP, p300, GCN5, and PCAF (Figures 2-3B and 2-6A), illustrating the role CIITA plays in the recruitment of these KATs to the locus. The binding of these KATs to *XL4* was also CIITA dependent. In agreement with the histone acetylation data (Figure 2-2B), a high level of GCN5 and PCAF were observed at the -600 region. Moderate levels of GCN5 and PCAF, but not CBP and p300, were associated within the coding region.

Recent studies in humans have defined two distinct GCN5/PCAF containing complexes, termed STAGA and ATAC that diverged from their shared common ancestor, the yeast SAGA complex (Guelman et al., 2009; Wang et al., 2008). To determine which of these complexes might participate in *HLA-DRA* gene expression, ChIP assays for a number of key subunits of each complex were conducted (Figure 2-6B). For the ATAC complex, ADA2a, ATAC2, YEATS2, and NC2-β were examined. The overall binding levels of these components were low and may be due to antibody affinity. The binding of ADA2a and YEATS2 displayed some level of binding across the locus in Raji and RJ2.2.5 cells, suggesting that their recruitment was dependent on RFX5 and CIITA. ATAC2 displayed statistically significant binding in all three cell types, suggesting that its recruitment was independent of RFX5 and CIITA. Binding of NC2-β was not detected for any of the regions. For STAGA complexes, ADA2b, TRRAP, and TADA1L were examined. ADA2b showed no statistical significance over background IgG in binding in any of the cell types (Figure 2-6B). TRRAP and TADA1L were bound in all three cell types, with TRRAP displaying significantly higher levels in Raji over RJ2.2.5 at several of the loci examined. Thus, while the presence of ADA2a versus ADA2b would suggest that the ATAC complex and not STAGA was bound, components of each of these complexes could be found associated with the *HLA-DRA* gene.

A similar analysis for the IFN-γ treated A431 cells was also conducted and is presented in Supplemental Figure 2-S4. Albeit at lower levels, GCN5 was found at significant levels from -600 through +3500 over background in both untreated and IFN-γ treated cells. A statistically significant increase in GCN5 was observed surrounding the promoter region (-300 through +300). Of the other factors examined, only ATAC2,

TRRAP, and TADA1L showed low but statistically significant binding and this binding was not dependent on IFN-γ.

siRNA knockdowns of GCN5 and MLL do not affect transcription or histone modification levels

To examine whether GCN5 or MLL play a non-redundant and critical role in the regulation of *HLA-DRA* gene expression, siRNA knockdowns of these proteins and WDR5 were conducted in Raji cells. Compared to a control non-targeting Dharmacon SMARTpool, siRNA SMARTpools to GCN5, MLL1, and WDR5 were able to reduce the levels of their respective proteins between 50 and 80% (Figure 2-7A). Analysis of *CIITA* and *HLA*-DRA gene mRNA levels at 3 days post transfection (Figure 2-7B) or 5 days post transfection with a second siRNA transfection at 48 hrs after the initial transfection (data not shown) showed no change in expression of either gene. Consistent with this result was the finding that histone H3K9ac, H3K18ac, H4K8ac, H3K4me2, H3K4me3 levels were not altered and CIITA binding was not affected (Figure 2-7C). A similar set of experiments conducted with A431 fibroblasts -/+ IFN-γ treatment and the siRNAs also did not provide a clear dependency on these factors for expression (data not shown). Thus, although these factors are readily detectable at the *HLA-DRA* gene, their roles are not essential to expression, there are functionally redundant factors, or once the gene is activated, the transcriptional complexes are stable.

Histone modifications are stably present after removal of IFN-γ

To address the possibility that once induced, the histone modifications associated with *HLA-DRA* expression are stable, A431 cells were treated with IFN-γ for 24 hrs as in

above experiments, then washed and supplied with culture media free of IFN-γ and cultured for an additional 24, 48, or 72 hrs. During the course of these experiments the cells double at least once per day. Within 24 hrs of IFN-γ removal, *CIITA* mRNA levels were substantially lower in these cells, whereas *HLA-DRA* mRNA levels did not significantly change up to 72 hrs (Figure 2-8A). CIITA binding at the *HLA-DRA* promoter was assessed by ChIP to determine whether IFN-γ removal also diminished recruitment of CIITA (Figure 2-8B). After removal of IFN-γ, CIITA levels at the promoter gradually decreased to ~49% of the initial induction. When histone modification levels were examined, none of the modifications tested showed a significant reduction up to the 72 hr time point. An additional set of experiments was carried out for 5 days after IFN-γ removal. At this time point, *CIITA* and *HLA-DRA* mRNA levels had fallen to ~7 and 400 fold over pre-stimulation levels, respectively. CIITA binding to the *HLA-DRA* WXY region was reduced by ~68%. Intriguingly, the histone modifications were for the most part unchanged even at these later time points (Figure 2-8B). These results illustrate the stability of CIITA binding at the *MHC-II* promoters, as well as the stability of the histone modifications.

Discussion

MHC-II genes, as a fundamental constituent of adaptive immunity, are highly regulated at the transcriptional level. While many essential cis-regulatory elements and transcription factors have been determined, only a few coactivating transcription factors have been identified. In this report, our efforts were focused on determining the location and distribution of active histone modifications associated with *MHC-II* gene transcription, and on identifying histone modifying coactivating factors that were recruited to the *HLA-DRA* gene, which served as a model MHC-II gene. Along with histone modifications, the relative

nucleosome density was examined across the gene. For the most part, the density was evenly distributed in cells that were not expressing *HLA-DRA*. However, when *HLA-DRA* was expressed, there was a notable decrease in the nucleosome density immediately upstream of the TSS. This decrease was not seen in either the CIITA- or RFX5-deficient cell lines or in control fibroblasts. This suggests that CIITA recruitment was necessary for altering the nucleosome density, and as discussed below, many of the other chromatin modulating events at the locus. The decrease in nucleosome density may maintain an accessible chromatin environment close to the TSS to allow efficient assembly of RNA polymerase components and subsequent transcription initiation.

With activation of transcription, histone modifications associated with active gene expression were present to varying degrees and patterns across the *HLA-DRA* locus. While the distribution patterns of each mark were largely similar between the IFN-γ induced cells and B cells, minor differences were observed. The difference in ChIP assay levels in general may reflect the difference between newly applied histone modifications as in the case of IFN-γ treated A431 cells, and constitutive levels of the same histone modifications in the B cells. The levels of histone modification observed in IFN-γ treated cells imply that histone modifying factors were newly recruited, whereas in both wild-type and mutant B cell lines, regulatory factors had the opportunity to establish a steady state of modifications by constant new application and removal of the marks. The dissimilarities of modification patterns were mostly at the *XL4* region, where higher levels of several modifications were observed in B cells compared to IFN-γ induced cells. H4K8ac was an exception to this as it was higher at *XL4* in the IFN-γ induced cells. These observations may reflect the potential for differential use of *XL4* as a regulatory element in the different cell types and conditions. The data collected here suggest that *XL4* is likely more important for B-cell specific than for

the IFN-γ induced expression. Alternatively, the use of *XL4* may correlate with higher levels of *HLA-DRA* transcription in Raji cells.

The three histone H3K4 methylation modifications had markedly distinct distribution patterns across the *HLA-DRA* gene. H3K4me3 was highly enriched in a focused region surrounding and just downstream of the TSS in both IFN-γ induced cells and B cells. This is an expected result as most active genes have a biphasic peak surrounding the TSS (Barski et al., 2007). Although diminished significantly, H3K4me3 was present in the CIITA-deficient cell line but not the RFX5-deficient line. Because the RFX-CREB-NF-Y ternary complex is assembled in RJ2.2.5 cells (Villard et al., 1999), the data suggest that these factors can at some level recruit the necessary KMT to write this mark close to the TSS. A similar observation was made for H3K4me2 as well. The H3K4me1 modification was highest in both CIITA- and RFX5-deficient cells, indicating that the factors bound in these cells are capable of recruiting the KMT responsible for this modification. Thus, in the most basal state, H3K4me1 is placed at the TSS region, and the assembly of the RFX-CREB-NF-Y factors and subsequent CIITA recruitment results in additional methylation of H3K4. The finding of any modifications in SJO (RFX5-deficient) was unexpected as previous reports had shown that in the absence of a functional RFX, the other DNA binding factors (CREB and NF-Y) do not assemble (Kara and Glimcher, 1991). However, the finding of the histone modifications in SJO cells could represent transient binding of NF-Y or CREB to these sites with the subsequent recruitment of KAT/KMTs and establishment of the observed histone modifications.

Intriguingly, in the *HLA-DRA* expressing B cells, there was an enrichment of H3K4me1 at +1500 similar to the levels observed at *XL4* in all three B cell lines. While this mark is known to be present within the body of genes (Heintzman et al., 2007), the +1500

did have significantly more of this mark than surrounding areas, suggesting the possibility of a novel regulatory region within the vicinity. Twenty-four years ago, a paper reported that this region of the *HLA-DRA* intron had tissue specific enhancer activity (Wang et al., 1987). This modification may reflect that activity. It is intriguing that the modification was not observed in the CIITA- or RFX5-deficient cells, suggesting that it is CIITA dependent. Similarly, in the IFN-γ induced fibroblasts H3K4me1 was enriched at -600 compared to the other sequences. This region has no known function at this time, but like the +1500 region, could represent a novel control element for this gene.

The addition of a secondary protein crosslinker to the standard ChIP assay allowed the identification and demonstration of the clear association of coactivators associated with the *HLA-DRA* gene. The KATs CBP, p300, GCN5, and PCAF have been previously described to associate with CIITA in cell lysates (Kretsovali et al., 1998; Spilianakis et al., 2003; Spilianakis et al., 2000), and CBP and GCN5 have been shown by ChIP to be bound to *HLA-DRA* WXY box regions in *MHC-II* expressing cells (Gialitakis et al., 2006; Zika et al., 2005). Here, each of these factors was shown to be recruited to the *HLA-DRA* WXY box region in a CIITA- and RFX5-dependent manner. Of the histone acetylation modifications observed, H3K9ac and H3K18ac were the most prominent, and these marks have been strongly associated with the KAT activities of GCN5/PCAF and CBP/p300, respectively (Horwitz et al., 2008; Jin et al., 2010; Kasper et al., 2010; Zsindely et al., 2009), and are likely responsible for placing these marks on *HLA-DRA*.

In B cells, there were significantly higher levels of enrichment within the body of the gene for GCN5 and PCAF, when compared to CBP and p300. GCN5 has been shown to bind within the body of genes and has been implicated in transcriptional elongation (Govind et al., 2007; Johnsson et al., 2009). Inducible genes can be heavily regulated at the elongation

step and GCN5 may be regulating transcription by acting as a gatekeeper for elongation under the appropriate signals. GCN5 and PCAF are also capable of acetylating transcription factors. PCAF was shown to acetylate CIITA and regulate its nuclear localization (Spilianakis et al., 2000). These reports support the notion that while these KATs are globally recruited to actively transcribed genes, they use different and/or multiple mechanisms to activate genes.

GCN5, the first KAT identified to be involved in transcriptional activation, functions as part of the well-characterized yeast SAGA complex (Brownell and Allis, 1995). In vertebrates PCAF shares 75% homology with GCN5 and both have somewhat redundant functions (Xu et al., 1998). Recent work in metazoans has further divided the SAGA complexes into STAGA and ATAC, which share a common core of GCN5/PCAF, ADA3, STAF36, and a homolog of the yeast ADA2, being either ADA2a in ATAC complexes, or ADA2b in STAGA complexes (Wang et al., 2008). STAGA and ATAC contain other unique subunits that allow them to function at separate target genes largely in a mutually exclusive manner (Krebs et al., 2011). While the results presented here showed enrichment for ADA2a, a component of the ATAC complex, but not ADA2b of the STAGA complex at *HLA-DRA* promoter and *XL4*, other components of the STAGA complex were present. The results presented here suggest other complexes for GCN5/PCAF may exist or that the components assemble independently of the larger complexes depending on the unique activating transcription factors available at each gene.

In sharp contrast to the other coactivators, the binding of WDR5 in B cells was completely independent of CIITA and RFX5. This suggests that WDR5 may be recruited to the region by the WXY box factors prior to CIITA and acts as a docking site for its partner histone modifying proteins. Although the other MLL complex proteins, MLL1, ASH2L,

RbBP5, and DPY-30 bound to varying levels in the absence of CIITA, their occupancy increased when CIITA was bound. This suggests that WDR5 is recruited through multiple mechanisms and potentially multiple complexes. WDR5 is reported to be present in both the MLL and ATAC complexes (Dou et al., 2005; Wang et al., 2008). A recent publication showed the NF-Y complex was able to recruit ASH2L to CCAAT containing promoters (Fossati et al., 2011). As NF-Y proteins are also part of the MHC-II promoter-binding complex, this could partially explain the CIITA-independent binding of MLL complex components. Also, a WDR5, ASH2L, and RbBP5 complex was shown to catalyze H3K4 mono- and di- methylation activity independent of MLL (Patel et al., 2011). By applying a basal level of H3K4 methylation independent of CIITA, these proteins may function to increase the accessibility of the chromatin at these sites, which can be modified further with the recruitment of MLL and other coactivators.

siRNA knockdown of GCN5, MLL1, and WDR5 could not provide evidence that these factors were essential to *HLA-DRA* gene expression. While the data could be interpreted that the components are not required or are redundant, another interpretation is also possible. This possibility involves the programming of this gene and the epigenetic stability of the histone modifications that were placed following activation. When IFN-γ was removed from the inducible system, the level of *HLA-DRA* was unaltered despite the loss of *CIITA* transcription. This may be partly due to the stability of the *HLA-DRA* mRNA itself. However, the data suggest that this is more likely a consequence of a number of events, including the presence of active histone modifications that are stably associated with the promoter. Albeit reduced, CIITA was also readily detectable at three and five days post IFN-γ removal from the system. Thus, there is an inherent stability of CIITA binding and the histone modifications associated with the locus once it is activated.

In summary, the results presented here illustrate the complexity in activating the *HLA-DRA* gene and showed that multiple KATs and KMTs are involved in the process. For histone acetylation, it is clear that CIITA is critically required for the placement of these marks. Even with this being the case, some of the factors can be recruited to the gene in the absence of CIITA, implying that their recruitment may not in its self be sufficient for them to catalyze these modifications. For histone methylation, CIITA-dependent and independent recruitment of factors occurs. However, some of these factors are active in the absence of CIITA, creating a state in which this region is open and accessible. Thus, their role may be to maintain a constitutively accessible state, such that these important adaptive immune response set of genes can be induced rapidly in response to infections.

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Figure Legends

Figure 2-1. IFN-γ treatment induced the deposition of active histone modifications throughout the *HLA-DRA* gene.

(A) RNA levels for *CIITA* and *HLA-DRA* were measured by qRT-PCR. Transcript levels for A431 cells are shown before and after treatment with 500U/ml of IFN-γ for 24 hrs.

The results of three independent experiments were averaged and plotted with standard error. (B) A schematic of the *HLA-DRA* gene and open reading frame is illustrated with exons (black boxes) and introns (clear) indicated. The bars below the gene represent the relative position of the PCR amplicons used. (C) ChIP and qPCR were conducted on untreated control (clear) or 24 hrs IFN-γ treated (black) A431 cells using the indicated antisera and amplicons described in B. The data are presented as an average of the percent input value derived from three to four biological replicates and error bars represent standard error. For unmodified histone H3, the asterisk (*) indicates a Student's t-test value of $p<0.05$ in comparing an upstream region (-600) and the WXY box region with IFN-γ treatment.

Figure 2-2. *MHC-II* expressing B cells have active histone modifications distributed across the *HLA-DRA* gene.

(A) The *HLA-DRA* gene region and additional PCR amplicons (-2000 and +5800) used in this and subsequent experiments are depicted. (B) Using the indicated antibodies, the distribution of unmodified H3, CIITA, and modified histones at the *HLA-DRA* gene was analyzed by ChIP-qPCR for the amplicons described in A in *MHC-II* expressing (Raji, black) and non-expressing (RJ2.2.5, CIITA-deficient, grey; SJO, RFX5-deficient, clear) B cell lines. The data are plotted as the average of the percent input values from three biological replicates and error bars represent standard error. In the unmodified histone H3 panel, an asterisk (*) indicates a Student's t-test value of $p<0.05$ comparing the -600 and -300/WXY region, and double asterisks (**) indicate a t-test value of $p<0.01$ between +300 and -300/WXY in Raji cells.

Figure 2-3. Dual crosslinking enhances the pulldown efficiency of coactivators CBP and p300.

(A) ChIP-qPCR was performed for CBP at the *HLA-DRA* promoter region (WXY) and a negative control region (-2000) after Raji cells were treated with disuccinimidyl glutarate (DSG) for the indicated time together with the conventional formaldehyde crosslinking. White columns indicate the percent input values for control IgG pulldown and black columns represent those for CBP. (B) ChIP-qPCR was performed for the indicated factors in Raji (black), RJ2.2.5 (grey; CIITA-deficient), and SJO (clear; RFX5-deficient) cells. Amplicons tested by qPCR were as illustrated in Figure 2-2A. The values plotted are an average of three biological replicates and error bars represent standard error. The double asterisks (**) in A denote a Student's t-test value of $p<0.01$ when compared to 0 min DSG crosslinked values.

Figure 2-4. Histone methyltransferase MLL1 core complex proteins are recruited to *HLA-DRA in B cells.*

MLL core complex proteins, MLL1, WDR5, ASH2L, RbBP5, and DPY-30 were assessed for their binding across the *HLA-DRA* gene in Raji (black), RJ2.2.5 (CIITA-deficient, grey), and SJO cells (RFX5-deficient, clear) by qPCR after DSG-ChIP. The negative control IgG ChIP pulldowns conducted concurrently with these experiments are shown in Figure 2-3B. The data represent the average of the percent input values from the pulldown of each indicated protein at each amplicon as indicated in Figure 2-2A. The values are an average of 3-5 biological replicates and the error bars represent the standard error. For RFX5-dependent factors, the asterisks represent Student's t-test values $p<0.05$ of statistical

significance for comparisons between RJ2.2.5 and SJO. For CIITA-dependent factors, the asterisks represent Student's t-test values $p<0.05$ between Raji and RJ2.2.5 cells.

Figure 2-5. MLL core complex proteins are recruited in IFN-γ treated cells

(A) Human epithelial A431 cells were treated with IFN-γ for 24 hrs and tested for the binding of CIITA and RFX5 to verify the experimental procedure in the IFN-γ inducible system. (B) MLL core complex proteins were tested for binding before and after IFN-γ treatment in A431 cells. The data presented represent the average percent input value at each amplicon (Figure 2-2A) after DSG-ChIP pulldown of three biological replicates from untreated (clear) and IFN-γ treated (black) cells. The error bars represent the standard error. Asterisks represent Student's t-test values $p<0.05$ of the IFN-γ treated cells compared with background IgG controls for that amplicon.

Figure 2-6. GCN5 and PCAF containing histone acetyltransferases ATAC and STAGA are recruited to *HLA-DRA*

(A) Dual crosslinking ChIP-qPCRs for histone acetyltransferases GCN5, and PCAF were conducted for regions across *HLA-DRA* in Raji (black), RJ2.2.5 (CIITA null, grey), and SJO (RFX5 null, clear) cells as above. (B) Binding of the ATAC (ADA2a, ATAC2, YEATS2, NC2-β) and STAGA (ADA2b, TRRAP, TADA1L) complex subunits was determined as in A. The average of percent input values from the DSG-ChIP for each indicated protein is shown with the values averaged from 3-5 biological replicates. The asterisks indicate a Student's t-test value of $p<0.05$ at each amplicon when compared to the control IgG values for each cell line.

Figure 2-7. GCN5, MLL1, and WDR5 knockdown does not affect *HLA-DRA* expression or histone modifications.

siRNA SMARTpools representing a control pool or the indicated gene were transfected into Raji cells using nucleofection. (A) At three days post transfection western blots for the indicated factor were performed along with β-actin control. (B) mRNA from similar cultures was analyzed by qRT-PCR for *CIITA* and *HLA-DRA* transcripts. The data from four biological replicates was plotted over the siRNA control transfection. (C) Chromatin was isolated from the indicated siRNA transfected cells at 3 days post transfection and analyzed for the presence of the indicated histone modifications at the WXY box region of the *HLA-DRA* gene. The results of three biological replicates were averaged and plotted with respect to the input chromatin. Error bars in B and C represent the standard error.

Figure 2-8. CIITA and modified histones are stably bound at the *HLA-DRA* promoter after IFN-γ removal.

(A) mRNA levels of *CIITA* and *HLA-DRA* were measured by qRT-PCR after A431 cells were treated with IFN-γ for 0 or 24 hrs (black bars), and after IFN-γ treated cells were washed and supplied with IFN-γ free media and cultured for an additional 24 to 72 hrs (grey bars). An additional but separate set of experiments were carried out for 120 hours (clear) and are shown. The results of three independent experiments were averaged and plotted with standard error. (B) ChIP for CIITA and each indicated histone modification, as well as a negative control IgG were conducted after A431 cells were treated as in A. ChIP assays were analyzed at the WXY box region. The qRT-PCR and ChIP values for the 0 and 24 hr IFN-γ treated samples in the left panels of each set are the same as those shown in Figure 2-

1 as the data were generated from the same set of experiments and chromatin preparations, and are provided again only for comparison purposes.

Supplemental Figure 2-S1. IFN-γ treatment induced the deposition of active histone modifications throughout the *HLA-DRA* gene, plotted with respect to histone H3 density. The data from Figure 2-1C were replotted as fold over the histone H3 percent of input chromatin values for each amplicon as determined by histone H3 ChIP.

Supplemental Figure 2-S2. *MHC-II* expressing B cells have active histone modifications distributed across the *HLA-DRA* gene, plotted with respect to histone H3 density. The data from Figure 2-2B were replotted as fold over the histone H3 percent of input chromatin values for each amplicon as determined by histone H3 ChIP**.**

Supplementary Figure 2-S3. Histone modifying proteins in Raji, RJ2.2.5, and SJO cells are expressed at similar levels.

Nuclear extracts from Raji, RJ2.2.5, and SJO cells were prepared and equally loaded on SDS-PAGE, blotted to PVDF membranes and stained with the indicated antibodies as described in materials and methods. Molecular weight (MW) are shown.

Supplemental Figure 2-S4. GCN5 complex component ChIP from IFN-γ treated A431 cells.

Dual crosslinking ChIP was performed on A431 cells -/+ IFN-γ for 24 hours as described in the text of the manuscript. These data represent the average of three biological replicates.

Asterisks represent data values that were statistically significant (Student's t-test $p<0.05$) when compared to the IgG control ChIP assays.

Table 2-1. DNA oligos used in real-time PCR reactions.

primer	forward	reverse
CIITA*	5'CTGAAGGATGTGGAAGACCTGGGAAAGC	5'GTCCCCGATCTTGTTCTCACTC
HLA-DRA*	GAGTTTGATGCTCCAAGCCCTCTCCCA	CAGAGGCCCCCTGCGTTCTGCTGCAAT
GAPDH*	CCATGGGGAAGGTGAAGGTCGGAGTC	GGTGGTGCAGGCATTGCTGATG
XL4	CAGAGAAAGGGAACTGAAAGTCATTT	TTATGACACTGTTTAGTCCTAGAACACTGA
-2000	CAACAACTTGGATTGAAGATGC	AGGTAAAGAGTCAGGAGAATGG
-600	ATGAGATACAATGCCAGCCATCC	ACAGTTGGAGAGTTTGCGTAAGG
-300	TGTCCCTTACGCAAACTCTCC	ACACAAGATACTCCGTTCATTGG
WXY	GATCTCTTGTGTCCTGGACCCTTTGCAAGAACCCT	CCCAATTACTCTTTGGCCAATCAGAAAAATATTTTG
+300	GGACGATAGACTACGAAGCATTGG	TGACTTACTTCAGTTTGTGGTGAGG
+600	AGCCCTGTTCTTATCTGAATACATG	GCCTTCCCTCCCCTTTTCC
+1500	CTCCGTCTCAAACAACCAAACC	ACCAACACCAAGGGAATAATGAAC
+3500	TTCCGCAAGTTCCACTATCTCC	CGAGTTTCACACAAGCATCATAGG
+5800	AGGTAAAGAGTCAGGAGAATGG	ATGATACAGCCAAGATGAAACC

* Primers used for Reverse Transcription PCR reactions.

Figure 2-1

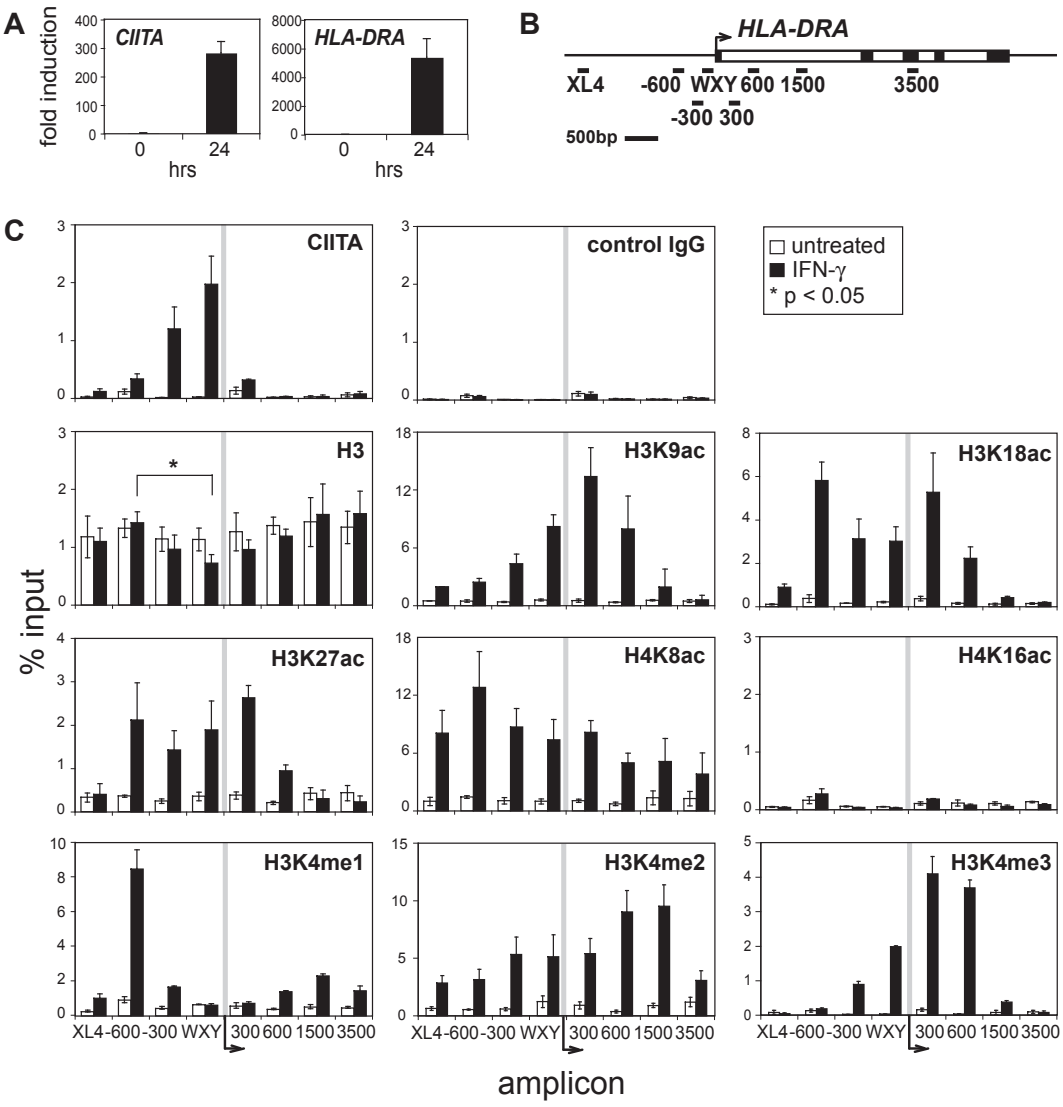

A
fold induction
CIITA
HLA-DRA
hrs
B
HLA-DRA
XL4
-600
WXY
600
1500
3500
-300
300
500bp
C
CIITA
control IgG
untreated
IFN-γ
* p < 0.05
H3
H3K9ac
H3K18ac
H3K27ac
H4K8ac
H4K16ac
H3K4me1
H3K4me2
H3K4me3
% input
XL4 -600 -300 WXY 300 600 1500 3500
amplicon

Figure 2-2

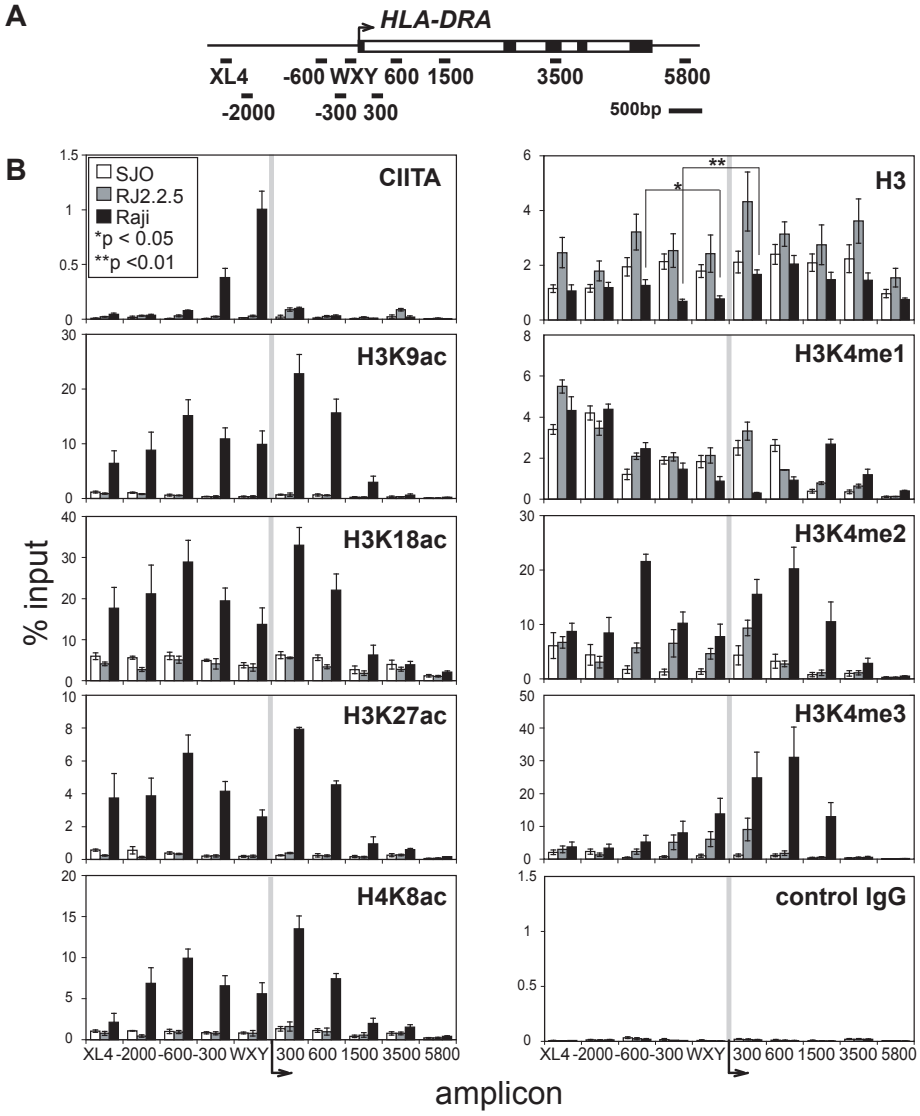

A
HLA-DRA
XL4
-2000
-600
-300
WXY
300
600
1500
3500
5800
500bp
B
SJO
RJ2.2.5
Raji
*p < 0.05
**p <0.01
CIITA
H3
H3K9ac
H3K4me1
H3K18ac
H3K4me2
H3K27ac
H3K4me3
H4K8ac
control IgG
% input
XL4 -2000 -600 -300 WXY 300 600 1500 3500 5800
amplicon

Figure 2-3

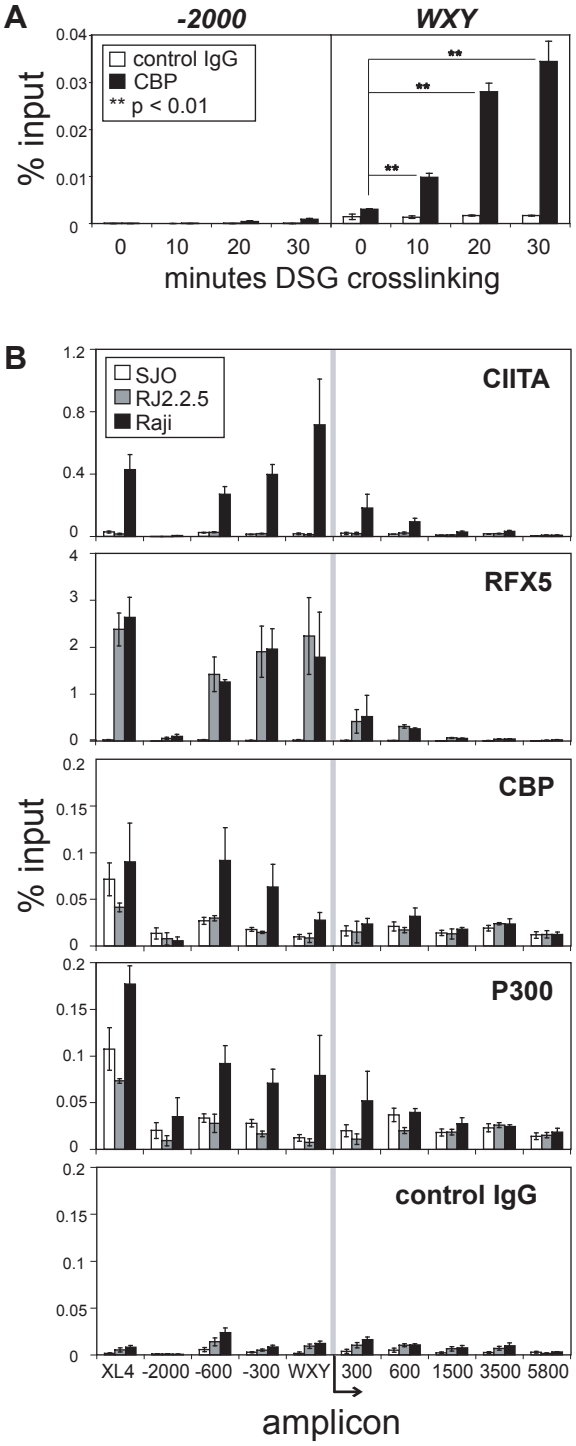

A
-2000
WXY
control IgG
CBP
** p < 0.01
% input
minutes DSG crosslinking
B
SJO
RJ2.2.5
Raji
CIITA
RFX5
CBP
P300
control IgG
% input
XL4 -2000 -600 -300 WXY 300 600 1500 3500 5800
amplicon

Figure 2-4

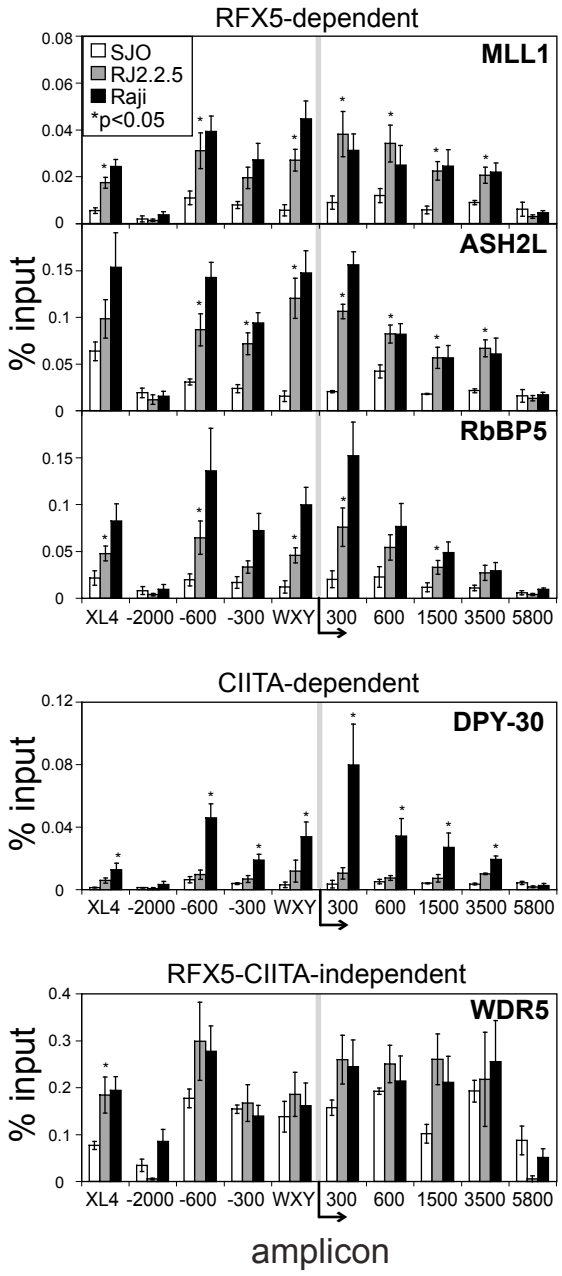

RFX5-dependent
SJO
RJ2.2.5
Raji
*p<0.05
MLL1
ASH2L
RbBP5
% input
XL4 -2000 -600 -300 WXY 300 600 1500 3500 5800
CIITA-dependent
DPY-30
RFX5-CIITA-independent
WDR5
amplicon

Figure 2-5

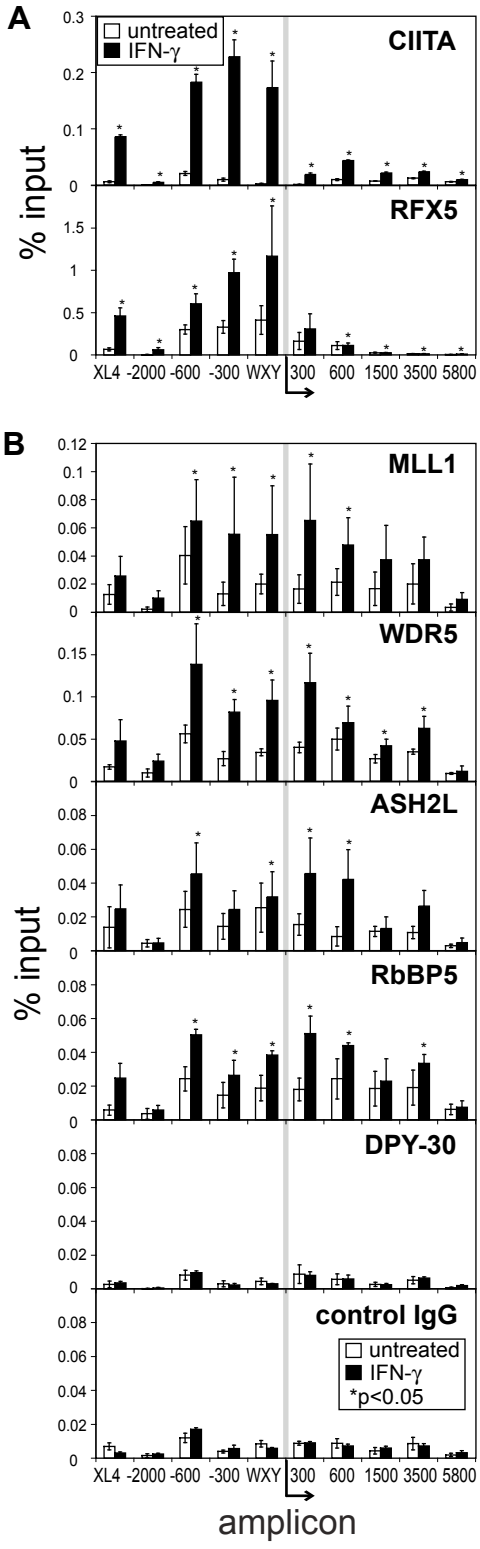

A
B
CIITA
RFX5
MLL1
WDR5
ASH2L
RbBP5
DPY-30
control IgG
untreated
IFN-γ
*p<0.05
% input
XL4 -2000 -600 -300 WXY 300 600 1500 3500 5800
amplicon

Figure 2-6

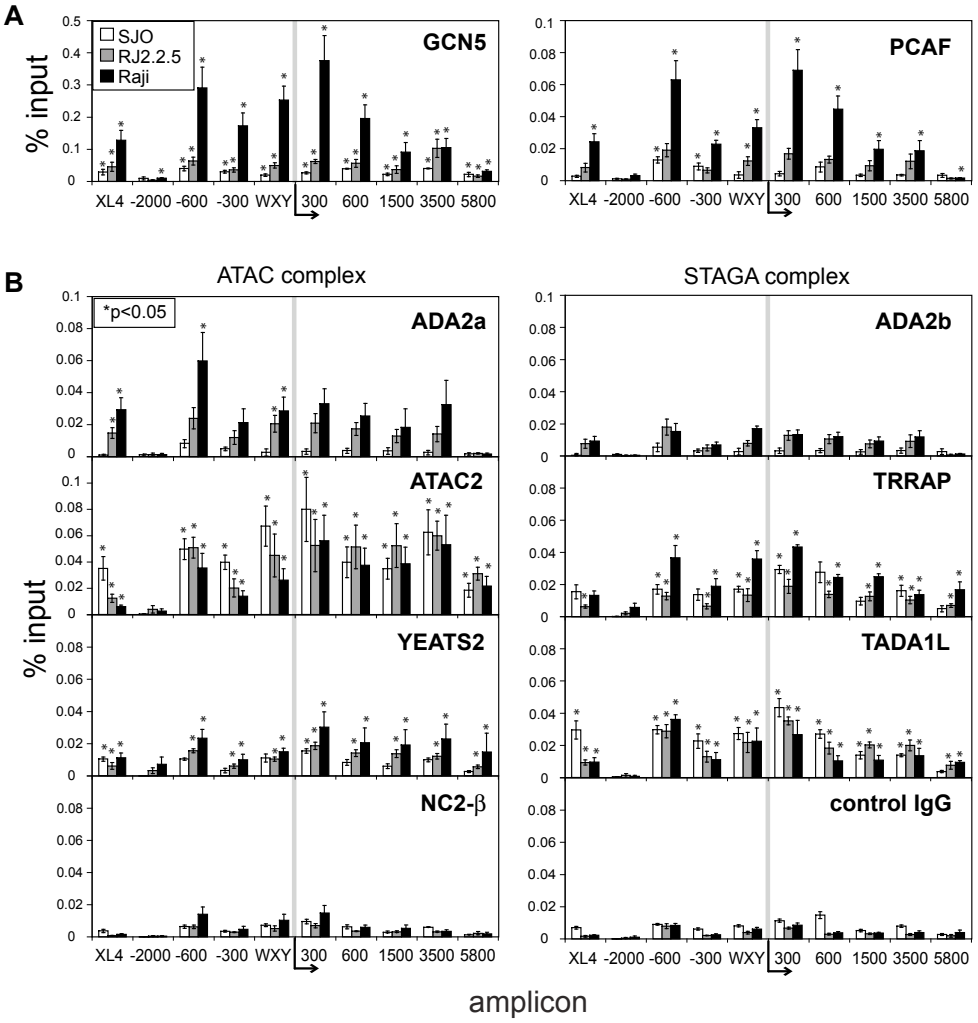

A
SJO
RJ2.2.5
Raji
GCN5
PCAF
% input
XL4 -2000 -600 -300 WXY 300 600 1500 3500 5800
B
ATAC complex
STAGA complex
*p<0.05
ADA2a
ADA2b
ATAC2
TRRAP
YEATS2
TADA1L
NC2-β
control IgG
% input
amplicon

Figure 2-7

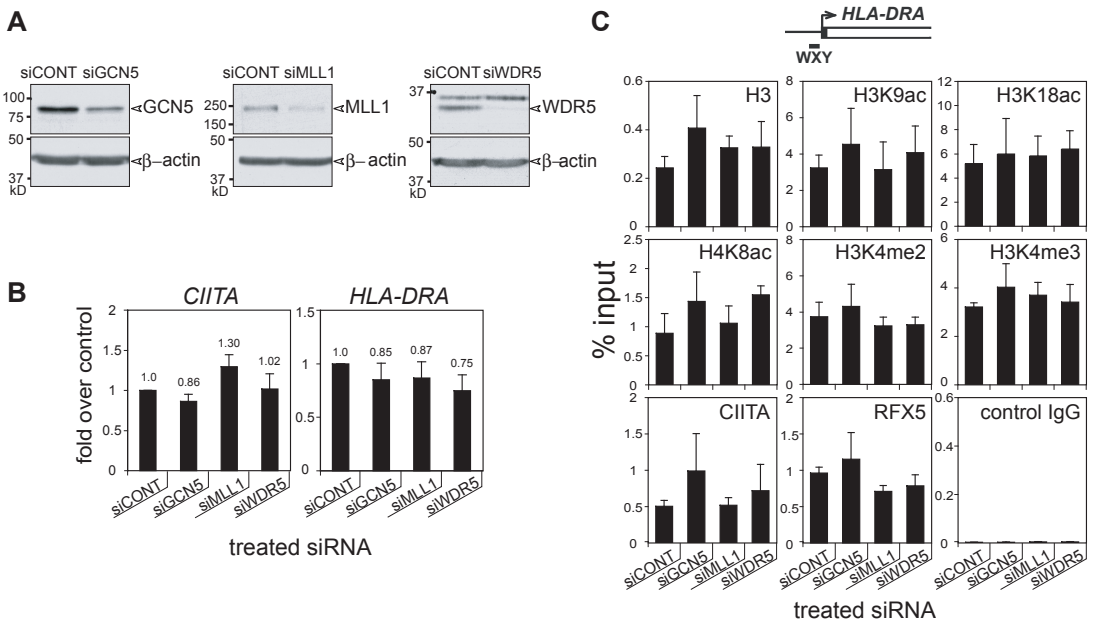
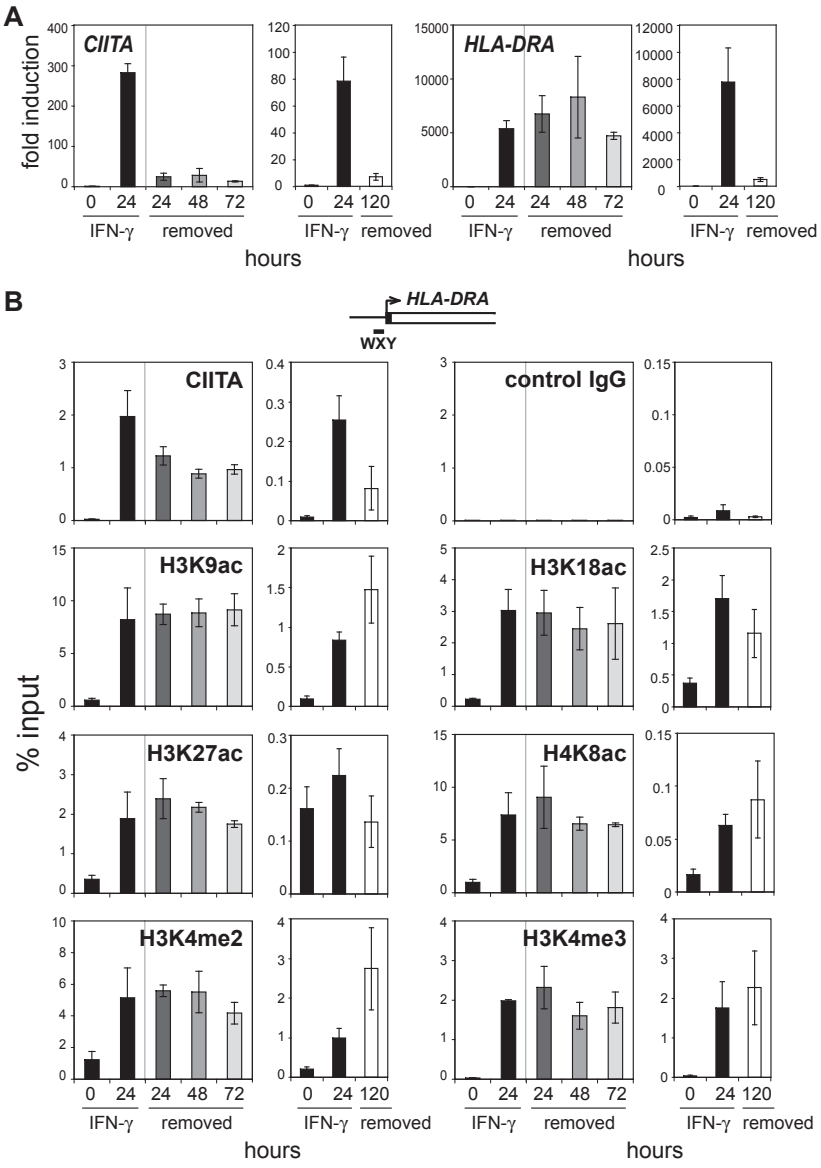
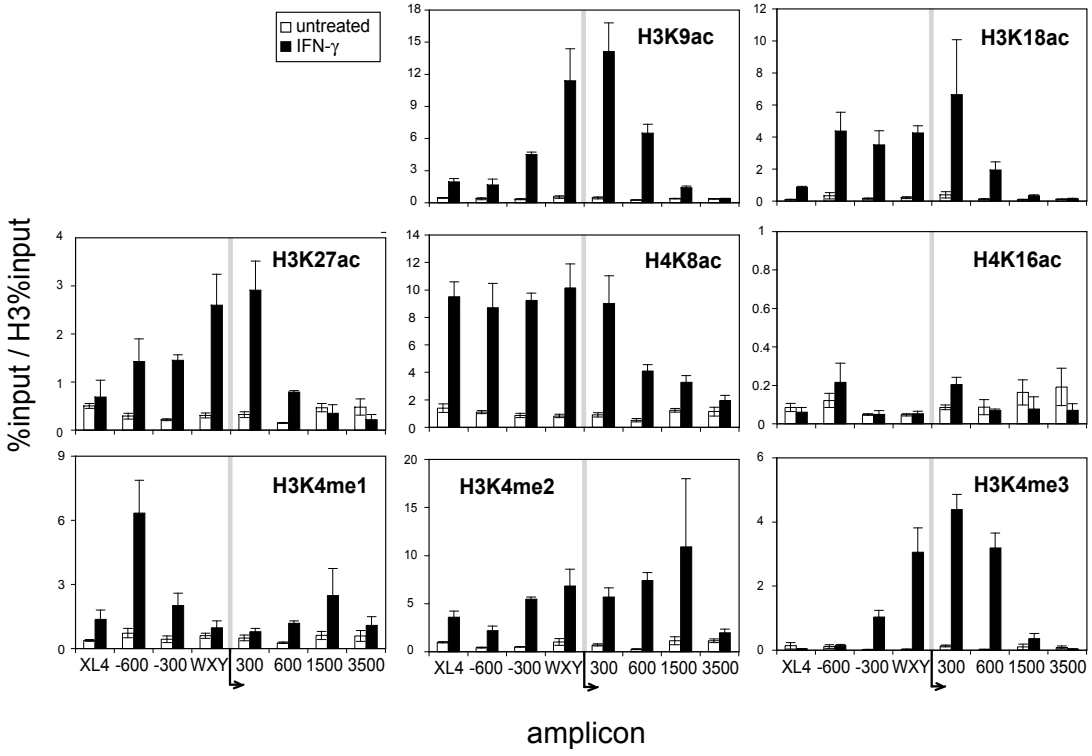

A
siCONT siGCN5
siCONT siMLL1
siCONT siWDR5
GCN5
MLL1
WDR5
β–actin
kD
B
CIITA
HLA-DRA
fold over control
treated siRNA
siCONT
siGCN5
siMLL1
siWDR5
C
HLA-DRA
WXY
% input
H3
H3K9ac
H3K18ac
H4K8ac
H3K4me2
H3K4me3
CIITA
RFX5
control IgG
treated siRNA

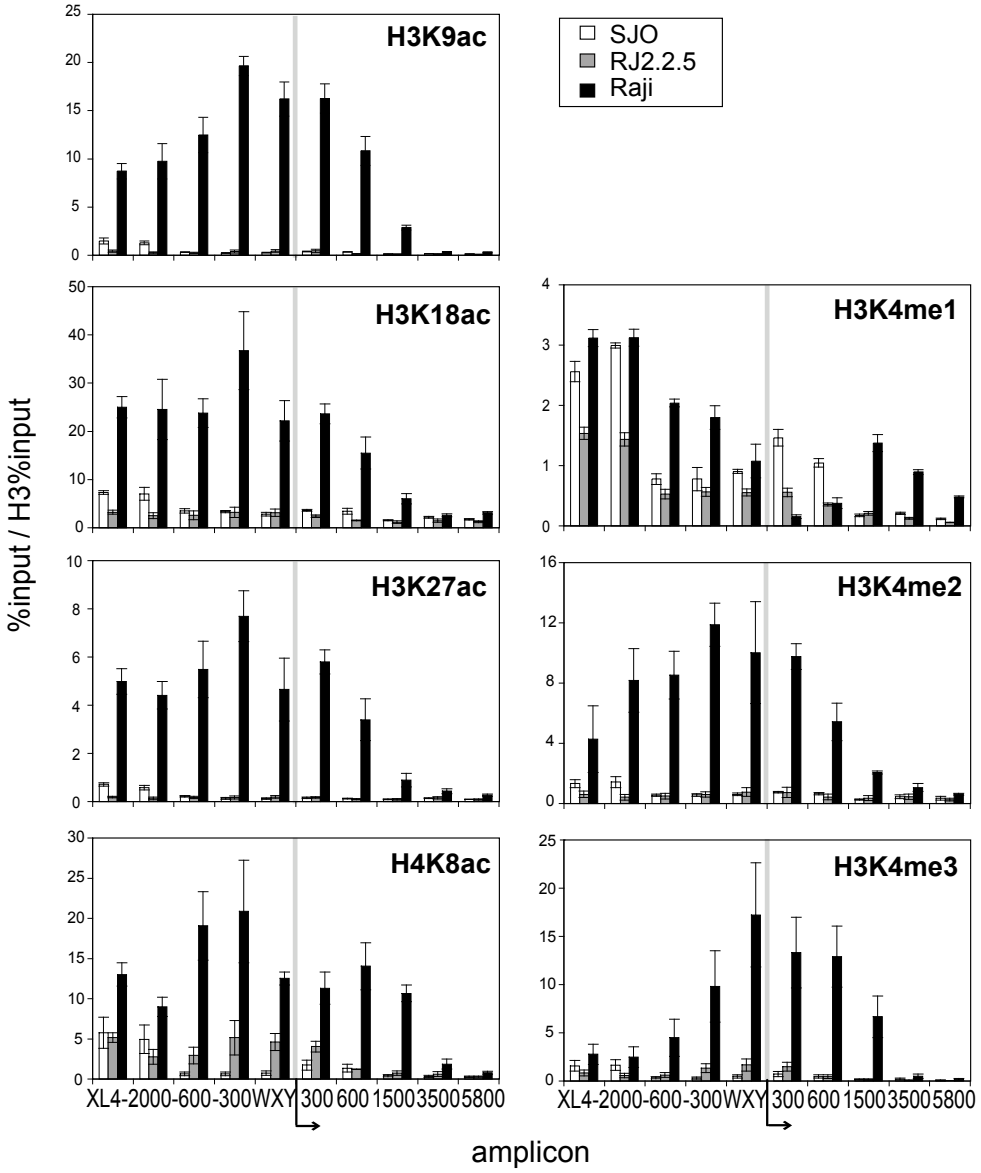
Figure 2-8


A
fold induction
CIITA
HLA-DRA
0 24 24 48 72
0 24 120
IFN-γ
removed
hours
B
HLA-DRA
WXY
CIITA
control IgG
H3K9ac
H3K18ac
H3K27ac
H4K8ac
H3K4me2
H3K4me3
% input

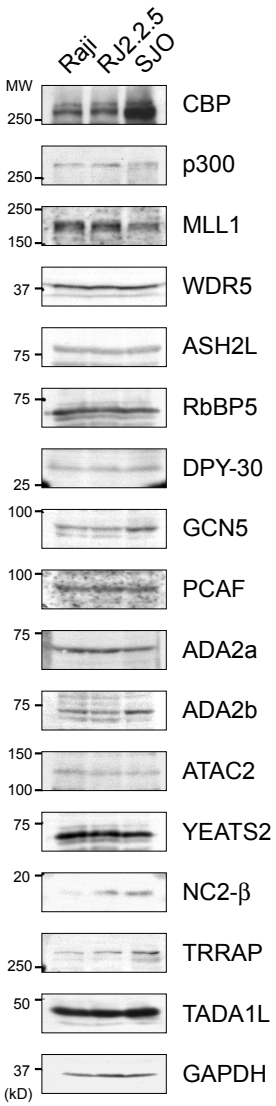
Supplemental Figure S1



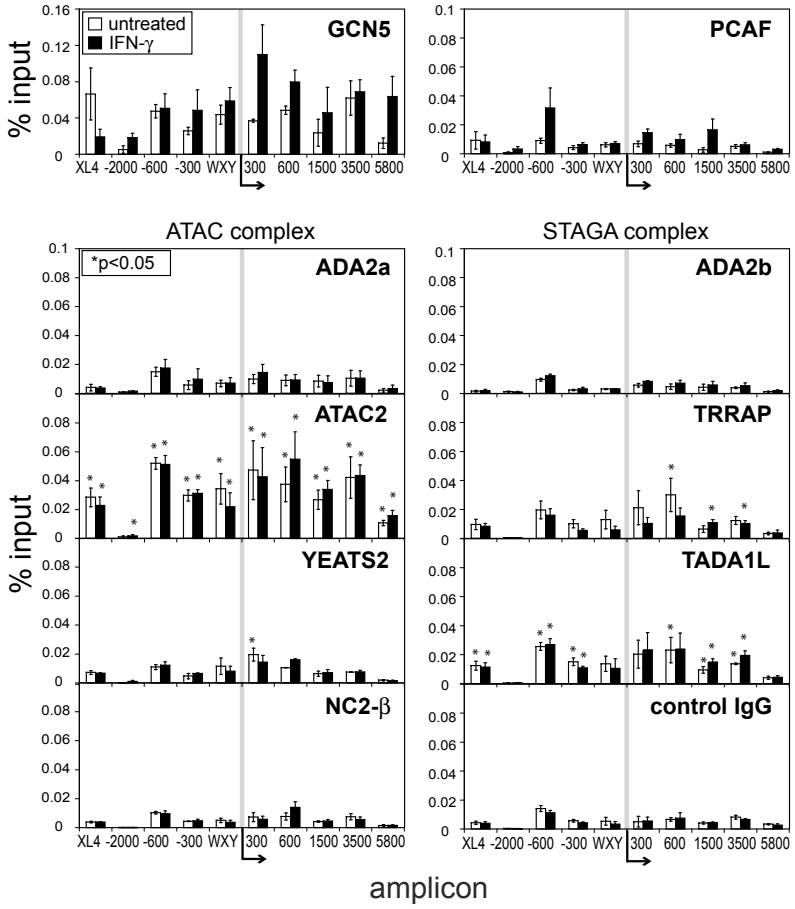
Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure 4



Chapter 3. Purification of the CIITA Interacting Protein Complex

Nancy M. Choi, Jeremy M. Boss

Conceived and designed the experiments: JMB NMC. Performed the experiments: NMC. Analyzed the data: NMC JMB. Wrote the chapter: NMC.

Introduction

CIITA is an essential transcription factor that is considered to be the master regulator of MHC-II expression. As discussed in previous chapters, it has been shown by multiple groups that CIITA interacts with the general transcription factors that form a PIC (pre initiation complex) with the RNA polymerase II. It also interacts with a variety of transcriptional coactivators such as CBP and PCAF. While a candidate approach to identifying CIITA interacting factors has been very fruitful in expanding our understanding of MHC-II gene regulation, there is the possibility of missing unexpected partners. For a more unbiased approach, a global proteomic methodology was employed. A biochemical strategy was taken to purify the CIITA interacting proteins and identify new interacting partners by mass spectrometric analysis.

Mass spectrometry (MS) coupled with 1D (by mass) or 2D (by mass and electric charge) gel electrophoresis is a widely used approach to identify individual proteins from a mixture of proteins. A protein sample would be separated, sequenced by first trypsinizing into peptides, followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS or electronic spray ionization (ESI)-MS (Mann et al., 2001). This procedure however, is inefficient in identifying proteins of very small or large masses, as they were not as easily isolated by gel electrophoresis. Similarly, by these methods, proteins of high degrees of hydrophobicity such as membrane proteins, or proteins extremely acidic or basic are difficult to identify. For global identification of proteins from a mixture, liquid chromatography followed by tandem mass spectrometry (LC/MS/MS) has greatly improved the efficiency and sensitivity of identification (Peng and Gygi, 2001). When further separation of proteins is desired, the liquid chromatography step can be extended to be multi-dimensional. Quantitative identification may also be achieved by isotope labeling of

proteins, in a method called stable isotope labeling with amino acids in cell culture (SILAC) (Trinkle-Mulcahy et al., 2008). Utilizing these various methods subsequent to the purification of a complex of proteins has greatly facilitated our knowledge of protein interaction networks.

Protein purification is most commonly conducted by using protein or epitope-tag specific antibodies and a binding matrix; usually agarose, sepharose, or more recently, magnetic matrices have been greatly utilized. However, antibodies with strong affinity with low levels of non-specific binding are difficult to generate. Also, in purifying protein complexes, the antigen that the antibody recognizes may be masked, hence unavailable for detection. Biotin and streptavidin have the strongest protein-to-protein affinity known to date, $K_d = 1x10^{-15}$ M. By designing a recombinant protein that is targeted by a biotin ligase, one can utilize this strong binding affinity to purify a protein and its interacting partners. This peptide is called the biotin ligase recognition peptide (BLRP), which gets recognized and biotinylated by the *E. coli* biotin ligase A (BirA) (Beckett et al., 1999; Furuyama and Henikoff, 2006). Utilization of the BLRP peptide has been proven a viable tool to purify protein interaction complexes (Furuyama and Henikoff, 2006).

To express recombinant proteins in mammalian cells, viral and non-viral vector tools are available. Viral vectors had gained great interest for their potential for therapeutic usage, as they have greater efficiency of transfection and long-term expression (Hanawa et al., 2002; Ma et al., 2003). However, non-viral vectors are an alternative option where EBV based vectors have been identified as a viable candidate (Conese et al., 2004). They are the most extensively studied extrachromosomal replicating systems used for stable transgenesis (Calos, 1996; Jackson et al., 2006). These vectors utilize an Epsteins-Barr virus (EBV) replication of origin (OriP) and the EBV nuclear antigen-1 (EBNA-1) gene to replicate within the host

nucleus and maintain itself for prolonged periods of time (Van Craenenbroeck et al., 2000). Despite multiple reports of improved long-term transgene expression in mammalian cells using EBV episomal plasmids its effectiveness does vary between cellular environments (Kameda et al., 2006; Sclimenti et al., 2003; Wade-Martins et al., 2000; Wendelburg and Vos, 1998). Since EBV vectors are predominantly present extrachromosomally within nuclei (Margolskee et al., 1988; Mũcke et al., 1997), this variability would not be due to positional effects seen with chromosomal insertions. There have been reports of DNA methylation occurring on episomal plasmids leading to silencing (Hong et al., 2001; Hsieh, 1999). This suggests that epigenetic silencing of these plasmids is a major hindrance of the system for further development in in vivo applications.

Insulator elements are genomic sequences that have an essential role in regulating proper gene expression. They have boundary element functions that block the spreading of heterochromatic environment, or interact with the insulator binding protein CTCF (CCCTC-binding factor) to function as enhancer-blocking elements (Bell et al., 1999; Gaszner and Felsenfeld, 2006). The Drosophila *gypsy* insulator and *cHS4* at the 5' of the chicken β-globin locus are the most extensively studied of these elements (Chung et al., 1993; Marlor et al., 1986). A human insulator within the MHC class II locus called *XL9* has also been determined to have insulator functions (Majumder et al., 2006). As a practical usage of these cis-regulatory elements, insulators have been used in various viral vectors as well as transposable elements to protect transgenes from becoming silenced due to genomic insertion into heterochromatic regions (Chung et al., 1993; Emery et al., 2000; Mori-Uchino et al., 2009; Tajima et al., 2006).

In this study, we have aimed to purify a CIITA interacting protein complex. To this goal we had designed N- or C-terminally BLRP tagged CIITA constructs that were co-expressed with BirA to utilize the strong protein interaction between biotin and streptavidin. In order to stably express this construct in a human B cell line, we made use of a modified non-viral EBV based vector that contains the human *XL9* insulator sequence. The *XL9* was included to enhance the duration of trans-gene expression by reducing the epigenetic silencing of the vector. Utilizing these tools, a potential CIITA interacting protein complex has been purified.

Materials and Methods

Plasmid construction

Long oligos including the Kozak sequence and N- and C-terminal BLRP-TEV-His6 (wild-type and mutant) tag, or the N-terminal double FLAG double BLRP (2F2B) tag were synthesized from Integrated DNA technologies (Coralville, Iowa) (Table 4-1). The forward and reverse oligos were resuspended in distilled deionized water and mixed to equimolar concentrations. The mixture was heated to 95 C° for 15 minutes and cooled to room temperature on the bench top. The double stranded DNA was digested with *Nhe*I and cloned into the pREP4 vector (Life technologies, Carlsbad CA). The full-length cDNA sequence of *CIITA* was amplified using the Phusion™ High-Fidelity DNA Polymerase (New England BioLabs Inc., Ipswich, MA). The *CIITA* PCR product was inserted into the *Not*I site. This and all subsequent DNA inserts were cloned using the In-Fusion™ Dry-Down PCR Cloning Kit (Clontech Laboratories Inc.) according to the manufacturers recommendations. For efficient biotinylation of the BLRP tag required in subsequent

applications, an internal ribosome entry site (IRES) and the *E. coli* biotin ligase, BirA, were also cloned downstream of the *CIITA* gene into the *Xho*I and *BamH*I sites respectively. The pREP4-BLRP-CIITA and pREP4-BLRP-CIITA-XL9 plasmids are identical up to this point. Only for the pREP4-BLRP-CIITA-XL9 plasmid was the 747bp sequence of *XL9* amplified and cloned into the above plasmid (Figure 3-1). The *XL9* fragment was inserted into the plasmid in the *Xba*I site present downstream of the polyadenylation signal of the CIITA-BirA coding sequence. Chemically competent bacterial cells, provided in the In-Fusion cloning kit, were transformed according to manufacturers instructions. The constructs were verified to be their correct sequences by restriction enzyme digestion and DNA sequencing. Transformed bacteria were grown on LB media supplemented with 100 μg/ml ampicillin. Plasmids were prepared by standard maxi prep followed by a CsCl gradient ultracentrifugation step to purify supercoiled DNA. The concentration of plasmid DNA was measured using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher). The plasmids to be used as negative controls that do not contain *CIITA*, pREP4-empty and pREP4-empty-XL9, were prepared simultaneously.

Cell lines, transfection, and magnetic cell separation

Raji anc Cos-7 cells were purchased from ATCC (American Type Culture Collection). RJ2.2.5 cells are *CIITA* absent mutants derived from Raji cells by γ-ray irradiation and negative for MHC-II expression (Accolla, 1983). Raji and RJ2.2.5 were cultured in RPMI (Life Technologies, Carlsbad, CA) supplemented with 5 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 5 % bovine calf serum (Life Technologies, Carlsbad, CA), and penicillin 200 U/ml streptomycin 200 μg/ml (Life Technologies, Carlsbad, CA). Cos-7 cells were cultured in DMEM supplemented with 10 % fetal bovine serum, and penicillin 200

U/ml streptomycin 200 μg/ml. Fugene 6 (Roche, Germany) was used for transfection of Cos-7 cells and the Amaxa® Nucleofector® kit V (Lonza, Switzerland) was used for transfection of B cells. For each transfection, 4 x 10^6 of RJ2.2.5 cells and 5 μg of CsCl prepared DNA was used and 2 μg of DNA and 6 μl of Fugene reagent were used for Cos-7 cells growing in a 6 well plate that were plated with 2.5 x 10^5 cells the previous day. For selection of successfully transfected cells, hygromycin (Millipore, Billerica MA) is added to the culture to a final concentration of 500 μg/ml 24 hours after transfection. Cells were grown in media supplemented with 500 μg/ml of hygromycin for 10 days until the selection is complete and then transferred to 100 μg/ml hygromycin to prevent loss of the episomal plasmid during prolonged maintenance of the stable cell lines. For the separation of HLA-DR expressing cells after stable transfection, anti-HLA-DR MicroBeads (Miltenyi Biotech, Germany) were used according to the manufacturers recommendations.

Flow cytometry

For flow cytometry, the experimental and control cells were collected and washed with cold PBS and resuspended in FACS sorting buffer (1 x Mg^{2+}/Ca^{2+}free-phosphate buffered saline, 25 mM HEPES pH 7.0, 1 mM EDTA, 1 % heat inactivated FBS, 0.2 μm filter sterilized). To measure the level of cell surface MHC-II expression, cells were labeled on ice with 1:20 dilution of APC conjugated anti-HLA-DR antibody (BD Biosciences, San Jose CA) and propidium iodide (0.5 ng/ml) was added to stain dead cells. Stained cells were finally washed twice with FACS sorting buffer. The BD FACSCalibur™ flow cytometer was used for data collection and the FlowJo computer software (Tree Star, Inc., Ashland, OR) was used for analyses.

SDS-PAGE and immunoblot

For immunoblot verification of trans-gene expression, at least 60 days after hygromycin selection, cells were collected and washed with cold PBS and nuclear extract was prepared using the NE-PER nuclear extract isolation kit (Pierce, Thermo Fischer, Rockford IL) for small scale nuclear extract preparation. Whole cell lysates of Cos-7 cells, the cells were washed with PBS and scraped with a cell scraper, then lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) supplemented with protease inhibitors. Protein content was measured using the BioRad protein assay solution. Proteins were separated by electrophoresis on an SDS-PAGE gel until fully resolved. Resolved proteins were then transferred to a PVDF membrane (GE Healthcare, Piscataway NJ) overnight at 4 C°. The membrane was blocked with 5 % non-fat dry milk in PBST (1 x PBS, 0.1 % Tween-20) for 1 hour at room temperature. Anti-BirA (abcam), anti-CIITA (7-1H), and anti-beta-actin (Santa Cruz Biotechnologies, Santa Cruz CA) were diluted in 5 % milk and hybridized with the membrane at 4 C° overnight. The membrane was washed with PBST three times for 5 minutes each and incubated with HRP conjugated secondary sheep-anti-mouse, goat anti-rabbit (Sigma-Alderich, St Louis MO) for 1 hour at room temperature. It was then washed three times, developed with ECL solution (GE Healthcare, Piscataway NJ), and exposed to Kodak BioMax autoradiography film (Kodak, Rochester NY).

Large-scale nuclear extract preparation

Cells were grown in BD TufRol roller bottles (Becton Dickinson, Franklin Lakes NJ) for large-scale cultures and continuously rotated at 37 C°. Approximately 2 x 10^9 cells were collected by centrifugation and kept on ice. The cells were swollen in 5 x pellet volume of

hypotonic buffer supplemented freshly with protease inhibitors, spermine, spermidine, and DTT (10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.05 % NP-40, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT) for 10 minutes on ice. The dounce homogenizers were autoclaved, pre-chilled, and stored in the -20 C° freezer until needed. Dounce homogenization is continued until ~95 % of the cells are lysed, checked by trypan blue staining observed under the microscope. 10 % volume of 75 % sucrose buffer (50 mM HEPES pH 7.9, 10 mM NaCl, 0.1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT) was mixed gently into solution and nuclei were precipitated in the JA-20 rotor for 1 min at 7500 rpm. The supernatant (cytosolic extract) was removed and the nuclear pellet was resuspended in nuclear resuspension solution without ammonium sulfate (20 mM HEPES pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 25 % glycerol, 1.5 mM $MgCl_2$, 0.75 mM spermidine, 0.15 mM spermine, 2 mM DTT), then transferred to polycarbonate ultracentrifuge tubes. After transfer, saturated ammonium sulfate (10 % volume saturated ammonium sulfate 4.1 M) was mixed in quickly, and rocked in a cold room for 30 min. The lysed nuclear preparation was centrifugated in an ultracentrifuge at 45000 rpm for 90 min in a Ti70.1 rotor, the upper nuclear extract layer was then dialyzed in dialysis buffer (20 mM HEPES pH 7.9, 150 mM NaCl 0.2 mM EDTA, 0.2 mM EGTA, 20 % glycerol, 1 mM $MgCl_2$, 0.75 mM spermidine, 0.15 mM spermine, 2 mM DTT) for two hours in at 4 C°. Insoluble particles were precipitated and cleared by centrifugation at top speed for 10 min, and stored at -80 C°.

Streptavidin purification of BLRP-tagged protein complex

Pierce High Capacity Streptavidin Agarose Resin (Thermo Fisher, Rockford IL) or Dynabeads® M-280 Streptavidin conjugated magnetic beads (Life Technologies, Grand

Island NY) were prepared, 50 μl/IP. The beads were washed three times with IP150 (0.3) buffer (20mM HEPES pH 8.0, 150 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 0.3 % NP-40, 1 μM DTT added fresh). The beads were resuspended in its original volume and left on ice until needed. If NE was frozen, after thawing on ice, the extracts were centrifuged at 10,000 rpm for 10 min at 4 C° in a microcentrifuge. 3 mg of NE were diluted it to 2 mg/ml concentration with IP150 (0.3) buffer. RNase A (1 μg/ml) and DNase I (0.5 U/ml) were added to each diluted NE. The prepared magnetic beads were added to the NE and rotated together O/N at 4 C°. The next day, the beads were washed with IP200 (0.3) buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 20 % glycerol, 1 mM EDTA, 1 mM EGTA, 0.3 % NP-40, 1 μM DTT added fresh) and one time with IP250 (0.3) (20 mM HEPES pH 8.0, 250 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 0.3 % NP-40, 1 μM DTT added fresh) for 5 minutes each by rotating in cold room and collecting the beads with a magnet. The collected samples were eluted and resolved by an SDS-PAGE gel and visualized by silver stain using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Fisher, Rockford IL), coomassie stain, or transfered to a PVDF membrane for WB detection.

Protein Identification by Mass Spectrometric Analysis

Protein samples were resolved on a SDS gel and stained with Pierce GelCode Blue Stain Reagent (Thermo Fisher, Rockford IL). Each lane of the SDS-PAGE gel was quartered, and each quartered piece was individually subjected to in-gel digestion. Reverse-phase liquid chromatography coupled with tandem mass spectrometry was conducted by using an LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA). After searching against a positive-orientation database of human peptide sequences, a reverse-orientation sequence

database was searched to eliminate incorrect matches and reduce false discoveries (Peng et al., 2002). Finally, only proteins that were matched by at least two peptides were accepted to further improve the confidence of identification. (The mass spectrometric analyses were performed by the Emory Proteomics Core Service Center.) Post analysis to determine difference between control and experimental samples were conducted by calculating the sum of spectral counts (SC) for both CIITA [C] and negative control [N] samples for each protein. Proteins that had a value $\Delta SC = \Sigma[C] - \Sigma[N] > 1$ were listed as potential interacting proteins. Outlier proteins ($\Delta SC > Q_3$ (third quartile) + $3(Q_3-Q_1)$) and proteins with values $\Sigma[C] / \Sigma[N] < 80$ % were eliminated. Gene ontology clustering was done by the most recent updateded version of the freely available online tool, DAVID (the Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources 6.7, which can be accessed at http://david.abcc.ncifcrf.gov/ (Jiao et al., 2012).

Results

CIITA is constitutively expressed in Raji cells, a human Burkitt's lymphoma B cell line. In a mutant cell line derived from Raji, RJ2.2.5, a truncation in the *CIITA* mRNA renders the mutant protein non-functional. This results in a total absence of MHC-II expression on the surface of these cells (Accolla, 1983). The ability of these two cell lines to express MHC-II genes is demonstrated by the HLA-DR expression of the two cell lines in Figure 3-2A. To stably re-introduce the *CIITA* cDNA into RJ2.2.5 cells, the EBV based episomal pREP4 vector was utilized as the backbone for plasmid cloning.

A BLRP (biotin ligase recognition peptide) - TEV (tobacco etch virus cleavage site) - His6 (hexahistidine) tagged CIITA construct was designed. A mutant BLRP tag that cannot be biotinylated and an empty vector was also cloned to use as a negative control. To ensure

the biotinylation of the BLRP tag within each cell, the BirA gene (*E. coli* biotin ligase) was cloned downstream of the *CIITA* gene, and the two genes were connected by an IRES (internal ribosome entry site; Figure 3-1). When the pREP4-BLRP-CIITA construct was transfected into RJ2.2.5 cells, initially at 20 days, there was a stably transfected population of high HLA-DR expressing cells compared to the empty vector transfected negative control cells (Figure 3-2B). This initial level of HLA-DR expression was equivalent to that of Raji cells showing that the CIITA transgene was fully functional and capable of driving the expression of the HLA-DR. However, after prolonged culture of 40 to 60 days, a gradual loss of HLA-DR expression was observed (Figure 3-2B).

In an effort to sustain the expression of the *CIITA* transgene, an insulator element was inserted into the plasmid. *XL9* is an insulator element present within the human MHC-II locus that has been characterized previously (Majumder et al., 2006). Within its endogenous cellular and genomic environment, it acquires high levels of histone acetylation, interacts with the human insulator binding protein CTCF, and associates with the nuclear matrix (Majumder et al., 2006). Its ability to enhance the stable expression of a transgene has never previously been determined. However, due to its observed qualities, it was hypothesized that *XL9* within the extrachromosomal plasmid would acquire acetylated histones to loosen the DNA structure, prevent the plasmid from acquiring DNA methylation, and possibly due to its interaction with CTCF, enhance transmission of the plasmid to daughter cells during cell division.

XL9 was added to the pREP4-BLRP-CIITA plasmid downstream of the poly-adenylation signal of the BLRP-CIITA-IRES-BirA sequences (Figure 3-1). At 20 days after hygromycin selection, there was a HLA-DR highly expressing population similar to that observed for the pREP4-BLRP-CIITA plasmid (Figure 3-2B). The stable transfectants for

pREP4-empty-XL9 plasmid were HLA-DR null as expected. When the pREP4-BLRP-CIITA-XL9 stable transfectants were maintained in culture for up to 60 days, there was a minimal loss of HLA-DR expression compared to the pREP4-BLRP-CIITA stable transfectants.

The protein expression level of CIITA was determined for the cells cultured for 60 days by immunoblot. While CIITA was undetectable in the pREP4-BLRP-CIITA stable transfectants, there was a clear abundant signal in the pREP4-BLRP-CIITA-XL9 transfectants (Figure 3-3A). This high level of expression is also detected for the BirA gene that is present downstream of *CIITA* on the same transcript, separated by an IRES. The degree of expression with the *XL9* containing construct is greater than the endogenous expression levels of CIITA in Raji cells. In a separate attempt to enhance the expression of the recombinant CIITA, the BLRP tag was added to the C-terminus of CIITA. This construct, when expressed in Cos-7 cells, had significantly less CIITA expression as determined by an immunoblot assay (Figure 3-3B). The mutant BLRP tag did not bind to the streptavidin matrix and functioned as a negative control for the streptavidin purification of the CIITA complex.

To determine the CIITA interacting protein complex, nuclear extracts were collected from the negative control pREP4-empty-XL9 and pREP4-BLRP-CIITA-XL9 stably transfected cells. Initially, the construct was designed for tandem purification: biotin purification, site-specific protein cleavage by TEV protease, followed by purification with the His6 tag. However, even with multiple attempts to optimize purification conditions, the TEV cleavage and His6 purification steps for this construct were inhibitingly inefficient. Hence, only the BLRP tag was utilized in the purification process.

After purification using streptavidin conjugated agarose resin, unique bands were found in the CIITA expressing nuclear lysate sample, visualized by silver staining (Figure 3-4A). To determine, whether CIITA and known CIITA-interacting proteins can be detected in the purified complex, immunoblot for RFX5, CTCF, and WDR5 were conducted (Figure 3-4B). Both of these proteins were found only in the CIITA sample and not the negative control. The rest of the same samples that were checked by silver staining (silver stain : MS = 1 : 9) were analyzed by LC/MS/MS to identify the proteins within the mixture.

After LC/MS/MS analysis and database search, an initial list of proteins from the mass spectrometry results was derived. There were 388 proteins with at least one spectral count (SC) detected in the purified CIITA complex sample, and 224 proteins that had one or more SC than in the negative control sample. A spectral count is defined as the total number of MS/MS scans matched to an identified protein (Lundgren et al., 2010). In general, the SC reflects the relative abundance of the protein. However, other factors, such as protein size or charge may influence the number. Larger proteins, for instance, often result in higher spectral counts as more peptides may be derived from a single protein. To compare the protein mixtures from the BLRP-CIITA and negative control transfected samples, the number of SC for each protein were compared and those with at least more than one greater are listed in Table 4-2. This experiment was repeated, and the results that overlapped in the second set of results are shown in Table 4-2 in red.

The proteins can be categorized into functional groups using bioinformatic tools made available to the scientific community by the National Institutes of Health. Gene ontology functional annotation clustering results are shown in Table 4-3. Of the 37 proteins that were recognized and annotated by the tool, ~60 % of the proteins were classified as nuclear lumen proteins ($p = 1.78 \times 10^{-13}$). An enrichment of the proteins involved in

chromatin related processes were observed; proteins were involved in chromosome organization ($p = 6.64 \times 10^{-7}$) and transcription ($p = 5.60 \times 10^{-5}$). Other functional categories were cell cycle ($p = 6.34 \times 10^{-3}$), zinc ion binding ($p = 0.02996$), and nuclear import ($p = 0.01548$). In terms of protein domain structure and protein complex incorporation, there was a great enrichment for nucleotide binding proteins ($p = 7.65 \times 10^{-4}$), bromodomain proteins ($p = 0.0028$), NuRD complex proteins ($p = 3.63 \times 10^{-6}$), and histone deacetylase complex proteins ($p = 1.29 \times 10^{-4}$).

To improve the purification of the CIITA protein complex, a magnetic bead matrix was also tested. In the silver stain results, the magnetic beads appeared to improve the pulldown efficiency and reduce the background level of binding in the negative control sample (data not shown). However, when the samples were analyzed by LC/MS/MS, there were no proteins that had significantly greater presence in the CIITA purified samples compared to negative control. In another construct, two tandem FLAG and two tandem BLRP tags (2F2B) were added to the N-terminus of CIITA. When the expression of these proteins were compared to that of the single N-terminal BLRP tagged CIITA, neither of these constructs were capable of expressing equivalent levels of *CIITA* or *HLA-DRA* in the RJ2.2.5 cells, nor were the HLA-DR+ cells selected by magnetic bead separation (Figure 3-5).

Discussion

CIITA is regarded the master regulator of MHC-II transcription. Many interacting protein partners have been determined previously, all of which were identified one-by-one through hypothesis driven investigations. While this approach greatly enriched our understanding of the MHC-II transcriptional machinery, a global non-biased approach may allow us to identify novel interacting partners.

The strong interaction affinity between biotin and streptavidin was utilized for our protein complex purifications. One unexpected hurdle was the gradual loss of expression of the recombinant CIITA construct. To overcome this, an *XL9* insulator sequence was added to the construct. This greatly enhanced the retention of high levels of expression. This is likely due to the protection of the episomal vector from epigenetic silencing by the insulator sequence. There have been reports of reduced DNA methylation and deacetylation of transgene insertions when they were flanked with the insulators (Pikaart et al., 1998; Tajima et al., 2006). This strategy of flanking transgenes with insulators has been utilized in various viral vectors, as well as transposon based gene delivery systems. They also seem to provide less variation in gene expression due to positional effects as well as improved proper promoter function (Markstein et al., 2008; Tian and Andreadis, 2009). *XL9* can bind to the mammalian insulator binding protein CTCF and associate with the nuclear matrix (Majumder et al., 2006). These characteristics could possibly allow *XL9* to function as an additional anchor to the host chromosome, allowing better nuclear retention and transmission to daughter cells during cell division, or guiding the transgene into transcriptionally active regions within the nucleus. However, the CTCF binding ability of the cHS4 has been described as unnecessary for elevated expression of transgenes (Moreno et al., 2009). The exact functional mechanism is still elusive and will need further investigation.

The proteins identified in the LC/MS/MS analysis were functionally annotated and categorized. A clear enrichment for nuclear proteins involved in transcription and chromatin organization was observed. Interestingly, both proteins of transcriptionally activating and repressing complexes were found to be interacting with CIITA. This was not entirely unexpected since histone deacetylases were previously determined to interact with

CIITA (Zika et al., 2003). However, because CIITA is well known for its role in transcriptional activation, it would be interesting to further uncover how these repressive complexes balance the activating function of CIITA.

Despite repeated efforts to enhance the CIITA complex purification efficiency, the magnetic bead matrix did not yield significant results. The different CIITA constructs led to less functional proteins, either due to being on the C-terminus of the protein or perhaps by hampering protein interactions due to the greater bulk of the epitope tag. The degree of sensitivity to detect proteins by LC/MS/MS may also be a reason that no proteins greater than background were observed. To gain a global perspective of the CIITA interacting proteins, technological advances in MS peptide detection, and protein purification may be required.

Figure Legends

Figure 3-1. Plasmid maps of the CIITA expression constructs.

The pREP4-BLRP-CIITA plasmid contains the N-terminally BLRP (wild-type and mutant) tagged CIITA and BirA expression sequences along with a bacterial origin of replication, antibiotic resistance, EBNA-1 and OriP for extrachromosomal maintenance in the eukaryotic cell, and a hygromycin resistance gene for selection after eukaryotic cell transfection. The pREP4-BLRP-CIITA-XL9 construct contains an additional *XL9* sequence, downstream of the BirA gene. pREP4-CIITA-BLRP-XL9 is a construct that expresses CIITA with a C-terminal BLRP tag. pREP4-2F2B-CIITA-XL9 was constructed to have two tandem FLAG epitope tag sequences and two tandem BLRP sequences (wild-type only) at the N-terminus of CIITA.

Figure 3-2. Inclusion of the *XL9* sequence enhances long-term retention of the episomal plasmid.

Cells stained with anti-HLA-DR and propidium iodide (PI) were gated for PI- cells and the contour plots for forward scatter and HLA-DR are presented. (A) Raji and RJ2.2.5 were tested as positive and negative controls to determine the range of HLA-DR expression levels. (B) RJ2.2.5 cells transfected with pREP4-BLRP-empty, pREP4-BLRP-CIITA, pREP4-BLRP-empty-XL9, or pREP4-BLRP-CIITA-XL9 and cultured for 20, 40, and 60 days.

Figure 3-3. *XL9* enhances expression of the N-terminal BLRP tagged CIITA construct.

(A) Expression of CIITA and BirA were compared in pREP4-BLRP-CIITA and pREP4-BLRP-CIITA-XL9 stably transfected RJ2.2.5 cells by immunoblot detection. Untransfected RJ2.2.5 and Raji are shown as negative and positive controls. Anti-beta-actin immunoblots are shown as the loading control for each lane. Molecular weight markers are shown on the right side of each panel. (B) Different BLRP tagged CIITA constructs are compared for CIITA expression levels as shown by immunoblot. Constructs were expressed in Cos-7 cells and prepared as whole cell lysates.

Figure 3-4. CIITA interacting proteins complex purification and verification.

(A) Nuclear extracts prepared from RJ2.2.5 cells stably transfected with pREP4-BLRP-empty-XL9 or pREP4-BLRP-CIITA-XL9 were precipitated with streptavidin coated agarose beads. 5 % and 10 % of the pulldown was loaded in the wells as indicated and visualized by silver staining. (B) These nuclear extracts and pulldown samples were also subjected to

immunoblot of CIITA, RFX5, CTCF, and WDR5. 10 % input nuclear extract loaded lanes show that the amount of starting materials was equal.

Figure 3-5. Expression of *CIITA* is reduced when tagged with 2F2B.

The mRNA expression levels were measured by qRT-PCR. RJ2.2.5 and Raji cells are shown as negative and wild-type controls. Expression levels of *CIITA* and *HLA-DRA* are shown by % 18S rRNA x 10^5 as a relative value. DR+ samples indicate that the cells expressing HLA-DR were selected by magnetic bead separation.

Table 3-1. Cloning primers for the BLRP constructs.

Table 3-2. Candidates for a CIITA interacting *protein* complex.

Proteins identified by LC/MS/MS and determined as more abundant in the samples purified from the CIITA expressing cells are listed in order of greatest ΔSC. Blue shaded rows are proteins that were previously reported to interact with CIITA. Red shaded proteins were found in two independent MS results.

Table 3-3. Gene ontology of the candidate CIITA complex proteins.

CIITA interacting proteins determined by LC/MS/MS were characterized for their known functions by the gene ontology clustering online tool, DAVID (the Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources 6.7.

Figure 3-1

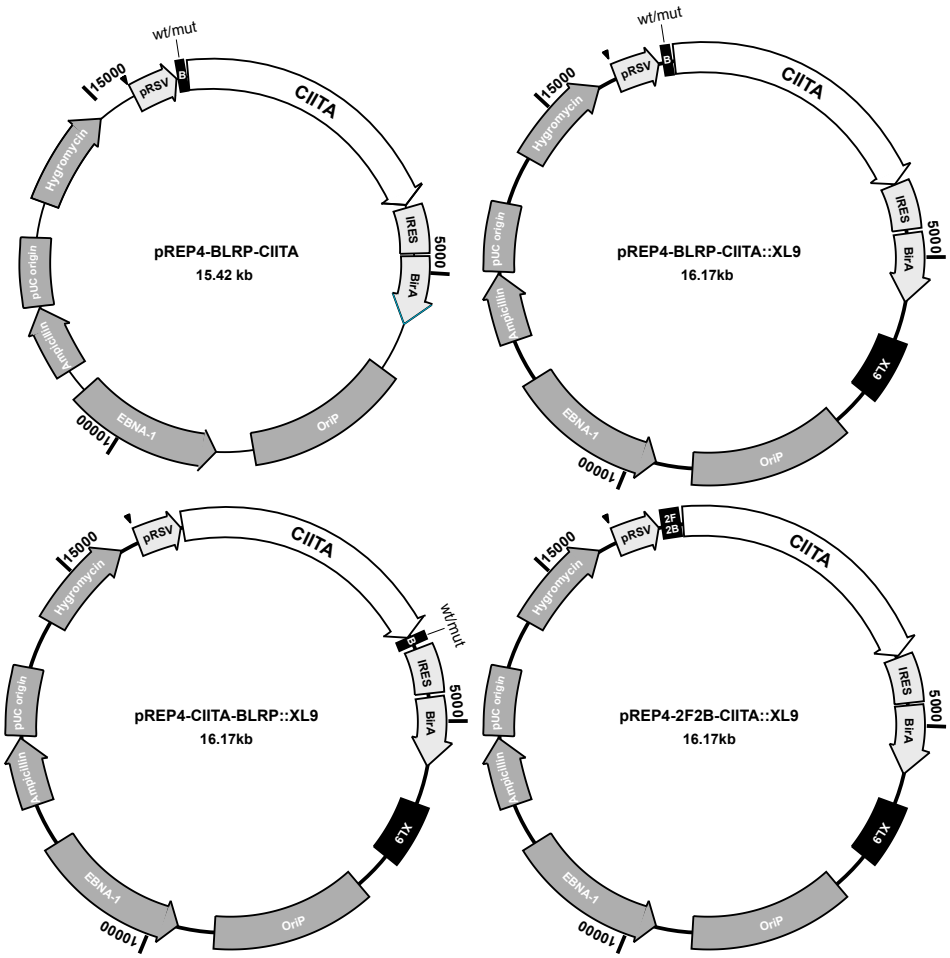

pREP4-BLRP-CIITA
15.42 kb
pREP4-BLRP-CIITA::XL9
16.17kb
pREP4-CIITA-BLRP::XL9
16.17kb
pREP4-2F2B-CIITA::XL9
16.17kb
wt/mut
pRSV
CIITA
IRES
BirA
6'L9
OriP
EBNA-1
pUC origin
Ampicillin
Hygromycin
15000
5000
10000
2F
2B

Figure 3-2

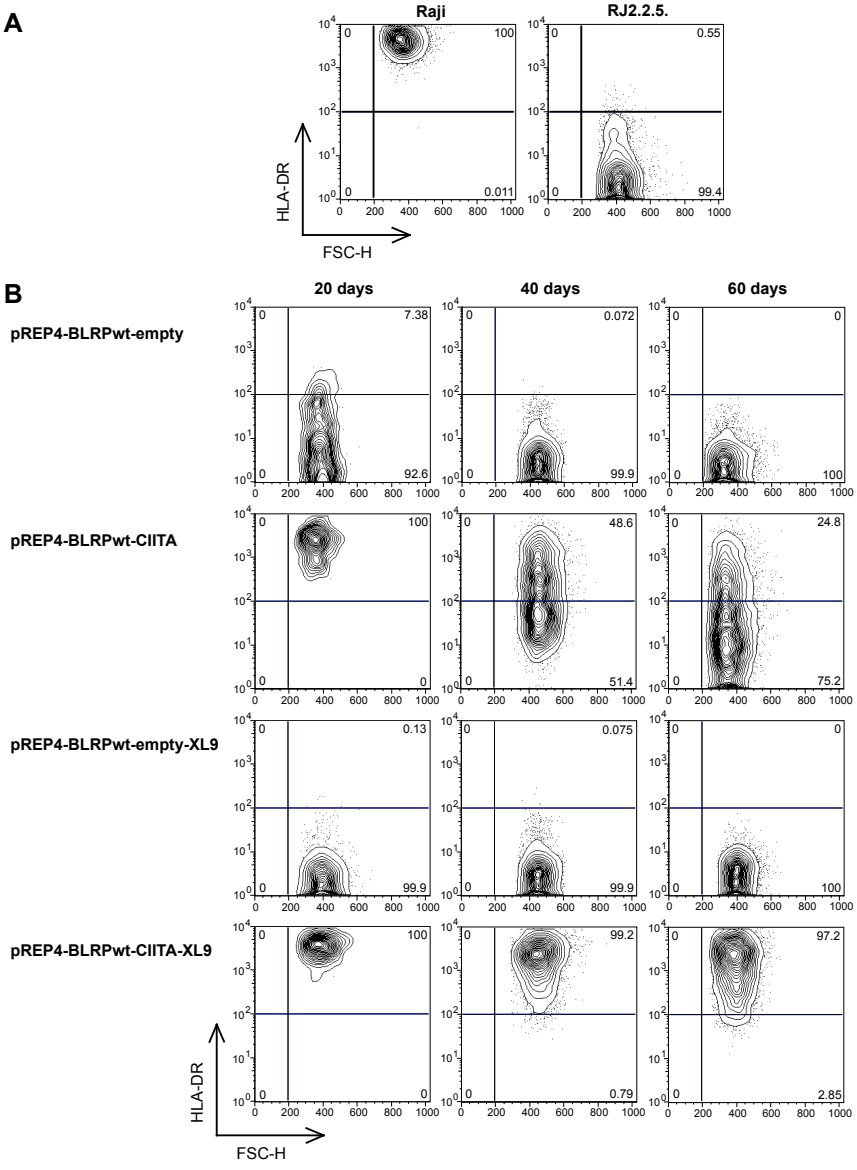

A
Raji
RJ2.2.5.
100
0.011
0.55
99.4
HLA-DR
FSC-H
B
20 days
40 days
60 days
pREP4-BLRPwt-empty
7.38
92.6
0.072
99.9
0
100
pREP4-BLRPwt-CIITA
100
0
48.6
51.4
24.8
75.2
pREP4-BLRPwt-empty-XL9
0.13
99.9
0.075
99.9
0
100
pREP4-BLRPwt-CIITA-XL9
100
0
99.2
0.79
97.2
2.85
HLA-DR
FSC-H

Figure 3-3

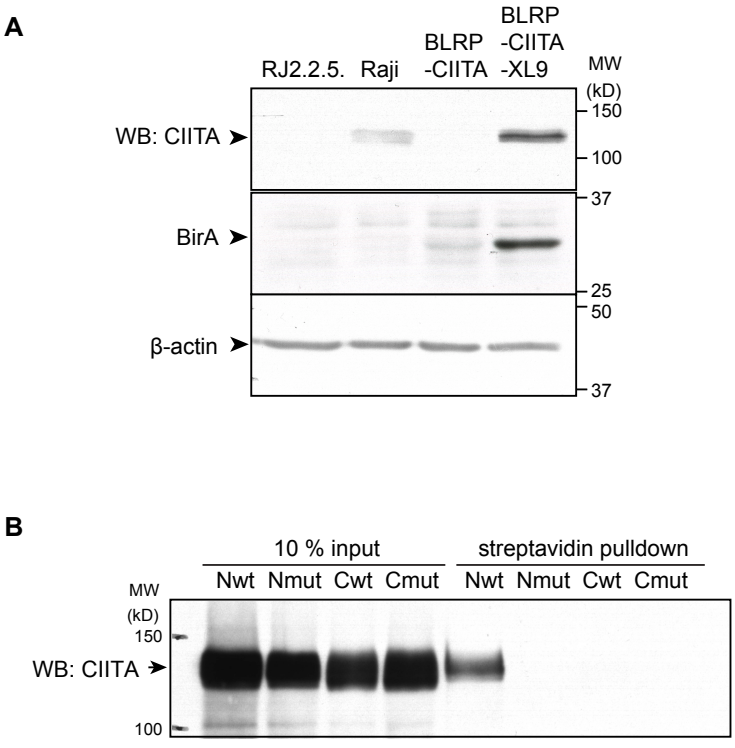

A
RJ2.2.5.
Raji
BLRP -CIITA
BLRP -CIITA -XL9
MW (kD)
WB: CIITA
BirA
β-actin
150
100
37
25
50
37
B
10 % input
streptavidin pulldown
Nwt Nmut Cwt Cmut
Nwt Nmut Cwt Cmut
MW (kD)
150
WB: CIITA
100

Figure 3-4

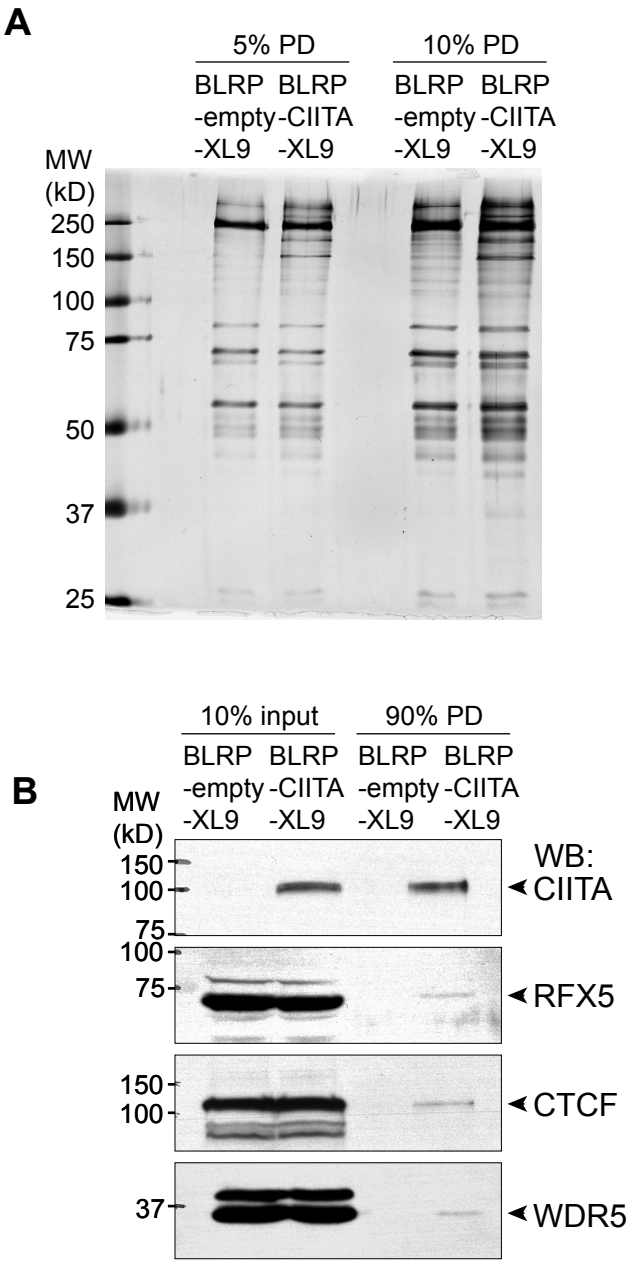

A
5% PD
10% PD
BLRP -empty -XL9
BLRP -CIITA -XL9
BLRP -empty -XL9
BLRP -CIITA -XL9
MW (kD)
250
150
100
75
50
37
25
B
10% input
90% PD
BLRP -empty -XL9
BLRP -CIITA -XL9
BLRP -empty -XL9
BLRP -CIITA -XL9
MW (kD)
150
100
75
100
75
150
100
37
WB:
CIITA
RFX5
CTCF
WDR5

Figure 3-5

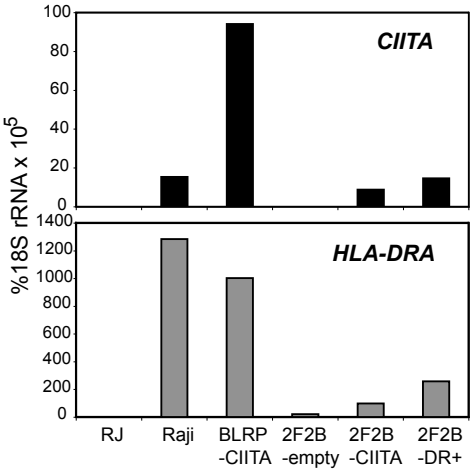

CIITA
HLA-DRA
%18S rRNA x 10^5
100
80
60
40
20
0
1400
1200
1000
800
600
400
200
0
RJ
Raji
BLRP -CIITA
2F2B -empty
2F2B -CIITA
2F2B -DR+

Table 3-1

BLRP cloning primers

BLRPwt-fwd	5'-TGG GTA CCA GCT GCT AGC ACC ATG GCT GGT GGC CTC AAC GAC ATC TTC GAG GCC CAG AAG ATC GAG TGG CAT GAA GAT ACT GGT GGA TCA GAG AAT TTG TAT TTT CAG TCT CAT CAT CAC CAT CAC CAT GCT AGC GGC CGC ATG CGT-3'
BLRPwt-rev	ACG CAT GCG GCC GCT AGC ATG GTG ATG GTG ATG ATG AGA CTG AAA ATA CAA ATT CTC TGA TCC ACC AGT ATC TTC ATG CCA CTC GAT CTT CTG GGC CTC GAA GAT GTC GTT GAG GCC ACC AGC CAT GGT GCT AGC AGC TGG TAC CCA
BLRPmut-fwd	TGG GTA CCA GCT GCT AGC ACC ATG GCT GGT GGC CTC AAC GAC ATC TTC GAG GCC CAG AGG ATC GAG TGG CAT GAA GAT ACT GGT GGA TCA GAG AAT TTG TAT TTT CAG TCT CAT CAT CAC CAT CAC CAT GCT AGC GGC CGC ATG CGT
BLRPmut-rev	ACG CAT GCG GCC GCT AGC ATG GTG ATG GTG ATG ATG AGA CTG AAA ATA CAA ATT CTC TGA TCC ACC AGT ATC TTC ATG CCA CTC GAT CCT CTG GGC CTC GAA GAT GTC GTT GAG GCC ACC AGC CAT GGT GCT AGC AGC TGG TAC CCA
BirA fwd	GCC GGC AAG GCC GGA TCC ACC ATG AAG GAT AAC ACC GTG CCA C
BirA rev	CTT ATC ATG TCT GGA TCC TTA TTT TTC TGC ACT ACG CAG GG
IRES fwd	TGC TAG CGG CCG CTC GAG GAA TTC TCT CCC TCC CCC CCC CCT AAC GTT A
IRES rev	GGC CTT GCC GGC CTC GAG TGT GGC CAT ATT ATC ATC GTG TTT TTC
NotI-CIITA fwd	TCA CCA TGC TAG CGG CCG CAT GCG TTG CCT GGC TCCA
NotI-CIITA rev	GAA TTC CTC GA GCG GCC GCT CAT CTC AGG CTG ATC CGT GAA TC
NheI-CIITA fwd	TGG GTA CCA GCT GCT AGC CAC CAT GCG TTG CCT GGC TCC A
CtermBLRPwt rev	GAA TTC CTC GAG CGG CCG CTC AAT GCC ACT CGA TCT TCT GGG CCT CGA AGA TGT CGT TGA GGC CCT GAA AAT ACA AAT TCT CAT GGT GAT GGT GAT GAT GTC CTC TCA GGC TGA TCC GTG AAT C
CtermBLRPmut rev	GAA TTC CTC GAG CGG CCG CTC AAT GCC ACT CGA TCC TCT GGG CCT CGA AGA TGT CGT TGA GGC CCT GAA AAT ACA AAT TCT CAT GGT GAT GGT GAT GAT GTC CTC TCA GGC TGA TCC GTG AAT C
XL9 fwd	CAT GAG GTC GAC TCT AGA TGC TTC CTT TCA GTG TCC AAG TG
XL9 rev	GGG GAT CGA TCC TCT AGA GGC CAG CCA CAC AGA GTT AGG GC
2F2Bwt fwd	GGG TAC CAG CTG CTA GCT ATG GCT GAC TAC AAA GAC GAT GAC GAC AAG GAC TAC AAA GAC GAT GAC GAC AAG GCT GGT GGC CTC AAC GAC ATC TTC GAG GCC CAG AAG ATC GAG TGG CAT GGC CTC AAC GAC ATC TTC GAG GCC CAG AAG ATC GAG TGG CAT GCT AAG CAG ATC TAC CGG TTG GCT AGC GGC CGC ATG CG
2F2Bwt rev	CGC ATG CGG CCG CTA GCC AAC CGG TAG ATC TGC TTA GCA TGC CAC TCG ATC TTC TGG GCC TCG AAG ATG TCG TTG AGG CCA TGC CAC TCG ATC TTC TGG GCC TCG AAG ATG TCG TTG AGG CCA CCA GCC TTG TCG TCA TCG TCT TTG TAG TCC TTG TCG TCA TCG TCT TTG TAG TCA GCC ATA GCT AGC AGC TGG TAC CC

Table 3-2

Mass Spectrometry Results Summary

score	gene ID	description
37	SMARCA5/ISWI/hSNF2H	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a5
29	DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9
15	TCP1/TCP-1-alpha	T-complex protein 1 isoform a
10	PPP1R10/PNUTS/PP1R10	protein phosphatase 1, regulatory subunit 10
10	CHD4/Mi-2b	chromodomain helicase DNA binding protein 4
10	CIITA	class II transactivator
8	HDAC1/RPD3L1	histone deacetylase 1
8	ESF1	ABT1-associated protein
8	KIF20B/CT90/MPP1	M-phase phosphoprotein 1
7	DDX46/Prp5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46
7	HDAC2/RPD3	histone deacetylase 2
7	ZBTB33/ZNF-kaiso	kaiso
6	CTCF	CCCTC-binding factor
6	ZFR/ZFR1	zinc finger RNA binding protein
5	TOX4/LCP1	epidermal Langerhans cell protein LCP1
5	THRAP3/TRAP150	thyroid hormone receptor associated protein 3
4	ACIN1/CAN	apoptotic chromatin condensation inducer 1
4	BAZ1B/WSTF	bromodomain adjacent to zinc finger domain, 1B
4	WDR82	WD repeat domain 82
4	RBBP7	retinoblastoma binding protein 7
3	GATAD2A/p66alpha	GATA zinc finger domain containing 2A
3	RBM26	RNA binding motif protein 26
3	PPP1CC/PP1gamma	protein phosphatase 1, catalytic subunit, gamma isoform
3	NOLC1	nucleolar and coiled-body phosphoprotein 1
3	NEK9	NIMA related kinase 9
3	SMC3	structural maintenance of chromosomes 3
2	RANBP2/NUP358	RAN binding protein 2
2	USP36/DUB1	ubiquitin specific protease 36
2	RRP12	ribosomal RNA processing 12 homolog
2	BRD4	bromodomain-containing protein 4 isoform long
2	HCFC1/HCF1	host cell factor 1
2	WDR11/BRWD2	bromodomain and WD repeat domain containing 2
2	ZNF22	zinc finger protein 22 (KOX 15)
2	RPL23	ribosomal protein L23
1	MAP7D3	MAP7 domain containing 3
1	LRWD1	leucine-rich repeats and WD repeat domain containing 1
1	SMC1A/SMC1	structural maintenance of chromosomes 1A
1	PRSS1	protease, serine, 1 preproprotein
1	USP13	ubiquitin specific protease 13 (isopeptidase T-3)
1	BAZ2A	bromodomain adjacent to zinc finger domain, 2A

Table 3-3

Gene Ontology clustering results

Annotation Cluster 1	Enrichment Score: 10.81			
Term	Count	%	PValue	Genes
GO:0031981~nuclear lumen	22	59.46	1.78E-13	DHX9, ZBTB33, RRP12, HCFC1, CTCF, RBBP7, PPP1CC, ZNF22, SMC3, ESF1, NOLC1, HDAC1, RPL23, THRAP3, SMARCA5, KIF20B, GATAD2A, USP36, ACIN1, BRD4, BAZ2A, CHD4
Annotation Cluster 2	Enrichment Score: 3.15			
GO:0000166~nucleotide binding	14	37.84	7.65E-04	CIITA, DHX9, TCP1, SMC3, BAZ1B, NOLC1, THRAP3, SMARCA5, KIF20B, NEK9, ACIN1, SMC1A, CHD4, RBM26
Annotation Cluster 3	Enrichment Score: 2.58			
IPR001487:Bromodomain	3	8.11	2.83E-03	BAZ1B, BRD4, BAZ2A
Annotation Cluster 4	Enrichment Score: 2.50			
GO:0006350~transcription	15	40.54	5.60E-05	CIITA, ZBTB33, PPP1R10, HCFC1, CTCF, RBBP7, ZNF22, ESF1, HDAC1, BAZ1B, THRAP3, SMARCA5, GATAD2A, BAZ2A, CHD4
GO:0051276~chromosome organization	10	27.03	6.64E-07	BAZ1B, HDAC1, SMARCA5, CTCF, ACIN1, RBBP7, SMC1A, BAZ2A, SMC3, CHD4
GO:0016581~NuRD complex	4	10.81	3.63E-06	HDAC1, GATAD2A, RBBP7, CHD4
GO:0000118~histone deacetylase complex	4	10.81	1.29E-04	HDAC1, GATAD2A, RBBP7, CHD4
Annotation Cluster 5	Enrichment Score: 2.14			
GO:0007049~cell cycle	7	18.92	6.35E-03	NOLC1, KIF20B, HCFC1, NEK9, PPP1CC, SMC1A, SMC3
Annotation Cluster 6	Enrichment Score: 1.78			
IPR002464:DNA/RNA helicase, ATP-dependent, DEAH-box type, conserved site	3	8.11	2.69E-03	DHX9, SMARCA5, CHD4
Annotation Cluster 7	Enrichment Score: 1.73			
GO:0000793~condensed chromosome	5	13.51	2.52E-04	BAZ1B, SMARCA5, CTCF, SMC1A, SMC3
Annotation Cluster 8	Enrichment Score: 1.67			
IPR019781:WD40 repeat, subgroup	4	10.81	1.20E-02	WDR11, WDR82, RBBP7, LRWD1
Annotation Cluster 9	Enrichment Score: 1.45			
GO:0016563~transcription activator activity	6	16.22	2.28E-03	CIITA, HDAC1, THRAP3, SMARCA5, HCFC1, CTCF
Annotation Cluster 10	Enrichment Score: 1.27			
GO:0008270~zinc ion binding	11	29.73	3.00E-02	ZBTB33, BAZ1B, GATAD2A, PPP1R10, CTCF, RANBP2, BAZ2A, CHD4, ZNF22, RBM26, USP13
Annotation Cluster 11	Enrichment Score: 1.00			
GO:0006606~protein import into nucleus	3	8.11	1.55E-02	RPL23, PPP1R10, RANBP2

Chapter 4. The role of a single nucleotide polymorphism within the MHC-II gene, *HLA-DRA*, in Parkinson's disease

Nancy M. Choi*, George T. Kannarkat*, Jae Kyung Lee*, Malú G. Tansey, Jeremy M. Boss

Conceived and designed the experiments: JMB MGT JKL NMC GTK. Performed the experiments: JKL NMC GTK. Analyzed the data: JKL NMC GTK. Wrote the chapter: NMC.

* These authors equally contributed to this work

Introduction

In a recent genome wide association study (GWAS) of Parkinson's disease (PD), a single nucleotide polymorphism (SNP) was identified to have high correlation with late-onset sporadic PD (Hamza et al., 2010). This SNP (rs3129882; *PARK18*) was located within the first intron of *HLA-DRA*, which suggested a role for *HLA-DRA* or other MHC-II genes in the disease progression of PD. This prompted further inquiring of whether there were any differences in the expression of MHC-II genes in PD patients in relation to the sequence of the SNP, and if so, what regulatory mechanisms would be involved.

PD is one of the most common neurodegenerative diseases, only second to Alzheimer's disease (Tansey and Goldberg, 2010). The most distinguishing pathological manifestation is the formation of proteinaceous inclusions called 'Lewy bodies' in the substantia nigra (SN) of the midbrain, primarily formed of the protein α-synuclein, and a mixture of other ubiquitylated proteins (Goldman et al., 1983; Kuusisto et al., 2003). While certain protein mutations have been associated with the development of Lewy bodies, how the accumulation of these proteins start to occur or whether they are the cause of the neurological degeneration are still under investigation. The immune reaction towards Lewy bodies and a sustained inflammatory cytokine environment are thought to be a cause of the severe loss of dopaminergic neurons associated with PD (Castaño et al., 1998).

Chronic inflammation of the central nervous system (CNS) has been associated with many of these neurodegenerative diseases of aging (Block and Hong, 2005). Neuroinflammation is also another hallmark of PD. Early in the disease process, a transient activation of the glial cells occurs, and due to environmental or genetic cues, this activation becomes sustained (McGeer and McGeer, 1998). This prolonged activation of glial cells leads to accumulation of pro-inflammatory cytokines, activation of the nuclear factor kappa

B (NFκB) pathway, and induced production of reactive oxygen species (McGeer and McGeer, 1998). These processes together are suggested to be possible causes of death for the dopaminergic neurons and degeneration of the SN.

While it was previously considered that the CNS was 'immune privileged' and impenetrable to immune cells, this is no longer considered to be the case. In normal conditions, mature B and T lymphocytes cross the blood brain barrier (BBB) and circulate throughout the CNS (Stolp and Dziegielewska, 2009). When exposed to pro-inflammatory cytokines, the permeability of the BBB is enhanced and an elevated number of immune cells are observed in the CNS (Rite et al., 2007). It is hypothesized that the accumulation of α-synuclein may lead to an increased pro-inflammatory environment, causing infiltration of macrophages and lymphocytes, as well as IgG that recognize α-synuclein within the CNS. This in turn enhances the opsonization of dopaminergic neurons and other damaged cells leading to a greater degree of neuronal cell death. These suggest a role of the adaptive immune system having a role in disease progression of PD. There are reports of elevated levels of HLA-DR and HLA-DQ in monocytes in patients with PD (Lampe et al., 2003). However, how these are connected to the pathological symptoms of PD is unknown.

From the finding of *PARK18* as a highly correlative genetic marker of PD (Hamza et al., 2010), it was hypothesized that there would be a novel cis-regulatory element associated with the SNP that regulates the MHC-II genes, further supporting the role of the adaptive immune system during PD development. Whether *PARK18* regulates *HLA-DRA* itself, or acts as a long-range enhancer or repressor element adjusting the expression of another gene will have to be determined. The goal of this project was to first determine whether there are any significant differences in MHC-II mRNA or surface protein expression between normal subjects and PD patients. Simultaneously, the first intron of *HLA-DRA* was sequenced in

multiple cell lines and dissected into smaller fragments to determine if there were any regulatory elements within this region. These investigations will not only lead us to a better understanding of the MHC-II locus in general, but also allow us to explain the role of the adaptive immunity in Parkinson's disease.

Materials and Methods

Patient Samples and Cell Lines

The Collaborative Research In Neuroscience (CRIN) consortium collected whole blood samples from Parkinson's disease and control subjects. The subjects were genotyped for rs3129882 (*PARK18*) and demographical data was collected and stored in the CRIN database. Cell lines used in the study, Raji, THP-1, U937, TUR, and H929, were acquired from the American Type Culture Collection (ATCC). Monomac 6 cells can be obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). RJ2.2.5 cells are CIITA null mutants derived from Raji cells by γ-ray irradiation and negative for MHC-II expression (Accolla, 1983). Raji and RJ2.2.5 cells were cultured in RPMI (Life Technologies, Carlsbad, CA) supplemented with 5 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 5 % bovine calf serum (Life Technologies, Carlsbad, CA), and penicillin/streptomycin (Life Technologies, Carlsbad, CA). THP-1, U937, TUR, and H929 cells were cultured in RPMI supplemented with 10 % fetal bovine serum, penicillin 100 U/ml streptomycin 100 μg/ml (Life Technologies, Carlsbad, CA), non-essential amino acids (Life Technologies, Carlsbad, CA), and 1mM sodium pyruvate (Life Technologies, Carlsbad, CA). Monomac 6 cells were cultured in RPMI 1640 supplemented

with 10 % fetal bovine serum, penicillin 200 U/ml streptomycin 200 ug/ml, non-essential amino acids, and OPI-supplement (Sigma-Aldrich, St. Louis, MO).

Cell separation and interferon-γ treatment

From each control and PD subject, 50 ml of whole blood is drawn. This is diluted in a 1:1 ratio with Dulbecco's Phosphate Buffered Saline (DPBS) and layered on top of ficoll in a conical tube. The samples are centrifugated at 500 x g for 30 min and the layer at the interface is collected; these are the peripheral blood mononuclear cells (PBMC). These cells were further separated by CD19 or CD14 positive selection MACS beads (Miltenyi, Germany) according to the manufacturers recommendations to purify the B and monocyte population of cells, respectively. The monocytes are further divided into two populations, one untreated and one population treated with interferon-γ (IFN-γ) for 24 hours, and also cells that were not plated onto culture cells and passed on directly to the next steps. The IFN-γ treatment concentrations ranged from 12.5 – 1000 U/ml and are indicated in each figure. These separated cells are then either stained immediately with cell type specific antibodies for flow cytometry analysis, or collected for RNA and DNA samples.

Flow cytometry

The PBMC cells were washed in FACS buffer (1 x PBS pH 7.4, 2 mM EDTA, 1 % BSA, 0.1 % sodium azide, 0.2 μm filter sterilized). Then stained with isotype control antibodies or the combination of CD19-PerCP, HLA-DR-APC, HLA-DQ-FITC or CD14-PE, HLA-DR-APC, HLA-DQ-FITC. The separated cell types were verified for the purity of the population and MHC-II expression by staining for CD19-PerCP, HLA-DR-APC, and HLA-DQ-FITC for B cells, and CD14-PE, HLA-DR-APC, and HLA-DQ-FITC for monocyte

populations. Isotype control antibodies, IgG2a Mouse Fc APC, IgG1 Mouse Fc FITC, IgG1 Mouse Fc PerCP, and IgG2a Mouse Fc PE, and PerCP anti-human CD19, PE anti-human CD14 antibodies were purchased from Biolegend (San Diego, CA). APC anti-human HLA-DR and FITC anti-human HLA-DQ were purchased from BD Biosciences (San Jose, CA). The BD FACSCalibur™ flow cytometer was used for data collection and the FlowJo computer software (Tree Star, Inc., Ashland, OR) was used for data analyses. Statistical analyses were also conducted using the statistical software program GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA).

RNA preparation and reverse transcription PCR

The purified B cells, monocytes, and the residual non-BM cells were each washed and collected in 1 X PBS. Cells were resuspended in RLT buffer provided in the RNeasy mini kit (QIAGEN, Germany) and lysed by passing through the QIAshredder column (QIAGEN, Germany), and was used to extract RNA. RNA concentration was measured using the Nanodrop 2000 (Thermo Fisher, Wilmington, DE) spectrophotometer. 0.5 – 2 μg of RNA were used for each reverse transcription reaction in a final volume of 20 μl. RNA was treated with 0.5 U DNase (RQ1 RNase-Free DNase, Promega, Madison, WI) for 30 min at 37 C° with 20 U RNAse inhibitor (Roche, Germany) in RT buffer 1 (50 mM KCl, 10 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$, 0.001% gelatin). After deactivation of the DNase by heating to 75 C° for 5 min, a second RT buffer 2 (50 mM KCl, 10 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$, 0.001% gelatin, 10 mM dNTP, 2.5 μM random hexamer, 2.5 μM oligo dT_{16}) and 100 U SuperScript® II Reverse Transcriptase (Life Technologies, Carlsbad, CA) is added to each reaction. This reaction is heated to 24 C° for 10 min for the annealing, 42 C° 40 min for elongation, and 95 C° 5 min for the inactivation step. The reactions were diluted to 50 –

200 μl with DNase/RNase free dionized water, according to the amount of starting material. To quantify the level of specific mRNA Real-time PCRs using the primers listed in Table 4-1 were performed using the RT reaction product as 10% of the reaction volume. qPCR for 18S rRNA was conducted simultaneously for data normalization, calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

Genomic DNA extraction and plasmid cloning

Cells are washed with PBS and resuspended in 1 x TE (10 mM Tris pH 8.9, 1 mM EDTA). Lysis buffer (20 mM Tris pH 8.0, 4 mM EDTA, 20 mM NaCl, 1 % SDS) supplemented with proteinase K (1 mg/ml) and RNase A (1 mg/ml) is added. This mixture is incubated in a 65 C° water bath overnight to fully digest the cells with proteinase K. This is sonicated briefly for 2 seconds to facilitate the phenol chloroform extraction. An equal volume of PCIA (25:24:1) is added to the digest and genomic DNA is cleaned; the upper layer is taken and this step may be repeated until the interface becomes clear. The genomic DNA is precipitated and washed with 70 % ethanol and stored in 1 x TE. The genomic DNA (gDNA) is used as a template for PCR reactions to clone the first intron of *HLA-DRA* into either pCR2.1®-TOPO® TA vector (Life Technologies, Carlsbad, CA) or the pGL3-promoter luciferase plasmid (Promega, Madison, WI). A high fidelity polymerase is used for the cloning PCR reactions, Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA), according to manufacturers recommendations using 50 ng of gDNA or 10 ng BAC DNA (RP11-974L24) as template. When cloning into the TA vectors, conventional Taq polymerase is added to the final reaction and an incubated at 68 C° for an additional 10 min on the thermocycler to ensure the addition of adenine residues to the 3' end of the amplicons. Primers used for cloning are listed in Table 4-1. The PCR is optimized to

produce a single band, but if unobtainable, a gel extraction is performed by electrophoresis of the PCR sample on a 1 % agarose gel, excising the band, and extract DNA using the QIAEX II Gel Extraction Kit (QIAGEN, Germany). These fragments are inserted into the TA vector using the TOPO® TA Cloning® Kit (Life Technologies, Carlsbad, CA) according to the manufacturers recommendations. Fragments were inserted into pGL3-promoter vector between restriction enzyme sites *Kpn*I and *Nhe*I. The clones are verified by restriction enzyme digestion and by sequencing the insert.

Transfections and dual luciferase assays

THP-1 and Jurkat cells were transfected using the Lipofectamine® 2000 (Life Technologies, Carlsbad, CA). The reaction mixture is prepared in Opti-MEM® Reduced Serum Medium (Life Technologies, Carlsbad, CA) with 1 μg of plasmid DNA and 3 μl of transfection reagent for transfection of 5 x 10^5 cells. For Raji cells, 1 x 10^6 cells were transfected using the Cell Line Nucleofector® Kit V (Lonza, Switzerland) with 2 μg of plasmid DNA and 100 μl of nucleofection reagent. Transfection efficiency was tested with pcDNA3.1-HA-EGFP plasmid. For the dual luciferase assays, Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) was used according to the manufacturers recommendation.

Results

Cell line characterization and sequencing

Microglial cells are the resident macrophages of the brain. As these cells are difficult to isolate from human subjects, in all initial molecular characterizations, the circulating immune cells collected by a blood draw would be used. Therefore, it was important to establish a model macrophage cell line to use for in vitro experiments. Human macrophage cell lines

available from ATCC were purchased and tested for the expression level of MHC-II and the degree of gene induction when treated with IFN-γ. MHC-II expression in U937, TUR, monomac 6, and THP-1 cells were measured by both qRT-PCR and flow cytometry for MHC-II expression. When the cells were treated with IFN-γ, the monomac 6 and THP-1 cells had the greatest degree of gene induction by both measures (Figure 4-1A, B).

The transfection efficiency of the cell lines was also tested for transgene expression. A plasmid encoding the human enhanced green fluorescent protein (EGFP) was transfected into the above cell lines and degree of EGFP expression was measured by flow cytometry. THP-1 had the greatest level of EGFP expression (Figure 4-1C). Therefore, together with the gene induction results, the THP-1 cells were chosen as the model monocytic cell line for further in vitro experiments.

The dominant base for *PARK18* (rs3129882) is adenine and the risk allele a guanine, with a risk allele frequency of 0.40 ($p = 2 \times 10^{-10}$). To determine whether the different cell lines carried an adenine or guanine at *PARK18*, the first intron of *HLA-DRA* were cloned and sequenced for a B cell, monocyte, and plasma cell line, Raji, THP-1, and H929. Also, the first introns were cloned from a bacterial artificial chromosome (BAC) that includes the *HLA-DRA* gene, as well as a genomic DNA sample from a human subject and sequenced. As shown in table 5-1, the different alleles from each sample contain a diverse combination of single nucleotide polymorphisms, including *PARK18*.

Reporter constructs were designed to search for internal cis-regulatory fragments

In a manuscript published in 1987, there was a report on the first intron of *HLA-DRA* containing a cell type specific transcriptional enhancer (Wang et al., 1987). The enhancer was able to enhance transgene expression in B and T lymphocytes regardless of their normal

expression levels of HLA-DR, but not in fibroblast cells despite being capable of induction with IFN-γ treatment. To verify and possibly find novel cis-regulatory elements within intron 1 of *HLA-DRA*, this region was divided into smaller fragments and cloned upstream of the luciferase gene (Figure 4-2).

The plasmids including the fragments 1 through 6 were transfected into Jurkat, THP-1, and Raji cells. When the entire first intron is present, the expression of luciferase is reduced in the tested cell types. Fragments 4 and 5 had activation activity in the Jurkat cells. However, fragment 2 that contains the region that was reported previously as the cell type specific enhancer (Wang et al., 1987) did not have any enhancer activity. Additional fragments 7 and 8 were constructed but are yet to be tested.

MHC-II gene expression in Parkinson's disease patients

In collaboration with the Emory University CRIN (Collaborative Research In Neuroscience) consortium, whole blood samples were collected from PD and age matched control subjects. At the current stage of sample collection, a total of 7 females and 1 male participated as control volunteers; 6 female and 13 male PD participants provided blood samples. The demographic data are shown in Table 5-5. There was no significant difference in age of onset or diagnosis (Figure 4-3). The distribution between sexes and different age groups were distributed equally for these criteria. The total number of lymphocytes collected was not significantly different between control and PD participants. Isolated B cells were significantly lower in PD patients when measured as number of B cells per volume of whole blood, but this reduction was not significant when measured as percent PBMC. Monocyte numbers were not significantly different between the two groups.

HLA-DR and HLA-DQ expression in PD patient samples

The surface expression of HLA-DR and HLA-DQ were assessed in B cells and monocytes, with and without IFN-γ treatment. HLA-DR expression levels were essentially equal in the two participant groups in both B cells and untreated monocytes (Figure 4-4A). The monocytes were treated with increasing concentrations of IFN-γ, and while there was a trend of lower DR expression with increasing IFN-γ there was no significant difference. Similarly, there was no significant difference between the B cells and untreated monocytes in HLA-DQ surface expression (Figure 4-4B). Again, while there was a trend of contrastingly higher expression of HLA-DQ in IFN-γ treated monocytes there was no statistically significant difference between the two groups.

There was no difference in the percentage of B cells or untreated monocytes expressing only one or both MHC-II complexes (Figure 4-5). Interestingly, when monocytes were treated with increasing concentrations of IFN-γ, there were fewer cells expressing HLA-DR or HLA-DQ only (Figure 4-5A, B), and more cells expressing both MHC-II molecules (Figure 4-5C).

MHC-II gene expression was also examined by quantitative RT-PCR to measure the steady state mRNA levels (Figure 4-6). In B cells, a higher level of HLA-DRB1 expression was observed but no difference in the other observed genes. In monocytes, both unstimulated and IFN-γ stimulated cells, there was also a higher level of HLA-DRB1, but also greater levels of HLA-DQB1. There were no other observed differences of MHC-II gene expression between the two groups of participants.

Discussion

PARK18, a single nucleotide polymorphism, was found highly correlated with the diagnosis of late-onset Parkinson's disease in patients. As this SNP was located within the first intron of an MHC-II gene, *HLA-DRA*, it was hypothesized that misexpression of *HLA-DRA* or another MHC-II gene may be associated with PD disease progression. To determine the role of MHC-II genes in PD in relation to their *PARK18* genotype, patient and control samples were collected and tested for MHC-II expression.

In the current state of an ongoing project, there does not seem to be a great difference in the HLA-DR or DQ expression levels in B cells or resting monocytes. When the monocytic lineage cells are treated with IFN-γ however, there is a trend of less HLA-DR surface expression and more HLA-DQ expression, as seen from the flow cytometry data. This is also reflected in the results where a lower percentage of cells expressing HLA-DR only were observed, while cells expressing only HLA-DQ or both HLA-DR and DQ increased in proportion to the population of cells extracted from whole blood. The mRNA levels revealed similar results, as there was enrichment for HLA-DQB1 expression in PD patients. However, HLA-DRB1 levels were also increased in monocytes and B cells rendering the results difficult to explain. The greatest limitation to the study in its current state is the small number of subjects in both patient and control groups. To obtain greater statistical power in the analyses, there will have to be a larger number of participants for both groups of approximately fifty subjects total for each group,

To examine the possibility of a novel cis-regulatory element within the first intron of *HLA-DRA*, reporter constructs were cloned. The results from the dual luciferase assay suggest a possible negative regulating element within this region, however, the results need to be repeated and are inconclusive at the current stage. There may also be a need to expand

the region of study to other conserved intergenic regions or regions that have high levels of histone acetylation, which may be found in ENCODE histone modifications datasets available on the UCSC genome browser (http://genome.ucsc.edu/ENCODE/).

From the sequencing of the first intron of *HLA-DRA* in the different cell lines, it is easily noticeable that there are unique as well as shared SNPs between the different alleles of the cell lines. If the sequencing is expanded to regions outside of the intron 1, it may become apparent that certain alleles or SNPs are more frequently associated with *PARK18*. Efforts to find other SNPs highly associated with *PARK18* are being made by cloning and sequencing conserved regions of the MHC-II gene promoters and other regulatory regions from both control and PD patient DNA samples. However, finding linked SNPs by this method may prove to be difficult as the MHC-II locus is one of the most polymorphic regions of the human genome. It is possible that mining the data provided by the 1000 genomes project (http://www.1000genomes.org/) may facilitate the search for a highly linked regions of the MHC-II locus, and provide further evidence for a novel cis-regulatory element.

Figure legends

Figure 4-1. Monocytic cell line characterization.

(A) Level of *CIITA* and *HLA-DRA* expression were determined for U937, TUR, monomac 6 (mm6), and THP-1 cells by qRT-PCR. Cells were either untreated or treated with IFN-γ (500 U/ml) for 24 hrs and represented as relative value to 18S rRNA multiplied by 10^5. The columns represent the average value from three biological replicates. Error bars depict standard error. (B) In the monocytic cell lines, HLA-DR surface expression was measured before and after IFN-γ treatment (500 U/ml, 24 hrs) by flow cytometry. (C) Cell lines were

transfected with different amounts of pcDNA3.1-EGFP plasmid shown in grey, blue, green and orange for 0 – 2 μg of DNA. The levels of EGFP expression were analyzed by flow cytometry.

Figure 4-2. Dual luciferase reporter assay for fragments of the first intron of *HLA-DRA*.

(A) The first intron of *HLA-DRA* (2415 bp) was cloned from the human BAC clone (A at *PARK18*) upstream of the SV40 promoter and firefly luciferase gene of the pGL3 promoter vector. Seven shorter fragments of the first intron were also cloned from the BAC into the same restriction enzyme sites of the pGL3 plasmid. (B) pGL3 promoter, fragments 1 through 6 from the human BAC, and the first intron sequence cloned from Raji cells (G at *PARK18*) were transfected into Jurkat, Raji, and THP-1 cells. Cell lysates were used in a dual luciferase assay to measure activation of gene expression. Bars represent one experiment.

Figure 4-3. Demographic information and cell population characterization.

(A) Distribution of genders is shown for age of onset and diagnosis. (B) The distribution of age groups is shown for age of onset and diagnosis. (C) Total number of PBMC cells and purified B cells and monocytes from each patient are shown. The asterisk symbol illustrates a significant difference between control and PD group with a p value less than 0.05. (D) The percentage of B cells and monocytes of the total extracted PBMC cells is shown. Average and standard error values are shown for each category in all graphs.

Figure 4-4. HLA-DR and HLA-DQ surface expression of PD patients in B cells and monocytes.

B cell and monocyte expression of HLA-DR (A) and HLA-DQ (B) on the extracellular surface was measured by flow cytometry for both control and PD patients. Monocytes were treated with increasing concentrations of IFN-γ from 0 to 1000 U/ml. Average and standard error are shown for each group of cells.

Figure 4-5. Proportion of cells expressing HLA-DR and HLA-DQ in control and PD samples.

(A) The percentage of cells expressing only HLA-DR amongst all the HLA-DR expressing cells in the B cell and monocyte population. Monocytes were treated with increasing concentrations of IFN-γ. (B) The percentage of cells expressing only HLA-DQ in the total HLA-DQ expressing cell population is shown for B cells and monocytes treated with increasing concentrations of IFN-γ. (C) The proportion of cells expressing both HLA-DR and HLA-DQ amongst all the HLA-DR and HLA-DQ positive cells are shown. Average and standard error are shown for each group of cells.

Figure 4-6. MHC-II gene mRNA expression levels in B cells and monocytes.

Steady state mRNA expression levels of MHC-II genes were quantified by qRT-PCR. B cells and monocytes both untreated and IFN-γ treated for 24 hrs are shown for control and PD groups.

Table 4-1. Cloning primers for luciferase reporter assay constructs.

Table 4-2. RT-PCR primers for MHC-II genes and control genes.

Table 4-3. Cloning primers for CpG regions within the *HLA-DRA* gene.

Table 4-4. Sequencing results for a human BAC, cell lines, and a trial human sample. The numbers indicate the position within the first intron of *HLA-DRA*. The first letter is the major base for each SNP as reported on the UCSC human/H19 genome and the second letter is the base from the sequencing results. A minus symbol indicates a missing base. The black SNPs are SNPs reported in the UCSC human genome and blue SNPs have not been reported in this database.

Table 4-5. Demographic information of control and PD samples.

Figure 4-1

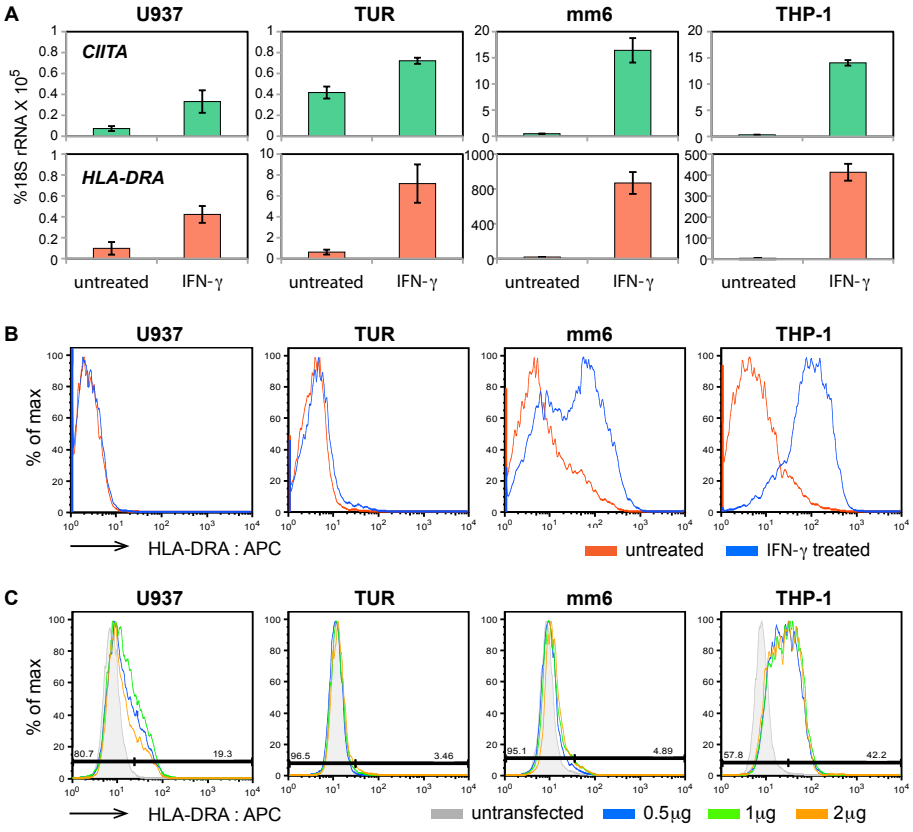
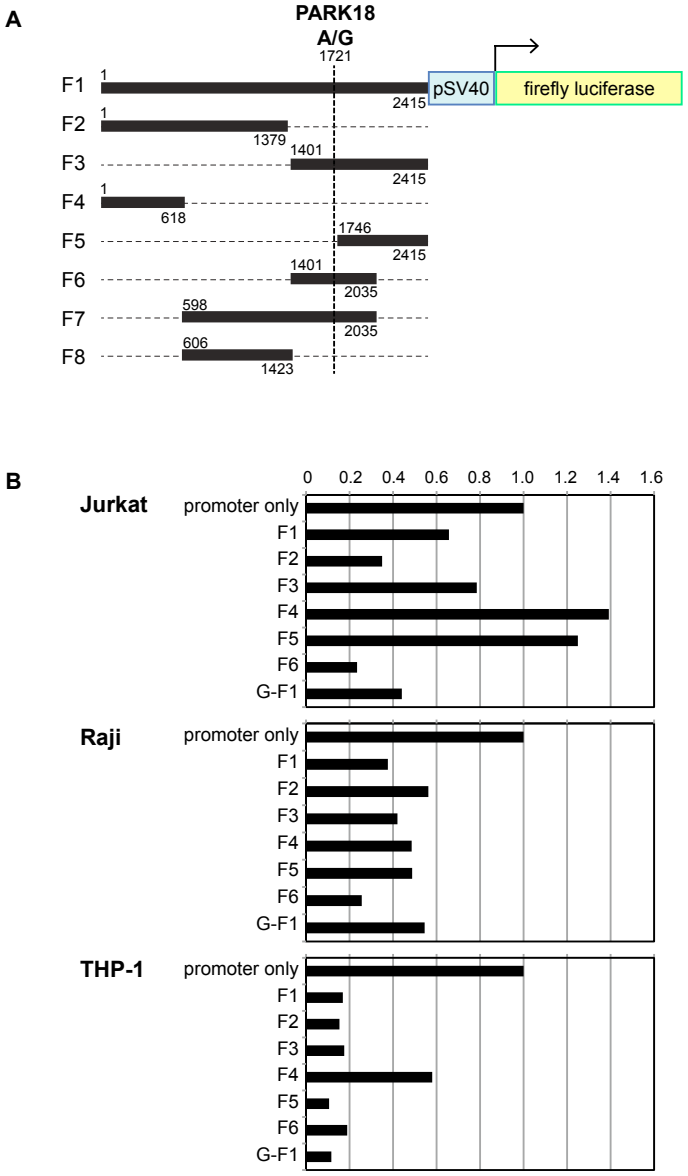

A
U937
TUR
mm6
THP-1
CIITA
HLA-DRA
%18S rRNA X 10^5
untreated
IFN-γ
B
% of max
HLA-DRA : APC
untreated
IFN-γ treated
C
80.7
19.3
96.5
3.46
95.1
4.89
57.8
42.2
untransfected
0.5μg
1μg
2μg

Figure 4-2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PARK18
A/G
1721
F1
F2
F3
F4
F5
F6
F7
F8
1
2415
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firefly luciferase
1
1379
1401
2415
1
618
1746
2415
1401
2035
598
2035
606
1423
B
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Jurkat
Raji
THP-1
promoter only
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F2
F3
F4
F5
F6
G-F1

Figure 4-3

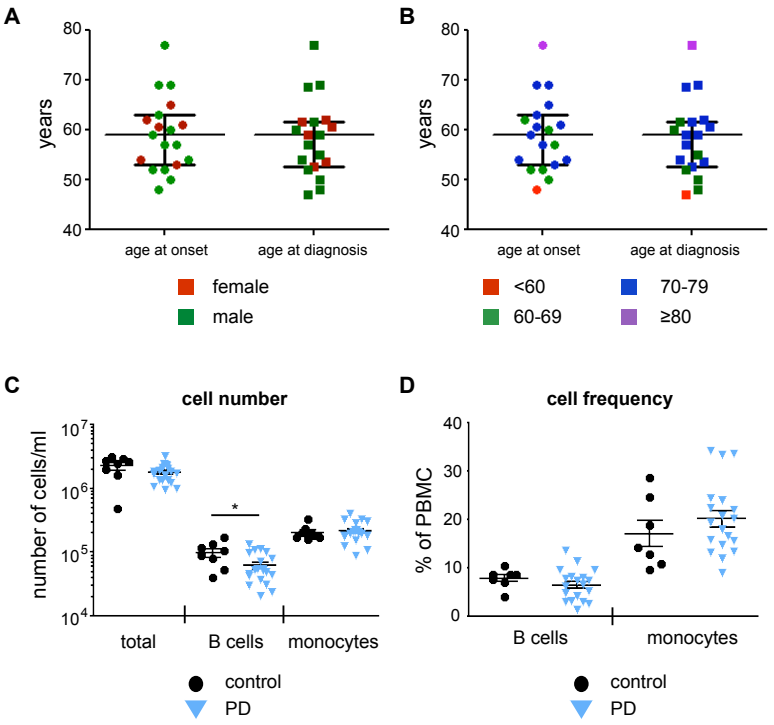
A
80
70
60
50
40
years
age at onset
age at diagnosis
female
male
B
80
70
60
50
40
years
age at onset
age at diagnosis
<60
60-69
70-79
≥80
C
cell number
10^7
10^6
10^5
10^4
number of cells/ml
*
total
B cells
monocytes
control
PD
D
cell frequency
40
30
20
10
0
% of PBMC
B cells
monocytes
control
PD

Figure 4-4

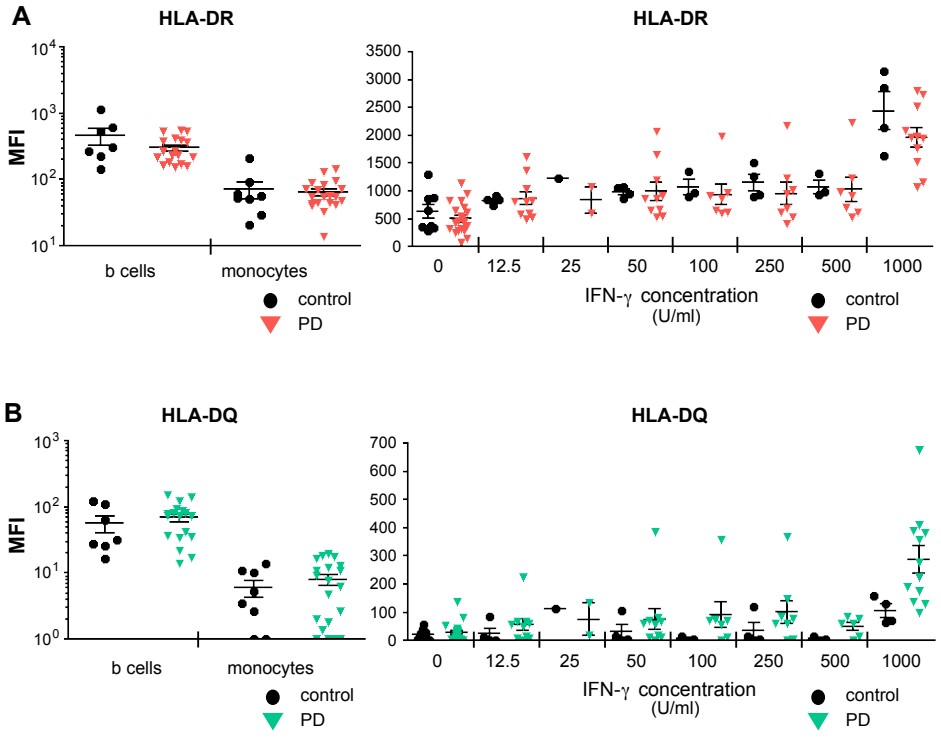
A
HLA-DR
MFI
b cells
monocytes
control
PD
HLA-DR
IFN-γ concentration
(U/ml)
0
12.5
25
50
100
250
500
1000
B
HLA-DQ
HLA-DQ

Figure 4-5

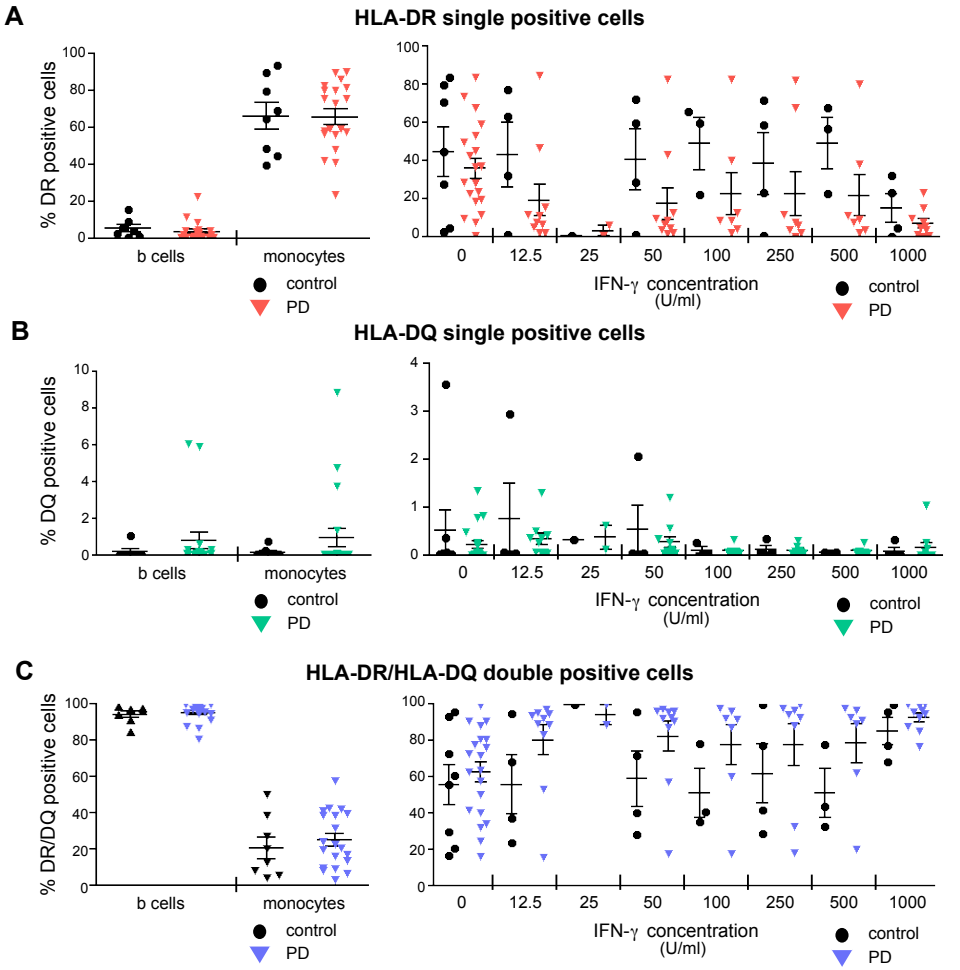
A
HLA-DR single positive cells
% DR positive cells
b cells
monocytes
control
PD
IFN-γ concentration (U/ml)
B
HLA-DQ single positive cells
% DQ positive cells
C
HLA-DR/HLA-DQ double positive cells
% DR/DQ positive cells

Figure 4-6

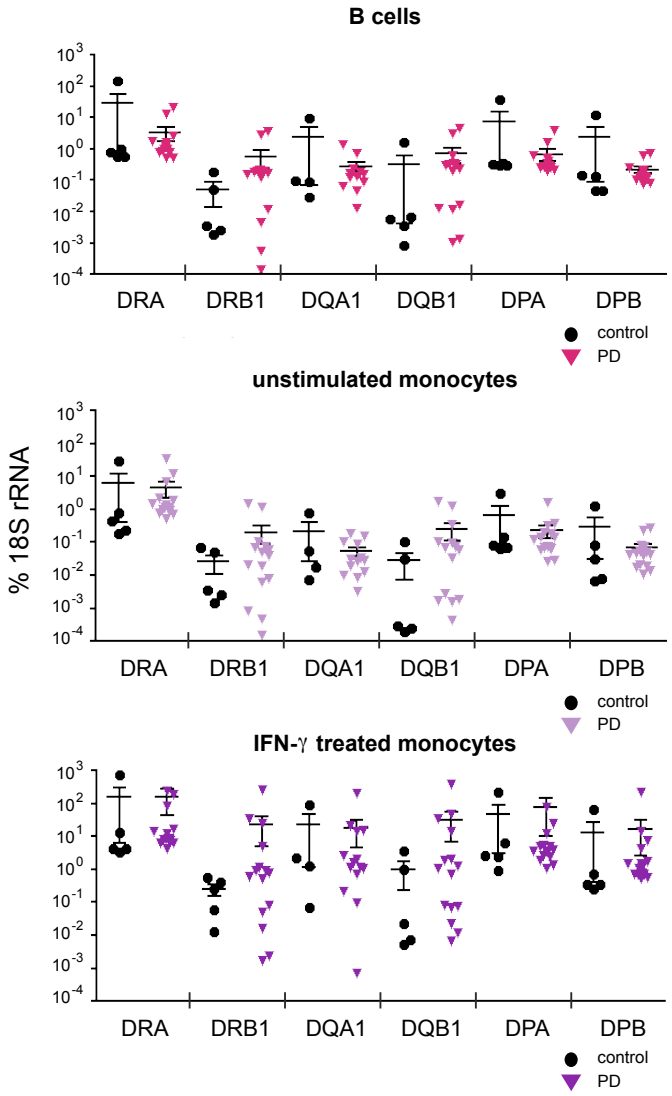
B cells
unstimulated monocytes
IFN-γ treated monocytes
% 18S rRNA
DRA
DRB1
DQA1
DQB1
DPA
DPB
control
PD

Table 4-1

PARK18 luciferase cloning primers

fragment 1	bases 1-2415 of intron 1
PARK18-F1	5'-GTCAGGTACCGTAGGTGCTGAGGGAATGAAATC-3'
PARK18-R2415	ACACCGCTAGCCTGAAAGGCAAGAAATGGAGAAAG
fragment 2	1-1379
PARK18-F1	GTCAGGTACCGTAGGTGCTGAGGGAATGAAATC
PARK18-R1379	ACACCGCTAGCCCTTAGTCTTCAAAGATAAG
fragment 3	1401-2415
PARK18-F1401	GTCAGGTACCTCGATACACTCCACAGAGGC
PARK18-R2415	ACACCGCTAGCCTGAAAGGCAAGAAATGGAGAAAG
fragment 4	1-618
PARK18-F1	GTCAGGTACCGTAGGTGCTGAGGGAATGAAATC
PARK18-R618	ACACCGCTAGCAAGGCAGGGAATGGCTATCAC
fragment 5	1746-2415
PARK18-F1746	GTCAGGTACCAACAGTGCTTGTTACAGTCTTG
PARK18-R2415	ACACCGCTAGCCTGAAAGGCAAGAAATGGAGAAAG
fragment 6	1401-2035
PARK18-F1401	GTCAGGTACCTCGATACACTCCACAGAGGC
PARK18-R2035	ACACCGCTAGCCACTTAGGCCAGAATTCCAGAG
fragment 7	598-2035
PARK18-F598	GTCAGGTACCGTGATAGCCATTCCCTGCCTTCC
PARK18-R2035	ACACCGCTAGCCACTTAGGCCAGAATTCCAGAG
fragment 8	606-1423
PARK18-F606	GTCAGGTACCCATTCCCTGCCTTCCCATCTCC
PARK18-R1423	ACACCGCTAGCGCTGCCTCTGTGGAGTGTATCG
sequencing primers	
PARK18-F1354	GTCAGGTACCTCTCACCTTATCTTTGAAGACTAAG
PARK18-F470	CCTGTCGGTATATATTGAGCAC
PARK18-F887	AGAGCCATACATAGGGATACTTAC
pGL3promoter-rev	GGGACTATGGTTGCTGACTAATTG
RVprimer3	CTAGCAAAATAGGCTGTCCC

* fragments were cloned in to *Kpn*I-*Nhe*I of the pGL3 promoter plasmid

Table 4-2

MHC-II RT primers

DRA fwd	5'-GAGTTTGATGCTCCAAGCCCTCTCCCA-3'
DRA rev	CAGAGGCCCCCTGCGTTCTGCTGCAAT
DRB1 fwd	TGCTGAGCTCCCTACTGGCT
DRB1 rev	CGCGTACTCCTCTCGGTTATAG
DRB3 fwd	TTGGCAGCGTTGACAGTG
DRB3 rev	GGAACTCCTCCTGGTTATGG
DRB5 fwd	AAGTATGAGTGTCATTTC
DRB5 rev	TCCTTCTGGCTGTTCCAG
DQA1 fwd	CACCTTTTCTCTGGGACTTAAGC
DQA1 rev	TGAGGAATTAGGTAGCCGGGT
DQB1 fwd	TATGCCTGCCCAGAATTCCC
DQB1 rev	AAACCCCTTGGGACCTGAGT
DPA1 fwd	CCATCAAGGCGGACCATGTGT
DPA1 rev	TCAAAGGAAAAGGCTTGGCCAA
DPB1 fwd	CAGCTCTTTTCATTTTGCCATCC
DPB1 rev	TCCCATTAAACGCGTAGCATTCC
DMA fwd	GGCTGGGTTGGTAGCTCCTA
DMA rev	TTTGCAGGTCATCTGGCCAC
DMB fwd	TGCGCAATGGGCTTCAGAAT
DMB rev	GAAGCCCCACACATAGCAGG
DOA fwd	GCTCTACCCAAAGCTCTGGC
DOA rev	TGGGCCAAATGGAGCAAGAC
DOB fwd	TCGTCATCCAGCTAAGGGCT
DOB rev	CCAGGGCCCAGACTACTCAT
18S rRNA fwd	GTAACCCGTTGAACCCCATT
18S rRNA rev	CCATCCAATCGGTAGTAGCG
beta-actin fwd	TGCGTGACATTAAGGAGAAGCTG
beta-actin rev	GCTCGTAGCTCTTCTCCAGGA

Table 4-3

HLA-DRA CpG rich region primers

DRA-1900 fwd	5'-TAGCGATCTAGGAGTTAATGCC-3'
DRA-1900 rev	CAGCCGTTCTCACAAGTTATG
DRA-1400 fwd	GCGACAGAGCAAGACTTCC
DRA-1400 rev	CAGTGAGGGAGCCCATCG
DRA-1000 fwd	GTTCCCAATAGAATAGGCTTTGC
DRA-1000 rev	CTCCTGACCTCGTGATTTGC
DRA+100 fwd	AGGAATCATGGGCTATCAAAGG
DRA+100 rev	ATTATCTTCCAAATGTCCATAGGTC
DRA+1100 fwd	GCTAGTATATTTGTGTGTGTTTGC
DRA+1100 rev	GTGCCCAGCCAAGATGAG
DRA+1800 fwd	TCAATCCAGACGAGAACCTTC
DRA+1800 rev	AATTAAGACTGAGACCTTGTAGC
DRA+2200 fwd	AAACCCAACCTTTCAAACAGC
DRA+2200 rev	AATGTTCTCTCACTTTCTTTACCC
DRA+2700 fwd	CATGTGGATATGGCAAAGAAGG
DRA+2700 rev	TTGGTGATCGGAGTATAGTTGG
DRA+3400 fwd	GAACTGAGAGAGCCCAACG
DRA+3400 rev	GGGAGATAGTGGAACTTGCG
DRA+4000 fwd	CCCAGAGACTACAGAGAACG
DRA+4000 rev	GACCACACCTAACTCACCTC
DRA+4200 fwd	GTGGCTCTTGATTTCTCTTTGC
DRA+4200 rev	ACTGGAGATGATTTCTAAGACTGG
DRA+5200 fwd	GGTGTTTAAGCCAGTTCTTTG
DRA+5200 rev	GACCAAGCCCAAATGAACC
H19 fwd	GCTCGGTCAACTGGATGG
H19 rev	ACTCGGATGGCACAGAGG
Xite fwd	AAGCGGGAGTCCTTTAACC
Xite rev	CGAAGCCTTGGCATAACG

Table 4-4

BAC	Raji allele 1	Raji allele 2	Raji allele 2 variant	THP-1 allele 1	THP-1 allele 2	H929 allele 1	Trial blood allele 1	Trial blood allele 2
A235C	A235C	G203A	G203A	G203A	A235C	A408-	G203A	A235C
C718G	A408-	A408-	A408-	A408-	A408-	A409-	A408-	A408-
T1108C	A409-	A409-	A409-	A409-	A409-	C718G	A409-	A409-
A1247G	C718G	G788A	G788A	G788A	T1108C	T967G	G788A	C718G
T1249C	T1108C	A926C	A926C	A926C	A1247G	T1108C	A926C	T1108C
A1496G	G1237A	G941A	G1098A	G1098A	T1249C	A1247G	G1098A	G1237A
G1721A	A1247G	G1098A	T1108C	T1108C	A1380-	T1249C	T1108C	A1247G
G1847A	T1249C	T1108C	A1247G	A1247G	A1496G	A1380-	A1247G	T1249C
C1972T	A1496G	G1237A	T1249C	T1249C	G1721A	A1496G	T1249C	A1496G
T1978C	G1847A	A1247G	A1380-	A1380-	C1972T	G1721A	A1380-	G1847A
T2328C	C1972T	T1249C	C1433A	C1433A	T1978C	G1847A	C1433A	C1972T
	T1978C	A1380-	C1675T	C1675T	T2187C	C1972T	C1675T	T1978C
	T2328C	C1433A	G1721A	G1721A	T2278C	T1978C	G1721A	T2328C
		C1675T	C1899T	C1899T	T2328C	T2328C	C1899T	
		G1721A	C2274A	T2328C			C2274A	
		G1847A	T2328C	C2274A			T2328C	
		C1972T	G2352-	G2352-			G2352-	
		T1978C						
		T1991C						
		T2328C						

Table 4-5

	Control	Parkinson's Disease
count, sex	7 females, 1 male	6 females, 13 males
median age	67 [57, 73]	71 [55, 85]
ancestry	English (4), Irish (3), Scottish (2), German (2), Lithuanian, Italian, Canadian, Polish	English (7), German(5), Irish (4), Scottish (3), Dutch (3), Russian (2), Unknown (2), Polish, Native american, Greek, Melungeon, Czech
age at diagnosis		59 [48, 77]
age at onset		59 [47, 77]
yrs with disease		12 [5, 18.5]

Chapter 5. Conclusions and Discussions

The major histocompatibility complex genes are a fundamental part of the adaptive immune system. Without expression of these genes in the proper cells in response to the correct developmental or environmental cues, our body fails to defend itself against infectious agents. This is well demonstrated in patients with genetic disorders that affect their cells to express MHC genes properly, the most severe example being the bare lymphocyte syndrome (BLS). Of the MHC genes, the research presented here has focused on the MHC class II genes. While the MHC-II genes are classically known for their role in our body's defense against extracellular pathogens, it has become increasingly clear that their influence is far reaching. Examples of the importance of properly functioning MHC-II genes are described for a plethora of diseases from cancer to various autoimmune diseases such as multiple sclerosis and rhumatoid arthritis, etc.

Research on BLS has led the scientific community to learn a great deal about the regulation of MHC-II genes (Glimcher and Kara, 1992). With the advancements of molecular biology and sequencing technologies in the 1970-80s, the MHC-II genes, the highly conserved proximal promoter element termed the *WXY* boxes, and other cis-regulatory elements have been identified and sequenced. Through studying the complementation cell groups of BLS, the essential transcription factors, RFX5, RFXAP, RFX-B, and CIITA, have been identified (Durand et al., 1997; Nagarajan et al., 1999; Steimle et al., 1995; Steimle et al., 1993). With these pieces of information, it was understood that while the MHC-II genes are greatly polymorphic, they have common cis- and trans-regulatory elements that allow them to be expressed in a synchronized manner.

Biochemical dissection of the transcription factors elucidated the domain structures of these proteins and their interaction networks. Through DNA footprinting experiments, it was determined that the RFX proteins, NF-Y proteins, and CREB, bound to the *WXY* box, but not CIITA. CIITA can interact with these transcription factors, and possibly other CIITA molecules to form the so-called MHC-II enhanceosome. CIITA interacts with the unique structure formed by the *WXY* box and its DNA binding factors to identify where within the genome the MHC-II genes are located (Masternak et al., 2000). The general transcription factors and transcriptional coactivators are recruited to the MHC-II promoters by their interaction with CIITA.

CBP/p300 and PCAF were some of the first proteins determined to have histone modifying activities (Ogryzko et al., 1996; Schiltz et al., 1999). They were observed to interact with CIITA, and in the case of PCAF, it was shown to acetylate CIITA directly to control the nuclear localization of CIITA and regulate transcription (Spilianakis et al., 2000). Until antibodies against histone modifications became available, it was difficult to demonstrate that histone modifications may be involved in MHC-II transcriptional activation. Using chromatin immunoprecipitation (ChIP), it was first shown that CIITA was indeed binding directly to the *WXY* box in vivo (Beresford and Boss, 2001), as ChIP allows one to capture a protein-DNA interaction in real time. Additionally, it was demonstrated that acetylation of histone H3 and H4 at the *HLA-DRA* promoter were concurrently increasing as the binding of CIITA was enhanced, providing further evidence that histone modification may have a role in transcriptional activation. However, while there was data showing interaction and cooperation between CIITA and histone modifying factors, there was little evidence showing that these interactions were occurring at the promoter when transcription was being activated.

Successively, the direct binding of histone modifying factors and general transcription factors at the *WXY* box were shown by ChIP and other matrix pulldown methods. CBP, CARM-1, Src-1, GCN5, etc. were shown to bind MHC-II promoters in either B cells or with IFN-γ treatment of non-MHC-II expressing fibroblast cells (Spilianakis et al., 2003; Tzortzakaki et al., 2003; Zika et al., 2005). The goal of the studies described in this thesis was to determine what specific histone modifications were induced with transcriptional activation, and identify novel factors responsible for these modifications that facilitated the transcriptional activation of the MHC-II genes.

Both in MHC-II expressing B cells and IFN-γ treated epithelial cells, various histone acetylation modifications, H3K9ac, H3K18ac, H3K27ac, H4K5ac, and H4K8ac, were induced, while some histone acetylation marks were not detected. H4K16ac was one of these undetected acetylation modifications, which may reflect the specific time point we collected the IFN-γ treated cells, or the quality of the antibody that was used. When *HLA-DRA* was not expressed, either in mutant B cell lines absent of RFX5 or CIITA expression, or in untreated epithelial cells, there was only background level of histone acetylation observed. The acetylation marks did not have much variation in their distribution across the *HLA-DRA* gene and upstream regions in B cells. However, in IFN-γ treated cells, while most of the acetylation marks were broadly positioned over the transcriptional start site (TSS), H3K9ac had a single prominent peak immediately downstream of the TSS. This suggests that this mark may be applied by a different factor than the other modifications, or that it signifies a distinct step during transcription, for example initiation of transcription vs. transition into transcriptional elongation. There was a report that H3S10 phosphorylation, H4K16ac, and H3K9ac together recruit a bromodomain containing protein BRD4 and its interacting partner P-TEFb, which is critical to progression into elongation (Zippo et al.,

2009). GCN5 and P-TEFb have been reported to interact resulting in the acetylation of P-TEFb (Sabò et al., 2008). This however, negatively regulated the transcriptional elongation activity of P-TEFb. There have also been reports of p300 acetylating P-TEFb in vitro to activate its activity (Fu et al., 2007). Together these suggest a complex network of protein acetylation and deacetylation, not only of the histone proteins but also the transcriptional regulators that may all be factors that influence the level of transcription.

Candidate coactivators that would likely be responsible for these histone acetylations were examined for binding at the *HLA-DRA* locus. Addition of a secondary crosslinker along with formaldehyde crosslinking significantly enhanced the signal to background ratio in ChIP experiments, and allowed for the detection of previously difficult protein-DNA interactions. CBP, p300, PCAF, and GCN5 were all shown to be directly binding to *HLA-DRA* promoter and the distal regulatory element, *XL4*. GCN5 and PCAF interacting protein complexes were purified at the time in human and multiple groups were reporting at least two distinct types of GCN5/PCAF containing complexes (Nagy and Tora, 2007). When the subunit components of these complexes were observed for recruitment at the *HLA-DRA* gene, we found that proteins from both STAGA and ATAC were present, albeit some at near background levels. Recently, a paper described STAGA and ATAC to be mostly mutually exclusive in the regions within the genome that they bind with little overlap (Krebs et al., 2011). It is still not fully understood how different the functions of the two distinct complexes are, nor is it understood whether the incorporation of GCN5 or PCAF endows the complexes to have distinct biological functions. Some of the subunits are shared with other multiprotein transcriptional regulatory complexes, such as WDR5 and HCF-1 of the ATAC complex. These proteins have also been purified with the histone methyltransferase complex MLL (Dou et al., 2005), suggesting WDR5 and HCF-1 may have

a role in functionally connecting the GCN5 and MLL complexes during MHC-II transcriptional activation. HCF-1 was also one of the proteins that were identified in the mass spectrometry results after the CIITA interacting proteins were purified. It would be interesting to examine whether HCF-1 mediates the interaction between CIITA and the histone modifying complexes.

Various degrees of histone H3K4 methylation were also observed with *HLA-DRA* transcription activation. The distribution of H3K4 mono-, di-, and trimethylation marks was not as similar to each other as the acetylation modifications were in their distributions throughout the *HLA-DRA* locus. H3K4me1 was most present at *XL4* in B cells, but at the -600 site in IFN-γ treated epithelial cells. The -600 site does not have any known enhancer or regulatory functions known to date. As H3K4me1 is widely accepted as the mark of enhancers when found in intergenic regions, this may be an indication of a novel regulatory element worth further investigation. The MLL1 complex of proteins was chosen as the most likely factor to be modifying the histones at *HLA-DRA,* as this was the most well studied histone H3K4 methyltransferase complex in mammals. Other H3K4 methylation proteins, Set1A, Set1B, MLL2, MLL3, and MLL4, as part of COMPASS-like H3K4 methylating protein complexes have since been further characterized (Cho et al., 2007; Hughes et al., 2004; Lee et al., 2007). Many of the subunit components are shared between the complexes with interchangeable Set domain containing catalytic subunits (Eissenberg and Shilatifard, 2009). They may be regulating different sets of genes or a gene under different activating conditions. However, it is not fully known how functionally different the different complexes are. A protein unique to the Set1 complex, WDR82, was copurified with the BLRP-CIITA construct in the mass spectrometry. It is possible that Set1 instead of MLL1 may be recruited under certain environmental cues. For example, one complex may

have a more dominant role in constitutive activation compared to IFN-γ activation. In any case, a COMPASS type methyltransferase complex seems to be important in activating MHC-II transcription.

One unexpected outcome from the histone modification data was that the modifications were maintained with a great degree of stability. Once the modifications were applied after IFN-γ treatment induced gene activation, they were present for up to 120 hrs. It is not known, whether it would be through a maintenance mechanism of constantly removing and reapplying new modifications, or through a preservation mechanism where the turnover for a modification is slowed down. The kinetics of turnover for any histone modification at the MHC-II genes has not been thoroughly investigated and was initially one of the objectives of the study. The protein half-lives of isoforms III and IV of CIITA, expressed from promoters III and IV respectively, are very short, both being approximately 30 minutes (Schnappauf et al., 2003). However, it is not clear if the half-life of a promoter bound CIITA would the same. When CIITA levels bound at the promoter were measured by ChIP after IFN-γ removal, there was still a significant level of CIITA bound after 120 hours and through several rounds of cell division. *HLA-DRA* mRNA levels were also maintained throughout 72 hours after IFN-γ removal but returned to basal levels by 120 hours. The reported half-life of *HLA-DRA* mRNA is 8 to 10 hours (Maffei et al., 1989). Therefore, this suggests that the prolonged *HLA-DRA* expression can be attributed to either the enhanced CIITA binding stability at the promoter or the maintenance of histone modifications at the promoter and surrounding regions.

A similar report of retaining a histone modification, H3K4me2, at the *HLA-DRA* promoter had shown that this may be due to the recruitment of promoters to PML bodies within the nucleus of the cell (Gialitakis et al., 2010). The researchers demonstrated direct

protein interaction between MLL and PMLIV. Also, WDR5 and ASH2L colocalized to PML bodies within the nucleus observed by confocal microscopy. These results together with the findings described in this thesis allude to a larger role of the MLL complex at the MHC-II genes beyond their histone methylating activity at the promoters and regulatory regions. They may have a role in organizing the MHC-II locus at a large-scale to direct them towards transcriptionally permissive environments within the nucleus. Such regions have also been referred to as transcription factories (Cook, 2010; Sutherland and Bickmore, 2009). It would be interesting to further explore these long-range chromosomal organization and nuclear translocation events at the MHC-II locus with chromatin conformation capture experiments coupled with next generation sequencing methods, 4C, 5C, or Hi-C (de Wit and de Laat, 2012).

Much of the long-range chromatin organization studies of the MHC-II locus have been focused on the role of CTCF. As mentioned in Chapter 2, CTCF was initially found to bind at an intergenic location named *XL9*, between *HLA-DRB1* and *HLA-DQA1*, and had since been shown to bind to at least 10 regions within the MHC-II locus (Majumder and Boss, 2010a; Majumder et al., 2006). CTCF interacts with CIITA, RFX, and cohesin proteins to regulate MHC-II genes (Majumder and Boss, 2011). As expected, amongst the CIITA interacting proteins purified with BLRP-CIITA, CTCF, SMC3, and SMC1A were present. Another CTCF interacting protein ZBTB33/kaiso was also found in the CIITA interacting protein complex. The exact mechanism of kaiso function is not well understood, but has been shown to bind to methylated CpG at consensus sites (Buck-Koehntop et al., 2012; Raghav et al., 2012), and mostly suppresses transcription of Wnt target genes (Park et al., 2005).

Other proteins identified in the mass spectrometry results are, HDAC1 and HDAC2, which both have been previously shown to interact with CIITA (Kong et al., 2009; Zika et al., 2003). These along with CHD4, RbBP7, and HCF-1 are part of the Mi2/NURD complex, which has been characterized as a negative regulator of transcription, that also contains methyl CpG-binding domain proteins MBD2 and MBD3 (Denslow and Wade, 2007). The role of the Mi2/NURD complex during B cell to plasma cell differentiation has been demonstrated previously and have a role in MHC-II regulation (Fujita et al., 2004). The Mi2/NURD complex has interestingly been previously shown to purify with hSNF2H/ISWI and cohesin complex proteins (Hakimi et al., 2002). ISWI is an ATP dependent chromatin remodeling complex, that has been shown to mostly bind regions with deacetylated histones to condense chromatin structure and repress gene activity (Cairns, 2009). In this report however, the authors described the hSNF2H interacting complex to be recruited to the CpG rich Alu repetitive DNA elements when the DNA was demethylated and histones H3 and H4 in the regions were acetylated. It was not described how this complex may regulate transcription in this report. The protein with the highest binding score from the CIITA interacting proteins was hSNF2H. WSTF and BAZ2A, other ISWI interacting proteins were also copurified. This suggests that these complexes may have a role in MHC-II transcription and chromosomal organization in cooperation with the cohesin proteins and CTCF. Since Mi2/NURD and ISWI are both repressive complexes, an interesting possibility would be, that while CIITA is recruiting coactivators, is at the same time recruiting repressors to set up the promoter for repression and preparing for the next stage of cellular development. These repressors may be kept inactive by the other CIITA interacting proteins until a certain signal arrives to shut down transcription of the MHC-II genes.

It is somewhat perplexing that kaiso and some of these CIITA interacting factors identified by mass spectrometry are more known for their transcriptional repressive roles. One possible explanation could be that when they are acting in combination with transcription activators, or interacting in a certain sequence of events, they may also acquire transcriptionally activating roles. CIITA may be relieving the repressive activity of these factors by facilitating the interaction of these repressors with other proteins that can postranslationally modify and 'switch' them to become active, or less repressive. As the histone modifying proteins are in most cases not limited in their activities to modify only histones, the CIITA interacting KATs and KMTs, may be modifying these repressive factors to become active. Another explanation could be that there is a cyclical turnover of posttranslational modification of histones and regulating factors that occur to maintain a controlled level of transcription, and CIITA may be acting as a hub where these proteins can interact. A similar cyclical turnover of histone modifications and recruitment of factors have been described for the estrogen receptor target genes (Métivier et al., 2003).

The complexity of the protein and DNA interacting networks is ever increasing. As next-generation sequencing methods evolved over the recent years, the amount of histone modification and transcription factor binding data available to the scientific community is staggering. The systems biologists are identifying protein-protein and gene interaction networks at an equally exuberant speed. To expedite the process of understanding how the MHC-II genes are regulated, this freely available knowledge must be utilized. It would be important to constantly search for novel interactions of the proteins that were found in these studies, especially as the function of these protein complexes are further uncovered. It would be useful to further utilize these techniques in the studies of MHC-II gene regulation. This is currently in progress in the form of CIITA and RFX5 ChIP-seq experiments, as well

as chromatin conformation capture experiments for the CTCF binding regions at the mouse MHC-II locus. These studies will greatly expand our understanding of how the MHC-II genes are regulated and how they are involved in various human pathologies. In the future, the uncovered protein and gene interactions may serve as therapy targets and allow us to improve the prognoses of these devastating diseases and improve the human condition.

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