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THE 26S PROTEASOME AND HISTONE MODIFYING ENZYMES REGULATE TRANSCRIPTION OF THE CLASS II TRANSACTIVATOR, CIITA

by

AGNIESZKA D. TRUAX

Under the Direction of Susanna Fletcher Greer, PhD

ABSTRACT

Major Histocompatibility Complex Class-II (MHC-II) molecules are critical regulators of adaptive immunity that present extracellular antigens required to activate $CD4^+$ T cells. MHC-II are regulated at the level of transcription by master regulator, the Class II Transactivator (CIITA), whose association with the MHC-II promoter is necessary to initiate transcription. Recently, much research focused on novel mechanisms of transcriptional regulation of critical genes like MHC-II and CIITA; findings that the macromolecular complex of the 26S-proteasome is involved in transcription have been perhaps the most exciting as they impart novel functions to a well studied system. Proteasome is a multi-subunit complex composed of a 20S-core particle

capped by a 19S-regulatory particle. The 19S contains six ATPases which are required for transcription initiation and elongation. We demonstrate that 19S ATPase-S6a inducibly associates with CIITA promoters. Decreased expression of S6a negatively impacts recruitment of the transcription factors STAT-1 and IRF-1 to the CIITA due to significant loss in histone H3 and H4 acetylation. S6a is robustly recruited to CIITA coding regions, where S6a binding coordinates with that of RNA polymerase II. RNAi mediated S6a knockdown significantly diminishes recruitment of Pol II and P-TEF-b components to CIITA coding regions, indicating S6a plays important roles in transcriptional elongation.

Our research is focused on the ways in which accessibility to and transcription of DNA is regulated. While cancers are frequently linked to dysregulated gene expression, contribution of epigenetics to cancers remains unknown. To achieve metastatic ability, tumors alter gene expression to escape host immunosurveilance. MHC-II and CIITA expression are significantly downregulated in highly metastatic MDA-MB-435 breast cancer cells. This suppression correlates with elevated levels of the silencing modification H3K27me3 at CIITA and a significant reduction in Pol II recruitment. We observe elevated binding of the histone methyltransferase to CIITApIV and demonstrate this enzyme is a master regulator of CIITA gene expression. EZH2 knockdown results in significant increases in CIITA and MHC-II transcript levels in metastatic cells. In sum, transcriptional regulation by the 19S-proteasome and histone modifying enzymes represents novel mechanisms of control of mammalian gene expression and present novel therapeutic targets for manipulating MHC expression in disease.

INDEX WORDS: Major histocompatibility class II, Class II transactivator, Transcription regulation, 26S proteasome, 19S ATPase, S6a, epigenetic, histone modifications, histone methyltransferase EZH2

THE 26S PROTEASOME AND HISTONE MODIFYING ENZYMES REGULATE TRANSCRIPTION OF THE CLASS II TRANSACTIVATOR, CIITA

by

AGNIESZKA D. TRUAX

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2011

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by

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Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2011

DEDICATION

Today's media often describes the life of a scientist as a person that makes a great discovery with relatively no effort. In reality, science is a profession filled with many more failures than successes. Most of time life of scientist is filled with lots of reading, followed by some well educated guesses and never ending experiments that provide you no results or the results that are completely opposite of what you have predicted. In short science requires lots of time, effort and sacrifice and would not be possible without the support of friends around you.

Therefore, I would like to dedicate this documents to all folks around me that have supported my journey throughout that degree; my husband Jon Truax whom I thank from the bottom of my heart, his parents Rita and Ric Truax who has been an excellent support throughout my studies, my brother Jacek and my dad, Jozef Groniecki who has always been there for me.

This thesis is dedicated to the memory of my mother, Grazyna, who never got to see me achieving my Doctoral Degree as she always dreamed. She has provided me guidance, patience and support of my learning and development throughout my childhood. Without the support of her and my dad I would not be an individual that I am.

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It is a pleasure to have this opportunity to acknowledge some of the people that have contributed to my degree. First and foremost I would like to thank Dr. Susanna Fletcher Greer for providing an excellent laboratory atmosphere that is positive and very supportive, where an independent thought is encouraged but guidance is always available. Her enthusiasm for science is definitely contagious. Greer lab has been definitely a place that I have matured as a scientist and had a chance to interact with a wonderful group of people.

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The idea of working in isolation is absolutely absurd. Therefore I would like to extent my thanks to all my labmates for their continuous support and encouragement. You are all so willing to help, and have helped me really grow during our time together. I look forward to our future not as fellow students, but as colleagues and friends.

.

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ABBREVIATIONS:

Acetylation (Ac)

Acetylated histone H3 (acH3)

Adenosine diphosphate (ADP)

Adenosine triphosphate (ATP)

Antigen Presenting Cells (APCs)

Arginine (R)

Bare Lymphocyte Syndrome (BLS)

Brahma-related gene (BRG-1)

Coactivator-associated arginine methyltransferase 1 (CARM1)

Camp Responsive Element Binding Protein (CREB)

Chromatin Immunoprecipitation (ChIP)

Class II Transactivator (CIITA)

Class II Transactivator promoter IV (CIITApIV)

CREB binding protein (CBP)

Co-Immunoprecipitation (Co-IP)

C-Terminal Domain (CTD)

Cyclin Dependent Kinase (CDK)

DRB-Sensitive Inducing Factor (DSIF)

Enhancer of Zeste homolog 2 (EZH2)

Gamma Activated Sequence (GAS)

Heterochromatin Protein 1 (HP1)

Hexamethylene Bisacetamide Inducible Protein (Hexim)

Histone 2A (H2A)

Histone 3 (H3)

Histone 4 (H4)

Histone H3 trimethylated at lysine 4 (H3K4me3)

Histone H3 acetylated at lysine 9 (H3K9ac)

Histone H3 acetylated at lysine 18 (H3K18ac)

Histone H3 trimethylated at lysine 27 (H3K27me3)

Histone H3 trimethylated at lysine 36 (H3K36me3)

Histone Acetyltransferase (HAT)

Histone Deacetylase (HDAC)

Human Leukocyte Antigen (HLA)

Histone Methyltransferase (HMT)

Immunoblot (IB)

Interferon-γ (IFN-γ)

Immunoprecipitation (IP)

Interferon Response Element (IRE)

Interferon regulatory Factor (IRF)

Janus Activated Kinase (JAK)

Lysine (K)

Major Histocompatibility Complex (MHC)

Messenger RNA (mRNA)

Methylation (Me)

Negative Transcription Elongation Factor (N-TEF)

Nuclear Factor-Y Complex (NFY)

Polymerase Chain Reaction (PCR)

Phosphorylation (P)

Positive Transcription Elongation Factor (P-TEFb)

Regulatory Factor X Complex (RFX)

RNA Polymerase II (RNA Pol II)

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Serine 5 (Ser5)

Serine 2 (Ser2)

Severe Combined Immunodeficiency (SCID)

Short Interfering RNA (siRNA)

Signal Transducer and Activator of Transcription (STAT)

Transcriptional Activation Domain (TAD)

TATA Binding Protein (TBP)

Transcription Factor (TF)

Transcription Factor II H (TFII H)

Tumor Necrosis Factor-α (TNF-α)

Ubiquitin (Ub)

Untreated (NT)

Upstream Regulatory Factor (USF)

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1 CHAPTER I: INTRODUCTION

1.1 MHC MOLECULES AND THE ADAPTIVE IMMUNE SYSTEM:

Human beings are constantly exposed to pathogens; thus the immune system is essential for survival as it functions to protect against invading pathogens. The immune system of higher vertebrates is composed of two responses, termed innate and adaptive, which collaborate to provide efficient and effective protection. Innate immunity is non-specific and provides the first line of defense against pathogens (Janeway and Medzhitov 2002). Adaptive immunity has evolved only in higher vertebrates and provides specific, although delayed, responses against pathogens. Cells of the adaptive immune response, unlike those of the innate response, have the capacity to develop memory against invading pathogens they have previously contacted and can provide long term protection against pathogens. Specificity and long term memory are therefore critical characteristics of the adaptive immune response (Janeway 2001) .

1.2 Innate immunity:

Innate immunity broadly includes molecular and cellular mechanisms present prior to infection capable of quickly developing a localized non-specific response against invading pathogens. Invading microorganisms initially encounter the host's physical barriers of defense including the skin and the epithelial linings of internal organs as well as secondary barriers including

the acidic stomach and perspiration. Beyond these initial barriers, the innate response includes groups of effector cells and antimicrobial compounds capable of neutralizing the invading pathogen based on common molecular markers present on pathogen surfaces. If the pathogen overcomes initial barriers, reactions of the innate system are activated based on recognition of the presence of the pathogen and recruitment of effector cells capable of killing and eliminating the pathogen (Janeway and Medzhitov 2002). Effector cells of the innate response include dendritic cells, natural killer cells, mast cells, basophils, and eosinophils which function together to recognize the invading pathogen and to prevent the infection from spreading.

1.3 Adaptive immunity:

A second form of immunity, the adaptive immune response develops in response to infection. Adaptive immunity differs from innate in that the adaptive response is highly specific and has the ability to develop immunological memory. Adaptive immunity is slower to develop in a primary response, but is able to recognize a much wider repertoire of foreign substances.

An effective adaptive immune response involves lymphocytes and antigen presenting cells. Lymphocytes are produced in primary lymphoid organs and, following development, circulate through the blood to various secondary lymphoid organs. Two major populations of lymphocytes are B cells and T cells. Both B and T lymphocytes have membrane bound receptors, the B cell receptor (BCR) and T cell receptor (TCR), respectively, which recognize specific antigens (Farber, Acuto et al. 1997; Gerloni and Zanetti 2005). Upon activation, B cells give rise to plas-

ma cells which produce antibody to aid in clearance of extracellular pathogens. T cells also participate in pathogen clearance, but unlike B cells, are able to clear infections of both intracellular and extracellular origin. There are three major types of T cells: cytotoxic $CD8^+$ T cells (T_c), $CD4^+$ T helper T cells (T_h), and regulatory T cells (T_{reg}). T lymphocytes are defined as CD4 or CD8 based on the presence of a CD4 or CD8 glycoprotein which is bound on the cell surface (Farber, Acuto et al. 1997). CD4 T cells are further subdivided into T helper 1 (Th1) and T helper 2 (Th2) cells, based upon cytokine secretion profiles (Gerloni and Zanetti 2005). Broadly speaking, cytotoxic T cells defend against intracellular infections and helper T cells fight extracellular infections. Both $CD4^+$ and $CD8^+$ T cells can, following activation, transition to a subset of memory cells which persist after an infection has been resolved and allow for quick expansion following re-exposure to the same pathogen (Jelley-Gibbs, Lepak et al. 2000; Seder and Ahmed 2003). CD4+ T cells also play roles in establishing immunological memory by promoting the generation of CD8+ memory T cells (Harbertson, Biederman et al. 2002).

In contrast to antibodies or B cell receptors which recognize free antigens, a majority of T cell receptors recognize antigen only when bound to cell surface Major Histocompatibility complex (MHC) molecules (Gerloni and Zanetti 2005). Recognition of antigens by a BCR or TCR complex together with MHC presentation and additional co-stimulatory signals is required to activate both B and T lymphocytes (Janeway 2001). Activated T and B cells induce multiple signaling cascades which activate transcription of genes and result in efficient proliferation of activated, antigen specific B and T cells, and initiation of the adaptive immune response (Janeway 2001; Parham 2009).

1.4 Major Histocompatibility Complex:

The Major Histocompatibility complex (MHC) was initially studied as a genetic component that determines the ability to accept or reject transplanted tissue from individuals of the same species (Kuo, Maruyama et al. 2005). All mammals have a tightly packed cluster of genes located on a chromosome 6 which determines the structure of the MHC. MHC molecules play important roles in the ability of the immune system to differentiate between 'self' and 'non-self,' and are thus crucial in the activation of immune responses against both pathogens and tumors. There are two major types of MHC molecules: MHC class I which present peptides derived from intracellular pathogens and MHC class II which present peptides derived from extracellular pathogens (Drozina, Kohoutek et al. 2005). MHC I are expressed on all nucleated cells with the level of expression differing between cell type, while MHC II are constitutively expressed on antigen presenting cells (APCs) and are inducible by inflammatory cytokine on all nucleated cells. MHC I present intracellular antigens to cytotoxic T cells via the CD8 receptor while MHC II molecules present extracellular peptides to $CD4^+$ T helper cells **(Figure 1.1)** (Rojo, Saizawa et al. 1989; Gerloni and Zanetti 2005).

MHC II molecules play important roles in activating adaptive immune responses and MHC II deficiencies lead to the development of bare lymphocyte syndrome, (BLS), which results in death in infancy (Reith and Mach 2001). Severe combined immunodeficiency (SCID) is also developed due to lack of MHC II expression. Patients with SCID are extremely vulnerable to infection because their lack of $CD4^+$ T cells also results in an inability to activate B cells, nor are they able to provide $CD4^+$ T help during $CD8^+$T cell responses (Reith and Mach 2001). Over

expression of MHC II molecules is also associated with disease development as all autoimmune diseases correlate with over expression of MHC II in the targeted tissues (Swanberg, Lidman et al. 2005). MHC II molecules play important roles in anti-tumor immunity. The presentation of tumor cell antigens by MHC class II molecules is particularly critical in the detection of a newly formed tumor as MHC class II proteins are capable of activating multiple arms of the anti-tumor immune response (Guy, Krajewski et al. 1986; Garrido and Ruiz-Cabello 1991). As MHC II molecules play critical roles in the activation of adaptive immune responses, and as deregulation of MHC II expression has such dire consequences, MHC II expression is tightly regulated, primarily at the level of transcription (Benoist and Mathis 1990).

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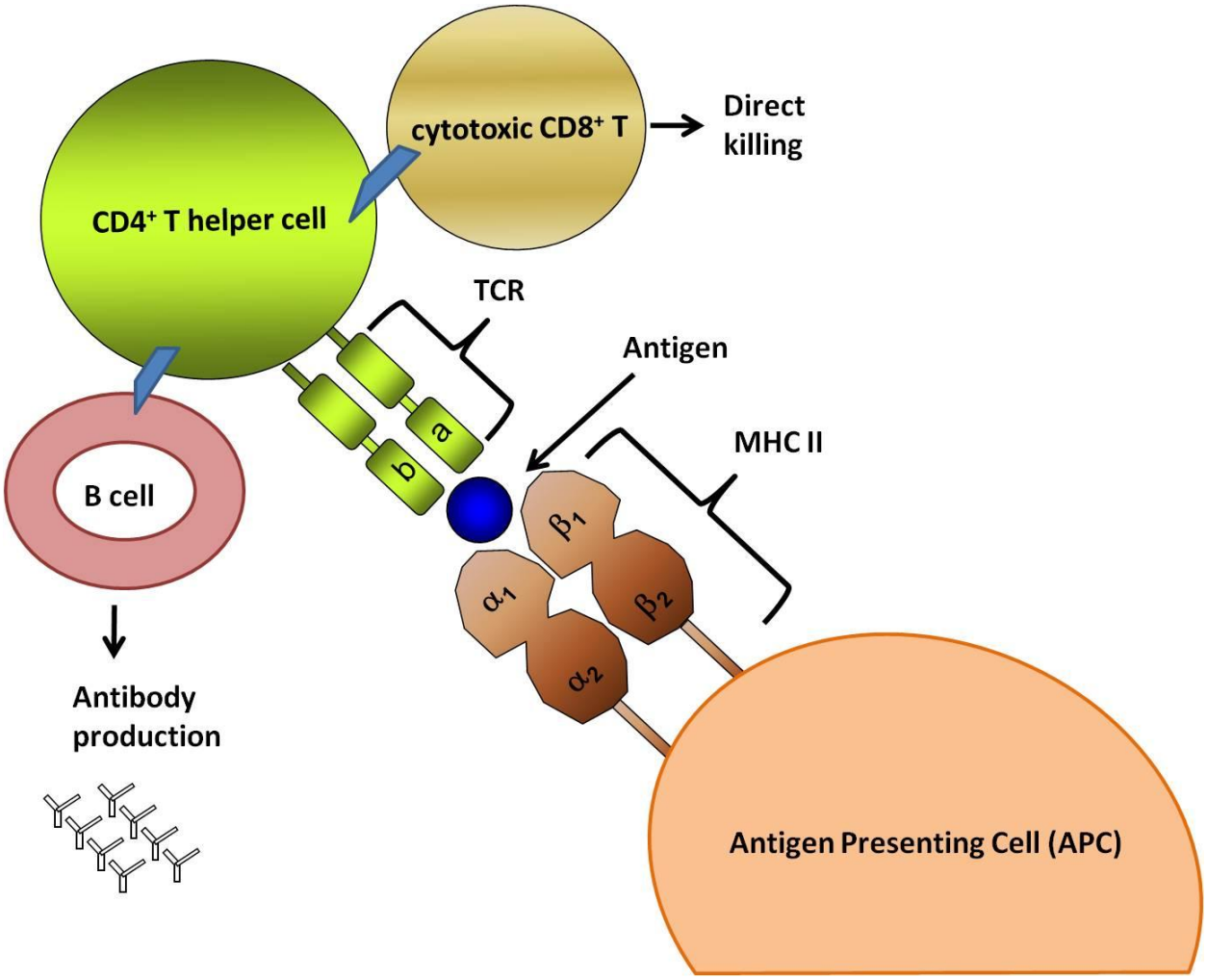

Figure 1.1 MHC II molecules are an indispensable arm of the adaptive immune response as they present processed extracellular antigens to CD4+T cells which in turn initiate adaptive immune responses.

Antigen presenting cells (APC) play an essential role in recognizing, processing, and presenting proteins. Processed antigens are presented by APCs and, by all interferon gamma (IFN-γ) stimulated cells, on MHC II molecules. MHC II are cell surface glycoproteins that present antigens to CD4+ T cells resulting in T cell activation. Activated CD4 T cells can activate B cells to produce specific antibodies and elicit adaptive immune responses. Activated CD4+ T cells can also activate cytotoxic CD8+ T cells to mediate direct killing (Kindt Thomas J 2007; Parham 2009).

1.5 Regulation of MHC II Transcription:

MHC II molecules are glycoproteins which are constitutively expressed on the surface of antigen presenting cells such as activated macrophages, B cells, dendritic cells, and activated T cells (Cresswell and Howard 1997; Morris, Beresford et al. 2002). Expression of MHC II can be induced in all nucleated cells by inflammatory cytokines, primarily the cytokine interferon gamma (IFN-γ) (Benoist and Mathis 1990; Kaufman, Salomonsen et al. 1994). IFN-γ is a type II interferon which, through binding to the type II IFN receptor, activates transcription of target genes including MHC II. In contrast to IFN-α and IFN-β, which can be expressed by all cells, IFN-γ secretion is restricted to T lymphocytes, dendritic cells, and NK cells (Mach, Steimle et al. 1996; Boss and Jensen 2003; Platanias 2005). Inducible expression of MHC II allows for enhanced antigen presentation and induction of localized immune responses. The expression of MHC II genes is tightly regulated by multiple transcription factors and by chromatin remodeling enzymes that bind to a conserved regulatory region in the MHC II gene (Ting and Trowsdale 2002; Boss and Jensen 2003; Drozina, Kohoutek et al. 2005). This regulatory region consists of conserved sequences designated as the X1 box, X2 box, and Y box (Benoist and Mathis 1990; Ting and Trowsdale 2002). These sequences are respectively recognized by ubiquitously expressed DNA binding factors. The X1 box is bound by the regulatory factor X (RFX) which is a trimer consisting of RFX5, RFXANK, and RFXAP (Masternak, Barras et al. 1998; Nagarajan, Louis-Plence et al. 1999). The c-AMP responsive element-binding protein (CREB) binds to the X2 box (Moreno, Beresford et al. 1999). The Y box is bound by the nuclear factor Y (NF-Y) trimeric complex which consists of NF-YA, NF-AB, and NF-YC (Mantovani 1999; Drozina, Kohoutek et al. 2005). Together, these factors form an enhanceosome, formation of which is necessary but

not sufficient for the initiation of MHC II transcription. Instead, the enhanceosome serves as platform for binding the master regulator of MHC II transcription, the class II transactivator, CIITA (Masternak, Muhlethaler-Mottet et al. 2000) **(Figure 1.2)**.

CIITA is a co-activator as it is not a DNA binding protein, but instead binds to the enhanceosome complex through interactions with RFX, NFY, and CREB (Masternak, Muhlethaler-Mottet et al. 2000; Ting and Trowsdale 2002). As seen in **Figure 1.2**, CIITA initiates transcription of MHC II genes by recruiting the basal transcriptional machinery including the TATA binding protein (TBP), TATA associated factors (TAFs) (Mahanta, Scholl et al. 1997), histone acetyltransferases (HATs) (Spilianakis, Papamatheakis et al. 2000; Wright and Ting 2006; Koues, Dudley et al. 2008), and histone methyltransferases (HMTs) (Koues, Mehta et al. ; Zika, Fauquier et al. 2005), which act together to change MHC II promoter accessibility. CIITA recruitment leads to enhanced levels of acetylation at the proximal promoter of MHC II genes that in turn promotes efficient assembly of transcription machinery and rapid activation of MHC II transcription (Koues, Mehta et al. ; Sisk, Gourley et al. 2000; Wang, Huang et al. 2001). Therefore, interactions of CIITA with the MHC II proximal promoter are crucial for the activation of MHC II transcription.

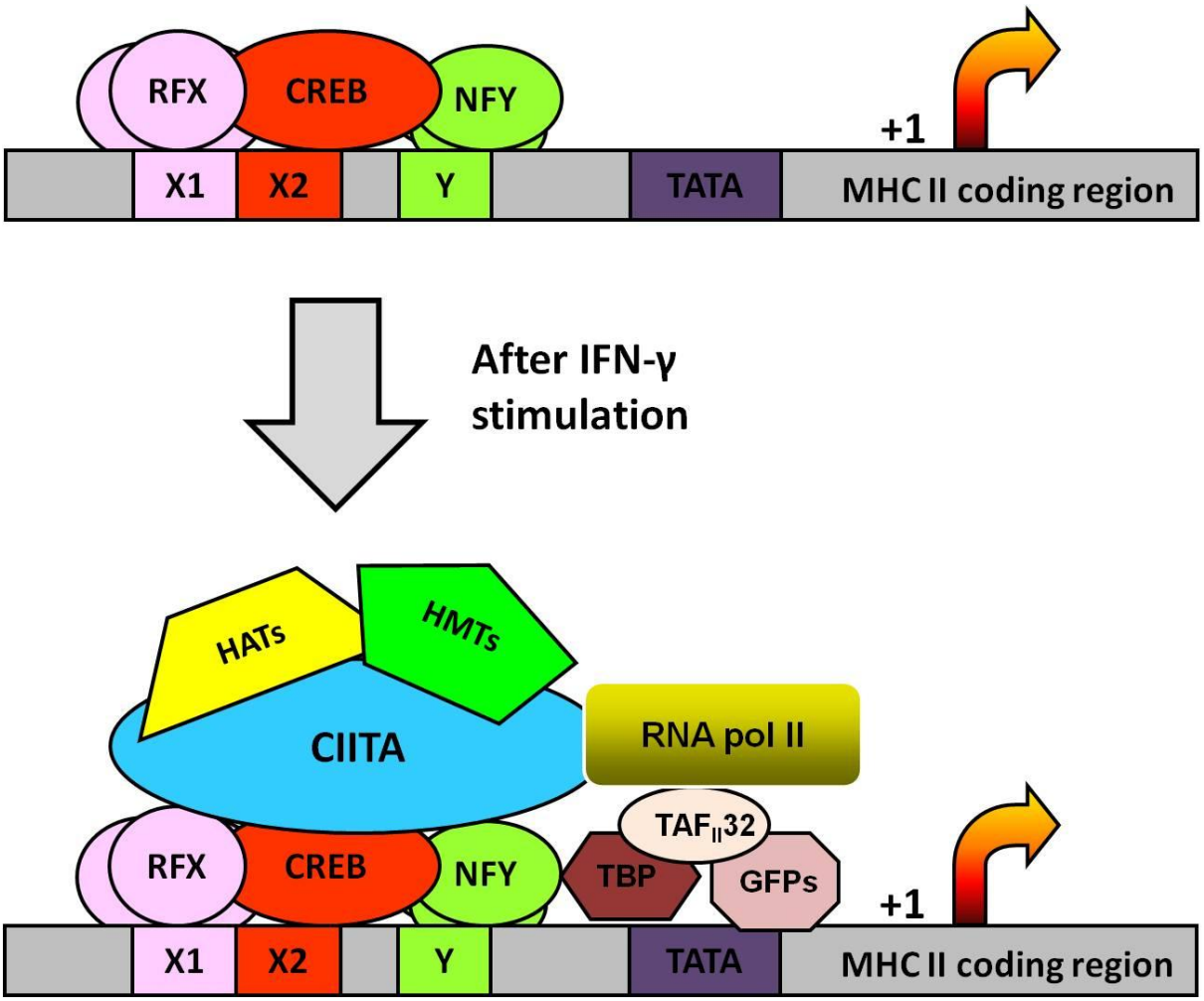

Figure 1.2 MHC II promoter region.

(Top) MHC II proximal promoter contains X1, X2 and Y boxes. The X1 box is bound by RFX which is a trimer consisting of RFX-5, RFX-ANK and RFX-AB. CREB binds X2 box. The Y box is bound by the NF-Y trimeric protein consisting of NF-YA, NF-YB and NF-YC. The binding of each of these constitutively expressed transcription factors forms the MHC II enhanceosome complex, which is necessary but insufficient to mediate cytokine inducible MHC II transcription (Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999; Zika, Greer et al. 2003; Zika, Fauquier et al. 2005).

(Bottom) The complex creates a surface necessary to recruit CIITA that is absolutely necessary for MHC II expression. After prolonged cytokine stimulation CIITA then recruits RNA pol II with the transcription machinery and histone modifying enzymes including histone acetyltransferases (HATs), histone methyltransferases (HMTs) (Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999; Zika, Greer et al. 2003; Zika, Fauquier et al. 2005).

1.6 CIITA, the Master Regulator of MHC II:

CIITA is the master regulator for MHC II gene expression (Mach, Steimle et al. 1996). Its activity is absolutely required for the expression of the MHC class II genes, however the regulation of CIITA itself is less understood (Chang, Fontes et al. 1994). In order to maintain tight regulation of MHC II, CIITA expression is also tightly regulated at the level of transcription (Ting and Trowsdale 2002). Transcription of CIITA is regulated in a cell specific manner by four different promoters: pI, pII, pIII and pIV (Muhlethaler-Mottet, Otten et al. 1997) **(Figure 1.3)**. By virtue of different transcriptional start sites illustrated in the top part of **Figure 1.3**, each promoter yields a different CIITA isoform that will have a unique first exon. The promoter I (pI) isotype is expressed primarily in dendritic cells and macrophages. Promoter II (pII) is not well conserved amongst different species and often considered to be inactive (Baton, Deruyffelaere et al. 2004). Promoter III generates the CIITA isotype (pIII) in constitutively expressed in B cells, and can also be enhanced by the IFN-γ stimulation (Piskurich, Gilbert et al. 2006).

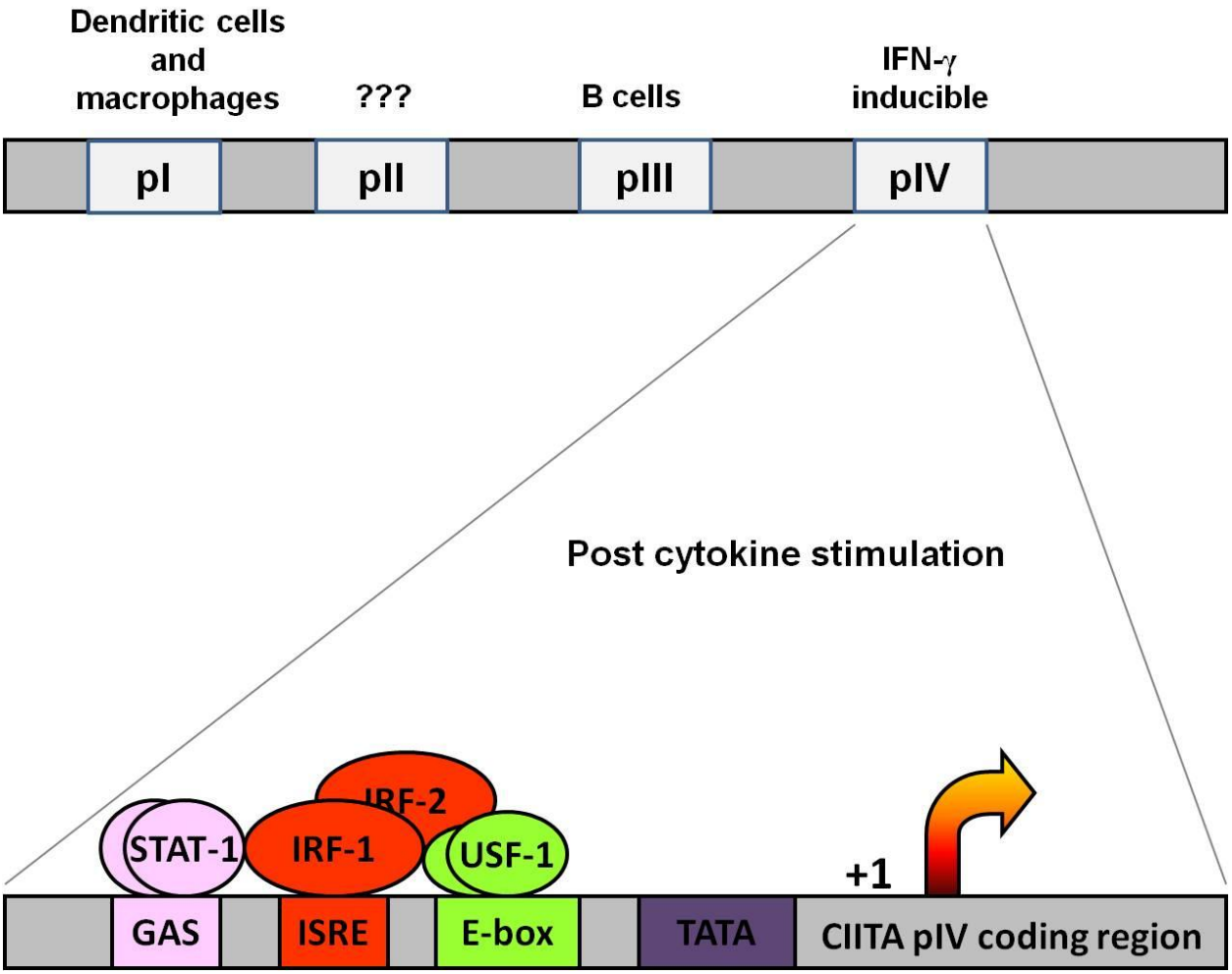

Figure 1.3 CIITApIV proximal region.

(TOP) CIITA is transcribed from 4 different promoter regions depending on a cell type. Promoter I drives expression of CIITA in dendritic cells. The function of promoter II is not conserved between species and the promoter is believed to be inactive. Promoter III primarily functions in B cells and promoter IV is responsible for the IFN-γ induced expression of CIITA.

(Bottom) The promoter region of CIITApIV isoform has 3 conserved sequences: GAS, ISRE and E-BOX. STAT-1 binds the GAS box within 30 minutes post IFN-γ stimulation, IRF-1 and IRF-2 gets recruited to ISRE within 4 hours of cytokine stimulation and USF-1 is the constitutively expressed transcription factor (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002).

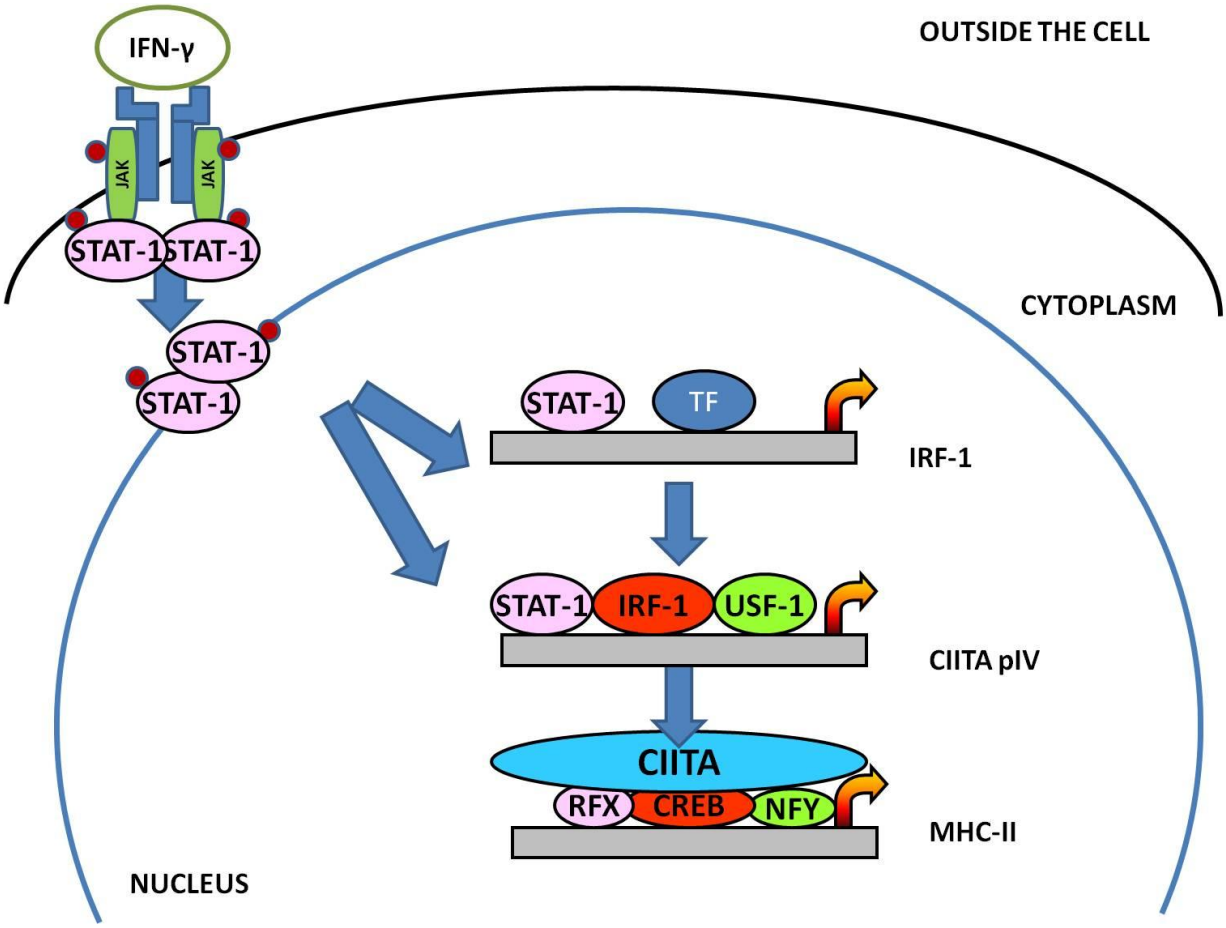

Figure 1.4 Activation of a JAK –STAT signal transduction pathway.

IFN-γ binds to its receptor and initiates the kinase function of JAK, which autophosphorylates itself. The STAT protein then binds to phosphorylated receptor. Once phosphorylated STAT-1 translocates to nucleus, where it binds to DNA and promotes transcription of genes that are responsive to STAT-1. STAT-1 binds to CIITA isoform IV and IRF-1 promoter regions. IRF- ones transcribed and translated binds to CIITApIV together with ubiquitously expressed USF-1 and inducibly recruited STAT-1and initiate expression of CIITA. CIITA protein, the master regulator of MHC II genes then binds ubiquitously expressed components of the enhanceosome complex and initiate expression of MHC class II genes (Piskurich, France et al. 1993; Morris, Beresford et al. 2002; Piskurich, Gilbert et al. 2006).

Promoter IV CIITA isotype (pIV) is the IFN-γ responsive form of CIITA which is predominantly expressed in IFN-γ stimulated cells (Piskurich, Gilbert et al. 2006) resulting in parallel INF-γ induction of MHC II expression (Muhlethaler-Mottet, Otten et al. 1997; Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002). It is critical that CIITA expression is tightly regulated because abbearant expression is associated with multiple diseases including autoimmune conditions and cancer (van den Elsen, Gobin et al. 2001; Satoh, Toyota et al. 2004).

Cytokine dependent MHC II transcription allows for enhanced antigen presentation by all nucleated cells and is achieved by binding of the transcription factors signal transducer and activator of transcription (STAT-1) and interferon regulatory factor 1 (IRF-1) to CIITApIV (Piskurich, France et al. 1993; Piskurich, Blanchard et al. 1995; Piskurich, Youngman et al. 1997; Piskurich, Wang et al. 1998; Piskurich, Linhoff et al. 1999). Cytokine activated CIITApIV is also occupied by the constitutively expressed transcription factor upstream stimulating factor 1 (USF-1) **(Figure 1.3)** (Muhlethaler-Mottet, Di Berardino et al. 1998; Piskurich, Linhoff et al. 1999; Pattenden, Klose et al. 2002). Activation of inducible CIITApIV is initiated when IFN-γ binds to its surface receptor and occurs via the Janus kinase signal transducer and activator of transcription (JAK-STAT) signal transduction pathway **(Figure 1.4)** (Morris, Beresford et al. 2002; Pattenden, Klose et al. 2002; Piskurich, Gilbert et al. 2006). Activated JAK1 and JAK2 phosphorylate STAT-1, causing STAT-1 to translocate to the nucleus. In addition to binding to the gamma activating sequence (GAS) box of CIITApIV, STAT-1 binds the IRF-1 gene STAT-1 binding site (Muhlethaler-Mottet, Di Berardino et al. 1998). CIITApIV also has conserved GAS and interferon stimulated response elements (ISRE) (Morris, Beresford et al. 2002) which bind

interferon regulatory factors (IRFs) that are globally involved in IFN responses (Piskurich, Linhoff et al. 1999; Piskurich, Gilbert et al. 2006; Wright and Ting 2006). In addition to promoting binding of transcription factors to pIV, IFN-γ induces acetylation of histones which loosens the chromatin structure and increases the accessibility of CIITApIV (Sterner and Berger 2000; Wright and Ting 2006). Because MHC II plays important roles in initiating, maintaining, and eventually terminating adaptive immune responses, the unique ability of CIITA to regulate the expression of MHC II genes makes CIITA a major player in the regulation of antigen presentation and adaptive immune responses.

1.7 The 26S Proteasome

The ubiquitin proteasome system (UPS) is well known for its role in the degradation of polyubiquitinated proteins, including transcription factors and chromatin remodeling proteins (Ciechanover 1994). However, recent investigations indicate subunits of the 26S proteasome are essential for transcriptional regulation of various genes independent of their roles in protein degradation (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003). Research in the Greer lab has shown that subunits of the UPS are necessary for transcriptional regulation of MHC II and CIITA genes (Koues, Mehta et al. ; Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). The role of the proteasomal subunit S6a in transcription will be described in detail in chapters 2 and 5 of this dissertation.

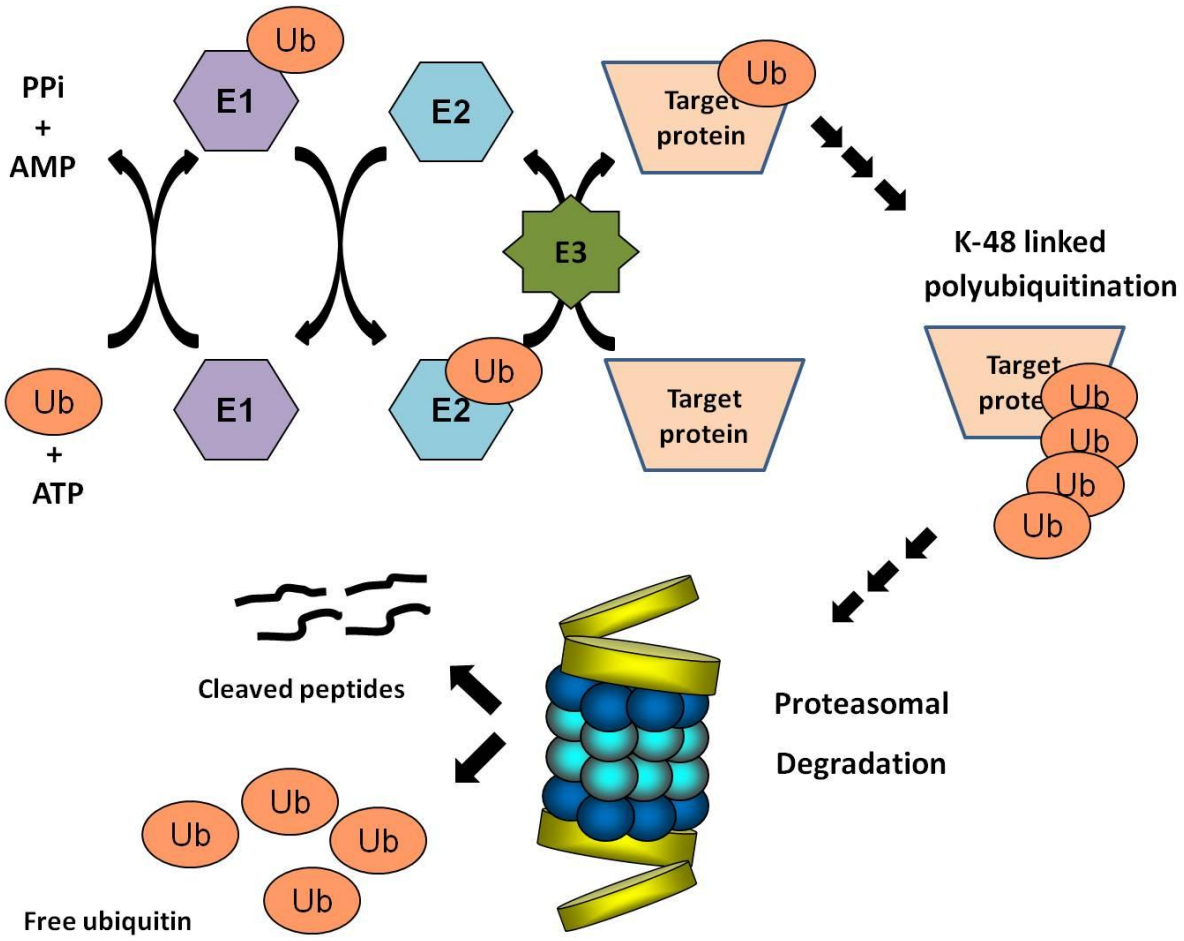

Figure 1.5 Enzymatic cascade of ubiquitination.

Molecule of Ubiquitin (Ub) is activated in ATP dependent manner and covalently linked to E1 ubiquitin activating enzyme. Activated ubiquitin is transferred from E1to E2 ubiquitin conjugating enzyme and then directly or with a help of E3 ubiquitin ligase to a lysine residue on a targeted protein. If the targeted protein is marked for degradation multiple Ub molecules will be added via K-48. Once when at least 4 Ub molecules are attached, the protein is recognized by the 26S proteasome and degraded. The 19S proteasome e recruits the polyubiquitinated proteins, cleaves of the Ub, unfolds and directs the proteins into the 20S core that cleaves proteins into peptides (Ciechanover 1994; Coux, Tanaka et al. 1996; Strickland, Hakala et al. 2000).

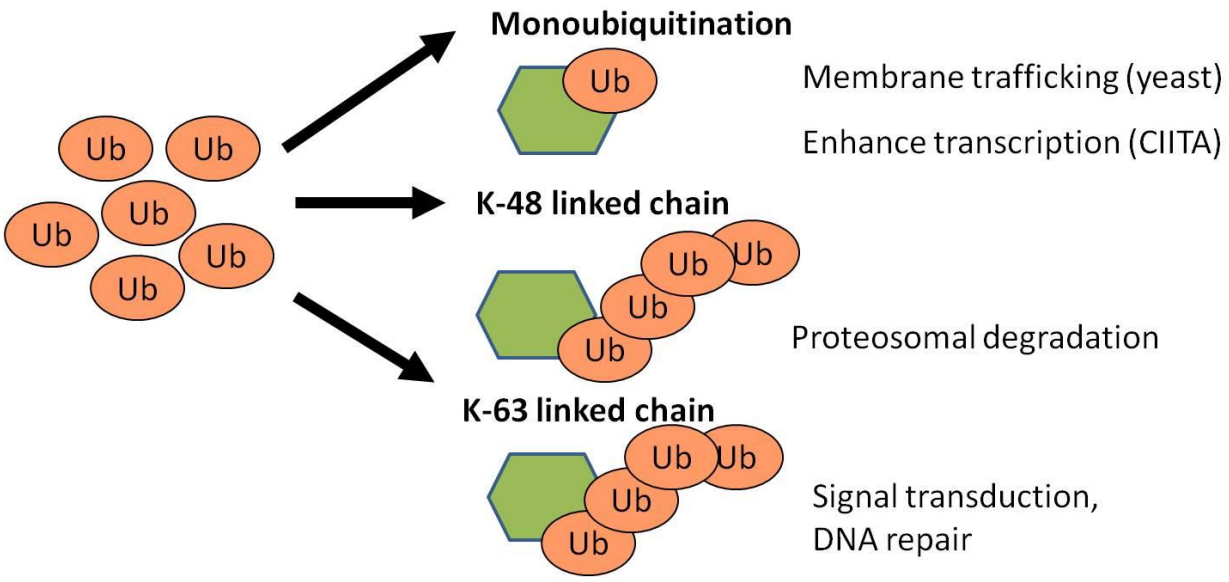

Figure 1.6 Ubiquitination regulates numerous cellular processes.

Ubiquitin (Ub) can be attached to lysine residues of cellular proteins either as a single unit (monoubiquitin) or in the form of branched, polyubiquitin chains. Several distinct structural forms of polyubiquitin can be assembled. Each modification confers different effect on the targeted protein they are attached to. In general, proteians that are monoubiquitinated are not degraded by the proteasome and play an important role in vesicle trafficking, gene activation and DNA repair. Polyubiquitination via K-48 tragets proteins for degradation via 26S proteasome and polyubiquitination via K-63 is important in various signaling pathways (Chau, Tobias et al. 1989; Finley, Sadis et al. 1994; Thrower, Hoffman et al. 2000; Kim, Kim et al. 2007).

It is well established that polyubiquitination targets proteins for degradation by the 26S proteasome (Pickart 2004; Haglund and Dikic 2005). The 8.5 Kd, 76 amino acid protein ubiquitin was initially described as a protein modification which is covalently attached to lysine residues of target molecules (Ciechanover 1994; Adams 2003; Shmueli and Oren 2005). Chains of four ubiquitin molecules are necessary for recognition and degradation by the 26S proteasome **(Figure 1.5)** (Ciechanover 1998; Conaway, Brower et al. 2002). Ubiquitin is attached to proteins via an isopeptide bond between the C terminus of the ubiquitin molecule and a lysine (K) side chain on the target protein. A protein targeted for degradation is tagged with ubiquitin molecules via a three step enzymatic cascade. Polyubiquitination occurs when these steps are repeated, resulting in the formation of a chain of four ubiquitin molecules on the target protein **(Figure 1.5)** (Thrower, Hoffman et al. 2000).

Ubiquitin has seven lysine residues (K-6, K-11, K-27, K-29, K-33, K-48 and K-63) and, while a majority of proteins are marked for proteasomal degradation by polyubiquitination via K-48, recent studies have shown that K-63 linked polyubiquitination also marks proteins for degradation (Chau, Tobias et al. 1989; Finley, Sadis et al. 1994) (Kim, Kim et al. 2007). Proteins polyubiquitinated via K-63 play roles in the activation of several cellular pathways and are eventually degraded in lysosomes (Tan, Wong et al. 2007). Alternatively, monoubiquitination regulates many cellular processes including nuclear export, DNA repair, protein interactions, and histone modifications **(Figure 1.6)** (Terrell, Shih et al. 1998; Lucero, Penalver et al. 2000; Lotocki, Alonso et al. 2003; Gupta-Rossi, Six et al. 2004; Sigismund, Polo et al. 2004; Haglund and Dikic 2005). Despite the multiplicity of possible ubiquitin combinations, relatively little is known of

the outcomes of alternative, ie, non-lysine 48 or 63 linked, ubiquitination; these alternative ubiquitination reactions will likely be a primary focus of ubiquitin research for years to come.

Once a protein is tagged via lysine 48 polyubiquitination with 4 or more molecules of ubiquitin, the protein is targeted to the 26S proteasome for degradation (Ciechanover 1998; Thrower, Hoffman et al. 2000; Finley, Ciechanover et al. 2004).

The 26S proteasome in mammalian cells is a 2.5 MDa multi-protein complex made of a 19S regulatory particle (RP) and a 20S proteolytic core (Baumeister, Walz et al. 1998) which can exist independently in both the nucleus and cytoplasm (Peters, Franke et al. 1994). The 19S can be further divided into two parts: a lid and a base. The lid is composed of eight non-ATPase subunits which are required for protein degradation (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998; Gorbea, Taillandier et al. 1999). The base of the 19S contains six ATPases belonging to the ATPases associated with a variety of cellular activities (AAA) family: S4, S6a, S6b, S7, Sug1 (S8) and Sug2 (S10b) which correspond, respectively, to the yeast homologous proteins Rpt 1-6, and four non-ATPase subunits, Rpn1, Rpn2, Rpn10, and Rpn13 (**Figure 1.7)** (Gorbea, Taillandier et al. 1999; Adams 2003; Bhat, Turner et al. 2008). The 20S catalytic core is a 700 kDa cylinder which consists of four stacked rings, with each ring containing seven α and β subunits in order of α β β α (Coux, Tanaka et al. 1996; Gorbea, Taillandier et al. 1999).

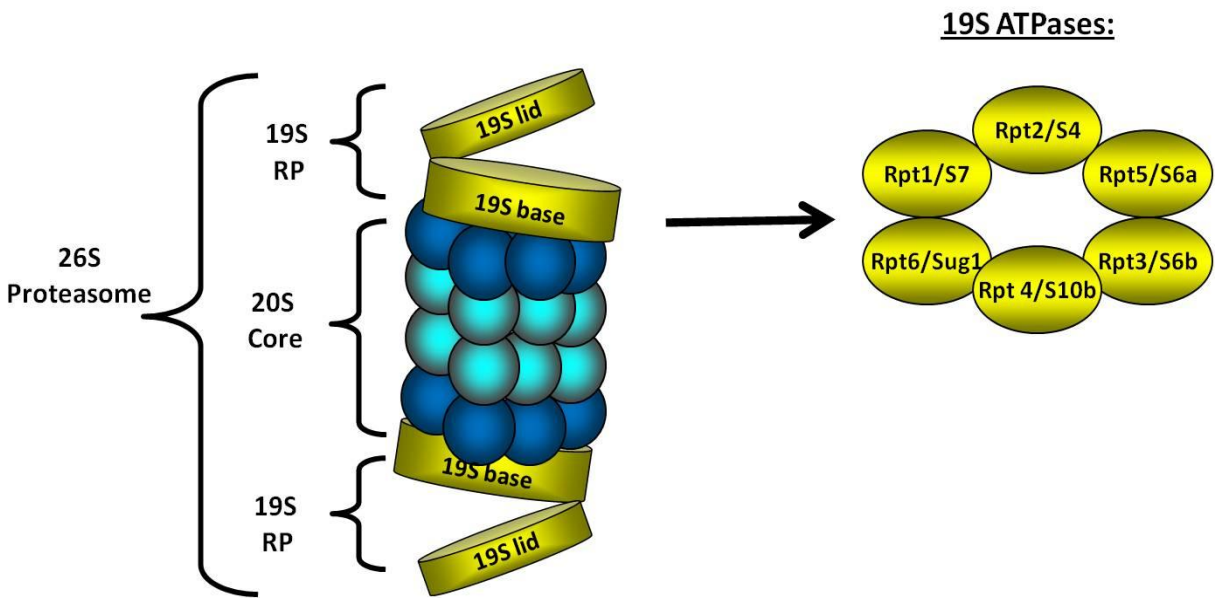

Figure 1.7 The 26S Proteasome.

The 26S proteasome is composed of a 20S proteolytic core and a 19S regulatory particle (RP). The 20S core particle is a hollow cylindrical structure composed of two heptameric rings of α subunits and two heptameric rings of β subunits (αββα). The 20S core particle is capped on one or both sides by a 19S regulatory particle to form the active 26S proteasome. Four non-ATPase (Rpn1, Rpn2, Rpn10 and Rpn13) and six ATPase subunits (Rpt1-6) are found in the 19S base.

The base ATPases contain a C terminal hydrophobic tyrosine X motif that docks into the pockets of the α rings of the 20S (Smith, Chang et al. 2007). In the presence of ATP, the 20S catalytic core associates with the 19S regulatory particle on both sides to form the 26S proteasome, allowing for the recognition of the ubiquitinated substrates which are marked for degradation (Coux, Tanaka et al. 1996; Gonzalez, Delahodde et al. 2002). The 19S regulatory particle recognizes the ubiquitin chains on targeted proteins, cleaves the chains, unfolds the protein, and directs the unfolded protein to the 20S core for degradation **(Figure 1.5)** (Coux, Tanaka et al. 1996; Strickland, Hakala et al. 2000). The result of proteasomal degradation is cleaved peptides with an average length of 8-12 amino acids and free ubiquitin, which is reactivated and recycled (Jung, Catalgol et al. 2009). Through these mechanisms the 26S proteasome eliminates a variety of unwanted and/or misfolded proteins, (Hilt and Wolf 1996; He, Qi et al. 1998) and thus regulates many important cellular processes.

1.8 Non-proteolytic roles of the proteasome:

Growing studies indicate the 26S proteasome plays critical regulatory, but degradation independent, roles in gene expression. While it is well accepted that 19S ATPases recognize and unfold ubiquitinated proteins for degradation, these ATPases have RNA/DNA helicase activity, indicating their potential involvement in cellular processes independent of proteolysis (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998; Adams 2003). Studies in yeast demonstrate the 19S ATPases associate with transcription factors, with RNA polymerase II, with activated promoters, and with transcriptional elongation complexes (Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Gillette, Gonzalez et al. 2004). Knockout studies in yeast and

in mammalian cells have shown that the 19S base is essential for initiation and elongation by RNA pol II, but that proteolytic activity of the 26S is not required (Ferdous, Kodadek et al. 2002). Work in the Greer has demonstrated the 19S ATPases associate with the MHC II proximal promoter, with CIITA, and with the enhanceosome complex where they play important role in regulating CIITA activity and MHC II expression (Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). Evidence also indicates inhibition of the 19S decreases elongation, while inhibiting the catalytic activity of the 20S increases elongation, thus the balance between the 19S/20S subunits is crucial for transcriptional regulation (Gillette, Gonzalez et al. 2004). These observations indicate the ubiquitin/proteasome pathway plays multiple roles in transcription and imply the non-degradative roles of the proteasome may more far reaching than its known roles in protein degradation.

1.9 ROLES FOR THE 26S PROTEASOME COMPONENTS IN TRANSCRIPTION:

Transcription is a first step in gene expression and is divided into 5 stages: pre-initiation, initiation, promoter clearance, elongation, and termination. During pre-initiation, general transcription factors guide RNA pol II to proximal promoter regions. In eukaryotes transcription requires special sequences, known as TATA boxes, located around 30 base pairs upstream of start sites. The TATA box is a binding site for the TATA binding protein (TBP) which is itself a part of Transcription Factor II B (TFIIB), here illustrated on the MHC II proximal promoter **(Figure 1.2)**. The core domain of TFIIB associates with TBP at the TATA box and interacts with the DNA both upstream and downstream of the TATA box, thus recruiting RNA pol II to the correct site (Calvo and Manley 2003; Deng and Roberts 2007). Eukaryotic RNA pol II is not directly recruited to the DNA region of interest. Instead, groups of transcription factors create an initiation complex that recruits RNA pol II and initiates transcription. Although extensively investigated, the full mechanisms that control transcriptional initiation, elongation, and termination remain unknown.

In 1992, Swaffield and colleagues first indentified roles for proteasome subunits in transcriptional regulation. These researchers demonstrated 19S ATPase subunits Rpt6 (Sug1) and Rpt4 (Sug2) rescued a mutant transactivator Gal4 in *Saccharomyces cerevisae* (Swaffield, Bromberg et al. 1992; Rubin, Coux et al. 1996). Soon thereafter, the intact 26S proteasome, as well as the 19S regulatory particle and the 20S core, were demonstrated to localize in both the nucleus and the cytoplasm (Peters, Franke et al. 1994). These early experiments were the first to link the 26S proteasome to transcription, and in the years that followed, researchers have ob-

served that the 19S component of the proteasome also plays important roles in regulating transcription at a variety of different genes (Koues, Mehta et al. ; Kinyamu, Chen et al. 2005; Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). Subunits of the proteasome are required in the formation of initiation complexes, in maintaining elongation, and in executing termination. Roles for the 19S ATPases in transcriptional initiation at CIITApIV will be described in this dissertation in Chapter 2 and in Chapter 5, we demonstrate how 19S subunits maintain transcriptional elongation of CIITA genes.

1.10 Roles in transcription initiation:

Recent publications have demonstrated the 19S ATPases play important roles in initiating transcription of various yeast and mammalian genes. In yeast, the 19S proteasome has been shown to be responsible for the recruitment of the histone acetyltransferase (Spt-Ada-Gcn5-acetyl-transferase) SAGA to promoter regions. Lack of the 19S ATPase Rpt6, the yeast homolog of mammalian Sug1, results in reduced recruitment of the HAT SAGA and in lower levels of histone H3 acetylation, indicating that 19S ATPases are important for recruiting HATs to promoter regions (Lee, Ezhkova et al. 2005). Furthermore, as mentioned above, alleles of Rpt6 rescue mutations in the Gal4 activation domain (Rubin, Coux et al. 1996). Rpt6 also mediates the recruitment of transcription factors to TBP (Swaffield, Melcher et al. 1995) and binds to various actively transcribing genes (Gonzalez, Delahodde et al. 2002). Overall, these observations demonstrate the 19S proteasome plays important roles in transcriptional initiation of yeast promoters. The ubiquitin proteasome system is also linked to transcription initiation in mammalian cells. In

2006, Rasti and colleagues demonstrated the 19S ATPase Sug1 plays positive roles in adenovirus E1A dependent transcription (Rasti, Grand et al. 2006). In 2007, Zhu determined the 26S proteasome enhances recruitment of p53 to p21 *wafl* responsive promoters (Zhu, Wani et al. 2007). Concurrent observations from Lassot and colleagues demonstrated 19S ATPases play important roles in regulating Tat dependent transcription of HIV-1 genes (Lassot, Latreille et al. 2007). The 19S ATPase Sug-1 also plays an important proteolytic and non proteolytic role in regulating transcription mediated by retinoic acid (Ferry, Gianni et al. 2009).

The 19S ATPases play critical roles in regulating transcription initiation of MHC II and CIITA genes. The ATPase Sug1 is recruited to the MHC II proximal promoter following IFN-γ stimulation. Decreased expression of Sug1 results in decreased recruitment of CIITA to the MHC II promoter and in reduced expression of MHC II genes (Bhat, Turner et al. 2008). Sug1 exerts control over CIITA promoter binding by regulating histone H3 acetylation at the MHC II proximal promoter through interactions with acetylated histone H3. In cells treated with Sug1 specific siRNA, MHC II histone acetylation is decreased with a preferential impact on acetylation at histone H3 lysine 18. Sug1 also recruits the histone acetyltransferase CREB binding protein (CBP) to MHC II promoters, further implicating Sug1 as an important regulator of histone modifications at the MHC II proximal promoter (Koues, Dudley et al. 2008).

Sug1 is also important for regulating H3 lysine 4 trimethylation and H3 arginine 17 dimethylation at both MHC II and CIITA genes. However, Sug1 does not impact H3 lysine 36 trimethylation or histone H2B lysine 120 ubiquitination. As both arginine 17 dimethylation and lysine

4 trimethylation are important activating modifications, these observations further implicate Sug1 as being involved in activating transcription initiation (Koues, Dudley et al. 2009). Supporting a role for Sug1 in initiation are observations that the absence of Sug1 enhances levels of H3 lysine 27 trimethylation, resulting in repression of transcription initiation (Koues, Mehta et al.). Chapter 2 of this dissertation details observations that an additional 19S ATPase, S6a, plays critical roles in initiating transcription of CIITApIV. Decreased expression of S6a significantly impacts the recruitment of transcription factors STAT-1 and IRF-1 to the CIITApIV proximal promoter and diminishes levels of activating acetylation on histone H3 and histone H4 with a preferential loss of histone H3 lysine 18 acetylation and H4 lysine 8 acetylation (Truax, Koues et al.) Together, these observations provide evidence that the19S proteasome plays regulatory roles in transcriptional initiation of the cytokine inducible genes MHC II and CIITA.

1.11 Regulation of transcriptional elongation:

Following transcription initiation, the multi-component transcriptional machinery falls under the control of positive and negative transcription factors which regulate gene expression. During the early steps of elongation, RNA polymerase II is paused by negative transcription elongation factors (N-TEFs) (Sims, Belotserkovskaya et al. 2004). As illustrated in **Figure 1.8**, positive transcription elongation factor (P-TEF-b) is recruited to the N-TEF complex where it phosphorylates the carboxy terminal domain (CTD) of RNA pol II and N-TEF, thus permitting elongation (Sims, Belotserkovskaya et al. 2004). P-TEF-b exists in two distinct molecular forms as an active and inactive complex (Nguyen, Kiss et al. 2001; Yang, Zhu et al. 2001). P-TEF-b activity is inhibited by association with hexamethylene bisacetamide (HMBA) inducible protein

1 (Hexim-1) or 2 (Hexim-2) that is bound to 7SK small nuclear RNA (snRNA) **(Figure 1.8)** (Barboric, Kohoutek et al. 2005; Blazek, Barboric et al. 2005; Dulac, Michels et al. 2005; Schulte, Czudnochowski et al. 2005). The CTD domain of RNA pol II is composed of 52 repeats of the heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. During transcriptional initiation, the CTD domain remains hypophosphorylated and becomes hyperphosphorylated during elongation (Corden, Cadena et al. 1985). The switch from transcriptional initiation to elongation is dynamic and is controlled by interactions between positive and negative transcription factors. Additional regulation through pausing of RNA pol II allows the replacement of initiation factors with molecules required for transcriptional elongation and RNA processivity.

In addition to the above described roles in transcription initiation, the 26S proteasome also regulates transcription elongation. Initial observations that inhibition of proteasome activity reduces recruitment of RNA pol II to various yeast promoters first implicated the proteasome as involved in of transcriptional elongation (Lipford, Smith et al. 2005). Evidence has since accumulated that 19S ATPases play important roles in pol II dependent elongation that are independent of the presence of the 20S core (Ferdous, Gonzalez et al. 2001). Rpt 6, the yeast homolog of mammalian Sug1, has been demonstrated to link histone ubiquitination and methylation, which are both important steps in elongation (Sun and Allis 2002; Ezhkova and Tansey 2004; Pokholok, Harbison et al. 2005). 19S components also interact with the yeast elongation factor CDc8 (Ferdous, Gonzalez et al. 2001). Studies in mammalian systems are less extensive, but also support roles for proteasomal subunits in transcriptional elongation. 19S ATPases are recruited to coding regions of HIV-1 genes with a preferential abundance of the 19S ATPase S6a (Lassot, Latreille et al. 2007). Proteasome subunits also associate also with coding regions of estrogen

receptor responsive pS2 genes a complex with elongin and RNA pol II (Zhang, Sun et al. 2006), suggesting proteasomal subunit cooperation with transcriptional factors to mediate elongation. Observations in the Greer lab indicate robust occupancy of 19S ATPases in coding regions of CIITA and MHC II genes (Truax, Koues et al.), mechanistic details of these associations are described in detail in Chapter 5 of this dissertation.

More than a decade has passed since scientists first explored non-degradative roles for the proteasome. The discovery of nuclear proteasome, followed by preliminary research in yeast first indicated the proteasome is 'not just degrading' anymore. Recent studies in both mammalian and yeast systems indicates 19S ATPase components of the ubiquitin proteasome system play important roles in regulating transcription of various genes via both proteolytic and non proteolytic roles. Data described in detail in Chapters 2 and 5 of this dissertation adds to this growing body of evidence by demonstrating that the 19S ATPase S6a is a crucial regulator of both transcriptional initiation and elongation of CIITA genes. As MHC II and its master regulator CIITA are major players in adaptive immunity, tight transcriptional regulation of these genes is essential to a properly functioning immune system. The contributions of my data increase our understanding of the transcriptional regulation of not only these genes, but of all inducible genes where a rapid response to stimulation is essential.

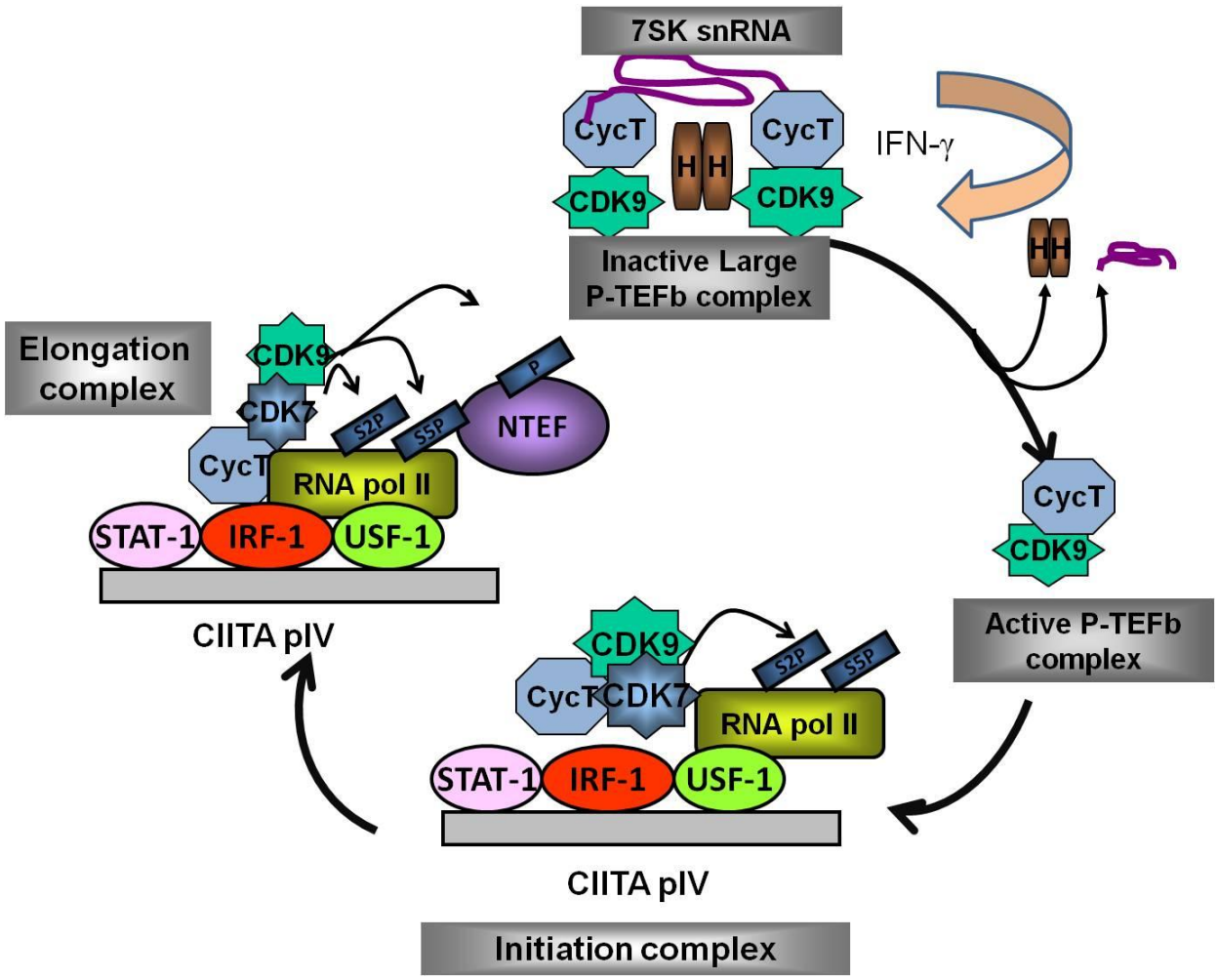

Figure 1.8 Dynamics at the transcriptional initiation and elongation of CIITA.

Transcriptional initiation and elongation at CIITA is regulated by interacting with specific transcription factors. P-TEF-b is in a large inactive complex with 7SK and Hexim dimmers. Once stimulated Hexim and 7S dissociates from the complex allowing the P-TEF-b to transform into active form that is recruited to proximal promoter. The CDK9 subunit of P-TEF-b then phosphorylates RNA pol II at Ser5 and also phosphorylates N-TEF to release the inhibition on RNA pol II and thus help in the transition from initiation to elongation(Corden, Cadena et al. 1985; Peng, Marshall et al. 1998; Michels, Fraldi et al. 2004; Sims, Belotserkovskaya et al. 2004; Barboric, Kohoutek et al. 2005) .

1.12 THE ACCESSIBILITY OF CHROMATIN:

DNA exists in a highly organized, tightly packed form termed chromatin (Berger 2007). Differential packaging of DNA with histone and non-histone proteins into chromatin determines DNA accessibility during transcription. Chromatin is dynamic and can be found in multiple forms, from condensed, tightly packed heterochromatin that is inactive to loosely organized, and transcriptionally accessible, euchromatin (Berger, Kouzarides et al. 2009). The fundamental unit of chromatin is the nucleosome (Mandel and Fasman 1976; Berger 2007). Histones (H) are integral components of nucleosome structures as they provide a scaffold for double stranded DNA to wrap around. There are four histone proteins: H2A, H2B, H3, and H4, two copies of which form the octameric nucleosome structure along with a fifth linker histone, H1 **(Figure 1.9)** (Kornberg and Thomas 1974; Luger, Mader et al. 1997). Histones are positively charged, while DNA is negatively charged due to phosphate groups in its sugar backbone. Because of this charge differential, DNA is wrapped tightly around the histone octamers (Ward, Bowman et al. 2009). As such, the base unit of chromatin are nucleosomes, consisting of histone proteins around which DNA is wrapped 1.67 times (Luger, Mader et al. 1997). Nucleosome cores consist of two H2A-H2B dimers and H3-H4 tetramers. Histone H1 stabilizes nucleosomes by interacting with double stranded DNA as it enters and exits the nucleosome (Kouzarides 2007) and aids formation of dinucleosomes (Widom 1998). Dinucleosomes fold over to form 30nm fibers that coil and wrap around to form chromatin.

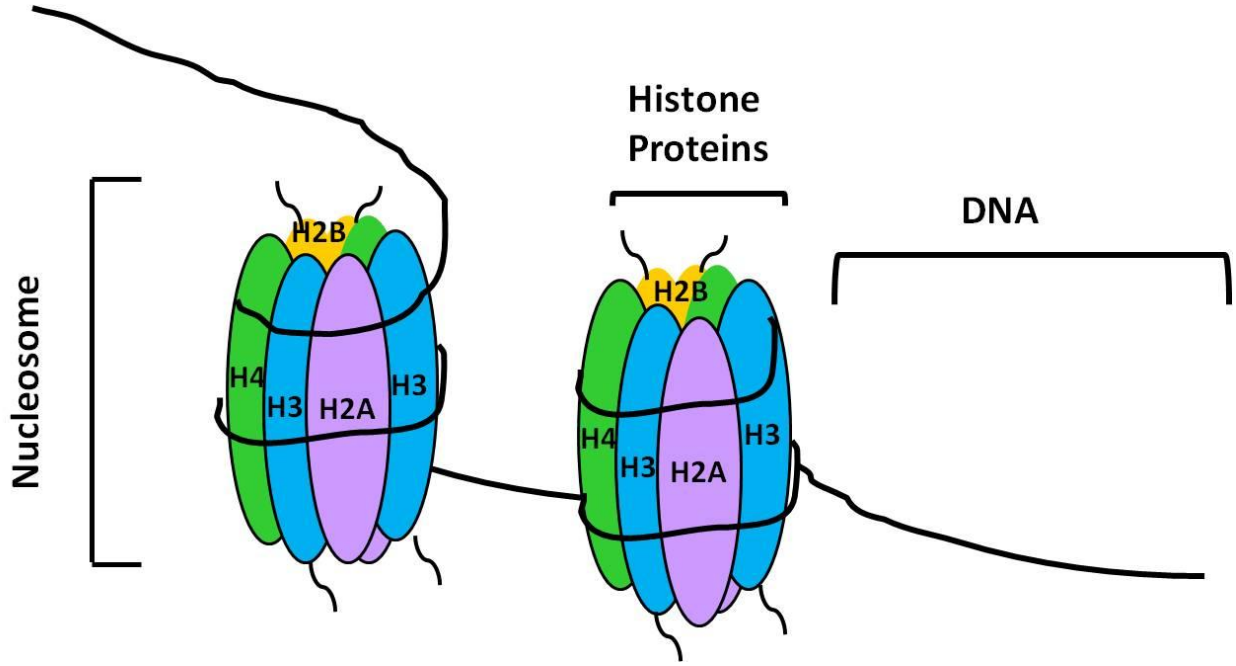

Figure 1.9 Structure of nucleosomes.

Histones are small proteins that provide a scaffold for the DNA to wrap around. There are four histone proteins which form an octomer structure. The nuclesome histone core consists of central H3/H4 tetramer that is surrounded on either side by a H2A/H2B dimer. DNA wraps tightly around the histone proteins to form the intact nucleosome structure. Chromatin is very dynamic and can be found in multiple forms depending on how tightly DNA wraps around those histone proteins (Kornberg and Thomas 1974; Luger, Mader et al. 1997; Clapier and Cairns 2009).

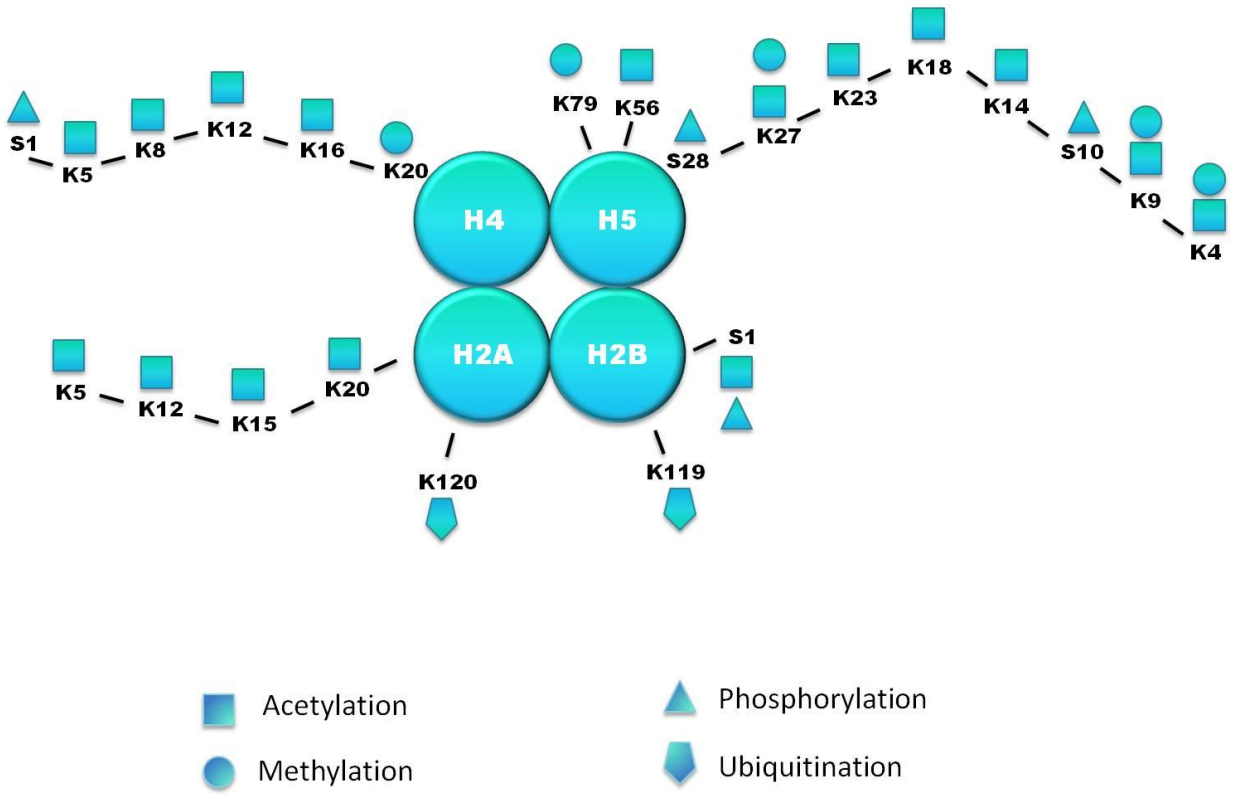

Figure 1.10 Histone code.

Posttranslational modifications of the histone molecules are located on the tail regions that are extended out of the nucleosome structure. Potential modifications occurring on histone tails include acetylation, methylation, ubiquitination and phosphporylation (Kornberg and Thomas 1974; Jenuwein and Allis 2001; Zhang and Reinberg 2001; Shilatifard 2006).

In inactive heterochromatin, histone proteins bind tightly to DNA and regulate access to DNA of transcription factors and the general transcription machinery. Structural changes to histones are facilitated by posttranslational modifications that render chromatin more or less available for transcription. The N- and C-terminal tails of histones are regions available for posttranslational modifications (Felsenfeld and Groudine 2003). Modifications to histone tails include acetylation (Morris, Rao et al. 2007), phosphorylation (Ivaldi, Karam et al. 2007), methylation (Li, Lin et al. 2002), sumoylation (Nathan, Sterner et al. 2003), ADP ribosylation (Boulikas 1990) and ubiquitination (Davies and Lindsey 1994; Ng, Xu et al. 2002; Ting and Trowsdale 2002; Shukla, Stanojevic et al. 2006; Shukla and Bhaumik 2007). These modifications have been linked to gene expression through a histone code hypothesis which proposes that the combinatory effects of these modifications determines the “open” and “closed” state of chromatin and thereby aids in determining the expressivity of genes (Wray, Hahn et al. 2003). Histone modifications create a variety of modifications that alter N terminal tails of histones and create the epigenetic code that, in part, determines the accessibility of chromatin.

Contributions from histone modifications are just one element of regulation that determines the overall structure of chromatin. The state of chromatin packaging is determined by a combination of factors in addition to histone modifications including DNA methylation and nucleosome remodeling, which together define gene expression patterns and regulate transcription. In eukaryotes, DNA methylation occurs by covalent modification of cytosines to CpG dinucleotides (Illingworth and Bird 2009). Promoter regions in the human genome are embedded with CpG islands, where methylation occurs and provides an additional mechanism of transcription regulation through silencing (Sandelin, Carninci et al. 2007; Illingworth and Bird 2009). While

multiple mechanisms contribute to gene expression, histone modifications are the most studied of these mechanisms. Due to the complex relationship between DNA and the histones around which it is wound, modifications to histones are a key component of transcriptional regulation. Of histone modifications, acetylation and methylation are the most critical as they impart significant structural alterations in histones and dramatically change interactions with DNA.

1.13 Histone acetylation:

One of the most studied histone modifications is acetylation of lysine residues on histones which loosens interactions between histone proteins and DNA, and contributes to an "open" state of chromatin (Cosgrove 2007). Acetylation primarily occurs on lysine residues located on histones H3 and H4 **(Figure 1.10)**. A deacetylated tail of histones H3 and H4 are positively charged and, as DNA is negatively charged; deacetylated histones tightly bind DNA and render it inaccessible to transcriptional activation. The addition of acetyl groups to targeted lysine residues neutralizes the positive charge on histone tails and loosens interactions with DNA (Struhl 1998). Alterations in histone acetylation result from changes in the balance between the enzymes which catalyze histone acetylation: histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), which remove acetyl groups (Utley, Ikeda et al. 1998; Yang 2004). HATs and HDACs are recruited to chromatin as transcriptional coactivators by transcription factors and are generally found in large remodeling complexes. There are a variety of known HATs, including several which are discussed in this dissertation: general control of amino acid synthesis 5 (GCN5), CREB binding protein (CBP), p300 and p300/CBP associated factor (PCAF), (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996; Chan and La Thangue 2001;

Legube and Trouche 2003). Acetylation is reversed by histone deacetylases (HDACs) which remove acetyl groups from lysine residues (de Ruijter, van Gennip et al. 2003). Both HATs and HDACs influence a myriad of cellular processes including signal transduction, apoptosis, cell cycle regulation, cell growth and transcription (Utley, Ikeda et al. 1998; Yang 2004).

1.14 Histone methylation:

Methylation plays dual roles in regulating histone accessibility as it has been linked to both activation and silencing of transcription (Daujat, Bauer et al. 2002; Naeem, Cheng et al. 2007). Methylation confers additional levels of complexity as methylation can be mono, di- or tri- methylation and occurs at lysine (K) and arginine residues (R) **(Figure 1.10)**. Methylation of arginine residues has been linked to gene activation (Bauer, Daujat et al. 2002) while lysine methylation has been linked with gene silencing and activation (Jenuwein and Allis 2001; Nakayama, Rice et al. 2001; Berger 2002; Lehnertz, Ueda et al. 2003; Shilatifard 2006). Arginine is a positively charged amino acid and the nitrogen of arginine can be modified by the addition of one or two methyl groups (Gary and Clarke 1998). Currently eight mammalian protein arginine methyltransferases (PRMTs) have been identified (Bedford and Richard 2005). While the mechanism through which arginine methylation enhances transcripition remains unknown, evidence exists that methylated arginines collaborate with other transcriptional activators and enhance their activity to promote transcriptional activation (Stallcup 2001). Methylation on lysine residues generally correlates with gene suppression but can also contribute to gene activation. One, two or three methyl groups can be added by histone methyltransferases (HMTases) (Lachner and Jenuwein 2002). Trimethylation of histone 3 lysines 9 and 27 or histone 4 lysine 20

is associated with gene silencing, while H3K4 di- and trimethylation, H3K36 trimethylation, and H3K79 di- and trimethylation methylation contributes to gene activation (Varier and Timmers 2011).

The histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2) belongs to Polycomb Group (PcG) and is involved in gene repression (Laible, Wolf et al. 1997; Satijn and Otte 1999). EZH2 catalyzes trimethylation of H3K9 and K27 with a strong preference for K27 (Cao, Wang et al. 2002; Czermin, Melfi et al. 2002; Muller, Hart et al. 2002). EZH2 has a cysteine rich SET domain which is involved in binding to the Polycomb repressive complex (Cao, Wang et al. 2002). Data in Chapter 3 of this dissertation demonstrates EZH2 binds to the CIITA proximal promoter and is a "master regulator" of the silencing histone modifications at CIITApIV, thus implicating roles for EZH2 in regulating other silenced, but inducible genes (Mehta, Truax et al. 2011). Of note are recent studies indicating expression of EZH2 is elevated in multiple human cancers, including breast cancer, which will be described in detail in this dissertation in Chapter 4.

While the mechanisms by which histone methylation contributes to gene silencing are not known, cross talk between various modifications has been observed (Muller and Verrijzer 2009). H3K4 methylation blocks methylation of H3K9 and induces the dimethylation of H3K27, resulting in an opening of chromatin structure (Wang, Huang et al. 2001; Agger, Cloos et al. 2007; Lee, Villa et al. 2007). H3K36 methylation recruits histone deacetylases and reestablishes closed structure resulting in decreased gene expression (Carrozza, Li et al. 2005; Lee and Shilatifard

2007). Similar to reversible acetylation, histone methylation can also be reversed by histone demethylases (HDMTs). One of first identified HDMT is LSD1 which demethylates mono- and di- H3K4in a flavin adenine dinucleotide (FAD) dependent oxidative reaction (Wysocka, Milne et al. 2005). This observation led to discovery of highly conserved Jumonji C containing proteins which remove methyl groups from lysine residues (Fodor, Kubicek et al. 2006; Klose, Yamane et al. 2006; Tsukada, Fang et al. 2006; Whetstine, Nottke et al. 2006; Yamane, Toumazou et al. 2006). This recent observation that methylation is reversible by histone demethylases provides new levels of regulation for gene expression. There are also additional interactions between methylated histones and the DNA methylation machinery which further determines the availability of DNA for gene expression. While histone methylation and the impact on DNA structures is reversible, DNA methylation is permanent and leads to stable repression of gene expression. The interactions between methylated histones and methylated DNA are complex as histone methylation mediates DNA methylation and DNA methylation also serves as a template for modifications to histones (Ooi, Qiu et al. 2007; Mohn, Weber et al. 2008). Greater understanding of the crosstalk between modifications to histones and modifications to DNA provide new, and largely unexplored, levels of regulation of gene expression (Cedar and Bergman 2009).

1.15 Additional Histone Modifications:

Although histone acetylation and methylation are the most studied, there are a variety of additional histone modifications including phosphorylation, ubiquitination, sumoylation, and poly ADP ribosylation which contribute to the status of chromatin. While phosphorylation of histone tails is indicative of active transcription, the mechanism by which this modification effects

gene expression is unknown (Nowak and Corces 2000). Phosphorylation of histone H3 at serine 10 **(Figure 1.10)** plays an important role in the very early events of transcriptional elongation that occur prior to P-TEF-b recruitment (Ivaldi, Karam et al. 2007). Furthermore, several histone acetyltransferases have enhanced activity towards phosphorylated histone substrates (Cheung, Tanner et al. 2000; Lo, Trievel et al. 2000). Histone sumoylation is generally associated with transcriptionally silenced genes (Nathan, Sterner et al. 2003) (Shiio and Eisenman 2003) and histone ubiquitination, similarly to histone methylation, plays dual roles in transcription activation and silencing, depending on the residue targeted. For example, histone H2A ubiquitination (ubH2A) plays roles in both gene activation and in H3K27me3 mediated silencing (Cao, Tsukada et al. 2005; Wei, Zhai et al. 2006; Lee, Norman et al. 2007). Finally, poly (ADP ribosylated) histones are important in the assembly of histone complexes during DNA replication (Boulikas 1990). Thus, the structure of chromatin and the availability of DNA for transcription is determined by various posttranslational modifications.

1.16 Histone modifications and DNA accessibility:

Posttranslational modifications to N terminal tails of histones affect chromatin structure and availability for gene expression **(Figure 1.10)**. The state of chromatin is determined by a combination of modifications on single histones which orchestrates a histone code that regulates transcriptional activation. While this field remains in its infancy, a histone code has begun to emerge. Methylation at histone H3K9 and H3K27 are considered to be silencing modifications, while active chromatin is characterized by acetylation at H3K9, H3K14, and H3K18, as well as

trimethylation at H3K4. Poised chromatin is identified by the activating modification H3K4me2 and the silencing modification H3K27me3 (Jenuwein and Allis 2001; Lachner, O'Carroll et al. 2001; Zhang and Reinberg 2001; Bernstein, Humphrey et al. 2002; Santos-Rosa, Schneider et al. 2002; Shilatifard 2006). The full mechanisms which determine interactions between various histone modifications and regulate their effects on chromatin remain unknown and are areas of intense investigation. Thus, the interplay between those various modifications creates a so-called "epigenetic landscape" which determines the expressivity of genes.

1.17 Chromatin structure of MHC II and CIITA promoters:

Extensive work has established the epigenetic map of MHC II and CIITA genes as knowledge of the epigenetic modifications to histones of these genes is critical to understanding their transcriptional regulation. Among the most well documented modifications associated with active transcription of MHC II genes is acetylation of lysine residues on histone H3 (primarily K9 and K18) and histone H4 (primarily K5 and K8) (Beresford and Boss 2001; Masternak, Peyraud et al. 2003; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005; Rybtsova, Leimgruber et al. 2007). Basal levels of acetylation are constitutively observed on MHC II histones H3 and H4 which dramatically increases upon recruitment of CIITA (Beresford and Boss 2001; Zika, Fauquier et al. 2005). Multiple histone modifying enzymes are recruited to MHC II with CIITA including histone acetyltransferases CBP/p300, pCAF, and Src-1, to establish an open state of chromatin. In the absence of CIITA, basal levels of acetylation are observed, but the lack of recruitment of HATs diminishes the availability of MHC II promoter DNA, indicating HATs are required to fully open MHC II chromatin (Zika, Greer et al. 2003; Zika, Fauquier et al. 2005;

Koues, Dudley et al. 2008). Histone H3 acetylation at K9, K14, K18, and K27, and histone H4 acetylation at K8, are the active marks of MHC II genes (Gomez, Majumder et al. 2005). Changes in methylation are also associated with active transcription of MHC II genes including CARM1 mediated dimethylation of histone H3R17 and trimethylation of histone H3K4 and K36, K9, and K27 (Zika, Greer et al. 2003; Zika, Fauquier et al. 2005; Chou and Tomasi 2008; Koues, Dudley et al. 2008).

CIITA is the master regulator of MHC II genes, thus CIITA expression correlates with that of MHC II. Relatively few studies have analyzed the epigenetic status of CIITApIV. However we and others have documented significant increases in acetylation of histone H3 and acetylation of histone H4 at CIITApIV within moments of IFN-γ stimulation (Morris, Beresford et al. 2002). Specific increases in acetylation have been observed at H3K9, H3K18, and H4K8 (Ni, Karaskov et al. 2005). Trimethylation of CIITApIV H3K4, indicative of active transcription, is also observed after prolonged stimulation with IFN-γ (Ni, Karaskov et al. 2005). Histone modifying enzymes that have been mapped to the CIITA promoter region include the HATs CBP/p300, the HMT CARM1, and the chromatin remodeler BRG-1 (Koues, Mehta et al. ; Pattenden, Klose et al. 2002; Ni, Karaskov et al. 2005; Mehta, Truax et al. 2011). Detailed characterization of histone modifying enzymes which mediate additional MHC II and CIITA histone modifications remains to be completed. Increased knowledge of epigenetic regulation of MHC II and CIITA genes will allow us to better understand dysregulated expression of MHC II genes in various disorders and diseases.

1.18 DYSREGULATION OF MHC II MOLECULES IN CANCER:

During an immune response, foreign antigens are detected by $CD4^+$ T cells via the T cell receptor and MHC II molecules. As MHC II plays a central role in a properly functioning immune system, its faulty expression is related to many diseases. Expression of MHC II genes and its master regulator CIITA is thus tightly regulated at multiple levels (Handunnetthi, Ramagopalan et al. 2010) with a majority of regulation focused at the level of transcription. Understanding the regulatory mechanisms that dictate MHC II and CIITA gene expression is an important step towards developing better and more specific therapies to regulate MHC II expression in disease states. While it is well understood that over expression of MHC II leads to many autoimmune disorders, it has recently become obvious that lack of MHC II expression is equally destructive, resulting in a severe combined immunodeficiency disorder when expression is blocked (de Preval, Hadam et al. 1988) and in tumor formation and metastasis when MHC II expression is suppressed (Chamuleau, Ossenkoppele et al. 2006).

Cancer develops from a deadly combination of activation of oncogenic pathways and inactivation of tumor suppressor pathways. Initially cancer was believed to result exclusively from genetic events including mutations, gene rearrangements, and gene deletions. However, we now know that dysregulated epigenetics and DNA methylation profiles are equal contributors to the development of cancer growth. These malfunctions include global hypomethylation (Eden, Gaudet et al. 2003) and deacetylation, hypermethylation at specific promoters, and altered ex-

pression of a multiplicity of enzymes involved in epigenetics. Despite these observations, global histone profiling in cancer is still quite limited. Great effort has been devoted to understanding the role of DNA methylation in cancer cells, but these efforts provide insight into only one level of regulation amongst many, including those provided by various posttranslational modifications to histone tails. Although histones modifications play important roles in regulating gene expression, knowledge of histone modifications in cancers, and correlation to various stages of cancer, remains poor.

Of cancers, the most lethal are those that have gained metastatic ability. In general, the ability of cancer cells to metastasize is determined by their ability to travel to distal sites through the bloodstream. Once established in a new location, cancer cells proliferate and give a rise to secondary tumors that are a major cause of death in cancer patients. Metastasized cancers often down regulate expression of various genes to avoid recognition and response by the immune system. It has rapidly become appreciated that epigenetic alterations play critical roles in aberrant expression of genes which contribute to tumor cell metastasis. Tumor associated antigens (TAA), which are selectively expressed by tumors, can be taken up by antigen presenting cells and presented via MHC II to $CD4^+$ T cells which in turn activate $CD8^+$ cytotoxic T cells to eliminate the tumor cell (Chamuleau, Ossenkoppele et al. 2006; Meissner, Whiteside et al. 2008). Tumor cells expressing MHC II molecules can also initiate an efficient antitumor immune response. Activated $CD4^+$ T cells multiply and activate B cells to initiate a killing response via antibodies. $CD4^+$ T cells also secrete cytokines to activate $CD8^+$ T cells to mediate direct killing of tumor cells.

Recent evidence indicates one mechanism utilized by tumor cells to escape recognition and elimination by the immune system is suppression of cell surface expression of MHC II. The absence of MHC II expression results in tumor escape and tolerance (Chamuleau, Ossenkoppele et al. 2006). Studies performed in mouse tumor models indicate $CD4^+$ T cells are essential for initiating effective anti-tumor immune responses (Greenberg, Cheever et al. 1981; Fujiwara, Fukuzawa et al. 1984; Hock, Dorsch et al. 1991; Lauritzsen, Weiss et al. 1994). Loss of tumor cell MHC II expression is associated with decreases in tumor infiltrating T cells and increased aggressiveness of multiple carcinomas (Garrido and Ruiz-Cabello 1991; Warabi, Kitagawa et al. 2000). These studies suggest MHC II presentation of tumor derived antigens generates critical targets for activation of the anti-tumor immune response and emphasizes the importance of understanding transcriptional regulation of MHC II. Observations that metastatic tumor cells specifically suppress CIITA genes expression resulting in decreased expression of MHC II molecules and tumor immune evasion are described in Chapter 4 of this dissertation.

1.19 SUMMARY:

The regulation of gene expression is one of the most intensely studied processes in science. Dysregulated gene expression has profound effects on cellular function and is the cause of a majority of human disease. Although there are many processes that modulate gene expression, transcription is the most direct. While it has long been understood that the molecular and functional characterization of chromatin modulating enzymes and transcription factors is impor-

tant, recent observations of dysregulated chromatin dynamics and transcription factor activity in disease have made clear that transcription offers unlimited potential in medicine and in cancer treatment in particular.

Induction of an antigen specific immune response is critical for eliminating infections and for recognizing cancer cells. MHC II molecules present peptides derived from extracellular proteins to activate the adaptive immune response. As a direct result, dysregulated expression of MHC II results in serious illness. Over expression of MHC II leads to autoimmune disease while the loss of MHC II results in a severe combined immune deficiency. MHC II is also very important in regulating anti-tumor response. Because MHC II plays such an important role in initiating the adaptive immune response to infections and tumors, it is critical to fully understand transcriptional regulation of MHC II and CIITA.

It is known that the "master of degradation," the 26S proteasome, plays important roles in transcriptional regulation of MHC II and CIITA genes. Therefore, multiple roles likely exist for the ubiquitin/proteasome pathway in initiating transcription, in stabilizing transcriptional complexes, and in regulating removal of transcription factors from promoters leading to transcriptional termination. We have learned much regarding the transcription of protein coding genes in eukaryotes, but much remains to be learned if we are to harness the power of transcription and, as in the case of CIITA and MHC II, regulate the regulators.

In order to better understand the transcriptional regulation of CIITA, the master regulator of MHC II genes, we have investigated novel regulatory mechanisms of CIITA. As described above, expression of mammalian genes is regulated at multiple levels, including transcriptional control. Chapters two and three of this dissertation focus on the novel roles played by the 19S ATPase S6a in regulating transcription initiation and elongation of CIITApIV. S6a is an important ATPase known for binding polyubiquitinated proteins that are marked for degradation. Our studies have shown that following cytokine stimulation, S6a rapidly binds to MHC II and CIITApIV proximal promoters where S6a mediates chromatin accessibility and recruitment of transcription factors STAT-1 and IRF-1 to CIITApIV. S6a also binds robustly to CIITA coding regions where it mediates the recruitment of the proteins RNA pol II and cyclin T1, both of which are required for transcriptional elongation. In these chapters we therefore demonstrate novel ways of regulating transcription of CIITApIV, where the 19S ATPase S6a plays important roles in transcription initiation and elongation.

Chapters four and five of this dissertation focus on transcriptional regulation of CIITApIV via histone modifying enzymes and the histone methyltransferase EZH2. As CIITA is the master regulator of the critical inflammatory gene MHC II, transcription at CIITApIV must be rapidly activated. Therefore, I in Chapter four, I determined the order and timing of epigenetic events that occur at the CIITA proximal promoter. Silencing modifications of trimethylation on histone H3 lysine 9 and lysine 27 are significantly reduced within the first 20 minutes post cytokine stimulation and reach basal levels within two hours. The opposite trend was observed with the activating methylation at CIITApIV histone H3 lysine 4. Together, these results suggest that changes observed in chromatin structure occur very rapidly post stimulation with IFN-γ to allow

transcription factors like STAT-1 to bind CIITApIV. Binding of the histone methyltransferase EZH2 to CIITApIV also declines rapidly following IFN-g stimulation. Work in Chapter five delves further into the role played by EZH2 and demonstrates EZH2 is a master regulator of CIITA. We determined that eliminating EZH2 expression through knockdown restores expression of CIITA and MHC II in MDA MB 435 breast cancer cells. These results hold exciting promise for the use of EZH2 knockdown in tumor therapies to reconstitute expression of CIITA and MHC II.

The overall focus of this dissertation is the transcriptional regulation of CIITA genes. We hypothesize that the 19S ATPase S6a and the histone methyltransferase EZH2 each play critical roles in regulating transcriptional expression of CIITA genes. Understanding expression of CIITA and MHC II genes will allow us to reach new horizons of understanding the mechanisms which determine the immune response to pathogen, to self and to tumors.

2 CHAPTER II: THE 19S ATPASE S6a (S6'/TBP1) REGULATES TRANSCRIPTION INITIATION OF THE CLASS II TRANSACTIVATOR

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2.1 ABSTRACT:

Class II transactivator (CIITA) is the master regulator of the major histocompatibility class II transcription complex (MHC II) and is critical for initiation of adaptive immune responses. We have previously demonstrated that the 19S proteasome ATPase Sug1 plays a significant role in regulating CIITA activity and MHC-II expression. We now show that an additional component of the 19S complex, the 19S ATPase S6a (S6′/Tat-binding protein 1), is crucial for regulating cytokine-inducible transcription of CIITA. Lack of S6a negatively impacts CIITA activity and CIITA expression. Decreased expression of S6a significantly diminishes the recruitment of transcription factors to the CIITA interferon-γ-inducible promoter IV (CIITApIV) and significantly decreases CIITApIV histone H3 and histone H4 acetylation, with a preferential loss of acetylation at H3 lysine 18 and H4 lysine 8. In addition, we provide evidence for the involvement of the 19S AAA (ATPases associated with diverse cellular activity) ATPase hexamer as the 19S ATPase S6b binds CIITApIV in an S6a dependent fashion and has effects similar to S6a on CIITApIV histone acetylation. These analyses demonstrate the importance of 19S ATPases in the assembly of CIITApIV transcription machinery and provide additional insight into the regulatory mechanisms of the 19S proteasome in mammalian transcription.

2.2 BACKGROUND

Major histocompatibility class II (MHC II) molecules are cell surface glycoproteins which present antigenic peptides to $CD4^+$ T lymphocytes and play important roles in initiating, maintaining and terminating adaptive immune responses against invading pathogens and tumors. MHC II genes are induced by the inflammatory cytokine interferon gamma (IFN-γ) (Cresswell and Howard 1997; Ferdous, Gonzalez et al. 2001) and the expression of MHC II molecules is primarily regulated at the level of transcription. Class II transactivator (CIITA) is a master regulator that drives activation of MHC II genes (Chang, Fontes et al. 1994; Mach, Steimle et al. 1996). CIITA does not bind DNA, but interacts with the MHC II promoter via direct binding to the requisite MHC II transcription factors Regulatory Factor X (RFX), cAMP Response Element Binding (CREB) and Nuclear Factor Y (NF-Y) at the respective X1, X2 and Y promoter elements of MHC-II genes. Once bound, CIITA stabilizes this enhanceosome complex and recruits other components of the basal transcriptional machinery (Moreno, Beresford et al. 1999; Masternak, Muhlethaler-Mottet et al. 2000; Zhu, Linhoff et al. 2000). Transcription of CIITA itself is regulated in a cell specific manner by four distinct promoters: pI, pII, pIII and pIV (Muhlethaler-Mottet, Otten et al. 1997; Morris, Beresford et al. 2002). CIITA promoter IV (pIV) is the primary IFN-γ responsive form of CIITA and is predominantly induced in non-antigen presenting cells in response to IFN-γ stimulation via the JAK-signal transducer and activator of transcription (STAT) signal transduction pathway (Morris, Beresford et al. 2002; Piskurich, Gilbert et al. 2006).

Epigenetic analysis of CIITApIV has shown that, prior to IFN-γ stimulation, acetylation of histones H3 and H4 occur at low to moderate levels (Ni, Karaskov et al. 2005). Following cytokine stimulation, transcription of CIITA is induced at CIITApIV and is correlated with increased acetylation of histones H3 and H4 (Morris, Beresford et al. 2002) and recruitment of histone acetyltransferases, CIITApIV specific transcription factors, and the basal transcription machinery (Ni, Karaskov et al. 2005).

The 26S proteasome is a large, multimeric complex made of a 19S regulatory particle and a 20S proteolytic core (Baumeister, Walz et al. 1998). The 19S regulator is further divided into two parts: a lid and a base. The lid is composed of eight non-ATPase subunits which are required for protein degradation (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998). The 19S base contains six ATPases termed S4, S6a (S6', TBP1), S6b, S7, Sug1 (S8), and Sug2 (S10b), and three non-ATPase subunits (Gorbea, Taillandier et al. 1999; Adams 2003). During protein degradation, the 19S regulatory base contacts the 20S catalytic core and regulates the access of substrates to the core by recognizing and hydrolyzing ubiquitin from polyubiquitinated proteins (Coux, Tanaka et al. 1996; Glickman, Rubin et al. 1999; Rabl, Smith et al. 2008). Recent evidence in yeast and mammalian cells has supported novel, non-degradative functions for components of the 26S proteasome in regulating gene transcription (Baumeister, Walz et al. 1998; Ciechanover 1998; Muratani and Tansey 2003; Lee, Ezhkova et al. 2005; Sulahian, Johnston et al. 2006). In yeast, the 19S regulatory complex directly associates with the Gal4 activator and is recruited to the *GAL* responsive promoter (Chang, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Archer, Burdine et al. 2008). Knockout studies in yeast and mammalian cells have shown that the 19S is essential for initiation and elongation by RNA polymerase II,

while the proteolytic activity of the 26S is not required (Ferdous, Kodadek et al. 2002). Our previous studies have demonstrated that the 19S ATPase Sug1 associates with MHC II proximal promoter where it plays an important role in regulating CIITA recruitment to the *HLA-DRA* MHC II promoter and in subsequent MHC II gene expression (Bhat, Turner et al. 2008). We have further shown that Sug1 plays a role in recruitment of multiple histone modifying enzymes where Sug1 positively regulates histone acetylation and methylation of MHC II and CIITApIV (Koues, Dudley et al. 2008; Koues, Dudley et al. 2009).

Despite these findings, it remains unclear if additional 19S ATPases regulate mammalian gene expression and whether or not the regulatory mechanisms we have seen at the MHC-II promoter occur at additional genes. S6a (S6'/TBP-1) was first isolated through direct interactions with the human immunodeficiency virus type 1 (HIV-1) Tat transactivator (Nelbock, Dillon et al. 1990) and has subsequently been shown to directly interact with multiple other proteins including TBP-1-interacting protein (GT198/Hop2), the von Hippel-Lindau protein, rat α-synuclein and the human ARF tumor suppressor. S6a was recently shown to cooperate with TBP interacting protein to cooperatively enhance nuclear receptor (NR)-mediated transactivation, however the mechanisms that support the activating effects of S6a on NR transcription (Satoh, Ishizuka et al. 2009) remain unknown. We demonstrate here that S6a plays critical roles in the initiation of transcription from CIITApIV. S6a is critical for the recruitment of requisite transcription factors STAT-1and interferon regulatory factor 1 (IRF-1) to CIITApIV and for the acetylation of histones H3 and H4 preceding transcription factor recruitment. These results are novel, as they indicate previously uncharacterized roles for the 19S ATPases in regulating transcrip-

tion of inducible genes and provide further insight into the roles of the 19S proteasome in mammalian transcription.

2.3 RESULTS

The 19S ATPase S6a associates with CIITApIV and MHC II proximal promoters.

We have recently shown that the 19S ATPase Sug1 positively regulates MHC II transcription by stabilizing CIITA binding (Bhat, Turner et al. 2008) and by regulating histone H3 acetylation at the MHC II proximal promoter (Koues, Dudley et al. 2008). Specific roles for additional 19S subunits in mammalian transcription remain largely unknown. As the 19S ATPase, S6a has recently been shown to enhance transcription, we first investigated roles of S6a in MHC gene regulation by determining if S6a binds to MHC II promoter element and to the promoter of CIITApIV. HeLa cells were stimulated with IFN-γ, subjected to immunoprecipitation (IP) with antibody to endogenous S6a, and were analyzed by real-time PCR with primers spanning the proximal promoters of MHC II *HLA-DRA* and CIITApIV. Upon IFN-γ stimulation, S6a demonstrated rapid and robust association with both the MHC-II proximal promoter **(Figure 2.1 A)** and with CIITApIV **(Figure 2.1 B)**. ChIP analyses in yeast have determined association of the proteasome with elongation and coding regions of transcribed genes (Gillette, Gonzalez et al. 2004). Likewise, we observed the robust binding of S6a to MHC II exon III **(Supplementary Figure 2.11 B)**, MHC II exon V **(Supplementary Figure 2.10 C)** and CIITA coding region **(Supplementary Figure 2.10 E)** indicating a strong potential for parallel pathways in mammalian cells.

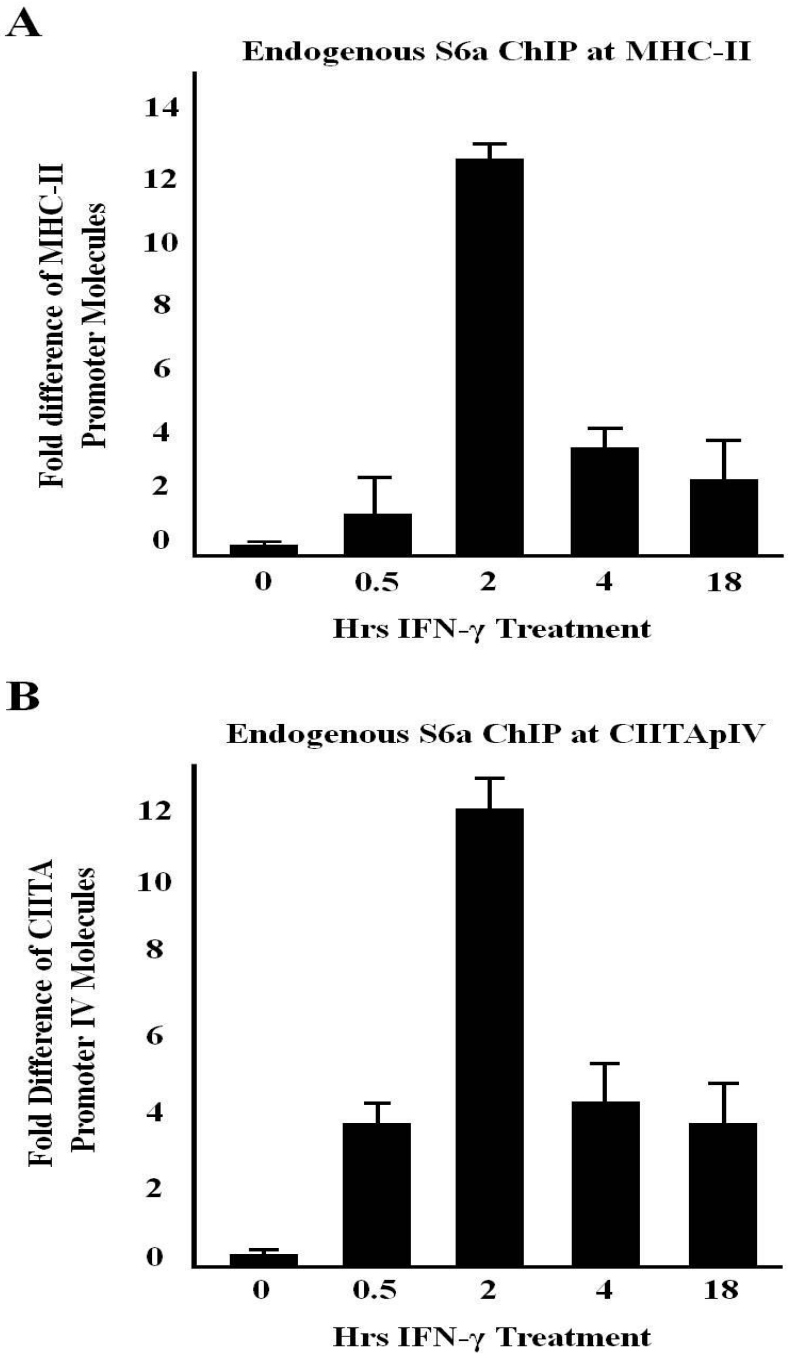


Figure 2.1 The 19S ATPase S6a associates with MHC-II and CIITApIV proximal promoters.

(A-B) ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous S6a and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC-II *HLA-DRA* (A) and primers spanning IRF-E-GAS box of CIITApIV (B) promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as

fold increase in the MHC-II and CIITA promoter DNA relative to unstimulated S6a IP samples. Control IP values for (A) and (B) were 0.5 ± 0.05. Values for control IPs and S6a IPs represent mean ± SEM of five independent experiments.

Decreased expression of S6a decreases CIITA expression and activity.

As S6a inducibly bound MHC II and CIITA promoters, we next investigated the role of S6a in regulating activation of these cytokine inducible genes by using short interfering RNA (siRNA) duplexes to specifically knockdown endogenous S6a. siRNA mediated reduction of endogenous S6a decreased S6a protein expression by 90% relative to control siRNA transfected cells, but did not impact the expression of any of the other five 19S ATPases **(Figure 2.2 A)**. We next addressed the role of S6a in regulating MHC II expression by using real-time PCR to measure endogenous levels of mRNA in S6a siRNA transfected cells. Decreased expression of S6a dramatically reduced the IFN-γ inducible levels of endogenous MHC II mRNA **(Figure 2.2 B, white bars)**, in comparison to cells transfected with control siRNA **(Figure 2.2 B, black bars)**.

CIITA expression and activity drives MHC II expression. In order to determine if depletion of S6a affected CIITA transactivation, we employed dual luciferase reporter assays. Transfecting cells with control siRNA, CIITA and *HLA-DRA*-Luc resulted in increased MHC II *HLA-DRA* promoter activity **(Figure 2.2 C, black bars)**. By comparison, in cells similarly transfected with S6a siRNA or predesigned MISSION Vérité S6a siRNA, CIITA mediated activation of the MHC II *HLA-DRA* promoter was significantly reduced **(Figure 2.2 C, white bars)**. Notably, levels of CIITA activity in S6a siRNA and MISSION Vérité S6a siRNA treated samples were comparable indicating that our predesigned S6a siRNA is very specific with minimum of off target effects. As an additional control for siRNA specificity, we knocked down S6a, reconstituted S6a expression by over expression of Myc-S6a, and restored the MHC-*HLA-DRA* promoter activity in these cells **(Figure 2.2 C, grey bars)**.

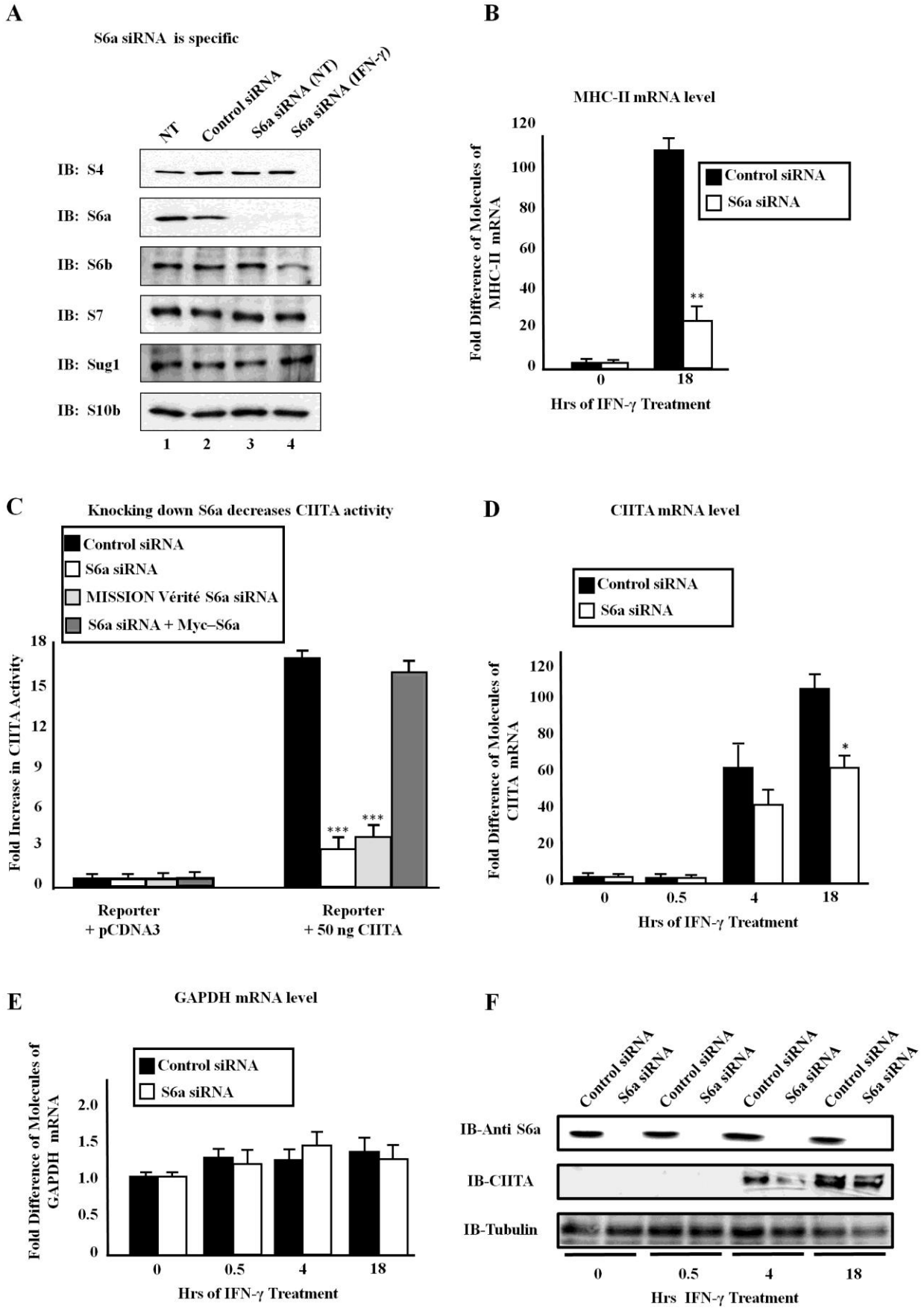

A
S6a siRNA is specific
NT
Control siRNA
S6a siRNA (NT)
S6a siRNA (IFN-γ)
IB: S4
IB: S6a
IB: S6b
IB: S7
IB: Sug1
IB: S10b
1 2 3 4
B
MHC-II mRNA level
Fold Difference of Molecules of MHC-II mRNA
Control siRNA
S6a siRNA
**
Hrs of IFN-γ Treatment
C
Knocking down S6a decreases CIITA activity
Control siRNA
S6a siRNA
MISSION Vérité S6a siRNA
S6a siRNA + Myc–S6a
Fold Increase in CIITA Activity
***
***
Reporter + pCDNA3
Reporter + 50 ng CIITA
D
CIITA mRNA level
Control siRNA
S6a siRNA
Fold Difference of Molecules of CIITA mRNA
*
Hrs of IFN-γ Treatment
E
GAPDH mRNA level
Control siRNA
S6a siRNA
Fold Difference of Molecules of GAPDH mRNA
Hrs of IFN-γ Treatment
F
Control siRNA
S6a siRNA
IB-Anti S6a
IB-CIITA
IB-Tubulin
Hrs IFN-γ Treatment

Figure 2.2 The 19S ATPase S6a is required for optimal CIITA activity and CIITA gene expression.

(A) S6a siRNA specifically and efficiently decreases S6a protein expression in the absence or presence of cytokine stimulation. HeLa cells were transfected with control or S6a siRNA, untreated or stimulated with IFN-γ, harvested and subjected to Western Blot analysis of endogenous expression of 19S ATPases S4, S6a, S6b, S7 Sug1 and S10b. Western Blot analysis shows 90% knockdown of S6a and stable expression of the other 19S ATPases. Results reported are representative data of three independent experiments.

(B) Reduced expression of S6a in siRNA transfected cells decreases endogenous MHC-II mRNA expression. Experimental cells were transfected with S6a specific siRNA and control cells were transfected with scrambled control siRNA. Twenty four hours following siRNA transfection, HeLa cells were left unstimulated or were stimulated with IFN-γ for 0 to 18 hours. Fractions of cell lysates were analyzed for S6a expression by Western blot (not shown) or for levels of MHC-II mRNA. Levels of mRNA were measured by real-time PCR and were normalized to 18S mRNA. Real-time PCR was performed in triplicate and results represent the mean ± SD of six independent experiments.

(C) Lack of S6a specifically decreases CIITA transcriptional activity. HeLa cells were transfected with MHC-II *HLA-DRA*-Luc reporter construct, *Renilla*, 100ng of Myc-S6a and 100ng of CIITA as indicated. Controls were transfected with the Luc reporter, empty pCDNA3 vector and were transfected with control siRNA (black bars), with S6a specific siRNA (white bars) or versite S6a specific siRNA (grey bars). Luciferase assays were performed in triplicate and data are presented as fold increase in the luciferase activity. Results presented represent the mean ± SD of three independent experiments.

(D and E) Reduced expression of S6a in siRNA transfected cells decreases endogenous CIITA mRNA (D) and does not affect GAPDH mRNA (E) expression levels. As above, cells were transfected with S6a specific or scrambled control siRNA and were stimulated with IFN-γ for 0 to 18 hours. Fractions of cell lysates were analyzed for S6a expression by Western blot (not shown) or for levels of CIITA normalized to 18SmRNA (D) or as a control for GAPDH (E) mRNA level. Real-time PCR was performed in triplicate and results represent the mean ± SD of six independent experiments. ***$P < 0.001$, **$P < 0.005$, *$P < 0.05$ versus control siRNA

(F) S6a siRNA decreases CIITA protein expression. HeLa cells were transfected with control or S6a siRNA, stimulated as indicated, harvested and subjected to IB for S6a (top), CIITA (middle) and tubulin (bottom). Immunoblots shows 95% decrease of S6a in stimulated and unstimulated cells, 60% decrease of CIITA (lane 6) 4hrs post cytokine stimulation and 35% decrease of CIITA (lane 8) 18hrs post stimulation**.** Results reported are representative of five independent experiments.

As S6a binds to CIITApIV, we next sought to determine if S6a was important for driving CIITA expression from the IFN-γ inducible CIITApIV. Reduced expression of S6a had a significant negative impact on CIITA transcript levels **(Figure 2.2 D, white bars)** and protein level **(Figure 2.2 F)** in comparison to cells transfected with control siRNA **(Figure 2.2 D, black bars)**. As controls for the specificity of the knockdown effects on CIITA and MHC II gene expression, we investigated the role of S6a in regulating expression of constitutively expressed glyceraldehyde-3-phosphate dehydrogense GAPDH **(Figure 2.2 E)**. Real-time PCR analysis indicated that transcript levels of GAPDH were not affected by siRNA mediated depletion of S6a, indicating that the effects of decreased expression of S6a were specific to these inducible genes.

S6a knockdown decreases transcription factor recruitment to the CIITApIV proximal promoter.

CIITApIV contains three cis acting elements which are required for transcription in response to IFN-γ: an IFN-γ activated sequence (GAS), an IRF element (IRF-E), and an E-box. Ligand-induced dimerization of the IFN-γ receptor activates JAK1 and JAK2 tyrosine kinases which phosphorylate STAT-1 allowing STAT-1 to translocate to the nucleus where it induces the expression of the IRF-1 gene (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002; Ni, Karaskov et al. 2005). STAT-1 is also recruited to the CIITApIV GAS sequence, while a constitutively expressed dimer of USF-1 is recruited to the CIITApIV E-box (Muhlethaler-Mottet, Di Berardino et al. 1998). A heterodimer of transcribed and translated interferon regulatory factor 1 (IRF-1) and inducibly expressed IRF-2 is recruited to the IRF-E (Piskurich, Linhoff et al. 1999; Morris, Beresford et al. 2002) to assemble a transcription initiation complex with USF-1 and STAT-1. We therefore sought to determine if S6a interacts with

transcription factors critical for CIITA expression. Endogenous S6a and STAT-1 co-immunoprecipitated from HeLa cells with and without cytokine stimulation **(Figure 2.3, upper panel lane 3 and 4)**. Positive control samples were immunoprecipitated with endogenous STAT-1 **(Figure 2.3, upper panel lane 1)** or a negative control IgG antibody **(Figure 2.3, upper panel lane 2)**. Equal loading of cell lysates was confirmed by immunoblot analysis of total STAT-1 protein **(Figure 2.3, lower panel).**

To further address the role of S6a in regulating IFN-γ inducible CIITA expression, ChIP assays were performed to determine the level of STAT-1 and IRF-1 binding to CIITApIV in the absence of S6a expression. As previously demonstrated, (Morris, Beresford et al. 2002) initial ChIP mapping experiments show that STAT-1 rapidly and stably binds CIITApIV in an eighteen hour timecourse of IFN-γ stimulation **(Figure 2.4 A)**. In cells transfected with S6a siRNA, STAT-1 binding to CIITApIV was significantly decreased when compared to cells transfected with scrambled siRNA **(Figure 2.4 B)**. ChIP mapping experiments further demonstrated that IRF-1 transiently associated with CIITApIV following four hours of IFN-γ stimulation **(Figure 2.4 C)**. Similar to the effects of S6a depletion on STAT-1 binding, upon transfection with S6a siRNA, there was a significant decrease in IRF-1 binding to CIITApIV upon transfection with S6a siRNA **(Figure 2.4 D)**.

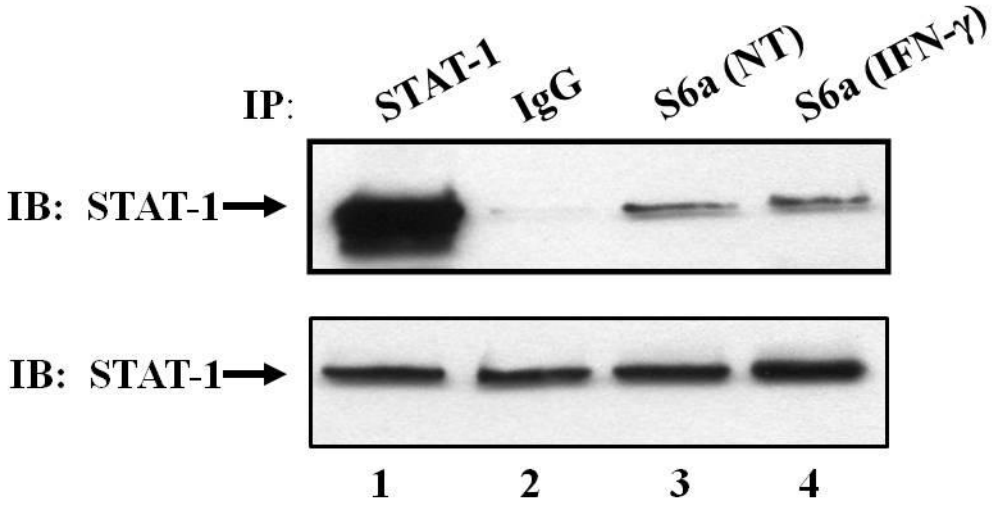


Figure 2.3 19S ATPase S6a associates with STAT-1

S6a associates with STAT-1. HeLa cells were stimulated with IFN-γ for 30 min, lysed and subjected to IP with STAT-1 (lane 1) as a positive control, with mouse isotype control IgG (lane 2), with S6a (lane 3) and with S6a under stimulation with IFN-γ (lane 4). IP and lysate control samples (bottom) were subjected to IB for STAT-1. Results reported are representative data of three independent experiments.

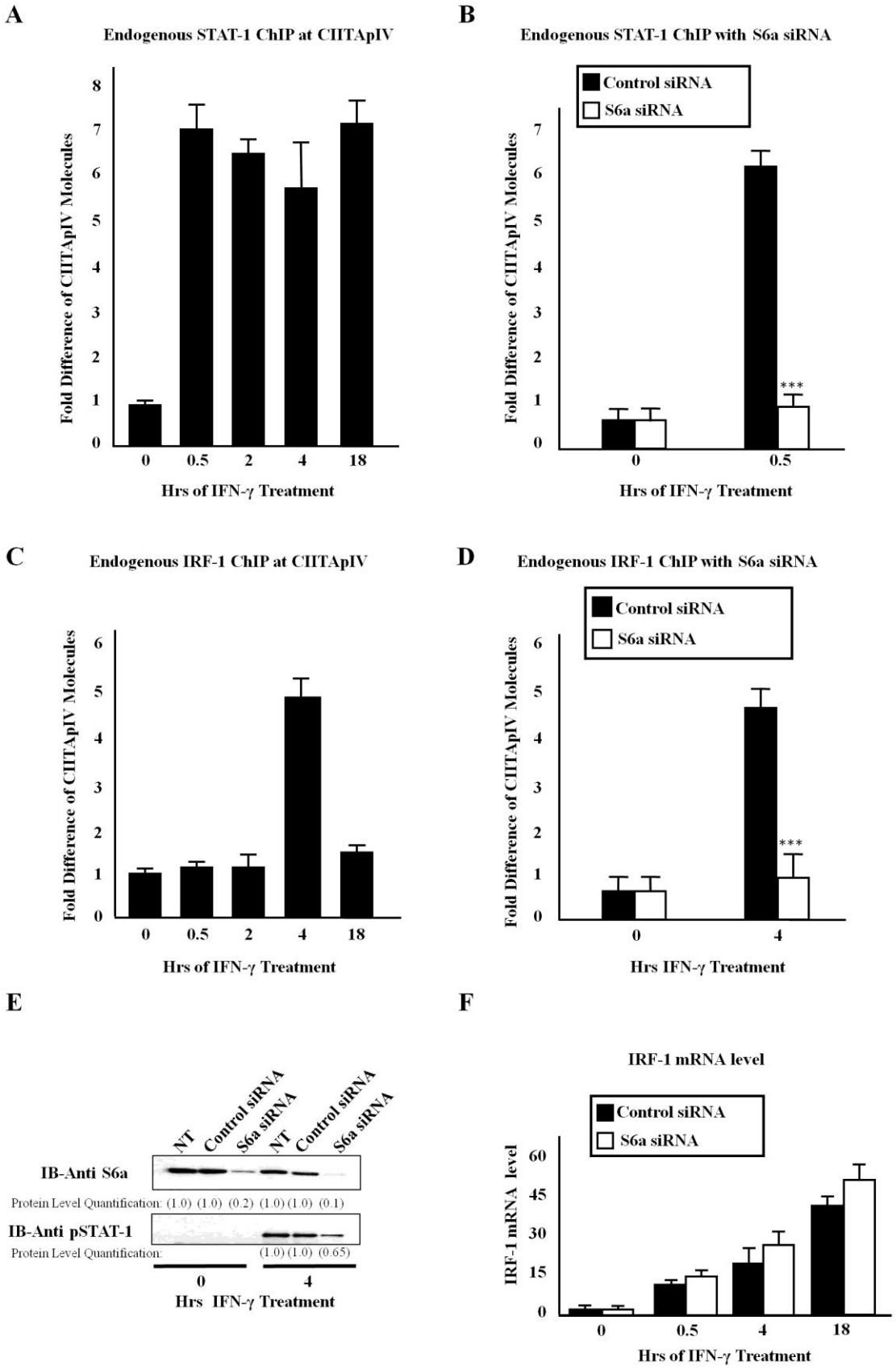

A
Endogenous STAT-1 ChIP at CIITApIV
Fold Difference of CIITApIV Molecules
Hrs of IFN-γ Treatment
B
Endogenous STAT-1 ChIP with S6a siRNA
Control siRNA
S6a siRNA
***
C
Endogenous IRF-1 ChIP at CIITApIV
D
Endogenous IRF-1 ChIP with S6a siRNA
Hrs IFN-γ Treatment
E
NT
Control siRNA
S6a siRNA
IB-Anti S6a
Protein Level Quantification: (1.0) (1.0) (0.2) (1.0) (1.0) (0.1)
IB-Anti pSTAT-1
Protein Level Quantification: (1.0) (1.0) (0.65)
F
IRF-1 mRNA level

Figure 2.4 STAT-1 and IRF-1 recruitment to the CIITApIV proximal promoter is dramatically decreased in the absence of S6a.

(A and C) STAT-1 and IRF-1 are recruited to CIITApIV upon IFN-γ stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, with antibody to endogenous STAT-1 (A) or with antibody to IRF-1 (C), and associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITA isoform IV proximal promoter (as described in Figure 1). IP values are presented as fold increase in CIITApIV promoter DNA relative to unstimulated STAT-1(A) and IRF-1(C) IP samples. Control IP values for (A) and (C) were 0.9 ± 0.4. Values for control IPs, STAT-1 and IRF-1 IPs represent mean ± SEM of two independent experiments.

(B and D) S6a knockdown decreases recruitment of STAT-1 and IRF-1 to the proximal promoter of CIITApIV. ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0.5hr (B) and 4 hours (D). Lysates were immunoprecipitated (IP) with control antibody, with antibody to endogenous STAT-1 (B) or with antibody to IRF-1 (D), and associated DNA was isolated and analyzed as above. IP values are presented as fold increase in CIITApIV DNA relative to unstimulated CIITA IP samples. Control IP values for (B) and (D) were 0.7 ± 0.5. Values for control IPs, STAT-1 and IRF-1 IPs represent mean ± SEM of four independent experiments.

(E) S6a knockdown does not significantly affect the protein level of phosphorylated STAT-1 (pSTAT-1)**.** HeLa cells transfected with control or S6a specific siRNA were stimulated with IFN-γ, harvested and subjected to Western Blot analysis of endogenous expression of pSTAT-1 (bottom) and S6a (top). Immunoblots show 90% decrease of S6a that slightly affects expression of pSTAT-1. Protein levels were quantified by Multi Gauge V3.1. Results reported are representative data of three independent experiments.

(F) Reduced expression of S6a in siRNA transfected cells does not decrease endogenous levels of IRF-1 mRNA. As described in Figure 2, cells were transfected with S6a specific siRNA and control cells were transfected with scrambled control siRNA. Following IFN-γ stimulation cell lysates were analyzed for S6a expression by Western blot (not shown) or for levels of IRF-1 mRNA. Real-time PCR was performed in triplicate and results represent the mean ± SD of three independent experiments. *** $P < 0.001$ versus control siRNA.

Upon IFN-γ stimulation, phosphorylated STAT-1 dimerizes and moves rapidly into nucleus where it binds to GAS elements of promoters of IFN-γ responsive genes including IRF-1 and CIITA (Hobart, Ramassar et al. 1997; Piskurich, Linhoff et al. 1999). To determine if S6a siRNA affected nuclear levels of phosphorylated STAT-1, we performed a Western Blot analysis to detect levels of phosphorylated STAT-1 in cells transfected with control or S6a specific siRNA **(Figure 2.4 E)**. As expected, phosphorylated STAT-1 was undetectable in unstimulated cells. In INF-γ stimulated cells, decreased expression of S6a did not significantly impact the level of phosphorylated STAT-1, indicating STAT-1 was phosphorylated and appropriately targeted in the absence of S6a. We next assayed IRF-1 transcript levels in S6a siRNA transfected cells. Real-time PCR analysis indicated that transcript levels of IRF-1 were not affected by depleting S6a **(Figure 2.4 F, white bars)**, indicating that STAT-1 was functional at the IRF-1 promoter in the absence of S6a expression. In total, these results demonstrate that the 19S ATPase S6a plays important and specific roles in recruiting the crucial transcription factors STAT-1 and IRF-1 to CIITApIV.

S6a knockdown diminishes the levels of histone H3 and histone H4 acetylation at the CIITA isoform IV proximal promoter.

Epigenetic studies of the IFN-γ inducible CIITApIV indicate that prior to cytokine stimulation; histone H3 and histone H4 are moderately acetylated (Morris, Beresford et al. 2002; Wright and Ting 2006). This basal acetylation correlates with promoter recruitment of ubiquitously expressed USF-1 (Muhlethaler-Mottet, Di Berardino et al. 1998). Following IFN-γ stimulation, STAT-1 and IRF-1bind to the CIITA proximal promoter, recruit the basal transcription

machinery and initiate CIITA transcription of CIITA (Morris, Beresford et al. 2002; Wright and Ting 2006). As STAT-1 and IRF-1 recruitment to CIITApIV is diminished in S6a depleted cells, we sought to determine if S6a is involved in regulating chromatin modifications to CIITApIV. To investigate the role of S6a in initial CIITApIV chromatin modifications, we analyzed promoter IV levels of acetylated histone H3 and acetylated histone H4 in cells transfected with either S6a or control siRNA. In order to stimulate maximum acetylation in the presence of S6a knockdown, cells were simultaneously treated with histone deacetylase (HDAC) inhibitors and IFN-γ and results were graphed as percent input to demonstrate levels of histone modification. S6a siRNA transfected HeLa cells lacked inducible enhancement of H3 or H4 acetylation **(Figure 2.5, A-B white bars)** compared to similarly treated control siRNA transfected cells **(Figure 2.5, A-B, black bars)**.

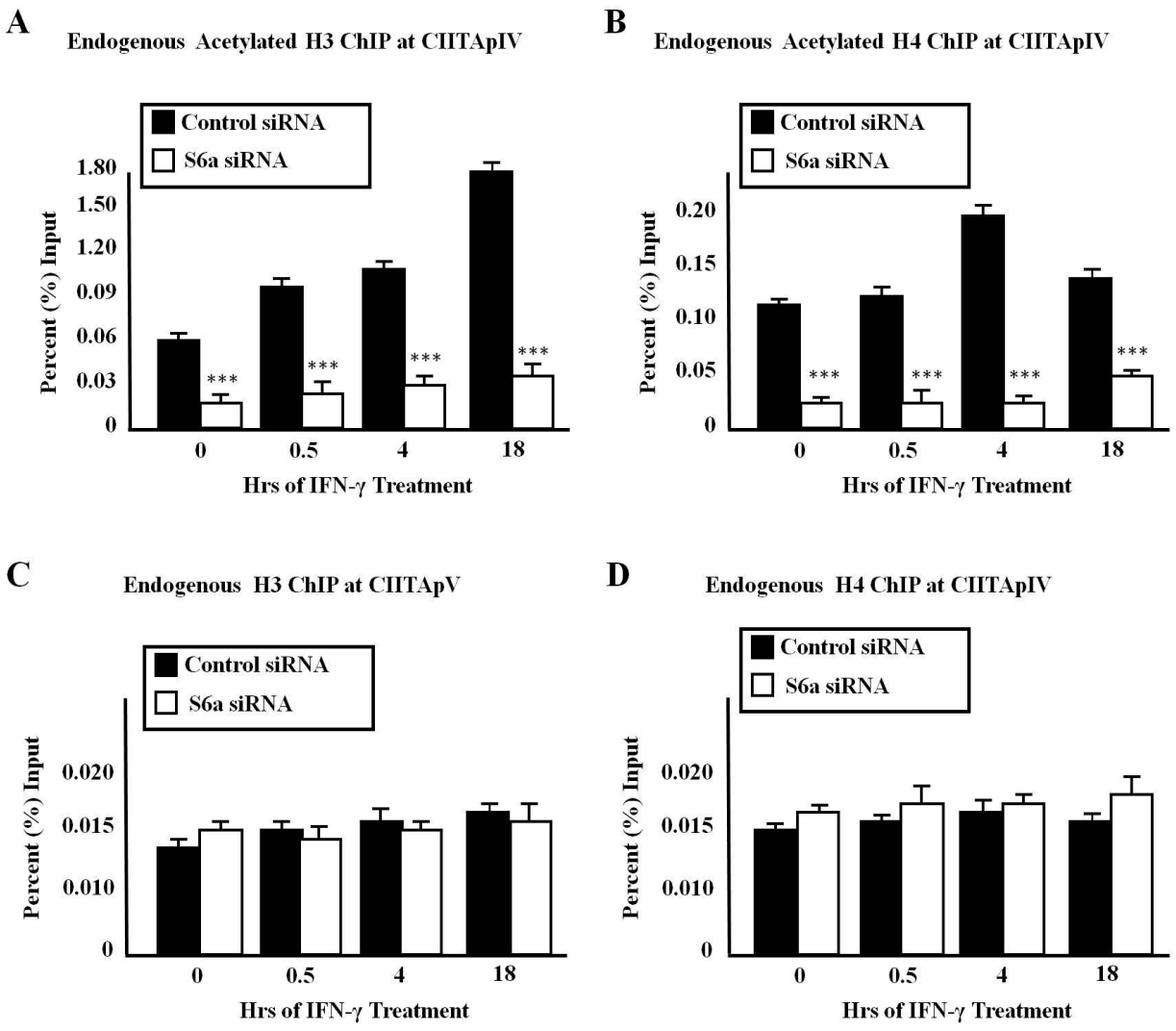


Figure 2.5 S6a knockdown decreases CIITApIV histone H3 and histone H4 acetylation.

(A and B) Lack of S6a decreases histone H3 and H4 acetylation at CIITApIV. ChIP assays were carried out in HeLa cells transfected with S6a specific or control siRNA. Cells were treated with HDAC inhibitors for 20hrs and were stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to acetylated histone H3 (A) or with antibody to acetylated histone H4 (B), and associated DNA was isolated and analyzed via real-time PCR using primers spanning the proximal promoter CIITA isoform IV proximal promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). IP values are presented as increase in CIITApIV promoter DNA relative to unstimulated samples. Control IP values for (A) and (B) were 0.04 ± 0.01. Values for control IPs, acetylated histone H3 and acetylated histone H4 IPs represent mean ± SEM of three independent experiments.

(C and D) S6a knockdown does not impact levels of histone H3 and histone H4 at CIITApIV. As above ChIP assays were carried out in HeLa cells transfected with S6a specific or control siRNA and stimulated with IFN-γ for 0-18 hours. Lysates were subjected to IP with control antibody or antibody to endogenous histone H3 (C) or H4 (D). Associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITApIV. IP values are presented as increase in CIITApIV DNA relative to unstimulated histone H3 (C) and histone H4 (D) IP samples. Control IP values for (C) and (D) were 0.002 ± 0.001. Values for control IPs, histone H3 and histone H4 IPs represent mean ± SEM of three independent experiments. *** $P < 0.001$ versus control siRNA.

Additional ChIP assays were performed to ensure that the loss of H3 and H4 acetylation observed at CIITApIV is not indicative of a total loss of H3 or H4 in the cells. Cells treated with S6a specific siRNA showed no significant changes in levels of histones H3 or H4 compared to the levels observed in control siRNA treated and untreated cells **(Figure 2.5, C-D)**. In sum, these data indicate that the 19S ATPase S6a plays important and specific role in regulating activating epigenetic modifications at the CIITApIV proximal promoter.

S6a knockdown decreases acetylation of histone H3 lysine 18 and histone H4 lysine 8 at the CIITA isoform IV proximal promoter.

Acetylation takes place on lysine residues of histone tails. Histone code of CIITA promoter has shown that only lysines (K) 9 and 18 of histone H3 and lysine 8 of histone H4 are acetylated (Ni, Karaskov et al. 2005). We have previously shown that Sug1 is crucial for regulating histone H3 acetylation at the MHC II proximal promoter. In the absence of Sug1, histone H3 acetylation is significantly decreased with a preferential loss of acetylation at H3K18 (Koues, Dudley et al. 2008). Next, we wanted to determine if lack of S6a will mirror the affect of Sug1 siRNA studies at MHC II promoter.

To evaluate H3K9, H3K18 and H4K8 acetylation levels at the CIITApIV proximal promoter in the presence and absence of 19S ATPase S6a, HeLa cells were transfected with control or S6a specific siRNA, were treated with HDAC inhibitor, stimulated with IFN-γ as indicated, and immunoprecipitated with antibodies specific for H3K9 **(Figure 2.6 A)**, H3K18 **(Figure 2.6 B)**, and H4K8 **(Figure 2.6 C)** acetylation. ChIP assay showed elevated levels of acetylated

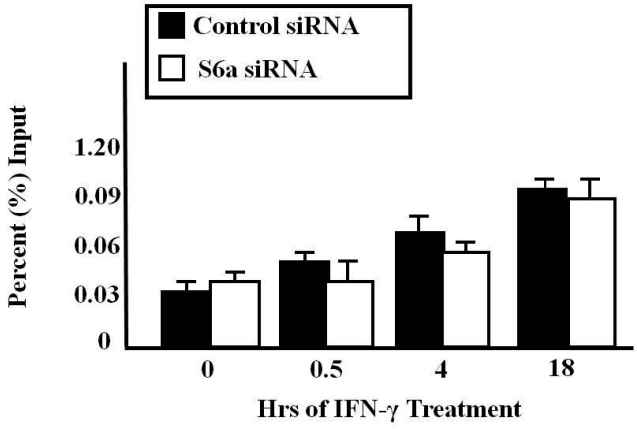
H3K9 **(Figure 2.6 A)** upon cytokine stimulation that were not affected is S6a siRNA treated samples **(Figure 2.6 A white bars)** when comparing to control siRNA samples **(Figure 2.6 A black bars).** Similarly, when evaluating levels of H3K18 and H4K8 acetylation, HeLa cells were transfected, stimulated and IP with antibody to endogenous acetylated H3K18 **(Figure 2.6 B)** and H4K8 **(Figure 2.6 C)**. Real time PCR analysis showed elevated levels of H3K18 and H4K8 upon IFN-γ stimulation that were significantly decreased in S6a siRNA treated samples **(Figure 2.6 B-C, white bars)** when comparing to control samples **(Figure 2.6 B-C, black bars)**. These data indicate that S6a acts similarly to Sug1 and affects the acetylation levels of similar and specific lysine residues.

The role of S6a in transcription initiation is gene specific and independent of degradation

To ensure that decreased acetylation at the CIITApIV in the presence of S6a siRNA is not indicative of a global decrease in acetylated H3 and H4, S6a and control siRNA transfected cells were lysed and lysates were immunoblotted for endogenous levels of acetylated H3 and acetylated H4. Although levels of S6a were successfully reduced **(Figure 2.7 A, bottom panel, lane 2)**, the levels of acetylated H3 and acetylated H4 remained unchanged when compared to control siRNA treated and untreated cells **(Figure 2.7 A top panels, lane 1 and 3 versus lane 2)**. To determine if S6a knockdown influences histone acetylation at additional genes, ChIP assays were performed to determine the levels of acetylated H3 and H4 at the GAPDH promoter. S6a knockdown had no impact on levels of acetylated H3 **(Figure 2.7 B)** or acetylated H4 **(Figure 2.7 C)** at the GAPDH promoter.

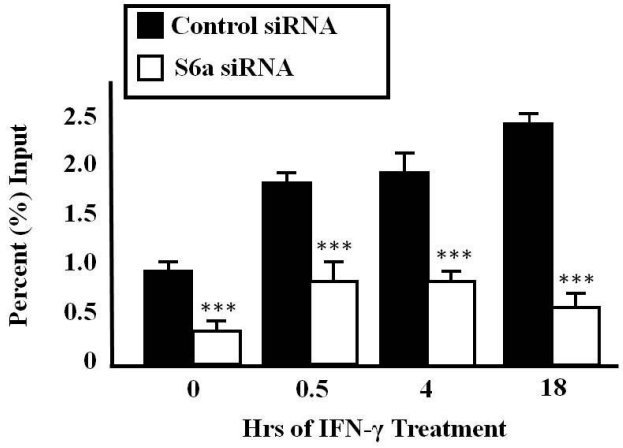
A

Endogenous Acetylated H3 K9 ChIP at CIITApIV



B

Endogenous Acetylated H3 K18 ChIP at CIITApIV



C

Endogenous Acetylated H4 K8 ChIP at CIITApIV

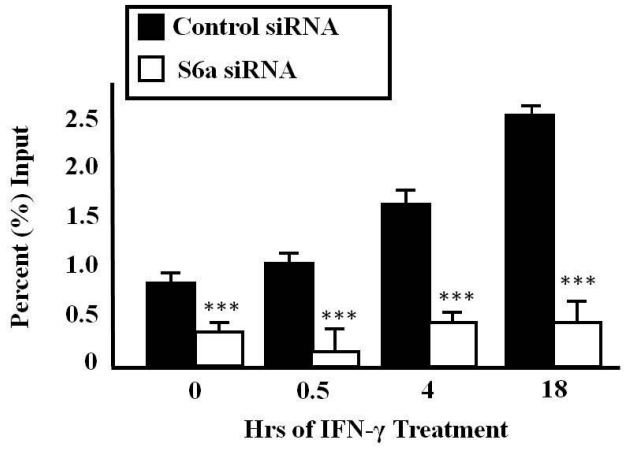


Figure 2.6 S6a knockdown decreases histone H3K18 and H4K8 acetylation but has no effect on H3K9 acetylation.

(A) Histone H3K9 acetylation at CIITApIV is enhanced upon IFN-γ stimulation and HDAC inhibition and unaffected by the loss of S6a expression. HeLa cells were transfected with scambled control or S6a specific siRNA. 24 hrs later were treated with HDAC inhibitor and stimulated with IFN-γ from 0-18hrs. Lysates were subjected to IP with control antibody or antibody to endogenous acetylated H3K9, and associated DNA was isolated and analyzed as described in Figure 5.

(B and C) S6a knockdown specifically decreases H3K18 (B) and H4K8 (C) acetylation at CIITApIV. HeLa cells were transfected with scrambled or S6a specific siRNA and 24 hrs later were treated with HDAC inhibitor and stimulated with IFN-γ for 0 to 18hrs. Lysates were subjected to IP with control antibody or antibody to endogenous acetylated H3K18 (B) and H4K8 (C). DNA was isolated and analyzed as described in Fig. 5. Data are presented as increase in the CIITApIV DNA relative to unstimulated IP samples.

Control IP values were 0.1 ± 0.09. Control and acetylated histone H3K9, H3K18 and H4K8 IP values represent mean ± SEM of three independent experiments. *** $P < 0.001$ versus control siRNA.

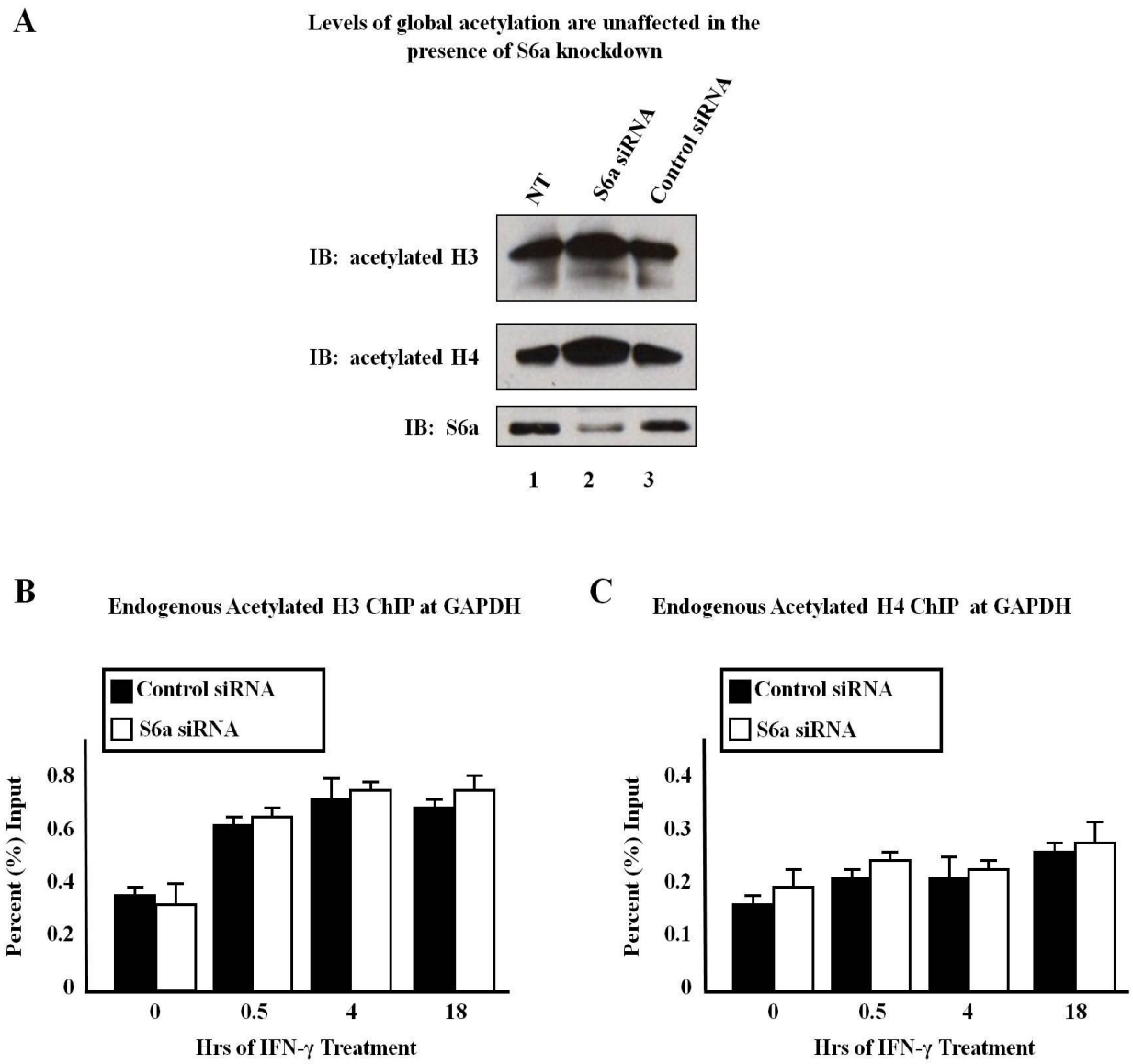


Figure 2.7 S6a knockdown decreases histone H3 and H4 acetylation in a promoter specific manner.

(A) Global histone H3 and H4 acetylation is unaffected by S6a siRNA knockdown. HeLa cells were left untreated (NT), (lane 1) or were transfected with either S6a specific siRNA (lane 2) or with control siRNA (lane 3). Lysates were subjected to IB for acetylated histone H3 (top), acetylated histone H4 (middle) or for endogenous S6a (bottom). Results reported are representative data of three independent experiments.

(B and C) Lack of S6a does not affect levels of acetylated histone H3 (B) and acetylated histone H4 (C) at the GAPDH promoter. ChIP assays were carried out in HeLa cells transfected with S6a specific or with control siRNA. Cells were treated with HDAC inhibitors for 20hrs and were stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control

antibody, with antibody to acetylated histone H3 (B) or to acetylated histone H4 (C), and associated DNA were analyzed via real-time PCR using primers spanning GAPDH promoter. IP values are presented as increase in GAPDH promoter DNA relative to unstimulated samples. Control IP values for (B) and (C) were 0.05 ± 0.02. Values for control IPs, acetylated histone H3 and acetylated histone H4 IPs represent mean ± SEM of three independent experiments.

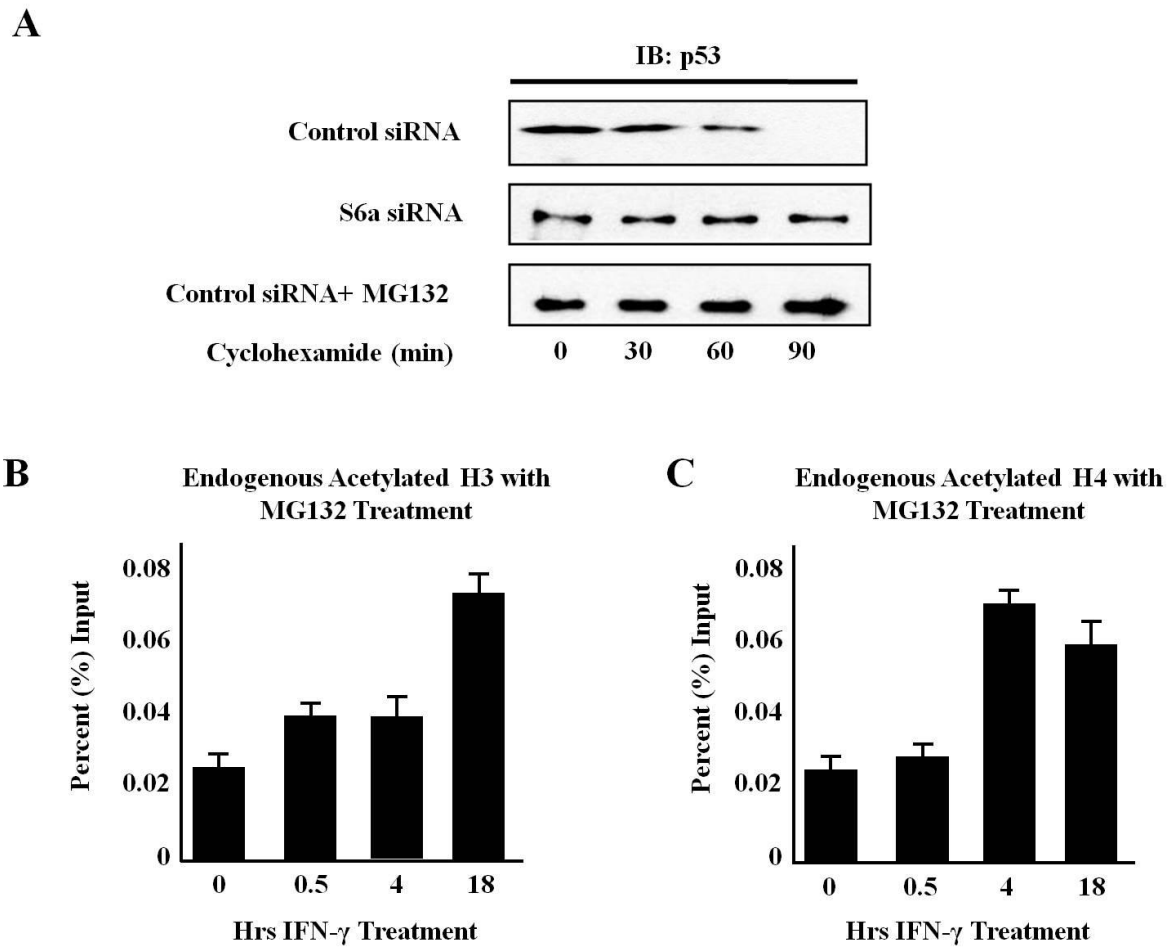


Figure 2.8 S6a dependent regulation of CIITApIV histone acetylation is independent of the proteolytic activity of 26S proteasome.

(A) S6a is essential for 26S mediated protein degradation. HeLa cells were transfected with S6a specific (middle panel) or with control siRNA (top and bottom panel) and were treated with proteasome inhibitor MG132 (bottom panel). Following cyclohexamide treatment cells were harvested and analyzed for p53 degradation. Results are representative of three independent experiments.

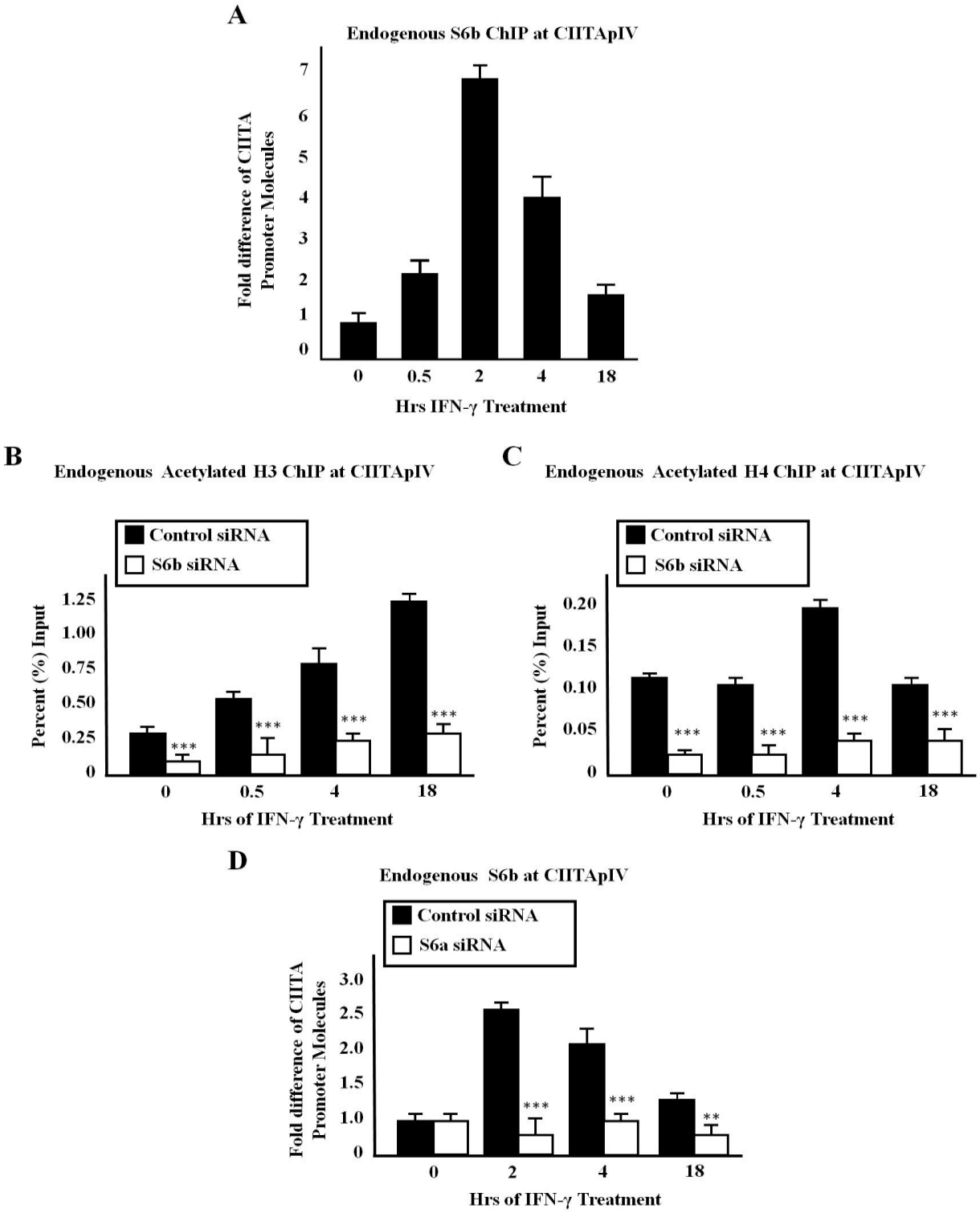
(B and C) ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours and treated with proteasome inhibitor MG132. Lysates were immunoprecipitated (IP) with control antibody or antibody to acetylated histone H3 (B) and acetylated histone H4 (C). Associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITApIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as increase in CIITApIV promoter DNA relative to unstimulated samples. Control IP values for (A) and (B) were 0.002 ± 0.001. Values for control IPs and for acetylated histone H3 IPs represent mean ± SEM of three independent experiments.

S6a is a critical component of the 19S proteasome whose primary function is to recruit and degrade polyubiquitinated proteins. In a p53 half-life assay described previously (Koues, Dudley et al. 2008), we observed that cells transfected with S6a siRNA showed elevated levels of p53 expression upon cyclohexamide treatment when compared with control siRNA treated samples, indicating an expected decrease in 26S proteasome activity in the presence of S6a siRNA **(Figure 2.8 A)**. These data indicate that S6a knockdown blocks the degradative function of the proteasome, we next questioned if the opposite was true as well: Does proteasome inhibition affect activating histone modifications in similar manner to S6a siRNA? To answer this question, we determined if the loss of histone H3 and histone H4 acetylation in S6a knockdown samples was independent of proteolysis. Cells were treated with the proteasome inhibitor MG132 and ChIP experiments were performed to detect endogenous levels of CIITApIV H3 and H4 acetylation. MG132 treated samples showed elevated levels of acetylated H3 **(Figure 2.8 B)** and acetylated H4 **(Figure 2.8 C)** at CIITApIV upon IFN-γ stimulation. Therefore, although S6a function is required for degradation mediated by the 26S proteasome, the proteasome is not required for inducing activating transcription marks on CIITApIV. These data indicate specific, proteolysis independent roles for the 19S ATPase S6a in regulating transcriptional activation of CIITApIV.

S6b knockdown also decreases CIITA specific histone H3 and H4 acetylation.

As a component of the 26S proteasome, S6a works with other 19S ATPases (S4, S6b, S7, Sug1 and S10b) to recruit polyubiquitinated proteins for degradation (Glickman, Rubin et al. 1999). It is therefore feasible that the intact 19S holoenzyme complex-not the individual ATPas-

es acts to regulate chromatin dynamics, independent of roles for the 19S in degradation. First, we have determined the binding pattern of the additional 19S ATPase, S6b to CIITApIV **(Figure 2.9 A)**. HeLa cells were stimulated with IFN-γ, subjected to chromatin immunoprecipitates (IP) with antibody to endogenous S6b, and analyzed by real time PCR with primers spanning the proximal promoters of CIITApIV. Upon cytokine stimulation, S6b has shown robust association to CII-TApIV **(Figure 2.9 A)**. Therefore, next we sought to determine the contribution of the additional 19S ATPase, S6b to histone H3 **(Figure 2.9 B)** and histone H4 **(Figure 2.9 C)** acetylation. Treatment of HeLa cells with S6b specific siRNA decreased S6b proteins levels by 90% **(Supplementary Figure 2.11 A top)** relative to control siRNA transfected cells, but did not impact protein level of S6a **(Supplementary Figure 2.11 A middle)** nor tubulin **(Supplementary Figure 2.11 A bottom)**. Similar to our prior observations in S6a knockdown cells **(Fig. 2.5 A-B)**, acetylated H3 and H4 ChIPs performed in the presence or absence of S6b exhibited significantly decreased acetylation of histone H3 **(Figure 2.9 B, white bars)** and acetylation of histone H4 **(Figure 2.9 C, white bars)** at CIITApIV when compared to control siRNA transfected cells **(Figure 2.9, B-C black bars)**. Additional ChIP assays were performed to make sure that the loss of acetylation of histone H3 and H4 was not caused by the total loss of histone H3 and histone H4 in the cells **(Supplementary Fig. 2.11 B-C).** Cells treated with siRNA specific against S6b showed no changes in levels of histones H3 and H4 when compared to control siRNA treated cells.


A
Endogenous S6b ChIP at CIITApIV
Fold difference of CIITA Promoter Molecules
0
1
2
3
4
5
6
7
0
0.5
2
4
18
Hrs IFN-γ Treatment
B
Endogenous Acetylated H3 ChIP at CIITApIV
Control siRNA
S6b siRNA
Percent (%) Input
0
0.25
0.50
0.75
1.00
1.25
***
0
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18
Hrs of IFN-γ Treatment
C
Endogenous Acetylated H4 ChIP at CIITApIV
Control siRNA
S6b siRNA
Percent (%) Input
0
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0.15
0.20
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0
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18
Hrs of IFN-γ Treatment
D
Endogenous S6b at CIITApIV
Control siRNA
S6a siRNA
Fold difference of CIITA Promoter Molecules
0
1.0
1.5
2.0
2.5
3.0
***
**
0
2
4
18
Hrs of IFN-γ Treatment

Figure 2.9 S6b dependent regulation of CIITApIV histone acetylation.

(A) ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous S6b and associated DNA was isolated and analyzed via real-time PCR using primers spanning IRF-E-GAS box of CIITApIV promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as fold increase in the CIITA promoter DNA relative to unstimulated S6a IP samples. Control IP values were 0.7 ± 0.05. Values for control IPs and S6a IPs represent mean ± SEM of four independent experiments.

(B and C) Lack of S6b 19S ATPase decreases histone H3 and H4 acetylation at CIITApIV. ChIP assays were carried out in HeLa cells transfected with S6b specific or control siRNA. Cells were treated with HDAC inhibitors for 20hrs and were stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to acetylated histone H3 (B) or with antibody to acetylated histone H4 (C), and associated DNA was isolated and analyzed via real-time PCR using primers spanning the proximal promoter CIITA isoform IV proximal promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). IP values are presented as increase in CIITApIV promoter DNA relative to unstimulated samples. Control IP values for (B) and (C) were 0.05 ± 0.02. Values for control IPs, acetylated histone H3 and acetylated histone H4 IPs represent mean ± SEM of two independent experiments. *** $P < 0.001$, **$P < 0.005$ versus control siRNA.

(D) S6a siRNA decreases S6b binding to CIITApIV. As described above, ChIP assays were carried out in HeLa cells with S6a specific or control siRNA and stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous S6b, ad associated DNA was isolated and analyzed via real-time PCR using primers spanning IRF-E-GAS box of CIITApIV promoter. IP values are presented as fold increase in CIITApIV promoter DNA relative to unstimulated samples. The control IP values for (D) were 0.1 ± 0.05. Values for control IPs and S6b IPs represent mean ± SEM of two independent experiments. *** $P < 0.001$ versus control siRNA.

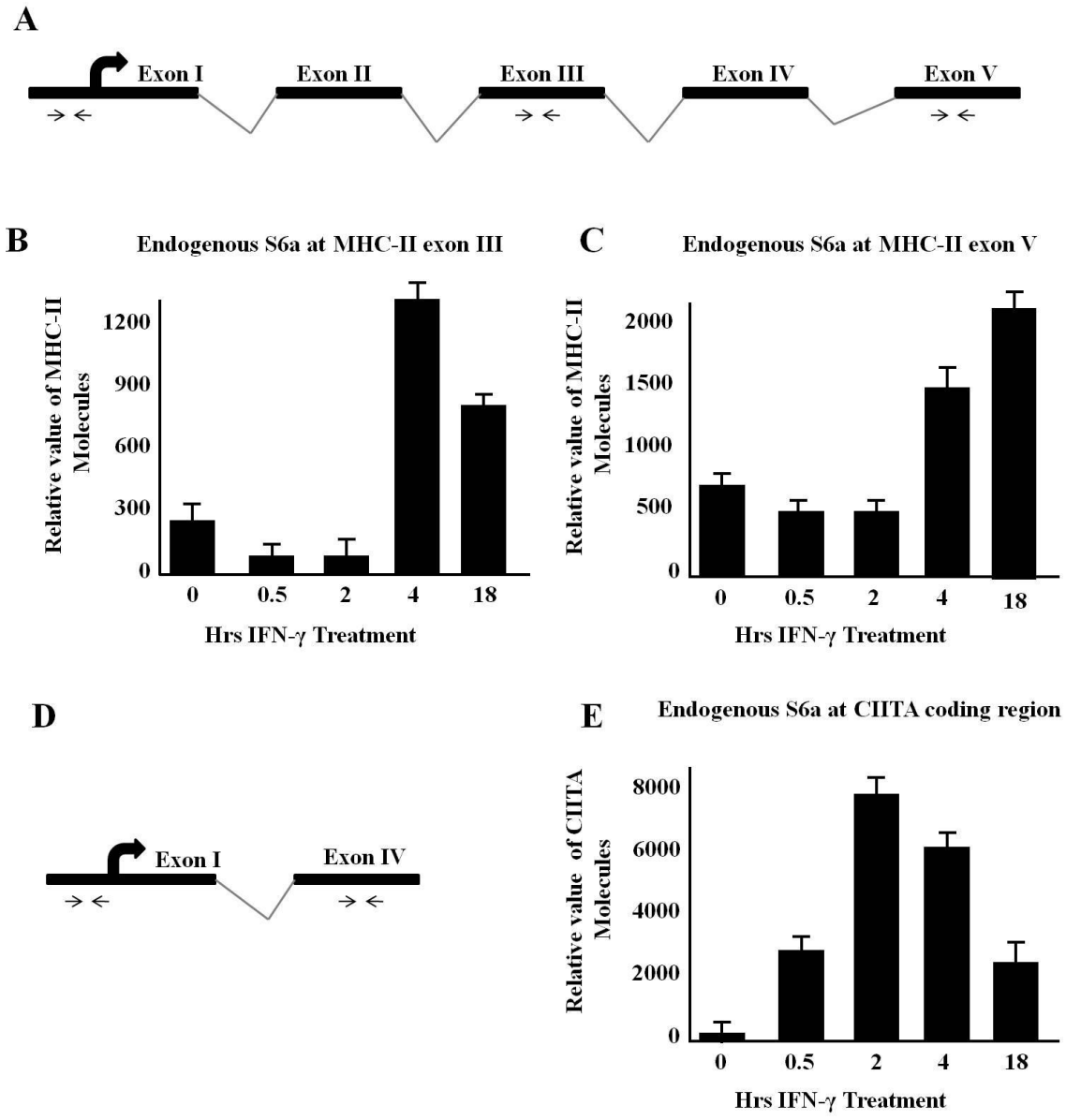


Figure 2.10 (Supplementary figure 1) The 19S ATPase S6a associates with MHC-II exon III, exon V and CIITA coding regions upon IFN-γ stimulation.

(A) Location of MHC II primer/probe sets.

(B-C) ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous S6a and associated DNA was isolated and analyzed via real-time PCR using primers spanning exon III (B), exon V (C) of the MHC-II *HLA-DRA*. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total

cell lysate. IP values are presented as fold increase in the MHC-II DNA relative to unstimulated S6a IP samples. Control IP values for (B) and (C) were 0.4 ± 0.06. Values for control IPs and S6a IPs represent mean ± SEM of three independent experiments.

(D) Location of CIITA primer/probe sets.

(E) ChIP assays were carried as above. DNA was isolated and analyzed using primers spanning coding region of CIITA. IP values are represented as fold increase in CIITA DNA relative to unstimulated samples. Control values were 0.5 ± 0.04. Values represent mean± SEM of three independent experiments.

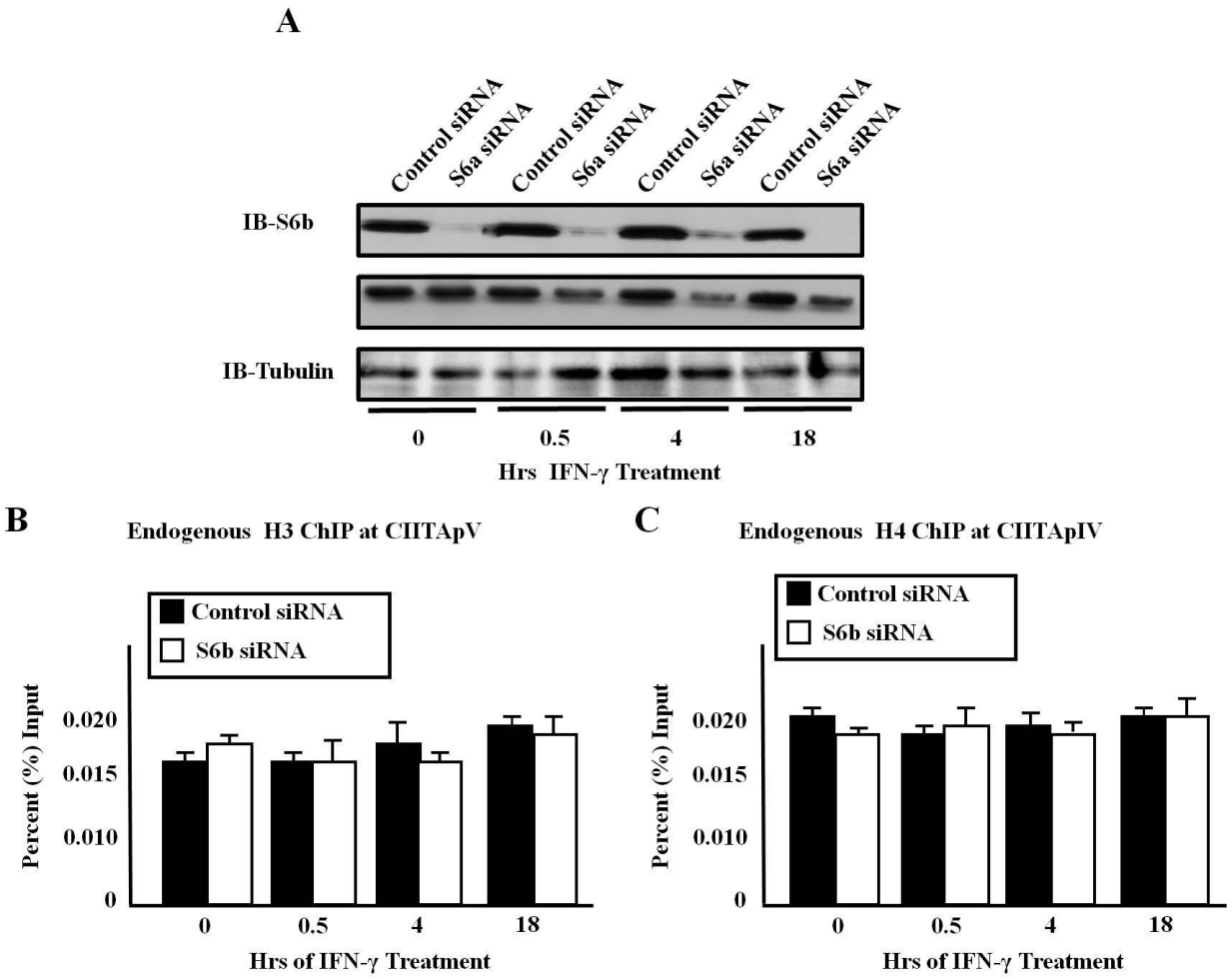


Figure 2.11 (Supplementary figure 2) The 19S ATPase S6b siRNA specifically and efficiently decreases S6b protein expression and does not influence levels of histone H3 and histone H4 at CIITApIV.

(A) S6b siRNA specifically and efficiently decreases S6b protein expression in the presence or absence of cytokine stimulation. HeLa cells were transfected with control or S6b specific siRNA, untreated or stimulated with IFN-γ for 0 to 18hrs, harvested and subjected to Western Blot analysis of endogenous expression of S6a, S6b and tubulin. Western Blot analysis shows 90% knockdown of S6b and stable expression of S6a and tubulin. Results reported are representative data of four independent experiments.

(B and C) S6b knockdown does not impact levels of histone H3 and histone H4 at CIITApIV. As above ChIP assays were carried out in HeLa cells transfected with S6b specific or control siRNA and stimulated with IFN-γ for 0-18 hours. Lysates were subjected to IP with control antibody or antibody to endogenous histone H3 (B) or H4 (C). Associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITApIV. IP values are presented as increase in CIITApIV DNA relative to unstimulated histone H3 (B) and histone H4 (C) IP samples. Control IP values for (B) and (C) were 0.001 ± 0.002. Values for control IPs, histone H3 and histone H4 IPs represent mean ± SEM of two independent experiments.

To determine whether S6bbinds to CIITApIV in the absence of S6a, we performed S6b ChIP assay in S6a siRNA treated cells. As previously described, initial ChIP experiment showed that S6b rapidly binds to CIITApIV **(Figure 2.9 A)**. However in cells transfected with S6a siRNA, S6b binding to CIITApIV was significantly decreased **(Figure 2.9 A white bars),** when compared to cells transfected with control siRNA **(Figure 2.9 A white bars)**. In sum these data and published Sug1 studies (Bhat, Turner et al. 2008; Koues, Dudley et al. 2009) indicate that the 19S ATPases play important and similar roles in regulating epigenetic modifications at CIITApIV and indicate intermolecular promoter interactions between 19S ATPases.

2.4 DISCUSSION

The 19S regulator of the 26S proteasome contains six proteasomal ATPases which belong to the AAA (*A*TPases *a*ssociated with diverse cellular *a*ctivity) superfamily of ATPases (Kinyamu, Chen et al. 2005). The 19S ATPases assemble into a hexameric ring that contacts the 20S core particle where it likely functions in substrate unfolding and translocation (Coux, Tanaka et al. 1996; Glickman, Rubin et al. 1999). The precise roles for the ATPases in ubiquitin mediated degradation remain unclear, but multiple studies have suggested that one or more of the 19S ATPases functions to unfold ubiquitinated substrates as they are targeted into the 20S catalytic core of the proteasome (Ciechanover 1998; Strickland, Hakala et al. 2000).

Recent studies have identified transcriptional activities of the 19S on yeast chromatin (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002;

Morris, Kaiser et al. 2003; Lee, Ezhkova et al. 2005; Sikder, Johnston et al. 2006; Sulahian, Johnston et al. 2006) Roles for 19S ATPases in mammalian transcription have likewise been previously reported (Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008), and the mechanisms of how 19S ATPases regulate transcription initiation of various genes remain similarly unclear. Transcriptional activity of eukaryotic genes is regulated at multiple levels, including modifications to histone tails, recruitment of transcriptional machinery and the assembly of preinitiation complexes (Lee and Young 2000). We have previously demonstrated that following IFN-γ stimulation, the 19S ATPase Sug1 rapidly binds the MHC-II proximal promoter and that diminished expression of Sug1 substantially inhibits MHC-II promoter histone acetylation and decreases recruitment of the transactivator CIITA, resulting in diminished MHC-II expression (Koues, Dudley et al. 2008). Despite these advances, evidence of roles for the remaining 19S ATPases in regulating mammalian chromatin structure remains to be demonstrated.

S6a is a critical ATPase subunit of the 19S regulator which we have previously reported to bind the promoter of the MHC II gene *HLA-DRA* (Koues, Dudley et al. 2008). As a part of the 26S proteasome complex, S6a has been shown to bind with the polyubiquitin chain of substrates targeted for proteasome mediated degradation (Lam, Lawson et al. 2002). Although the 19S ATPases likely form a ring structure, the fact that only the S6a subunit directly binds ubiquitin suggests delegation of specific tasks to specific ATPases and highlights the functional diversity of the 19S ATPase subunits (Rubin, Glickman et al. 1998; Kohler, Cascio et al. 2001). The aim of the present work was therefore to determine the role of the 19S ATPase S6a in two key processes preceding transcriptional initiation of IFN-γ inducible genes: acetylation of core his-

tones and recruitment of the general transcription factors. Our current study indicates that the 19S ATPase S6a rapidly and robustly binds the IFN-γ inducible promoters of MHC II *HLA-DRA* and CIITApIV and that in the absence of S6a, MHC II message levels and CIITA transactivation and expression levels are substantially decreased. S6a siRNA resulted in a 70% reduction of MHC-II message level and a 30-40% decrease of CIITA message and protein level. The CIITA result is likely more severe than indicated as the primers used to detect CIITA message levels detect CIITA expressed from any of the three CIITA promoters. As IFN-γ stimulation results in expression of CIITA isotype III and IV (Piskurich, Gilbert et al. 2006), the smaller percent decrease in CIITA message level is likely due to the fact that we are assaying for a combination of both IFN-γ inducible isotypes of CIITA: CIITApIII and CIITApIV. Importantly, the effects of S6a siRNA are specific, as reconstituting S6a expression in S6a siRNA transfected cells restores CIITA driven *HLA-DRA* promoter activity and GAPDH message levels are not impacted by S6a knockdown.

The expression of CIITApIV upon IFN-γ stimulation depends on the binding of the transcription factor STAT1 to a GAS sequence, of IRF-1 and IRF-2 to an IRF-E element, and of the ubiquitously expressed USF-1 to an E box. Initial studies demonstrated that endogenous S6a and STAT-1 co-immunoprecipitated, suggesting functional interaction between these proteins. Roles for S6a in binding and recruiting coactivators are likely. Ishizuka et al have previously indicated that S6a is capable of enhancing transactivation via HAT activity containing steroid receptor coactivator 1 (Ishizuka, Yamaya et al. 2001) and we have shown that 19S ATPase Sug1 to play an important role in the initial binding of HAT CBP to the MHC II proximal promoter (Koues,

Dudley et al. 2008; Koues, Dudley et al. 2009). Cytokine inducible association of STAT-1 and IRF-1 with CIITApIV further correlates with significant increases in the levels of acetylated H3 and acetylated H4 at the CIITApIV proximal promoter. Binding of STAT-1 and IRF-1 was significantly reduced in the presence of S6a knockdown as was CIITApIV H3K18 and H4K8 acetylation, suggesting that S6a plays an important role in chromatin remodeling, recruitment of transcription factors and in assembly of the preinitiation complex on CIITApIV. Observed effects of S6a knockdown are also lysine specific, as acetylation of histone H3K18 and histone H4K8 at CIITApIV are diminished in cells transfected with S6a siRNA, whereas acetylation of histone H3K9 are unaffected at CIITApIV proximal promoter.

The 19S ATPases recognize ubiquitinated substrates for proteolysis and are therefore critical components of the 26S proteasome (Ciechanover 1994; Glickman, Rubin et al. 1999). To determine the effect of S6a knockdown on proteasome function, we assayed p53 half life in the presence of S6a knockdown and cyclohexamide treatment. As S6a is an important regulatory protein of the 19S proteasome, it was not surprising that S6a knockdown negatively impacted proteasome activity. However, when cells were treated with the proteasome inhibitor MG132 and assayed for levels of H3 and H4 acetylation, the acetylation values were comparable to those of untreated cells. Thus, the loss of CIITApIV histone acetylation observed in S6a deficient cells was not due to a lack of proteasome function. These experiments further emphasize that although S6a is required for the proteolytic functions of the 26S proteasome, the effects of S6a on CIITApIV acetylation are proteolysis independent. Furthermore, the effects on H3 and H4 acetylation mediated by S6a siRNA are specific as whole cell lysates show no global changes in

H3 or H4 acetylation upon S6a knockdown and as acetylation levels at the GAPDH promoter are likewise unaffected by S6a knockdown.

We show here that additional 19S ATPase, S6b inducibly binds CIITApIV and modulates histone H3 and histone H4 acetylation similarly to S6a. In data not shown, we have further seen that each of the 19S ATPases rapidly binds CIITApIV following IFN-γ stimulation. In fact, many of the above observations in regard to roles for S6a in chromatin remodeling and transcription factor recruitment correlate with our previous observations that the 19S ATPase Sug1 regulates specific activating chromatin marks and transcription factor recruitment at the MHC II proximal promoter (Koues, Dudley et al. 2009). Likewise, promoter binding of the 19S ATPases might be interdependent, as decreased exoression of S6a significantly decreased promoter recruitment of S6b. It is therefore likely that 19S AAA ATPase play overlapping and similar roles in initiating CIITA transcription, where they potentially bind CIITApIV as AAA ATPase hexameric complexes. That the 19S ATPase S6a robustly binds to the coding region gene *HLA-DRA* and CIITA indicates role in elongation processes at these genes, and it has been proposed that the 19S AAA ATPases play roles in the coding regions of yeast genes by remodeling paused RNA polymerase II (Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002). However, among the 19S ATPases, only S6a was shown to crosslink to the coding region of the human immunodeficiency virus type 1 gene in the presence of transcription factor Tat (Lassot, Latreille et al. 2007). Therefore, conflicting evidence remains as to individual and hexameric functions and the contributions of 19S ATPases to transcriptional processes. Despite mounting evidence for the roles of the 19S ATPases in the regulation of mammalian transcription, it remains unclear how these proteins may regulate multiple stages of transcriptional processes. A full understand-

ing of the roles played by the 19S ATPases in mammalian transcription will require studies of the molecular events occurring at these and additional promoters.

2.5 MATERIAL AND METHODS

Cells

HeLa (human epithelial) cells from ATCC (Manassas, VA) were maintained using high glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal calf serum (FCS), 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies

S4, S7, S6a, S6b and S10b antibodies were from Biomol International, L.P. (Plymouth Meeting, PA). Sug1 antibody was from Novus Biologicals (Littleton, CO). IRF-1 and STAT-1 antibodies were from Santa Cruz (Santa Cruz, CA). Phosphorylated STAT-1 (pY701) was from BD Biosciences (San Jose, CA). Histone H3, acetylated histone H3, histone H4, acetylated histone H4 and rabbit and mouse immunoglobulin G (IgG) isotype control antibodies were from Millipore (Lake Placid, NY). Histone 3 (acetyl K9) and histone 3 (acetyl K18) antibodies were from Abcam (Cambridge, MA); histone H4 (acetyl K8) was from Millipore (Lake Placid, NY). p53 antibody was from Abcam (Cambridge, MA). Tubulin and CIITA antibody was from Santa Cruz (Santa Cruz, CA). HRP conjugated mouse antibody was from Promega (Madison, WI) and HRP conjugated rabbit antibody was from Pierce (Rockland, IL).

Plasmids

The following plasmids were previously described: Flag-CIITA, pcDNA3 and *HLA-DRA*-Luc (Cressman, Chin et al. 1999; Cressman, O'Connor et al. 2001; Greer, Zika et al. 2003). The *Renilla* luciferase control vector (E2231) was from Promega (Madison, WI). pBluescript (pBS)-S6a was kindly provided by Dr. Martin Rechsteiner (Richmond, Gorbea et al. 1997; Gorbea, Taillandier et al. 1999) and subcloned into Myc tagged pCMV-3 using EcoRI restriction sites.

Endogenous Co-Immunoprecipitations

HeLa cells were plated at a cell density of $2x10^6$ in 15 cm tissue culture plates. Thirty minutes prior to harvesting, cells were stimulated with IFN-γ (500U/ml), lysed in RIPA lysis (1M Tris pH 8.0, 10% Nonidet P-40, 5% DOC, 10% SDS, 5M NaCl, 5mM EDTA, 1M DTT) supplemented with Complete EDTA-free protease inhibitors (Roche) buffer and were precleared with 30µl IgG beads (Sigma). Cell lysates were immunoprecipitated (IP) overnight with 5µg of anti S6a (Biomol), anti STAT-1 (Santa Cruz), or mouse isotype control IgG (Upstate). Immune complexes were isolated with 50µl protein G beads, denatured with Leammli buffer, boiled and subjected to SDS-PAGE. Co-immunoprecipitated complexes were detected by immunoblotting (IB) with anti STAT-1 (Santa Cruz). HRP conjugates were detected with Supersignal West Pico Chemiluminescent substrate (Pierce). Equal transfection and loading was determined in non-immunoprecipitated lysates by immunoblot of total protein.

siRNA Constructs and Transient Transfections

Short interfering RNA (siRNA) duplexes predesigned against human PSMC3 (Qiagen) were previously described (Koues, Dudley et al. 2008) and were used for transient knockdown of the 19S ATPase S6a. Versite specific siRNA was predesigned against human S6a (Sigma) to control the specificity of our siRNA complex. S6b siRNA sequence was designed with a G+C content of 35 to 55% containing dTdT overhangs and was compared to the NCBI BLAST nucleotide database. The target sequences of S6b siRNA used was 5′- CACG-CATTTCGAGCTCTACAA-3′ (Qiagen). HeLa cells were transfected with 0.7µg of S6a or S6b specific siRNA, versite specific or scrambled sequence control using RNAiFect transfection reagent (Qiagen) according to the manufacturer's instructions and were treated with IFN-γ (500U/ml) as indicated. Cells were lysed in NP-40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP-40, 0.5M EDTA, 5M NaCl, 1M DTT, dH_2O) with EDTA free protease inhibitor (Roche) and analyzed by western blot for knockdown efficiency and specificity.

Luciferase Reporter Assays

HeLa cells were plated in 6 well plates at a density of $5x10^4$ cells/well and transfected with 0.7µg of siRNA using RNAi transfection reagent (Qiagen) according to the manufacturer's instructions. Following twenty-four hours of siRNA transfection, Flag-CIITA, *HLA-DRA*-Luc, *Renilla,* Myc-S6a and pCDNA3 plasmids were transfected as indicated using Fugene 6 (Roche, Indianapolis, IN). Eighteen hours following transfection, cells were subjected to dual luciferase assays (Promega) according to the manufacturer's instructions.

RNA Expression

HeLa cells were plated at a cell density of $8x10^5$ cells, transfected with S6a siRNA and twenty four hours later were stimulated with IFN-γ (500U/ml) as indicated. Cells were harvested and 10% of the cells were lysed with 1% Nonidet P-40 lysis buffer and subjected SDS PAGE to determine the efficiency of the knockdown. The remaining fraction of cells was subjected to RNA extraction with 1ml of Trizol reagent (Invitrogen) as previously described (Bhat, Turner et al. 2008). The Omniscript Reverse Transcription Kit (Qiagen) was used to generate cDNA from extracted RNA. Isolated DNA was analyzed by Real-time PCR on an ABI prism 7900 (Applied Biosystems) using primers and probes for MHC-II (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008) and GAPDH (Medhurst, Harrison et al. 2000). Primers sequences for CIITA and IRF-1 were as follows: CIITA sense GGGAGAGGCCACCAGCAG**,** CIITA antisense GCTCCAGG-TAGCCACCTTCT and CIITA probe FAM-CTGTGAGCTGCCGCTGTTCCC-TAMRA, IRF-1 sense TGGCCAAGGGTGTGATACTG, IRF-1 antisense CCACTCCGACTGCTCCAAGA and IRF-1 probe FAM-AGTTGATAGCCTCTTGCTCTTCTGTCCC-TAMRA. Values generated from Real-time PCR reactions were calculated on the basis of standard curves generated.

Histone Immunoblotting

HeLa cells were transfected with 0.7µg S6a siRNA or control siRNA using RNAiFect transfection reagent (Qiagen). Forty eight hours following siRNA transfection, 10% of the total cell volume was lysed with 1% Nonidet P-40 lysis buffer and was analyzed by western blot for

knockdown efficiency. The remaining cell volume was lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, dH_20) for 30 minutes and sheared by sonication. Lysate samples were normalized for protein concentration, denatured with Leammli Buffer (BioRad) and were separated by SDS PAGE. Gels were transferred to nitrocellulose and were immunoblotted with anti acetylated H3 (Millipore) and anti acetylated H4 (Millipore). Secondary HRP conjugated anti-rabbit was detected with SuperSignal West Pico Chemiluminescent substrate (Pierce).

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). Briefly, HeLa cells were plated at a cell density of $2x10^6$. Following IFN-γ stimulation (500U/ml), cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature. Crosslinking was stopped by the addition of 0.125M glycine for 5 minutes at room temperature. Cells were lysed in SDS lysis buffer and were sonicated at constant pulse to generate an average of 500-750bp sheared DNA. Sonicated lysates were precleared with salmon-sperm coated agarose beads (Upstate) and half of the lysate was immunoprecipitated with 5μg of indicated antibody overnight at 4°C. The remaining half of the lysate was used as a control and was immunoprecipitated with isotype control antibody (Upstate). Following an additional twelve hour immunoprecipitation with 50μl of salmon-sperm coated agarose beads, samples were washed for 3 minutes at 4°C with the following buffers: low and high salt buffer, LiCl and 1xTE buffer and were eluted with SDS elution buffer . Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer's instructions. Isolated

DNA was analyzed by real-time PCR using previously published primers spanning the W-X-Y box of the MHC-II *HLA-DRA* promoter (Bhat, Turner et al. 2008). Primers spanning the GAS-IRF-E box of CIITApIV were as follows: sense CAGTTGGGATGCCACTTCTGA, antisense TGGAGCAACCAAGCACCTACT and probe FAM-AAGCACGTGGTGGC-TAMRA. Primers spanning exon III and exon V of MHC II were previously published (Koues, Dudley et al. 2009) and primers spanning. Values were calculated based on standard curves generated.

Chromatin Immunoprecipitation with siRNA

HeLa cells were plated at a cell density of $8x10^5$ and transfected with 0.7µg S6a, S6b specific or control siRNA (Qiagen). Cells were stimulated with IFN-γ (500U/ml), 5mM HDAC inhibitor sodium butyrate (Upstate) and 5.2µM proteasome inhibitor MG132 (EMD Biosciences, San Diego, CA) as indicated and 10% of the total cell volume was lysed with 1% Nonidet P-40 lysis buffer and was analyzed by western blot for knockdown efficiency. The remaining cell volume was lysed in SDS lysis buffer and was subjected to sonication and the above described ChIP assay.

***In vivo* proteolytic activity**

Hela cells were transfected with S6a specific or control siRNA (Qiagen) and treated with 5.2µM MG132 proteasome inhibitor (EMD Bioscience) and 100µM cyclohexamide (Sigma) as indicated. Cells were lysed with 1% NP-40 buffer with protease inhibitor and total cell lysates were analyzed for p53 degradation and S6a knockdown efficiency.

2.6 ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society, the Georgia Cancer Coalition and the Georgia Research Alliance (to S.F. Greer) and by Ph.D. Fellowships from the Georgia State University Molecular Basis of Disease Area of Focus (to A.D. Truax and O.I. Koues).

3 CHAPTER III: THE 19S ATPase S6a PLAYS A ROLE IN RECRUITMENT OF RNA POL II AND CYCLIN T1 TO CLASS II TRANSACTIVATOR, CIITA CODING REGION

PUBLICATION IN PREPARATION: AGNIESZKA D.TRUAX, MEGHNA THAKKAR, JULIE MORGAN AND SUSANNA F. GREER

3.1 ABSTRACT

RNA polymerase II (Pol II) mediated elongation has recently emerged as a critical control step in gene expression. We and others have shown that components of the 26S proteasome act as mediators of transcriptional initiation and play important roles in gene expression. We have previously demonstrated the 19S proteasome ATPase S6a (S6'/TBP1) plays significant roles in transcriptional initiation of CIITApIV. We now demonstrate RNAi mediated knockdown of S6a significantly diminishes occupancy of Pol II at both proximal promoter and coding regions of CIITA genes. We further demonstrate S6a is bound at elevated levels at CIITApIV coding regions where Pol II binding is markedly increased. Further linking the 19S to elongation are demonstrated interactions of 19S ATPases S6a, S7, and Sug1 with critical regulators of elongation, the CDK9 and Hexim-1components of the inactive form of positive transcription elongation factor (P-TEF-b). Decreased expression of S6a significantly diminishes recruitment of Cyclin T1 to CIITApIV promoter and coding region, indicating S6a contributes to efficient elongation. Transcriptional regulation by the 19S represents a novel mechanism of regulating mammalian gene expression and presents the proteasome as a therapeutic target for manipulating MHC II expression in cancer and in adaptive immune responses.

3.2 BACKGROUND

Major Histocompatibility class II (MHC II) genes encode cell surface glycoproteins that bind and present antigens to $CD4^+$ T cells. Cell surface expression of MHC II molecules is tightly regulated to ensure rapid activation of the immune response (Glimcher and Kara 1992; Gerloni and Zanetti 2005). MHC II is expressed constitutively on antigen presenting cells, but can be induced on nucleated cells that are stimulated with the inflammatory cytokine interferon gamma, (IFN- γ) (Kaufman, Auffray et al. 1984; Boss 1997). Following IFN-γ stimulation, a large ubiquitously expressed multi-protein enhanceosome complex binds the MHC II proximal promoter and recruits the Class II Transactivator, (CIITA) (Reith and Mach 2001; Ting and Trowsdale 2002). Transcription of CIITA is regulated in a cell specific manner by four distinct promoters: pI, pII, pIII, and pIV. CIITA promoter IV (CIITApIV) is the CIITA promoter that responds to IFN-γ stimulation via the Janus kinase signal transducer and activator of transcription (JAK-STAT) transduction pathway (Piskurich, Linhoff et al. 1999; Piskurich, Gilbert et al. 2006). Once bound to MHC II promoter complexes, CIITA stabilizes the enhanceosome and recruits basal transcription components, including the cyclin dependent kinase 7 (CDK7) subunit of transcription factor II H (TFIIH) and the cyclin dependent kinase (CDK9) subunit of positive transcription elongation factor (P-TEF-b). CDK7 and CDK9 phosphorylate serine residues on RNA polymerase II (RNA pol II) and are thus critical regulators of the switch from initiation to elongation (Mach, Steimle et al. 1996; Masternak, Barras et al. 1998; Kanazawa, Okamoto et al. 2000; Boss and Jensen 2003).

Recent studies from our lab and others have highlighted the central role played by CIITA in controlling MHC II expression by regulating transcriptional initiation and elongation through stabilizing the recruitment and phosphorylation of RNA Pol II (Bhat, Truax et al. ; Truax, Koues et al. ; Ting and Trowsdale 2002; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). Transcription in eukaryotes is initiated by the recruitment of RNA polymerase II (Pol II) to promoter localized start sites. RNA Pol II initiates transcription upon association with basal transcription factors, P-TEF-b and via its C terminal domain (CTD) phosphorylation. The CTD of mammalian RNA Pol II consist of a series of 52 heptad repeats of YSPTSPS located on the large subunit of pol II (Dahmus 1996; McCracken, Fong et al. 1997). Once the preinitiation compex is formed, RNA Pol II initiates synthesis of mRNA that is accompanied by phosphorylation at serine 5 on the RNA pol II CTD. Eukaryotic genes often stall after synthesizing only 20-50 bases of RNA, when bound by negative elongation factor (NTE-F) and 5.6-dichloro 1-β –D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factor (DSIF) (Hirose, Tacke et al. 1999; Hirose and Manley 2000). To enable elongation, the active form of P-TEF-b phosphorylates N-TEF and serine 2 of RNA pol II CTD and releases the paused complex, leading to transcriptional elongation (Mancebo, Lee et al. 1997; Zhu, Pe'ery et al. 1997).

P-TEF-b is a heterodimer composed of CDK9 and C type cyclins: cyclin T1 (Cyc T1) and cyclin T2 (Cyc T2) or cyclin K (Cyc K) (Price 2000) and exists in two distinct molecular forms as an active or inactive complex (Nguyen, Kiss et al. 2001; Yang, Zhu et al. 2001). P-TEF-b predominantly exists as an inactive complex composed of CDK9, cyclins, and small nuclear RNA (sn7SK) bound to dimers of hexamethylene bisacetamide (HMBA) inducible protein 1 (Hexim-1) and Hexim-2 (Price 2000; Michels, Fraldi et al. 2004). Upon initiation of transcrip-

tion, P-TEF-b dissociates from Hexim-1 and 7SK through unknown mechanisms and forms the small active form of P-TEF-b that is recruited to transcription initiation complexes (Yik, Chen et al. 2003; Peterlin and Price 2006). The switch from transcriptional initiation to elongation is thus a dynamic process regulated by multiple protein-protein interactions. In addition to negatively regulating P-TEF-b, Hexim-1suppresses the transcriptional activity of transcriptional regulators including the glucocorticoid receptor, the estrogen receptor, and the p65 subunit of NF-κB (Ouchida, Kusuhara et al. 2003; Shimizu, Ouchida et al. 2005; Wittmann, Fujinaga et al. 2005). Important to this study, recent findings demonstrate Hexim-1 sequesters P-TEF-b from CIITA (Kohoutek, Blazek et al. 2006).

Increasing evidence indicates the ubiquitin proteasome system (UPS) is involved in transcriptional regulation. The 26S proteasome is a large, multimeric complex composed of a 19S regulatory particle and a 20S proteolytic core (Coux, Tanaka et al. 1996; Gorbea, Taillandier et al. 1999; Pickart 2004). The 19S regulator is further divided a lid and a base. Within the 19S base are six ATPases termed S4, S6a (S6', TBP1), S6b, S7, Sug1 (S8), and Sug2 (S10b), and three non-ATPase subunits (Gorbea, Taillandier et al. 1999; Adams 2003). Observations in yeast that ATPase components of the 19S proteasome associate with actively transcribed genes and facilitate recruitment of various transcription factors first established non- proteolytic involvement for the proteasome in transcription (Ciechanover 1998; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Muratani and Tansey 2003; Lee, Ezhkova et al. 2005; Sulahian, Johnston et al. 2006; Archer, Burdine et al. 2008). Additional studies have indicated the 19S ATPase subunits Rpt6 (homolog of Sug1) and Rpt4 (homolog of Sug2 or S10b) participate in chromatin remodeling in yeast (Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005). In

addition, the 19S proteasome associates with RNA pol II (Ferdous, Gonzalez et al. 2001) and is critical for efficient RNA pol II mediated elongation in yeast cells (Ferdous, Gonzalez et al. 2001; Lassot, Latreille et al. 2007). While the proteasome has been demonstrated to regulate transcription at multiple yeast promoters, the role of the proteasome in initiation and elongation during mammalian transcription remains unclear.

Our previous studies have demonstrated the19S ATPase S6a associates with the CIITA-pIV proximal promoter, where it plays an important role in recruiting the requisite transcription factors STAT-1 and IRF-1 which are required for efficient transcription of CIITA. We have shown S6a regulates the addition of activating epigenetic modifications to CIITApIV, indicating the 19S ATPases play important roles in initiating transcription of inducible mammalian genes. We show here that the 19S ATPase S6a associates with regulatory components of P-TEF-b and plays novel roles in recruiting RNA pol II and cyclin T1 to CIITA coding regions. These data offer new insights into non proteolytic roles of the proteasome in regulating transcription elongation.

3.3 RESULTS

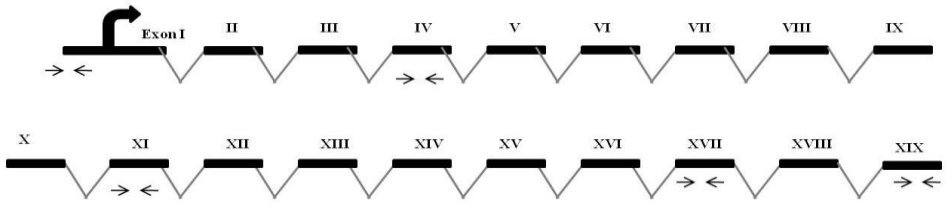
The 19S ATPase S6a associates with the promoter and coding regions of CIITApIV genes.

We have shown the 19S ATPase Sug1 positively regulates MHC II transcription by regulating CIITA binding and histone H3 acetylation at the MHC II proximal promoter (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). We have further demonstrated that the 19S ATPase S6a

plays similar roles in regulating transcriptional initiation at CIITApIV (Truax, Koues et al.) Each of the six 19S ATPases are recruited to the coding region of HIV-1 genes in the presence of the transactivator Tat, with S6a binding at significantly higher levels (Lassot, Latreille et al. 2007). As we have previously noted the 19S ATPase S6a binds to CIITA and MHC II coding regions (Truax, Koues et al.), we sought here to investigate the binding of the 19S ATPase S6a through-out the coding region of CIITA genes.

A

CIITA pIV primer design



B

Levels of S6a at CIITA proximal and coding regions

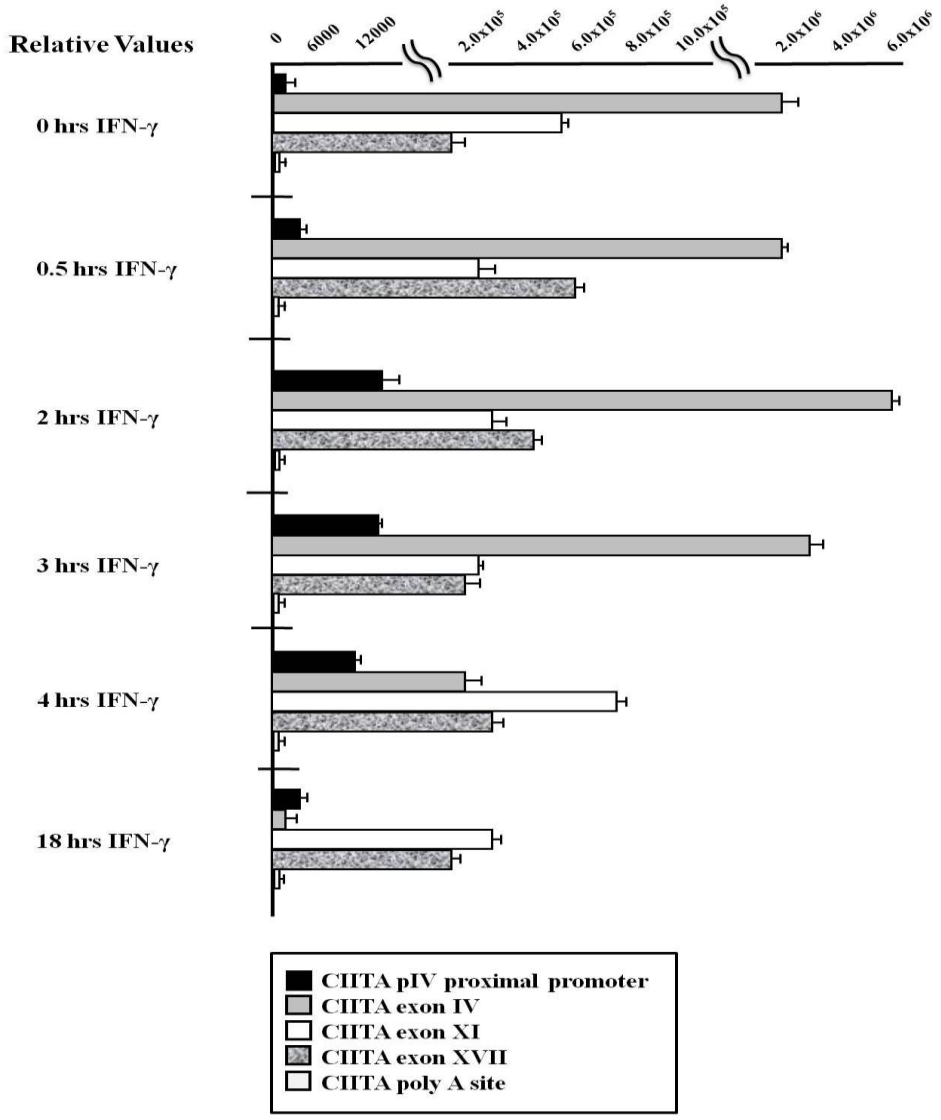


Figure 3.1 The 19S ATPase S6a binds to both promoter and coding region of CIITA.

(A) Schematic representation illustrating location of primers in the CIITA proximal promoter and coding regions.

(B) S6a binds robustly to multiple CIITA coding regions. ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody or antibody to endogenous S6a and associated DNA was isolated and analyzed via real-time PCR using primers spanning the IRF-E-GAS box of CIITApIV, exon IV, exon XI, exon XVII or the polyA site. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as a relative increase in the CIITA DNA. Control IP values for (A) and (B) were 1700 ± 500. Values for control IPs and S6a IPs are representative data of 3 independent experiments.

To investigate S6a binding to CIITA coding sequences, we designed sets of primers and probes spanning the exons of CIITA **(Figure 3.1 A)**. We performed chromatin immunoprecipitation (ChIP) assays in HeLa cells which were stimulated with IFN-γ as indicated, and crosslinked and sonicated lysates were subjected to immunoprecipitation (IP) with antibody against endogenous S6a. Following immunoprecipitation, associated DNA was isolated and analyzed by real time PCR with primers spanning the proximal promoter, exon IV, exon XI, exon XVIII, and the polyA site of CIITA. While S6a was inducibly recruited to CIITApIV, S6a demonstrated robust occupancy at CIITA exon IV in the absence cytokine stimulation that decreased with extended cytokine stimulation **(Figure 3.1 B)**. Constitutive S6a binding was also observed at CIITA exons XI and XVIII and no S6a binding was observed at the CIITA polyA site. These data indicate marked differences in S6a binding in CIITA genes, robust binding in CIITA exon IV, and reproduce our previous observation of inducible S6a CIITApIV binding.

S6a knockdown decreases RNA pol II occupancy at the CIITA proximal promoter and at exon IV.

Expression of protein coding genes is carried out by RNA pol II and various transcription factors (Buratowski 1994) and is controlled at multiple levels (Conaway and Conaway 1993; Roeder 1996). In addition to the mechanisms that control recruitment of RNA pol II to promoters, RNA pol II escape from promoter regions and the transition of RNA pol II to an elongating complex is also highly regulated (Saunders, Core et al. 2006; Chiba, Yamamoto et al. 2010).

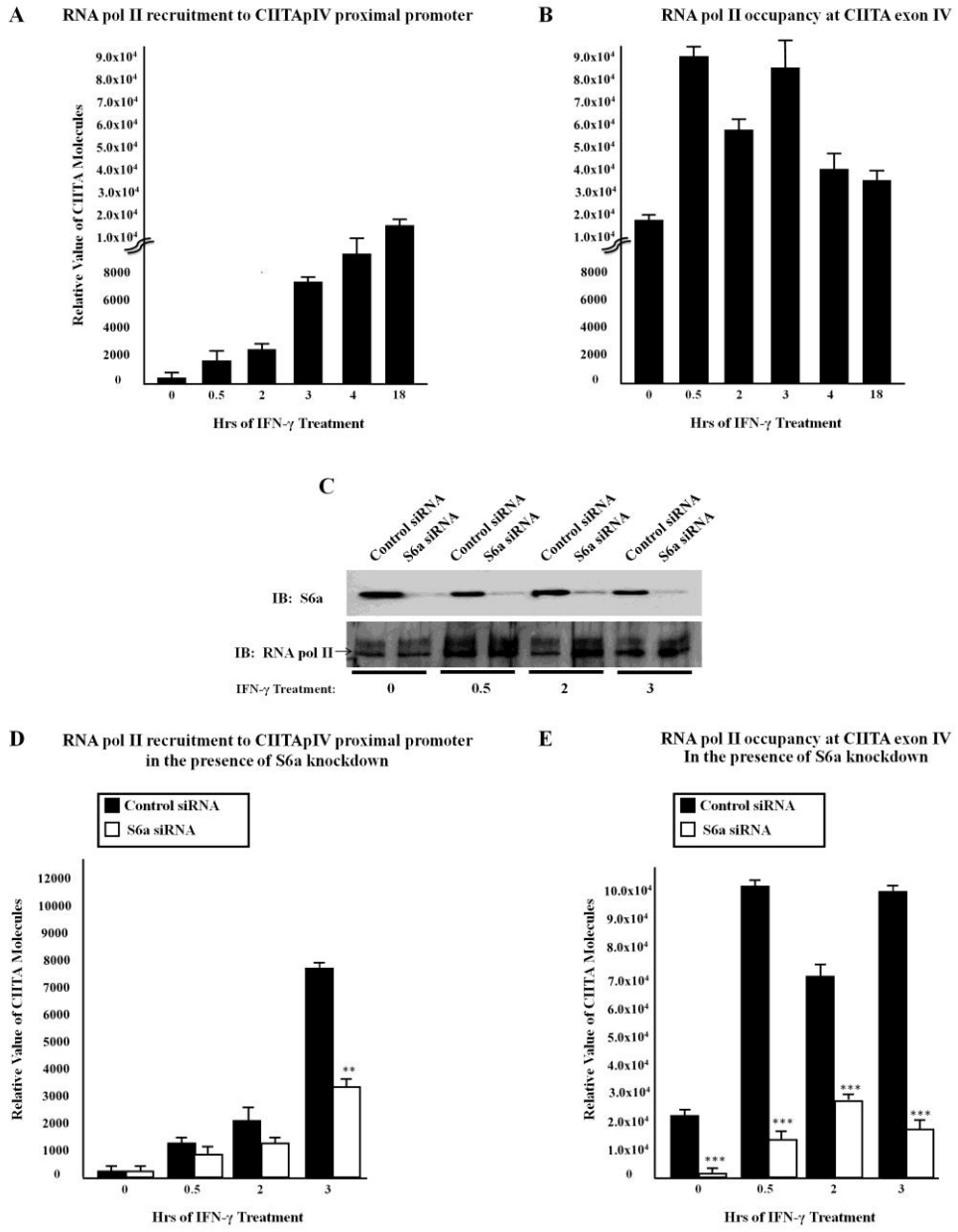


Figure 3.2 S6a knockdown reduces RNA pol II occupancy on CIITA promoters and coding regions.

(A-B) Timeline of RNA pol II binding to the promoter and coding regions of CIITA. ChIP assays were performed in HeLa cells stimulated with IFN-γ as indicated. Lysates were IP with control rabbit antibody or with antibody to endogenous RNA pol II. Associated DNA was isolated and analyzed using via Real Time PCR using primers spanning CIITA proximal promoter (A) and exon IV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as a relative increase in CIITA DNA. Control IP values for (A) and (B) were 2000. Real-

time PCR was performed in triplicate and results represent the mean ± SD of three independent experiments.

(C) S6a siRNA specifically and efficiently decreases S6a protein expression but does not affect endogenous levels of RNA pol II. HeLa cells were transfected with control or S6a specific siRNA, stimulated with IFN-γ as indicated, harvested and subjected to Western Blot analysis of endogenous expression of 19S ATPase S6a (top blot) and RNA pol II (bottom blot). Western Blot analysis shows 90% knockdown of S6a and stable expression of RNA pol II. Results reported are representative data of three independent experiments.

(D-E) S6a siRNA decrease RNA pol II occupancy at CIITA proximal promoter (D) and coding region (E). HeLa cells were transfected with S6a specific (white bars) or with control siRNA (black bars), stimulated as indicated, lysed, and IP with antibody against endogenous RNA pol II or control antibody. Associated DNA was analyzed via Real time PCR using primers and probe specific for the CIITA proximal promoter (D) or CIITA exon IV (F). IP values were normalized against the total DNA added to the reaction and are graphed as relative increase in CIITA DNA. Control IP values for (D) and (E) were 2300± 500. Experiments were performed in triplicate and graphed results are presented as a mean ± SD of three independent experiments. ***$P < 0.001$, **$P < 0.005$, *$P < 0.05$ versus control siRNA.

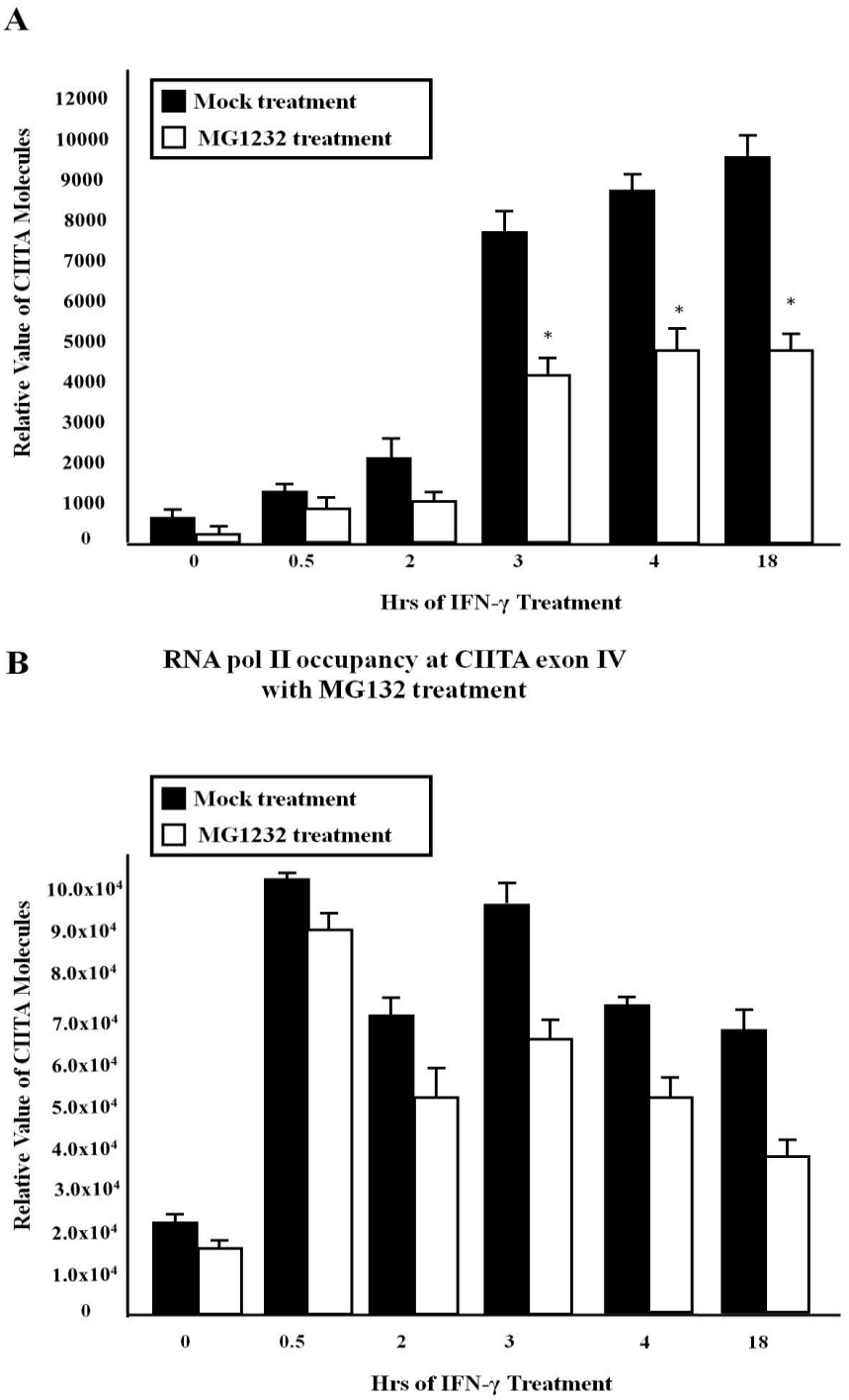


Figure 3.3 Proteasome inhibition and its effect RNA pol II occupancy at either the proximal promoter (A) or exon IV (B) of CIITA.

(A-B) ChIP assays were carried in HeLa cells stimulated with IFN-γ for 0-18 hrs (black bars) and pretreated with proteasome inhibitor MG132 (white bars) for 4 hrs prior harvesting. Lysates were subjected to IP with control antibody or with antibody against endogenous RNA pol II and

associated DNA was isolated and analyzed via Real time PCR using primers and probes spanning the proximal promoter (A) and exon IV (B) of CIITA. IP values are presented as a relative increase in CIITA DNA. Control values for (A) and (B) were 1800±300. Experiments were performed in triplicate and graphed results are presented as a mean ± SD of two independent experiments. *$P < 0.05$ MG132 treated samples versus IFN-γ treated only.

To determine if S6a plays roles in RNA pol II promoter escape from CIITApIV, ChIP assays were performed to assess levels of RNA pol II binding to CIITApIV in the absence of S6a. Initial experiments to map baseline RNA pol II occupancy at CIITApIV and CIITA exon IV showed RNA pol II binding inducibly the CIITApIV proximal promoter **(Figure 3.2 A)** and constitutive, elevated binding in the CIITA coding region **(Figure 3.2 B)** over a time course of IFN-γ stimulation. Having established baseline RNA pol II binding to the CIITApIV promoter and to exon IV, we next investigated roles of the 19S ATPase S6a in RNA pol II recruitment and occupancy. As shown in **(Figure 3.2 C)**, siRNA mediated reduction of endogenous S6a decreased S6a protein expression by 90% relative to control siRNA transfected cells, but did not impact the expression of the endogenous RNA pol II. In cells transfected with S6a siRNA, RNA pol II binding was decreased at the CIITA proximal promoter **(Figure 3.2 D)**. However, the lack of S6a had a more pronounced effect on RNA pol II occupancy in CIITA exon IV where cells treated with S6a specific siRNA **(Figure 3.2 D white bars)** demonstrated significant decreases in RNA pol II occupancy when compared with control siRNA treated samples **(Figure 3.2 D black bars).** Together these studies strongly indicate the 19S ATPase S6a plays roles in both recruitment and processivity of RNA pol II at CIITA genes.

The role of S6a in RNA pol II occupancy is independent of proteasomal degradation.

As S6a an ATPase of the 19S proteasome, S6a plays important roles in the degradation of polyubiquitinated proteins (Ciechanover 1994; Ciechanover 1998). While we have previously demonstrated that S6a siRNA does not block protein degradation, we have shown that S6a siR-

NA moderately decreases 26S mediated degradation (Truax, Koues et al.). To determine the effects on RNA pol II occupancy of complete inhibition of proteasome mediated degradation, we treated IFN-γ stimulated cells with the proteasome inhibitor MG132 and assayed by ChIP for RNA pol II occupancy on CIITA genes. Cells were either mock treated or were treated with the proteasome inhibitor MG132 in the presence or absence of cytokine stimulation and ChIP assays were performed to detect endogenous level of RNA pol II at CIITA proximal promoters **(Figure 3.3 A)** and coding regions **(Figure 3.3 B)**. MG132 treated cells showed slight decreases in the levels of RNA pol II at both the proximal promoter and coding region of CIITA. Therefore, although S6a plays roles in 26S mediated degradation (Truax, Koues et al.), proteasome inhibition does not prevent RNA pol II occupancy at CIITA promoters or coding regions, indicating the effects of S6a siRNA on RNA pol II occupancy are degradation independent.

The 19S ATPase S6a interacts with P-TEF-b components CDK9 and Hexim-1

Studies in yeast demonstrate components of the 19S proteasome associate with actively transcribing genes where they facilitate recruitment of various transcription factors (Ferdous, Gonzalez et al. 2001). As the 19S ATPase S6a binds both CIITA promoters and coding regions and impacts RNA pol II occupancy in these regions, we next sought to determine if S6a interacts with basal transcription factors required for elongation. RNA pol II is recruited to the CIITA proximal promoter and coding regions within hours of cytokine stimulation. It is known that specific phosphorylations of the RNA pol II CTD are required for transcription initiation and elongation (Sims, Belotserkovskaya et al. 2004; Hager, McNally et al. 2009). RNA pol II Ser 5 phos-

phorylation is required for the transition from initiation to elongation whereas Ser 2 phosphorylation is required for elongation (Hager, McNally et al. 2009). Phosphorylation is mediated by kinase TFIIH (CDK7), the mediator complex, and the kinase P-TEF-b (CDK9). P-TEF-b exists in two forms, active and inactive, where the inactive form of P-TEF-b is bound to Hexim1 and 7SK. We therefore investigated the interaction of the 19S ATPase S6a with components of P-TEF-b (CDK9) by transfecting HeLa cells with Myc S6a and either Flag-Hexim-1 or HA-CDK9. As shown in Figure 4, S6a co-immunoprecipitated with Hexim-1 **(Figure 3.4, lane 3 and 5)** and with CDK9 **(Figure 3.4, lane 4 and 6)**. Positive control was immunoprecipitated with Myc conjugated beads **(Figure 3. 4, lane 1)** and negative control with mouse IgG antibody **(Figure 3. 4, lane 2).**

Efficient transfections and equal loading of cell lysates was confirmed by immunoblot analysis of Myc, HA, and Flag tagged proteins. The association of S6a with Hexim-1 and CDK9 increases upon IFN-γ stimulation indicating that S6a may be important in the switch from the inactive to active form of P-TEF-b. **(Figure 3.6 Supplementary Figure 1)** demonstrates the 19S ATPases Sug1 and S7 also associate with Hexim-1 and CDK9, suggesting all 19S ATPases may promote the switch from inactive to active form of P-TEF-b.

A

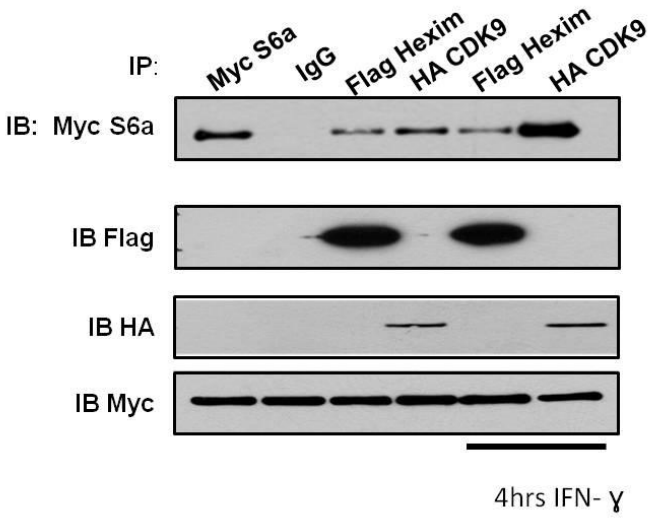


Figure 3.4 The 19S ATPase S6a associates with Hexim-1 and CDK9.

HeLa cells were transfected with Myc-S6a, Flag Hexim-1, and HA-CDK9, stimulated with IFN-γ for 4hrs as indicated, and were lysed and subjected to IP with Myc beads (lane 1) as a positive control, with mouse isotype control IgG (lane 2), with Flag Beads (lane 3 and 5), and with HA beads (lane 4 and 6). IP and lysate control samples (bottom) were subjected to IB for Myc, HA, and Flag as indicated. Results reported are representative data of three independent experiments.

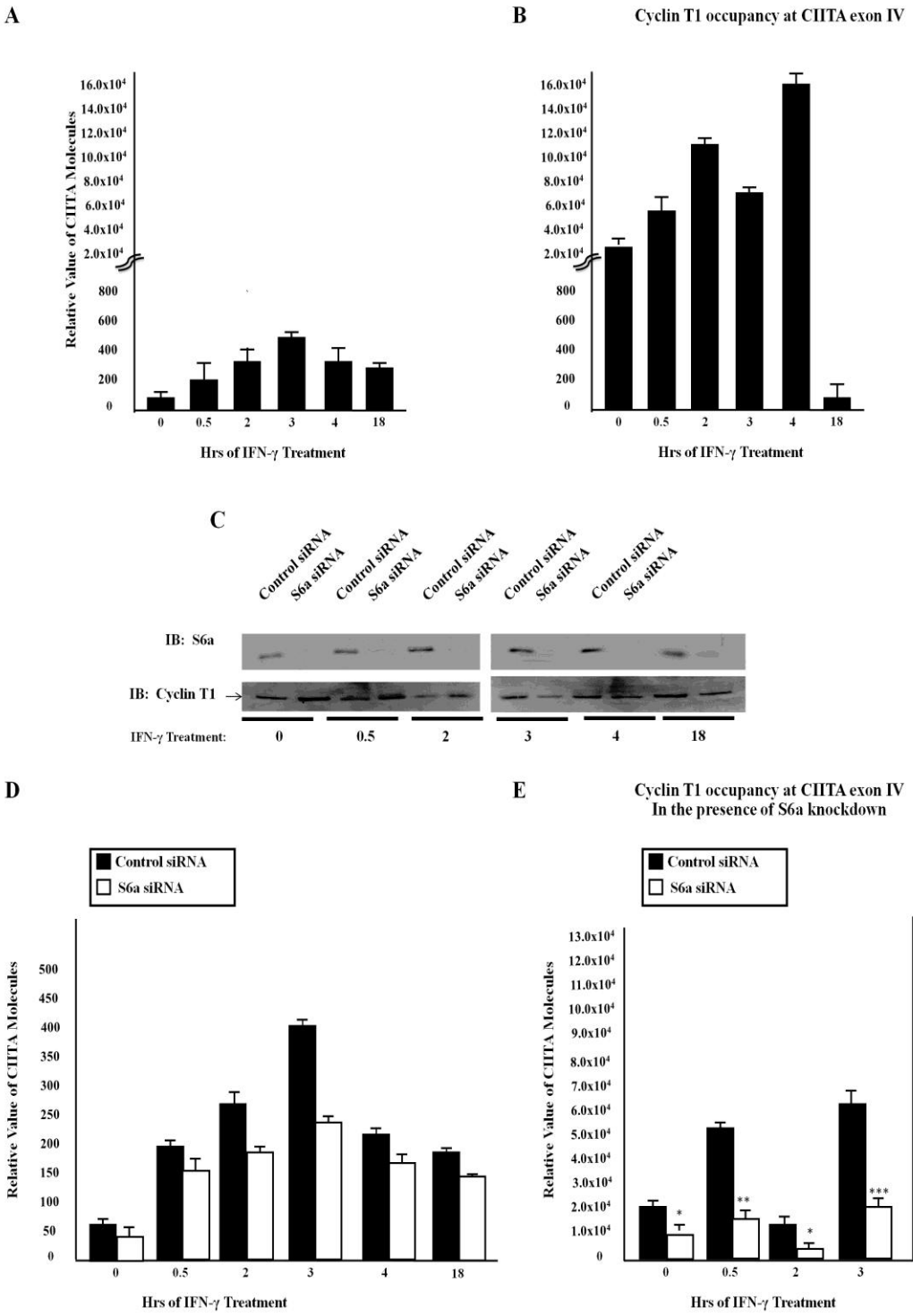

A
B
Cyclin T1 occupancy at CIITA exon IV
Relative Value of CIITA Molecules
16.0x10⁴
14.0x10⁴
12.0x10⁴
10.0x10⁴
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
800
600
400
200
0
0
0.5
2
3
4
18
Hrs of IFN-γ Treatment
C
Control siRNA
S6a siRNA
IB: S6a
IB: Cyclin T1
IFN-γ Treatment:
D
E
Cyclin T1 occupancy at CIITA exon IV
In the presence of S6a knockdown
Control siRNA
S6a siRNA
13.0x10⁴
12.0x10⁴
11.0x10⁴
10.0x10⁴
9.0x10⁴
8.0x10⁴
7.0x10⁴
6.0x10⁴
5.0x10⁴
4.0x10⁴
3.0x10⁴
2.0x10⁴
1.0x10⁴
500
450
400
350
300
250
200
150
100
50
*
**
***

Figure 3.5 Decreased expression of S6a decreases occupancy of cyclin T1 at CIITA proximal promoter and coding region

(A-B) Cyclin T1 inducibly binds the CIITA proximal promoter (A) and coding region (B). ChIP assays were carried out in HeLa cells as described in Fig 2 (A-B). Lysates were stimulated with IFN-γ for 0-18 hrs and subjected to IP against endogenous cyclin T1. Associated DNA was isolated and analyzed via Real Time PCR with primers spanning CIITA proximal promoter (A) and coding region exon IV (B). Control IP values for (A) and (B) were 1350±500. Control and IP values represent the mean ±SD of two independent experiments.

(C) S6a siRNA treatment significantly and specifically decreases S6a expression. HeLa cells were transfected with either control or S6a specific siRNA duplexes and were stimulated with IFN-γ as indicated. Lysates were subjected to IB for endogenous S6a (top blot) or endogenous cyclin T1 (bottom blot). Results reported are data representative of 2 independent experiments. Western blot shows specific 90-95% knockdown of S6a and stable expression of cyclin T1.

(D-E) S6a knockdown significantly decrease cyclin T1 occupancy at the CIITA proximal promoter (D) and CIITA exon IV coding region (E). ChIP analysis were carried out in HeLa cells that were stimulated as indicated with IFN-γ and transfected with control siRNA (black bars) or S6a specific siRNA (white bars). Lysates were subjected to IP with antibody against endogenous cyclin T1. Associated DNA was analyzed via Real Time PCR with primers specific for the CIITA proximal promoter (D) and CIITA coding region (E). IP values are presented as a relative increase in the CIITA DNA. Control IP values for (D) and (E) were 900±500. Control and cyclin T1 IP values represent the mean ±SD of 2 independent experiments. ***$P < 0.001$, **$P < 0.005$, *$P < 0.05$ versus control siRNA.

S6a knockdown decreases Cyclin T1 occupancy at the CIITA proximal promoter and coding region.

Studies have shown that P-TEF-b is recruited to an early elongation complex on the HIV long term repeats (LTR) and forms a complex with Tat *trans*-activator protein (Kanazawa, Okamoto et al. 2000) (Ping and Rana 1999) (Zhou, Chen et al. 1998). CIITA also recruits P-TEF-b containing cyclin T1 to the promoter of MHC II (Kohoutek, Blazek et al. 2006). P-TEF-b is also present at the transcribed regions of the HSP70 genes during heat shock activation (Lis, Mason et al. 2000) . Upon stimulation, P-TEF-b dissociates from the large inactive complex and forms a small complex that is recruited to transcriptional machinery (Nguyen, Kiss et al. 2001; Yang, Zhu et al. 2001). ChIP analyses were performed to assess cyclin T1 occupancy at the CIITA proximal promoter **(Figure 3.5 A)** and coding region **(Figure 3.5 B)**. Initial ChIP mapping experiments demonstrated cyclin T1 is inducibly recruited to both regions of CIITA. Levels of cyclin T1 at CIITA exon IV are significantly higher than at the proximal promoter, indicating cyclin T1 is an important elongation regulator at CIITA genes.

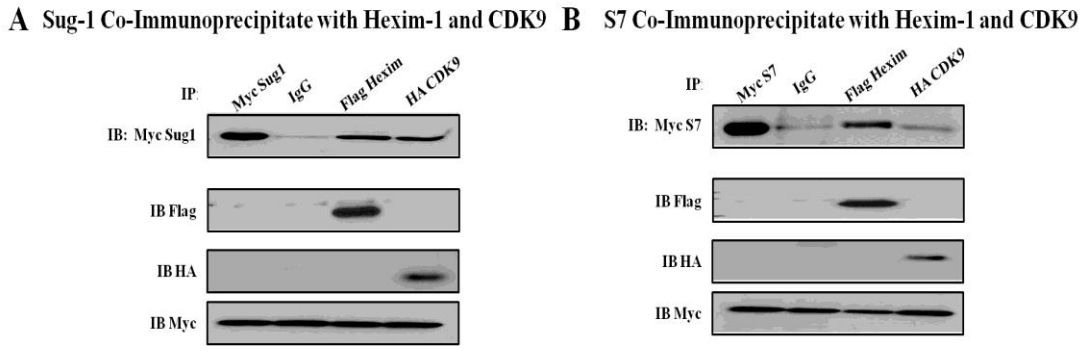


Figure 3.6 (Supplementary Figure 1) The 19S ATPase Sug1 (A) and S7 (B) associates with Hexim-1 and CDK9.

(A-B) HeLa cells were transfected with Myc-Sug1, Myc-S7 Flag Hexim-1, and HA-CDK9 as indicated, and were lysed and subjected to IP with Myc beads (lane 1) as a positive control, with mouse isotype control IgG (lane 2), with Flag Beads (lane 3), and with HA beads (lane 4). IP and lysate control samples (bottom) were subjected to IB for Myc, HA, and Flag as indicated. Results reported are representative data of three independent experiments.

To investigate roles for S6a in regulating occupancy of cyclin T1 at CIITA proximal promoters and coding regions, we utilized S6a siRNA specific duplexes to specifically knock-down endogenous expression of S6a in HeLa cells and then performed ChIP experiments to detect levels of cyclin T1 at the CIITA proximal promoter **(Figure 3.5 D)** and coding regions **(Figure 3. 5 E).** Although S6a was sufficiently reduced via S6a specific siRNA, level of endogenous cyclin T1 were unaffected **(Figure 3.5 C)**. S6a siRNA significantly impacted the recruitment of cyclin T1 to the CIITA proximal promoter and coding region, indicating S6a plays important roles in recruiting cyclin T1 to CIITA genes.

3.4 DISCUSSION

Critical roles in transcription initiation of various mammalian genes have been demonstrated for ATPases of the 19S proteasome (Truax, Koues et al. ; Kinyamu, Chen et al. 2005; Lassot, Latreille et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). We have previously demonstrated that upon cytokine stimulation, the 19S S6a is rapidly recruited to CIITA proximal promoters (Truax, Koues et al.). Promoter binding of S6a mediates initiation of transcription by stabilizing promoter binding of activating histone modifying enzymes and transcription factors. S6a, and other 19S ATPases, act as a critical regulator of the assembly of multi-subunit complexes on promoters and thus regulate in large part the mechanics of transcription initiation.

Evidence also exists that 19S ATPases play important roles in RNA pol II dependent elongation independent of the presence or function of the 20S core (Ferdous, Gonzalez et al. 2001). The 19S ATPase Rpt 6 interacts with the yeast elongation factor CDc8 (human FACT) and a loss of Rpt 6 blocks elongation in yeast cells (Xu, Singer et al. 1995; Ferdous, Gonzalez et al. 2001). Recent studies have documented the existence of a holo RNA pol II complex composed of a core RNA pol II and a mediator complex which functions to enable transcriptional activity of the core RNA pol II (Kim, Bjorklund et al. 1994). In these studies, the 19S ATPase Sug1 was identified as a subunit of mediator, indicting a mechanism by which Sug1 may regulate transcription initiation and elongation. We now provide evidence of novel roles for the 19S ATPase S6a in mediating transcriptional elongation of the critical mammalian gene CIITA. In the absence of S6a, RNA pol II binding to the CIITA promoter is decreased and RNA pol II binding to CIITA coding sequence is markedly reduced.

As DNA is wrapped around histone proteins that are then condensed into chromatin (Luger, Mader et al. 1997; Richmond and Davey 2003), there must be additional regulation of the availability of DNA for transcription, however the exact mechanisms by how 19S ATPases regulate the accessibility of DNA remains to be determined. The role of S6a in binding RNA pol II to the coding region of CIITA genes is much more pronounced than at proximal promoter regions. Perhaps 19S ATPases, which act as “unwindases” during proteasomal degradation, play similar roles in regulating the accessibility of coding region DNA. Evidence for roles of S6a in transcriptional elongation also comes from our observations that S6a associates with P-TEF-b components Hexim-1 and CDK9. Upon cytokine stimulation, P-TEF-b is dissociated from the inactive complex that was bound by Hexim-1 and 7SK and is recruited to actively transcribing

genes (Nguyen, Kiss et al. 2001; Yang, Zhu et al. 2001; Michels, Fraldi et al. 2004). 19S ATPases associate with components of the inactive (Hexim-1) and active (CDK9) form of P-TEF-b, indicating 19S ATPases may facilitate the switch from an inactive to active form of P-TEF-b. Of additional interest are our observations that S6a regulates CIITA promoter and coding region binding of the P-TEF-b component cyclin T1. However, the effects of S6a knockdown on cyclin T1 occupancy are more pronounced at CIITA coding regions than proximal promoters. Here 19S ATPases may play dual roles increasing DNA accessibility for elongation factors and in facilitating the switch from inactive to active P-TEF-b. The reduced levels of cyclin T1 in the absence of S6a are likely due to decreased release of Cyclin T1 from inactive P-TEF-b. Additional experiments will confirm and expand these findings. While S6a is an important regulator of degradation, the roles played by S6a in elongation are independent of the proteolytic activity of the proteasome, as proteasomal inhibition has minimal impact on RNA pol II promoter and coding region binding at CIITA genes. Studies in yeast using genome wide ChIP analyses indicate that while subunits of the 26S proteasome interact with a majority of genes, hundreds of genes associate with either the 19S or 20s components of the proteasome, but not with both, thus proteasome subunits have specific and independent function at different promoters (Sikder, Johnston et al. 2006).

Our studies are the first to show the 19S ATPase S6a binds robustly within coding regions of mammalian genes. S6a association is the strongest in CIITA exon IV while and is undetectable at polyA regions of CIITA genes. We are the first one to demonstrate that RNA pol II occupancy at the proximal promoter and coding regions of CIITA is dependent on S6a. P-TEF-b is a critical regulator of mammalian elongation. Our observation of S6a association with Hexim-

1 1 and CDK9 suggests the actions of S6a are important in the activation of P-TEF-b. Dissociation of Hexim-1 and 7SK promotes formation of the active form of P-TEF-b which is in turn recruited to actively transcribing genes (Nguyen, Kiss et al. 2001; Yang, Zhu et al. 2001; Michels, Fraldi et al. 2004). ChIP analyses demonstrated significantly reduced occupancy of the cyclin T1 component of P-TEF-b at CIITA coding regions in the absence of S6a.

In sum, we are the first to demonstrate 19S ATPases regulate transcriptional elongation of a mammalian gene. The mechanisms supporting this regulation remain unknown and the possibility remains that S6a regulates transcriptional elongation by controlling epigenetic modifications throughout coding regions. Our findings that 19S ATPases play critical roles in the regulation of transcriptional elongation advance our knowledge of the functions of the proteasome in gene expression. Full understanding of the role of the 19S proteasome in regulating elongation of mammalian genes will require additional studies of the molecular mechanisms regulated by the ATPases of the 19S proteasome.

3.5 MATERIAL AND METHODS

Cells

HeLa (human epithelial) cells were purchased from ATCC (Manassas, VA) and were maintained in high glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Hern-

don, VA) supplemented with 10% fetal bovine serum (FBS), 5mM L-glutamine, and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies

Antibodies recognizing RNA pol II and cyclin T1 were purchased from Abcam (Cambridge, MA). Mouse and rabbit immunoglobulin G (IgG) isotype control antibodies were purchased from Millipore (Lake Placid, NY). S6a antibody was purchased from Biomol International, L.P. (Plymouth Meeting, PA). HRP conjugated mouse antibody was purchased from Promega (Madison, WI); and HRP conjugated rabbit antibody was purchased from Pierce (Rockland, IL).

Plasmids:

Flag-Hexim-1 was kindly provided by Dr. David Price (Li, Price et al. 2005) and HA-CDK9 was a generous gift from Dr. Lou (Zhou, Chen et al. 1998). The pBluescript (pBS)-S6a and S7 plasmids were kindly provided by Dr. Martin Rochesteiner (Richmond, Gorbea et al. 1997; Rabl, Smith et al. 2008) and subcloned into Myc tagged pCMV-3 using EcoRI restriction sites. The Myc Sug-1 was kindly provided by Dr. A.A.Wani (Zhu and Ting 2001) and has been previously described (Koues, Dudley et al. 2008).

Co-Immunoprecipitations:

HeLa cells were plated at a cell density of $7x10^5$. Following adhesion, cells were transfected as indicated with 5 μg of Myc-S6a, Myc-S7, Myc Sug-1, pcDNA, Flag Hexim-1 and HA-CDK9 plasmids using Fugene 6 (Roche, Indianapolis, IN) according to manufacturer's instructions. Twenty four hours post transfections cells were harvested and lysed in RIPA lysis buffer (1M Tris pH 8.0, 10% Nonidet P-40, 5% DOC, 10% SDS, 5M NaCl, 5mM EDTA, 1M DTT) supplemented with Complete EDTA-free protease inhibitors (Roche) buffer and were precleared with 30μl IgG beads (Sigma). Cell lysates were immunoprecipitated (IP) overnight with 30ul of Myc conjugated beads (Sigma), Flag conjugated beads (Sigma), HA conjugated beads (Sigma), or mouse IgG beads (Sigma). Immune complexes were denatured with Leammli buffer, boiled and subjected to SDS-PAGE. Co-immunoprecipitated complexes were detected by immunoblotting (IB) with anti Myc horseradish peroxidase (HRP) conjugated antibody (Sigma), Flag HRP antibody (Sigma), and HA antibody (Santa Cruz). HRP conjugates were detected with Supersignal West Pico Chemiluminescent substrate (Denville). Bradford Assays were used to normalize the total protein in the sample. Equal transfection and loading was determined in non-immunoprecipitated lysates by immunoblot.

siRNA Constructs and Transient Transfections

Short interfering RNA (siRNA) duplexes predesigned against human S6a (Qiagen) were used for transient knockdown of S6a as previously described (Truax, Koues et al.). Cells were

transfected with 1ug of S6a specific siRNA,or All Star scrambled sequence control (Qiagen), using RNAiFect transfection reagent (Qiagen) according to the manufacturer's instructions and were treated with IFN-γ (500U/ml) as indicated. Cells were lysed in NP-40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP-40, 0.5M EDTA, 5M NaCl, 1M DTT, dH_2O) with EDTA free protease inhibitor (Roche) and were analyzed by western blot for knockdown efficiency and specificity.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Mehta, Truax et al. 2011). Briefly, cells were plated at a cell density of $3x10^6$, stimulated with IFN-γ as indicated (500U/ml), and were crosslinked with 1% formaldehyde for 10 minutes at room temperature. Crosslinking was stopped by the addition of 0.125M glycine for five minutes at room temperature. Cells were lysed in SDS lysis buffer and were sonicated at constant pulse to generate an average of 500-750bp sheared DNA. Sonicated lysates were precleared with salmon-sperm coated agarose beads (Millipore) and half of the lysate was immunoprecipitated with 5µg of indicated antibody overnight at 4°C. The remaining half of the lysate was immunoprecipitated with mouse or rabbit isotype control antibody (Millipore). Following a two hour immunoprecipitation with 60µl of salmon-sperm coated agarose beads, samples were washed for 3 minutes at 4°C with each of the following buffers: low salt buffer, high salt buffer, LiCl, and 1xTE and were eluted with SDS elution buffer. Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufactur-

er's instructions. Isolated DNA was analyzed by real-time PCR using previously published primers spanning the GAS-IRF-E box of CIITApIV and exon IV of CIITA (Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). Primers for the exon regions of CIITA coding region were as follows: CIITA exon XI sense GGGAAAGCTTGTGCAGACTC, antisense GGACAGATTGAGGGTTTCCA, and probe FAM- CTGCTGTTCGGGACCTAAAG-TAMRA; CIITA exon XVIII sense AACAGGATTCACGGATCAGC, antisense GCAGAGCAA-GATGTGGTTCA, and probe FAM- GGACACAGCTCTTCTCCAGG-TAMRA; and CIITA polyA sense AGCCTGCTAATTCCCCAGAT, antisense TCCCATTAGCTGTTTCCCTG, and probe FAM- AGTGGCAGCTGCTTTTGTCT-TAMRA. Values generated from Real-time PCR reactions were calculated based on standard curves generated.

Chromatin Immunoprecipitation with siRNA

ChIP with siRNA were performed as previously described (Truax, Koues et al. ; Koues, Dudley et al. 2008). Briefly, cells were plated at a density of $8x10^5$, transfected with 1µg S6a specific or control siRNA (Qiagen), and were stimulated as indicated with IFN-γ (500U/ml). 10% of the cell volume was lysed with 1% Nonidet P-40 lysis buffer and was analyzed by western blot for knockdown efficiency and specificity. The remaining cell volume was crosslinked, lysed in SDS lysis buffer, and subjected to sonication and the above described ChIP assay.

3.6 ACKNOWLEDGEMENTS:

Funding

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Acknowledgements

We thank Dr. David Price (Biochemistry Department, University of Iowa, Iowa City, Iowa) and Dr. Kunxin Luo (Department of Molecular and Cell Biology, University of California at Berkeley) for generously providing the Flag-Hexim 1 and HA CDK9 plasmids. We also appreciate Dr. Dr. Martin Rechsteiner (Department of Biochemistry, University of Utah, School of Medicine, Salt Lake City, UT) for sharing19S ATPase encoding plasmids.

4 CHAPTER IV: EARLY EPIGENETIC EVENTS REGULATE THE ADAPTIVE IMMUNE RESPONSE GENE, CIITA

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ABSTRACT

Precise regulation of Major Histocompatibility class II (MHC II) genes plays important roles in initiation, propagation, and termination of adaptive immune responses by controlling antigen presentation to $CD4^+$ T cells. MHC II genes are constitutively expressed in only a few cell types and are inducibly expressed by the inflammatory response cytokine interferon gamma (INF-γ) in all nucleated cells. The regulation of MHC II is tightly controlled by a Master Regulator, the class II transactivator (CIITA), which is a general regulator of both constitutive and inducible MHC II expression. Although much is known about the transcription factors necessary for CIITA expression, less is known about the epigenetic modifications and the requisite enzymes needed to provide these transcription factors access to DNA. We show here that multiple epigenetic changes occur at the IFN-γ inducible CIITA promoter within 20' of IFN-γ stimulation and that these changes correlate with the opening of the promoter and the initiation of transcription. Our study links these rapidly occurring epigenetic events at the inducible CIITA promoter to decreased promoter binding of the histone methyltransferase EZH2, and shows that decreased promoter binding of EZH2 transforms this previously tightly regulated and cytokine inducible promoter into a constitutively active and dysregulated gene.

4.1 BACKGROUND

MHC is an important gene family, whose protein product is expressed on the cell surface of all jawed vertebrates.(Gelin, Sloma et al. 2009) MHC II is constitutively expressed on antigen presenting cells, and is inducibly expressed on nucleated cells in response to pro-inflammatory cytokines.(Kaufman, Auffray et al. 1984; Chang, Fontes et al. 1994) Precise regulation of MHC II genes plays important roles in the control of adaptive immune responses, and in limiting infection, autoimmune disease, and tumor growth.(Adamski, Ma et al. 2004; Handunnetthi, Ramagopalan et al. 2010) The regulation of MHC II is controlled by a Master Regulator, the class II transactivator (CIITA), with multiple studies demonstrating that CIITA regulated expression of MHC II is controlled and induced by IFN-γ.(Murphy, Choi et al. 2004) As a functional CIITA gene is necessary for the expression of MHC II genes, CIITA is widely considered to be a required master regulator of both constitutive and inducible MHC II expression.(Collins, Korman et al. 1984; Boss and Jensen 2003; Reith, LeibundGut-Landmann et al. 2005).

Four distinct promoters regulate expression of CIITA in a highly regulated and cell type specific manner.(Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Linhoff et al. 1999) Promoter I drives CIITA expression in dendritic cells (Landmann, Muhlethaler-Mottet et al. 2001), CIITA promoter III is responsible for CIITA expression in B cells, (Lennon, Ottone et al. 1997) and promoter IV is responsible for the inducible expression of CIITA and is regulated by IFN-γ. (Piskurich, Gilbert et al. 2006) To date, the function of CIITA promoter II remains unknown. CIITApIV is composed of three *cis*-acting elements: an IFN-γ activated sequence (GAS), an E-box, and an IFN response element (IRE) site. (Piskurich, Linhoff et al. 1999) IFN-γ stimulation

leads to the activation of the JAK/STAT pathway by triggering the phosphorylation of signal transducer and activator of transcription 1 (STAT-1) homo-dimers, which then translocate to the cell nucleus and regulate the expression of multiple genes, including that of interferon regulatory factor 9 (IRF-1) (Reith and Mach 2001). Upon IFN-γ stimulation, STAT-1 and IRF-1 are recruited, along with the ubiquitous factor 1 (USF-1), to CIITApIV. STAT-1 binds to the GAS element, USF-1 localizes to the E-box, and IRF-1 is recruited to the IRE element. (Piskurich, France et al. 1993; Piskurich, Youngman et al. 1997; Piskurich, Linhoff et al. 1999; Piskurich, Gilbert et al. 2006) Following IFN-γ stimulation, CIITApIV histone acetylation is increased and the ATPase subunit of the SWI/SNF complex, Brahma-related gene 1 (BRG1), is necessary for chromatin remodeling. (Beresford and Boss 2001; Pattenden, Klose et al. 2002) Binding of these regulatory elements to CIITApIV leads to the appearance of CIITA transcripts, which have previously been observed two hours post IFN-γ stimulation.

Despite the requirement for CIITA expression in MHC II transcription, strikingly few studies have investigated the remodeling events occurring at CIITApIV and the early regulatory epigenetic modifications to CIITApIV. Increases in the levels of acetylation of histones H3 and H4 (Agalioti, Chen et al. 2002; Morris, Beresford et al. 2002) and tri-methylation of histone H3K4 has been observed upon prolonged cytokine stimulation.(Ni, Karaskov et al. 2005) Previous studies have shown that two histone modifying enzymes are recruited to CIITApIV: the HAT CBP/p300 and the ATP dependent chromatin remodeler BRG-1 which binds to the proximal promoter prior to STAT-1 recruitment. (Sisk, Gourley et al. 2000; Pattenden, Klose et al. 2002; Ni, Karaskov et al. 2005) Histone modification studies carried out at CIITApIV traditionally assay two distinct conditions: unstimulated cells and then a significantly later time period

post IFN-γ stimulation. In the case of CIITApIV expression, these later time points are less relevant as dynamic and regulatory epigenetic changes occur quickly following IFN-γ stimulation.(Morris, Spangler et al. 2000) Our previous observations of rapid induction of CIITA message levels from CIITAPIV demonstrate that this CIITA promoter is likely in an open conformation within two hours of IFN-γ stimulation (Truax, Koues et al. 2010) thus, an important area of study are the epigenetic events occurring before these early CIITA transcripts appear. The study of the epigenetic regulation of the Master Regulator CIITA is clinically relevant, as aberrant expression of CIITA leads to inappropriate expression of MHC II proteins. (Guy, Krajewski et al. 1986; Santin, Hermonat et al. 1998; Ting and Trowsdale 2002) Dysregulated expression of MHC II is associated with various disorders and diseases; in sum, over expression of MHC II is correlated with autoimmune diseases, while suppression of MHC II expression leads to tumor development and immune deficiency syndromes. (Guy, Krajewski et al. 1986; Santin, Hermonat et al. 1998).

4.2 RESULTS

Rapid epigenetic changes occur at CIITApIV following IFN-γ stimulation.

Acetylation of histone tails neutralizes the charge on histones and counters the natural tendency of DNA and histones to tightly interact. Histone acetylation occurs on lysine residues and is often targeted to lysine (K) residue 18 of histone H3. (Jenuwein and Allis 2001; Roth, Denu et al. 2001; Gorisch, Wachsmuth et al. 2005) Prior epigenetic studies of the IFN-γ inducible CIITA promoter, pIV, have shown weak to moderate acetylation of histones H3 and H4 in the absence of cytokine induction; (Morris, Beresford et al. 2002; Wright and Ting 2006) which allows binding of ubiquitously expressed USF-1 to CIITApIV. Following IFN-γ stimulation, CIITApIV is available for further transcription factor binding of STAT-1 and IRF-1, which in turn recruit the basal transcriptional machinery and, together with USF-1, drive robust transcription of CIITA. (Reith, LeibundGut-Landmann et al. 2005)

As prompt CIITA expression is required in the initial stages of an adaptive immune response, we sought to determine how quickly CIITApIV undergoes epigenetic remodeling in the presence of inflammatory cytokine. To evaluate levels of H3K18 acetylation at various time points following IFN-γ stimulation, HeLa cells were stimulated with IFN-γ, crosslinked, lysed, immunoprecipitated with antibodies specific to H3K18 acetylation, and analyzed by real-time PCR with primers and probe spanning the CIITApIV proximal promoter. ChIP assays showed elevated levels of H3K18 upon IFN-γ stimulation that were significantly increased within 20 mi-

nutes and that remained significantly elevated through 120 minutes of cytokine stimulation **(Figure 4.1 A, left panel)**. Of note, although H3K18 acetylation at CIITApIV is immediately increased by inflammatory cytokine, maximal levels of acetylation are not reached until 18h following IFN-γ stimulation **(Figure 4.1 A, right panel)**. To determine if histone acetylation is influenced by IFN-γ at all genes, ChIP assays were performed to determine the levels of acetylated H3K18 at the GAPDH promoter. **Figure 4.1 B** demonstrates that cytokine stimulation had no significant impact on the levels of acetylated H3K18 at the GAPDH promoter as the levels of acetylation remains constant throughout the IFN-γ time course.

We and others have previously reported that increases in acetylation of histone H3 frequently overlap spatially and temporally with increases in an additional activating epigenetic modification at histone H3: lysine 4 trimethylation (H3K4me3). (Wu, Wang et al. 2008; Koues, Dudley et al. 2009) In order to more precisely determine the timeline of IFN-γ induced changes that occur in regards to H3K4me3 at CIITApIV, chromatin immunoprecipitation experiments were performed to determine levels of H3K4me3 at CIITApIV at time points immediately following IFN-γ stimulation. As shown in **Figure 4.1C**, ChIP mapping experiments demonstrated that, similar to rapid changes in H3K18 acetylation, significant increases in H3K4me3 occur within 20 minutes of IFN-γ stimulation and continue to rise throughout the 120 minute time course. Additional ChIP experiments at the GAPDH promoter demonstrate the observed changes in Figure 1c are promoter specific as levels of H3K4me3 are not significantly altered by IFN-γ stimulation **(Figure 4.1 D)**.

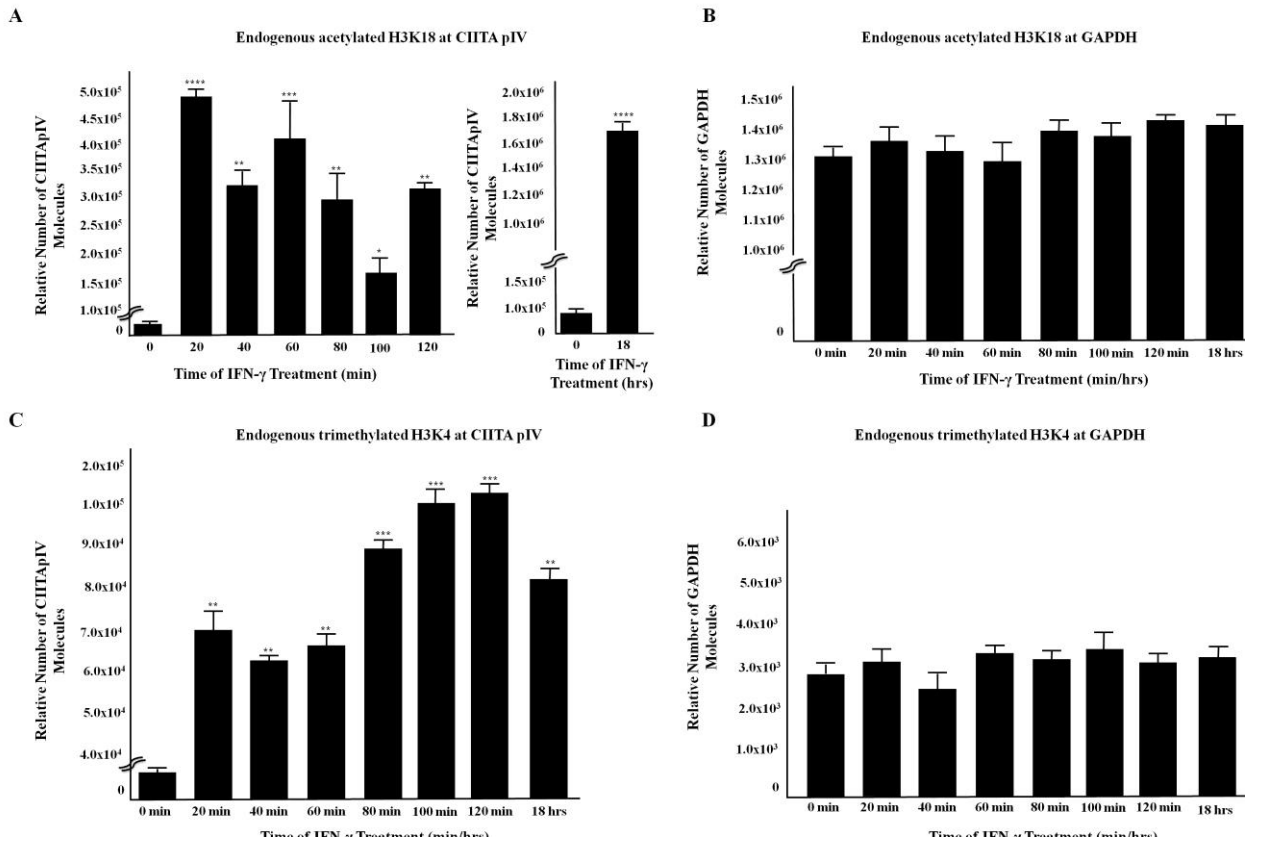

Figure 4.1 Rapid increases in histone 3 lysine 18 (H3K18ac) acetylation and in histone 3 lysine 4 (H3K4me3) are observed at CIITA promoter IV following IFN-γ stimulation.

(A and C) HeLa cells were stimulated with IFN-γ as indicated, crosslinked with formaldehyde, lysed and sonicated. Lysates were immunoprecipitated (IP) with control antibody or with antibody to H3K18ac (A) or to H3K4me3 (C). Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITAPIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input; 5% of the total cell lysate). IP values are presented as a relative increase in CIITApIV DNA relative to unstimulated samples. Control IP values for (A and C) were 350± 100. Values for control IPs, acetylated histone H3K18 IPs and for trimethylated H3K4 IPs represent mean ± SEM of three independent experiments. ****$P < 0.00005$, ***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$ versus unstimulated sample.

(B and D) H3K18 acetylation and H3K4me3 levels at the GAPDH promoter are not affected by cytokine stimulation. ChIPs were carried out as above in Hela cells stimulated with IFN-γ. Lysates were subjected to IP with control antibody or with antibody to endogenous H3K18ac (B) or H3K4me3 (D). Associated DNA was isolated and analyzed via real time PCR using primers spanning the GAPDH proximal promoter. IP values are presented as a relative increase in GAPDH DNA relative to unstimulated samples. Control IP values for (B and D) were 250± 50. Values for control IPs, acetylated histone H3K18 IPs, and for trimethylated H3K4 IPs represent mean ± SEM of three independent experiments.

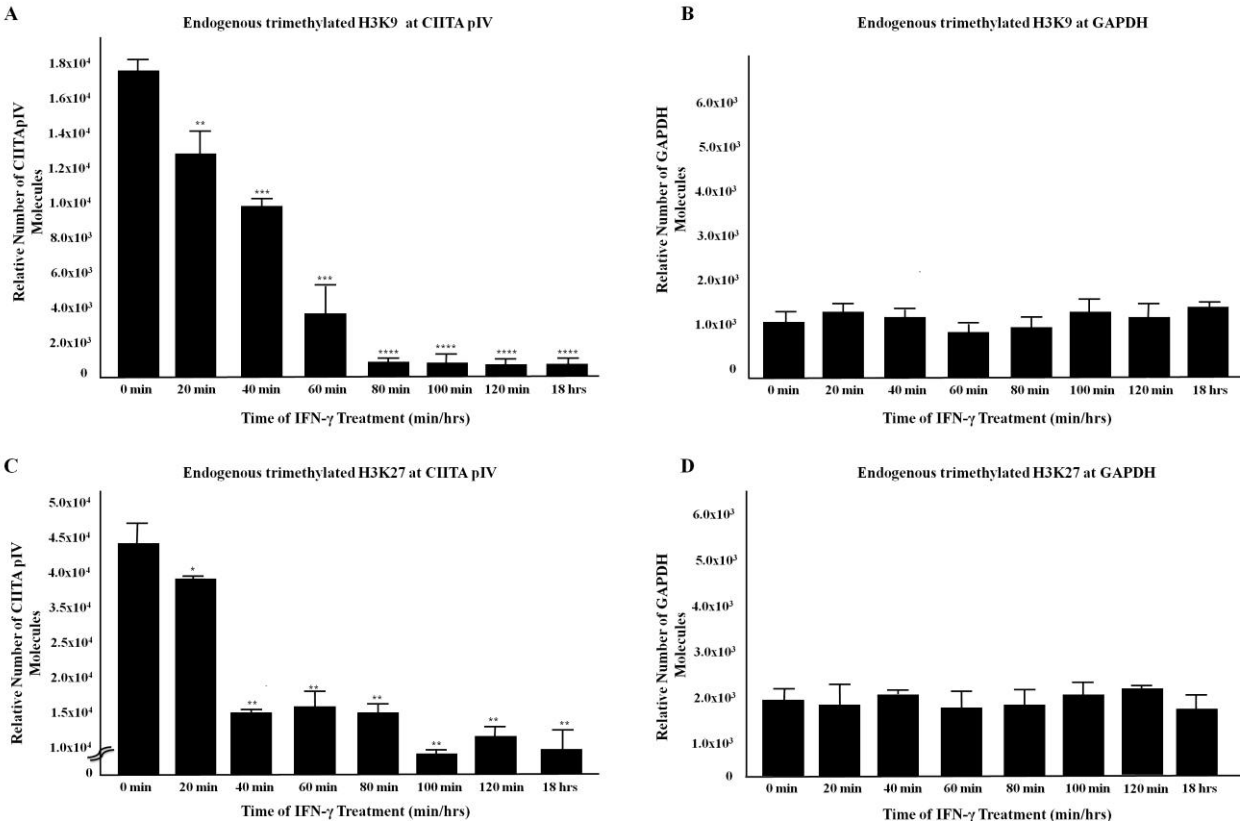

Figure 4.2 Rapid decreases in histone 3 lysine 9 (H3K9me3) and histone 3 lysine 27 (H3K27me3) trimethylation occur at CIITA promoter IV upon IFN-γ stimulation.

(A and C) HeLa cells were stimulated with IFN-γ, crosslinked with formaldehyde, and cells were lysed and sonicated. Lysates were immunoprecipitated (IP) with control antibody or with antibody to H3K9me3 (A) or to H3K27me3 (C). Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITAPIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input; 5% of the total cell lysate). IP values are presented as a relative increase in CIITApIV DNA relative to unstimulated samples. Average control IP values for (A) and (C) were 400± 80. Values for control IPs, for trimethylated H3K9 IPs, and for trimethylated H3K27 IPs represent mean ± SEM of three independent experiments. ****$P < 0.00005$, ***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$ versus unstimulated sample.

(B and D) H3K9me3 and H3K27me3 levels at the GAPDH promoter are not affected by IFN-γ stimulation. ChIPs were carried out as above in Hela cells stimulated with IFN-γ. Lysates were subjected to IP with control antibody or with antibody to endogenous H3K9me3 (B) or H3K27me3 (D). Associated DNA was isolated and analyzed via real time PCR using primers spanning the GAPDH proximal promoter. IP values are presented as a relative increase in GAPDH DNA relative to unstimulated samples. Control IP values for (B and D) were 200± 50. Results reported are represented by mean ± SEM of three independent experiments.

Converse to gains in lysine 4 hypermethylation, losses in histone 3 lysine 9 hypermethylation (H3K9me3) are associated with open chromatin structure and these losses are thus an indication of active transcription.(Agalioti, Chen et al. 2002) We next sought to determine levels of H3K9me3 at CIITApIV at early time points following IFN-γ stimulation. HeLa cells were stimulated with IFN-γ, crosslinked, lysed, subjected to IP with antibody against endogenous trimethylated H3K9, and analyzed by real-time PCR with primers and probe spanning the CIITApIV proximal region. ChIP assays showed significant decreases in H3K9me3 at CIITApIV within 20 minutes of IFN-γ stimulation **(Figure 4.2 A)**. Levels of H3K9me3 continued to decline until reaching baseline levels within 80 minutes, indicating that initial drops in H3K9me3 have marked effects on chromatin structure and transcriptional activity. ChIP assays and real time PCR analysis further indicated that promoter levels of H3K9me3 at GAPDH were not significantly affected by cytokine stimulation **(Figure 4.2 B)**.

Previous studies suggest that the addition of activating H3K4 methylation events similar to those observed in Figure 1c frequently correlate with the depletion of silencing hypermethylation events on histone H3 lysine 27 (H3K27me3). (Ng, Robert et al. 2003) To characterize changes in the levels of H3K27me3 at CIITApIV, we expanded our epigenetic map of this promoter by performing ChIP assays in IFN-γ stimulated HeLa cells and isolating H3K27me3 modifications. Stimulated HeLa cells were crosslinked, lysed and immunoprecipitated with antibody to endogenous H3K27me3, and isolated DNA was analyzed by real time PCR using primers and probe spanning proximal CIITApIV. As shown in Figure 2c, ChIP results demonstrate a significant decline in H3K27me3 at early time points post cytokine stimulation. In addition, further ChIP and real time PCR analysis indicate levels of H3K27me3 at the GAPDH proximal promo-

ter region are not significantly affected by cytokine stimulation **(Figure 4.2 D)**. In sum, these data indicate that following IFN-γ stimulation, significant regulatory epigenetic events occur at CIITApIV in rapid succession.

CIITApIV is rapidly activated for transcription by the inflammatory cytokine IFN-γ.

Following IFN-γ treatment, dimerization of the IFN-γ receptor activates JAK1 and JAK2 which phosphorylate STAT-1 and subsequently promote STAT-1 nuclear localization and binding to target promoters.(Chang, Fontes et al. 1994) STAT-1 binds the GAS element of CIITApIV and, along with USF-1, IRF-1, and the basal transcriptional machinery, assembles the transcriptional initiation complex necessary to promote transcription from CIITApIV. (Chang, Fontes et al. 1994; Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002) As binding of STAT-1 to CIITApIV occurs when the promoter is in an open conformation, we addressed the effectiveness of early activating epigenetic modifications to CIITApIV by performing ChIP assays to determine levels of STAT-1 bound to CIITApIV following IFN-γ stimulation. ChIP mapping assays demonstrate significant localization of STAT-1 to CIITApIV within 20 minutes of IFN-γ stimulation **(Figure 4.3 A)**. These data indicate the rapid alterations in CIITApIV chromatin structure are effectively opening the promoter and allowing assembly of initiation complexes on the proximal promoter of CIITApIV. Based on these observations, we conclude that significant activating epigenetic events occur at CIITApIV within the first 20 minutes of INF-γ stimulation.

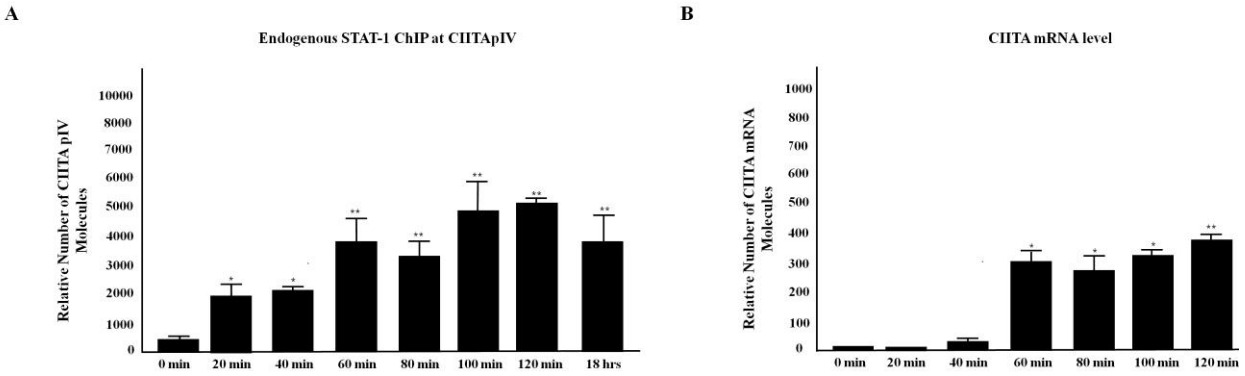

Figure 4.3 Figure 3. Transcriptional activation of CIITApIV occurs within 20' of IFN-γ stimulation.

(A) STAT1 binds CIITA promoter IV within 20' of IFN-γ stimulation. HeLa cells were stimulated with IFN-γ, crosslinked with formaldehyde, and cells were lysed and sonicated. Lysates were immunoprecipitated (IP) with control antibody or with antibody to STAT1. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITAPIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction. IP values are presented as a relative increase in CIITApIV DNA relative to unstimulated samples. Average control IP values were 532± 32. Values for control IPs and for STAT1 represent mean ± SEM of three independent experiments.

(B) Rapid increases in CIITA message levels occur within 40' post IFN-γ stimulation. CIITA and GAPDH mRNAs were prepared using TRIzol reagent and cDNA was generated using gene specific antisense primers for CIITA and GAPDH. cDNA was quantitated by real time PCR using gene specific antisense primers for CIITA and GAPDH. Data are graphed as relative value of CIITA mRNA and were determined after normalization to GAPDH mRNA. Values represent mean ± SEM of three independent experiments. ***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$ versus unstimulated sample.

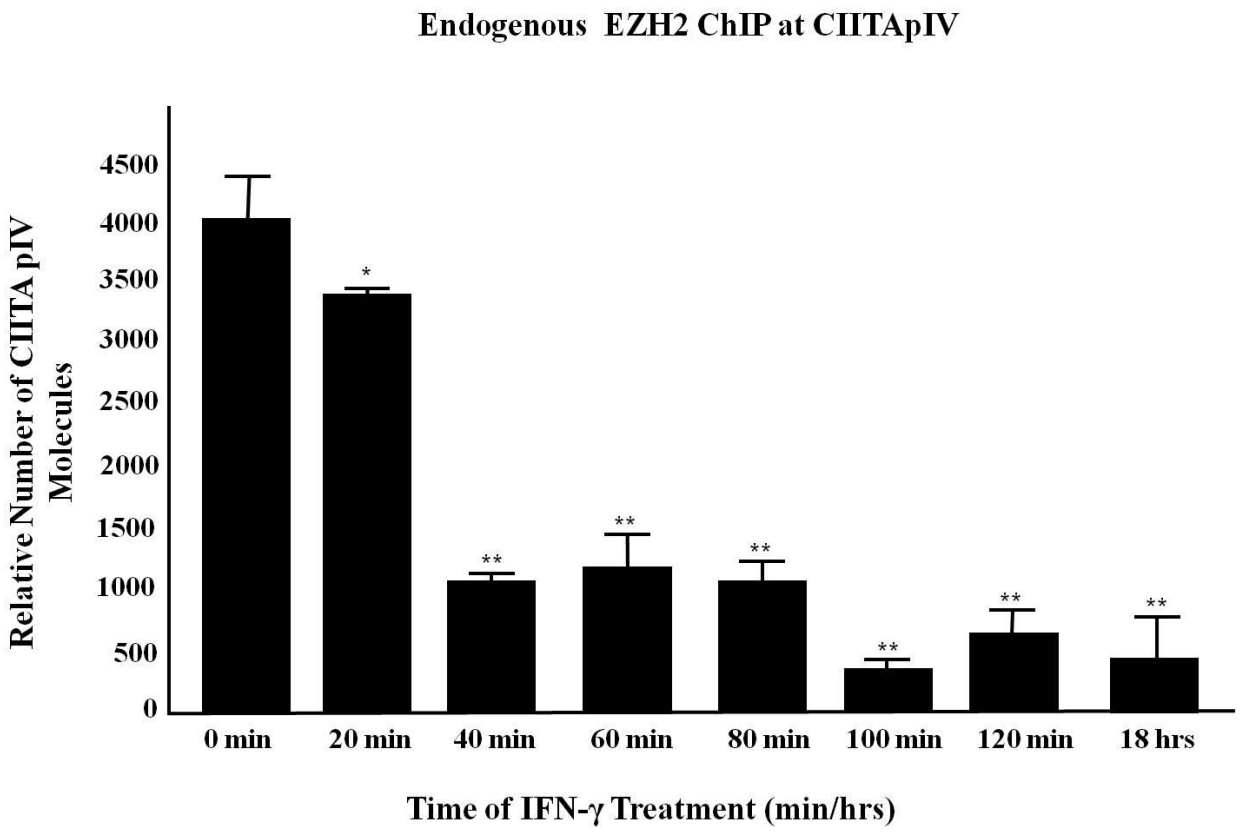

Figure 4.4 EZH2 binding to CIITApIV decreases significantly following IFN-γ stimulation.

HeLa cells stimulated with IFN-γ were crosslinked with DSG and formaldehyde and were then lysed and sonicated to generate fragments of approximately 750bp. Lysates were IP with control antibody or with antibody to EZH2. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITAPIV. IP values are presented as amount of CIITApIV DNA relative to unstimulated samples and have been normalized to the total amount of promoter DNA added to the reaction. Data are presented as the relative number of associated molecules analyzed in three cell lysate preps with each sample analyzed in triplicate. Control IP values were 435±30. **$P < 0.005$, *$P < 0.05$ versus unstimulated sample.

To address the effectiveness of these modifications in activating expression of CIITA, we assayed CIITA transcript levels in HeLa cells stimulated with IFN-γ at various time points. In contrast to previous studies demonstrating delayed CIITA mRNA expression; (Morris, Beresford et al. 2002; Boss and Jensen 2003) real-time PCR results in **Figure 4.3 B** indicate CIITA transcript levels increase to detectable levels as early as 40 minutes following IFN-γ stimulation.

Increases in CIITApIV transcription correlate with decreased promoter binding of the regulatory histone methyltransferase EZH2.

As a key epigenetic modifier, histone methylation frequently regulates other epigenetic alterations by generating binding sites for various chromatin remodeling complexes and histone modifying enzymes. Histone methyltransferases (HMTs) are chromatin remodeling enzymes capable of adding one, two, or three methyl groups to lysine residues on histones H1, H2A, H3, and H4, (Bannister and Kouzarides 2004; Schubeler, MacAlpine et al. 2004) and can activate (H3K4) or suppress (H3K27) transcriptional processes(Morris, Spangler et al. 2000). The HMT enhancer of Zeste homolog 2 (EZH2) is part of the Polycomb repressive complex 2, and is known to play important roles in gene silencing (Simon and Lange 2008) by catalyzing the trimethylation of H3K27 (Martinez-Garcia and Licht 2010) and H3K9 and has previously been shown to interact with CIITApIV. (Holling, Bergevoet et al. 2007) In order to determine if the loss of H3K9me3 and H3K27me3 observed at CIITApIV was consistent with changes in EZH2 binding to the promoter, we performed dual crosslinking ChIP assays by immunoprecipitating endogenous EZH2 from IFN-γ stimulated HeLa cells. Data in **Figure 4.4** demonstrates an abundance of EZH2 at CIITApIV that rapidly declines upon treatment of cells with IFN-γ and approaches base line levels 40 minutes following treatment. The drop in the level of EZH2 corre-

lates with our previous observations of decreases in H3K9me3 and H3K27me3, and with increases in activating modifications, promoter availability and CIITA transcript levels.

Loss of EZH2 leads to aberrant CIITA expression.

As EZH2 binding to CIITApIV was significantly and immediately decreased in the presence of IFN-γ, we next investigated the role of EZH2 in regulating activation of CIITApIV genes by using siRNA duplexes to specifically knockdown endogenous EZH2. siRNA mediated reduction of endogenous EZH2 decreased EZH2 protein expression by 90% relative to control siRNA transfected cells **(Figure 4.5 A, upper blots)** but did not impact the expression of tubulin **(Figure 4.5 A, lower blots)**. Analyis of EZH2 transcripts in EZH2 siRNA treated cells demonstrated 80-90% reduction of EZH2 mRNA **(Figure 4.5 B, white bars)** in comparison to control siRNA treated cells **(Figure 4.5 B black bars)**. As an additional control for specificity of the knockdown, we investigated the role of EZH2 in regulating expression of constitutively expressed GAPDH **(Figure 4.5 C)**. Real time PCR analysis indicated that transcript levels of GAPDH were unaffected by siRNA mediated depletion of EZH2. We next assayed CIITA transcript levels in EZH2 siRNA transfected cells. Real time PCR analysis indicated that transcript levels of CIITA were significantly increased in EZH2 depleted samples **(Figure 4.6, white bars)** in comparison to control siRNA samples **(Figure 4.6, black bars).**

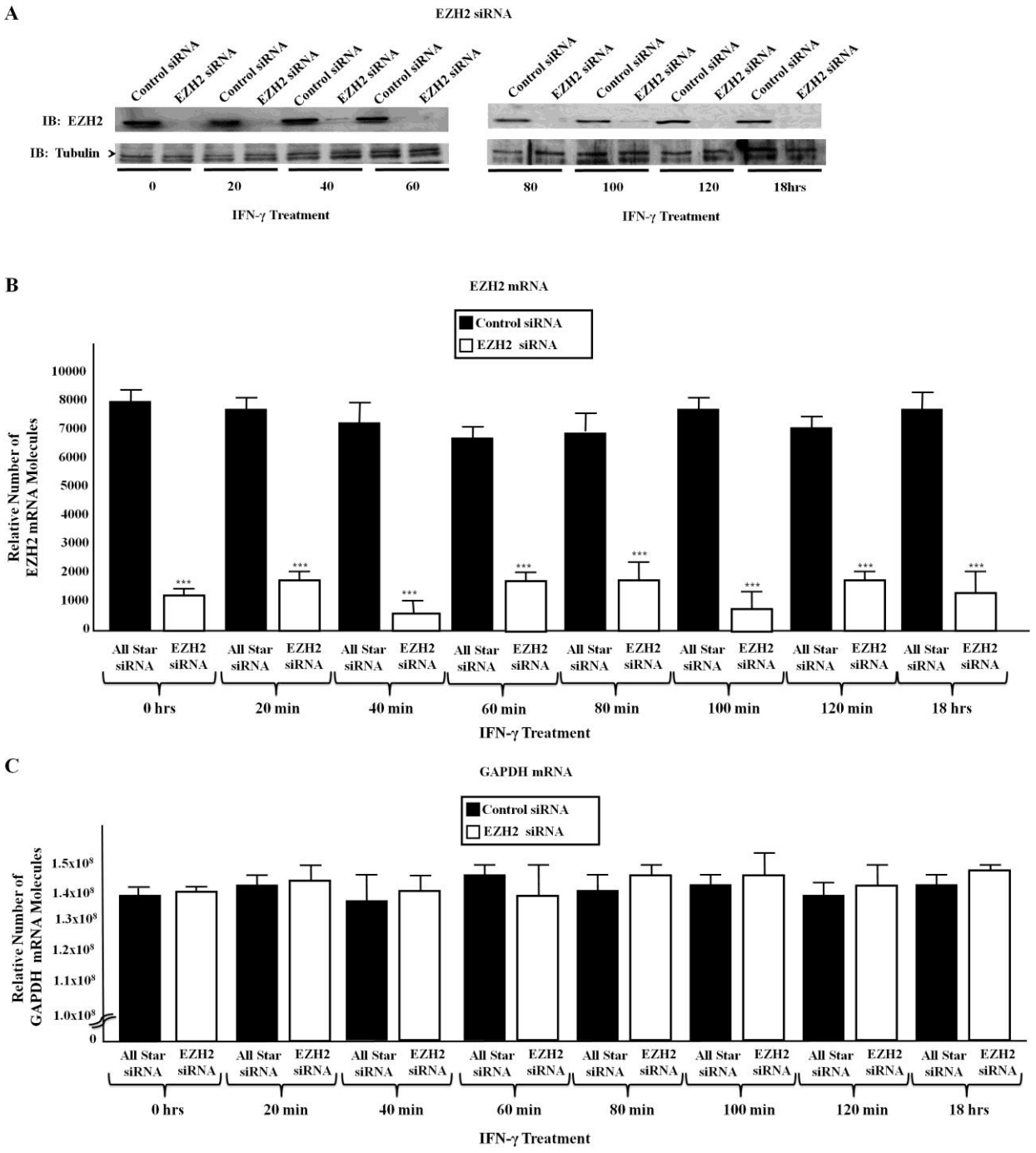

Figure 4.5 Figure 5. The EZH2 knockdown is specific.

(A) EZH2 siRNA efficiently and specifically decreases EZH2 protein expression. HeLa cells transfected with scrambled control or with EZH2 specific siRNA were stimulated with IFN-γ. Cells were harvested and were subjected to Western blot analysis for endogenous expression of

EZH2 and for tubulin. Western Blot analysis shows 90% knockdown of EZH2 and stable expression of tubulin.

(B-C) Reduced expression of EZH2 in siRNA transfected cells does not affect mRNA expression of GAPDH. EZH2 (B) and GAPDH (C) mRNA was prepared using TRIzol reagent and cDNA was generated using gene specific antisense primers for EZH2 and GAPDH. cDNA was quantitated by real time RT-PCR using gene specific primers for EZH2 and for GAPDH. Data are graphed as relative value of EZH2 mRNA in (B) determined after normalization to GAPDH mRNA in (D). Values represent mean ± SEM of three independent experiments. ***$P < 0.0005$, **$P < 0.005$ versus unstimulated sample.

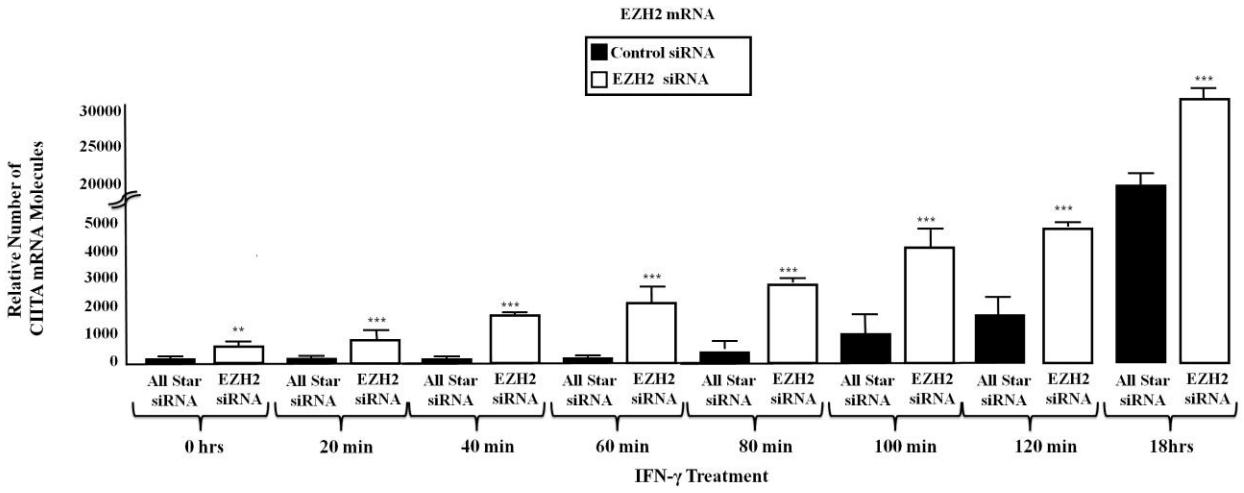

Figure 4.6 EZH2 knockdown significantly increases CIITA mRNA in the presence or absence of cytokine stimulation.

HeLa cells transfected with scrambled control or with EZH2 specific siRNA were stimulated with IFN-γ. Cells were harvested and CIITA mRNA was prepared using TRIzol reagent as described in Figure 5. Data are graphed as relative values of CIITA mRNA normalized against GAPDH mRNA. Values represent mean ± SEM of three independent experiments. ***P < 0.0005, **P < 0.005 versus unstimulated sample.

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The results in Figure 6 demonstrate that decreased expression of EZH2 results in the loss of suppression of CIITA.

The molecular mechanisms regulating EZH2 removal from CIITApIV, and other inducible promoters, is not well understood and the point at which EZH2 is actually removed from promoters remains unknown. As removal of EZH2 from CIITApIV strongly correlates with activation of transcription at CIITApIV, we addressed two mechanisms potentially involved in regulating removal of EZH2 from CIITApIV: degradation of EZH2 and BRG-1 binding**. Supplementary Figure 4.7 A** demonstrates that EZH2 has a 4 hour half life in HeLa cells and **Supplementary Figure 4.7 B** shows that EZH2 remains stable over the course of a 120 minute IFN-γ time course. Thus, EZH2 degradation in response to IFN-γ stimulation is an unlikely mechanism of regulating EZH2 binding to CIITApIV. Binding of Brahma-related gene 1 (BRG-1) to CIITApIV has previously been shown to be required for IFN-γ inducible pIV binding of STAT-1 and IRF and for increased chromatin accessibility of CIITApIV upon IFN-γ stimulation. (Ni, Karaskov et al. 2005) **Supplementary Figure 4.8** demonstrates that EZH2 is released from IFN-γ stimulated CIITApIV in the absence of BRG-1, indicating that BRG-1 binding is not required to regulate EZH2 removal from this inducible promoter.

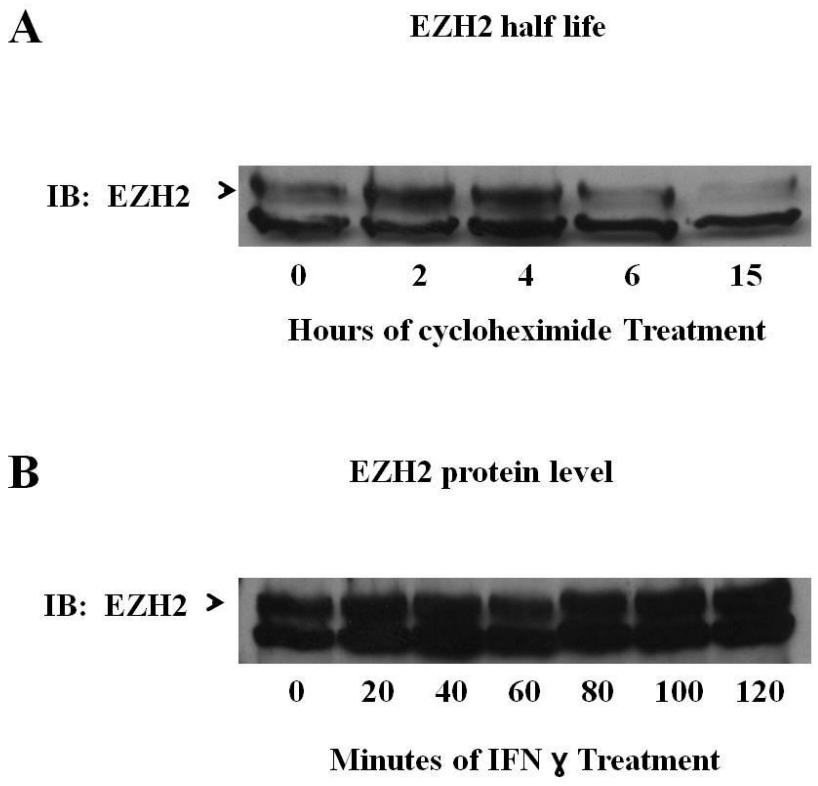
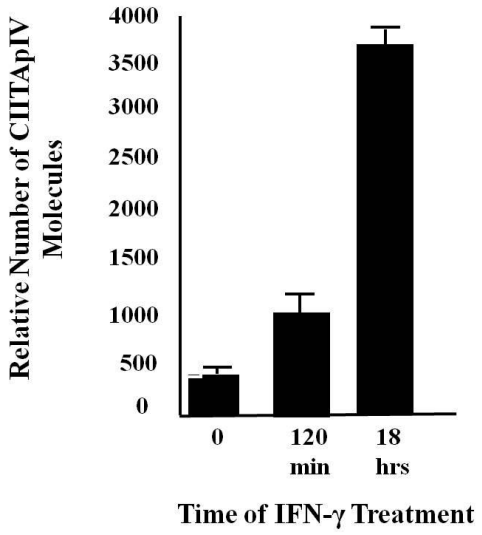


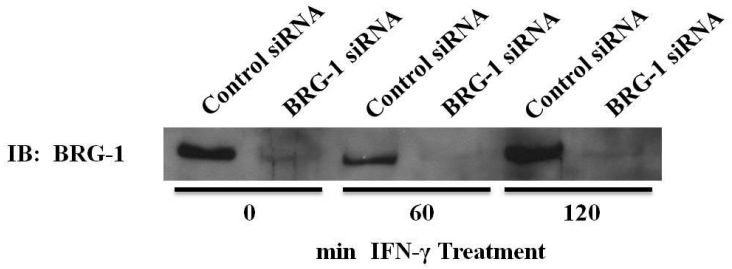
Figure 4.7 (Supplementary Figure) EZH2 half life is unchanged during a 120 minute IFN-γ time course.

(A) HeLa cells were treated with cycloheximide for 0-15hrs. Following cyclohexamide treatment, cells were lysed in 1% NP40 and western blot analysis was performed to determine the half life of endogenous EZH2. (B) HeLa cells were treated with IFN-γ for the indicated time points. Cells were harvested after stimulation and western blot analysis was performed to determine the level of endogenous EZH2 protein in whole cell lysates. Results shown are representative of two experiments.

A Endogenous levels of BRG-1 at CIITA pIV



B BRG-1 siRNA



C EZH2 recruitment to CIITA PIV in the presence of BRG-1 siRNA

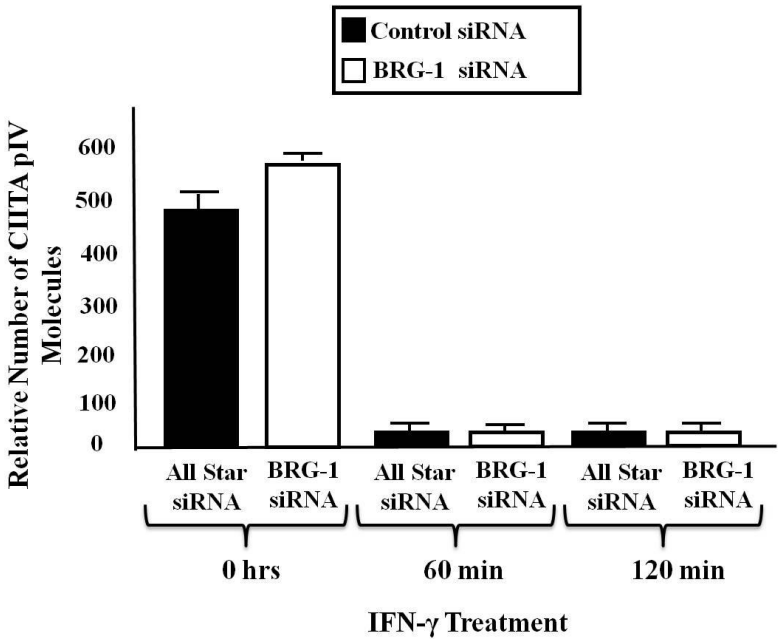


Figure 4.8 (Supplementary Figure) BRG-1 binding to CIITAPIV is not required for removal of EZH2 from CIITAPIV.

(A) Timecourse of BRG-1 binding to CIITApIV. HeLa cells were stimulated with IFN-γ, crosslinked with formaldehyde, lysed and sonicated. Lysates were IP with control antibody or with antibody to BRG-1. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITAPIV. IP values are presented as increases in CIITApIV DNA relative to unstimulated samples and have been normalized to the total amount of promoter DNA added to the reaction. Data are presented as the relative number of associated molecules analyzed in three cell lysate preps with each sample analyzed in triplicate. Control IP values were 650±50.

(B) BRG-1 siRNA efficiently decreases endogenous BRG-1 protein expression. HeLa cells transfected with scrambled control or BRG-1 specific siRNA were stimulated with IFN-γ for the time points indicated. Cells were harvested and subjected to Western blot analysis for endogenous expression of BRG-1. Western Blot analysis shows 90% knockdown of BRG-1.

(C) Binding of EZH2 to CIITApIV is unchanged in the absence of BRG-1. HeLa cells were transfected with BRG-1 specific siRNA or with control siRNA and were stimulated with IFN-γ for 0 to 18 hours. Double crosslinked lysates were IP with control antibody or with antibody to endogenous EZH2 and associated DNA was isolated and analyzed via real-time PCR using primers spanning IRF-1box of CIITApIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). IP values are presented as relative increase in CIITA DNA relative to unstimulated samples. Control IP values were 95±30. Values shown are representative data.

4.3 DISCUSSION

Our study provides an in depth description of the early epigenetic events occurring at IFN-γ stimulated CIITApIV. As the master regulator of MHC II genes, CIITA controls MHC II expression and is therefore tightly regulated. (Kaufman, Auffray et al. 1984; Guy, Krajewski et al. 1986; Adamski, Ma et al. 2004; Gerloni and Zanetti 2005) Previous studies from our lab and others have shown that epigenetic events play an important role in controlling activation of CIITApIV. (Boss and Jensen 2003; Morimoto, Toyota et al. 2004; Koues, Dudley et al. 2009; Truax, Koues et al. 2010) We observe here that following IFN-γ stimulation regulatory epigenetic events occur in rapid succession at CIITApIV. Although H3K18 acetylation levels rise post IFN-γ stimulation, the increase in H3K18 acetylation is non-linear, indicating an immediate, but non-maximal, loosening of the chromatin template following IFN-γ stimulation. Steady increases in the activating addition of methyl groups to histone H3 lysine 4 also occur at CIITApIV following IFN-γ stimulation. The levels of H3K4me3 significantly increase throughout the 120 minute time course, indicating an accumulation of epigenetic marks in addition to H3K18 acetylation for maximal methylation of lysine 4.

In contrast to K18 acetylation and K4 methylation is the silencing modification of H3K9me3, which is lost at CIITApIV upon IFN-γ stimulation. Levels of H3K9me3 significantly decline over the 120 minute IFN-γ time course and then remain at baseline for the duration of IFN-γ stimulation. A similar trend is observed in the case of H3K27me3 where loss of the inhibitory H3K27me3 modification occurs upon IFN-γ stimulation, with the decline in K27me3 mir-

roring gains in H3K4me3 and suggesting competitive targeting of lysines 27 and 4 in epigenetic regulation of CIITApIV.

Rapid changes in regulatory epigenetic modifications to histone H3 at CIITApIV following IFN-γ stimulation indicate chromatin is opened and transcription rapidly occurs following exposure of cells to inflammatory cytokine. This assumption is confirmed by observations that STAT-1 binds CIITApIV and drives expression of CIITA transcripts within 40 minutes of IFN-γ stimulation. Previous studies have demonstrated STAT-1 binding to the GAS sequence as one of the first trans-acting events occurring at CIITApIV, and have demonstrated STAT-1 binding within five minutes of IFN-γ stimulation.(Morris, Beresford et al. 2002) Based on these previous studies, it was anticipated that STAT-1 would rapidly associate with CIITApIV following IFN-γ stimulation. The present data demonstrate not only rapid STAT-1 binding, but also that binding is sustained at similar levels throughout IFN-γ induction, indicating initial chromatin remodeling events overcome critical thresholds required to activate CIITApIV expression. Additionally, both STAT-1 binding and low, but significant CIITA expression correlate with the rapid rise in the levels of H3K18ac and H3K4me3 and with the rapid drop in the levels of H3K9me3 and H3K27me3. Thus, the combination of rapidly occurring epigenetic modifications is sufficient to activate the CIITA gene.

It is important to note that levels of STAT-1 binding to CIITApIV do not significantly change after 60 minutes of IFN-γ stimulation, indicating that beyond this point CIITApIV maintains an open chromatin structure and active levels of transcription, despite greatly enhanced le-

vels of H3K18 acetylation at later time points. Observations that levels of H3K18ac do not peak until after 120 minutes of IFN-γ stimulation are in contrast to the rapid decreases in suppressing levels of H3K9me3 and H3K27me3, and suggest that the removal of methyl groups from CII-TApIV may play important roles in the initial control of CIITA activation. Similar observations that strong binding of the HMT EZH2 to CIITApIV rapidly declines following IFN-γ stimulation add further support to the regulatory control histone methylation exerts at CIITApIV.

Decreased expression of CIITA, and of MHC II, has also been previously described as a mechanism utilized by tumors to escape the immune response.(Holling, van Eggermond et al. 2006) Relevant to our study are observations that changes in chromatin structure (Jones and Laird 1999; Jones and Baylin 2002) and tissue specific changes in EZH2 expression are associated with various types of cancers. (Jones and Laird 1999; Schulz and Hatina 2006; Simon and Lange 2008; Cooper and Foster 2009) EZH2 was previously found to bind CIITAPIV chromatin in uveal melanoma cells where EZH2 siRNA resulted in increased CIITA expression in cells stimulated for 24h with IFN-γ. In contrast to our observations in HeLa cells, CIITA transcripts were not observed in unstimulated uveal melanoma cells treated with EZH2 siRNA.(Holling, Bergevoet et al. 2007) These differences could be due to several reasons including our increased efficiency of EZH2 knockdown and cell type specific differences. In data not shown, we have observed significant levels of IFN-γ independent CIITA mRNA expressed in multiple EZH2 siRNA treated human cancer cell lines, including variants of MDA MB 435, indicating cell type differences in the epigenetic status of the bivalent promoter CIITApIV. Thus, differences in cell lines may contribute to differing levels of suppression by EZH2 and differing states of chromatin packaging of CIITApIV. In addition, although DNA methyltransferases are known to associate

with EZH2 and the PRC2 complex, EZH2 mediated suppression in some cases may be sufficient to silence genes, as in the case of CIITApIV, where siRNA mediated depletion of EZH2 was successful in reactivating the silenced gene.

Few studies have been performed to determine how EZH2 is itself regulated and maintained in cells. EZH2 phosphorylation by cyclin-dependent kinase 1 (CDK1) and cyclin-dependent kinase 2 (CDK2) is required for EZH2 recruitment and H3K27me3 at target promoters. (Chen, Bohrer et al. 2010) As we and others have previously demonstrated the importance of prior phosphorylation in regulating ubiquitination of proteins, we sought to determine if EZH2 phosphorylation established a pattern of sustained phosphorylation leading to ubiquitination and degradation in response to IFN-γ stimulation. As the half life of EZH2 was unaltered in the presence of IFN-γ, it is unlikely that degradation is responsible for removal of EZH2 from CIITApIV in IFN-γ stimulated cells. Alternatively, the ATPase BRG1 is needed for chromatin remodeling and STAT-1 binding at CIITApIV and could provide a means of EZH2 removal from CIITApIV. However, the present study suggests that EZH2 is lost upon IFN-γ stimulation from CIITApIV in the absence of BRG-1, suggesting additional regulatory mechanisms are at play. In regards to the epigenetic regulation of CIITA, our study implicates EZH2 in regulating the initial chromatin remodeling events at the cytokine-inducible CIITA promoter. Knowledge that CIITApIV is rapidly released from surrounding chromatin following IFN-γ stimulation and departure of EZH2 strongly suggests that EZH2 is predominantly involved in initial chromatin remodeling in response to stimuli. Our study suggests EZH2 is a ‘master regulator’ of epigenetic modifications to histones and is the first to link the regulation of rapidly occurring epigenetic

changes to EZH2 and, as such, has strong implications for the regulation not only of CIITA, but for many other silenced but inducible genes.

MATERIALS AND METHODS

Cell Lines:

HeLa cells (human epithelial) from ATCC (Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies:

The ChIP antibodies recognizing H3K27me3, H3k9me3, and H3K18ac were from Active Motif (Carisbad, CA) and the H3K4me3 and EZH2 antibodies were from Abcam (Cambridge, MA). STAT-1 and BRG-1 antibodies were from Santa Cruz (Santa Cruz, Ca). Rabbit and mouse immunoglobulin G (IgG) isotype control antibodies were from Millipore (Lake Placid, NY). The antibodies recognizing EZH2 in western blots were from Millipore (Billerica, MA) and the anti-tubulin antibody was from Santa Cruz (Santa Cruz, Ca). HRP conjugated mouse antibody was from Promega (Madison, WI) and HRP conjugated rabbit antibody was from Pierce (Rockland, IL).

siRNA constructs and transient transfection:

Short interfering RNA (siRNA) duplexes were predesigned against human EZH2 (Qiagen) and BRG-1 (Qiagen) and were used for transient knockdown of EZH2 and BRG-1. All Star scrambled siRNA (Qiagen) was used as the negative control siRNA. HeLa cells were transfected with 0.7µg of control, EZH2, or BRG-1 specific siRNA using RNAiFECT transfection reagent (Qiagen) according to the manufacturer's instructions and were treated with 500 U/ml of IFN-γ from Peprotech (Rocky Hill, NJ) as indicated. Cells were lysed in NP-40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5 M EDTA, 5MNaCl, 1M DTT, dH_20) supplemented with Compete EDTA-free protease inhibitor from Roche (Florence, SC) and knock down efficiency and specificity was assessed by western blot or mRNA analysis.

Chromatin immunoprecipitation (ChIP):

ChIP assays were performed as described previously. (Truax, Koues et al. 2010) (Koues, Dudley et al. 2009; Koues, Mehta et al. 2010) Briefly, HeLa cells were plated at a density of 2×10^6 cells/15 cm plate and were stimulated with 500 U/ml IFN-γ. Formaldehyde cross-linking was performed with 1% formaldehyde for 10 minutes at room temperature and 0.125 M glycine was added for five minutes at room temperature to stop cross linking. Cells were lysed using SDS lysis buffer (50mM Tris pH 8.0, 10mM EDTA, dH_20, 1%SDS) with protease inhibitor (Qiagen, CA) for 20 minutes on ice. Samples were sonicated at constant pulse to generate DNA

fragments of 500-800 bp. Sonicated samples were run on 1% agarose gels and stained with Ethidium Bromide (EtBr) to determine sonication efficiency. Sonicated lysates were precleared with salmon-sperm coated agarose beads (Millipore) and half of the lysate was immunoprecipitated (IP) with 5µg of polyAb against H3K27me3, H3k9me3, H3K18ac, H3K4me3, STAT-1, or EZH2 overnight at 4°C. The remaining half of the lysate was used as a control and was IP with isotype control antibody (Millipore, MA). Following an additional one hour IP with 50µl of salmon-sperm coated agarose beads, samples were washed for three minutes at 4°C with the following buffers: Low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl, dH_20), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH_20), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH20) and 1xTE buffer and were eluted with SDS elution buffer (1% SDS, 0.1M NaHCO3, dH_2O). Following elution, crosslinks were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isoamyl alcohol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using primers spanning the GAPDH promoter and CIITApIV.(Morris, Beresford et al. 2002; Truax, Koues et al. 2010) Values graphed were calculated based on standard curves generated.

Dual Crosslinking Chromatin immunoprecipitation (Dual ChIP):

As described above for ChIP assays, HeLa cells were plated at a density of 2.5×10^6 cells/15 cm plate and were stimulated with 500 U/ml IFN-γ at indicated time points. Cells were harvested and washed with Phosphate Saline Buffer (PBS), and were resuspended in 10 ml of PBS (pH

8.0) containing 1mM $MgCl_2$ and 1μM of Disuccinimidyl Glutarate (DSG) and were incubated at room temperature for 45 minutes. 0.1 mM of Tris pH 7.4 was added for five minutes at room temperature to stop cross-linking. Cells were rinsed with PBS and resuspended in 10ml of PBS. Secondary formaldehyde cross-linking was performed with 1% formaldehyde for 10 minutes at room temperature, followed by the addition of 0.125 M glycine for five minutes at room temperature to stop further cross linking. Cells were lysed in SDS lysis buffer and were subjected to sonication and ChIP as above.

RNA expression:

HeLa cells were plated at a density of 8×10^5 cells/10 cm plate and were stimulated with 500 U/ml IFN-γ at experiment specific time points. Cells were harvested and total RNA was prepared using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions and as previously described(Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Bhat, Truax et al. 2010; Truax, Koues et al. 2010). The Omniscript Reverse Transcription Kit (Qiagen, CA) was used to generate cDNA from extracted RNA. Isolated DNA was analyzed by real time PCR using primers and probes for CIITA (Truax, Koues et al. 2010) and GAPDH (Truax, Koues et al. 2010) mRNA. Primer sequences for EZH2 were as follows: EZH2 sense TTCATGCAACACCCAACACT, EZH2 antisense GAGAGCAGCAGCAAACTCCT, EZH2 probe FAM- TTACCAGCATTTGGAGGGAG - TAMRA. Real-time PCR values were generated based on standard curves generated for each gene and levels of CIITA and EZH2 are presented as fold changes over GAPDH message levels.

RNA expression in cells treated with siRNA:

HeLa cells were plated at a cell density of $8x10^5$ cells, transfected with EZH2 siRNA and twenty four hours later were stimulated with IFN-γ as indicated. Cells were harvested and 10% of the cells were lysed with 1% NP-40 lysis buffer and subjected SDS PAGE to determine the efficiency of the knockdown. The remaining fraction of cells was subjected to RNA extraction with 1ml of Trizol as described above.

***In vivo* half life assay.**

HeLa cells were treated with 100 µM cycloheximide (Sigma) for 0-15hrs. The total cell volume was lysed with 1% NP-40 buffer with protease inhibitor and analyzed by Western blotting for EZH2 degradation.

4.4 ACKNOWLEDGMENTS

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5 CHAPTER V: THE HISTONE METHYLTRANSFERASE EZH2 IS A CRITICAL REGULATOR OF INDUCIBLE EXPRESSION OF CLASS II TRANSACTIVATOR, CIITA

PUBLICATION: AGNIESZKA D. TRUAX AND SUSANNA F. GREER

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5.1 ABSTRACT

One mechanism frequently utilized by tumor cells to escape immune system recognition and elimination is suppression of cell surface expression of Major Histocompatibility Class II (MHC II) molecules. Expression of MHC II is regulated primarily at the level of transcription by a Master Regulator, the Class II Transactivator, CIITA. Decreased expression of CIITA has been documented in multiple tumor cell types including breast tumors. We investigate here contributions of epigenetic modifications to transcriptional silencing of CIITA in variants of the human breast cancer cell line MDA MB 435. Increased histone H3 lysine 27 trimethylation correlated with significant reductions in RNA polymerase II recruitment to the interferon-γ inducible CIITA promoter, CIITApIV, and with increased CIITApIV occupancy by the histone methyltransferase enhancer of zeste homolog 2 (EZH2). Decreased expression of EZH2 in MDA MB 435 variants resulted in significant increases in CIITA and MHC II mRNA independent of cytokine stimulation. These data indicate EZH2 contributes critical regulatory marks to the silencing transcription of CIITA and to the regulation of MHC II expression.

5.2 BACKGROUND

Major histocompatibility class II (MHC II) genes encode cell surface proteins responsible for presenting peptides from extracellular spaces to activate $CD4^+$ T cells. As activated T cells are in turn responsible for driving adaptive immune responses, MHC II molecules play critical roles in regulating immune recognition of pathogens and tumors. Constitutive expression of MHC class II is limited to professional antigen presenting cells, but MHC II expression can be induced in most other cell types by inflammatory cytokines, of which IFN-γ is the most potent (Kaufman, Auffray et al. 1984; Chang, Fontes et al. 1994). Although $CD8^+$ T cells are more directly responsible for lysis of infected cells and tumor cells, recent studies have shown that peptide immunization in the presence of $CD4^+$ T cells enhances $CD8^+$ T cell responses (Langlade-Demoyen, Garcia-Pons et al. 2003). Further, several murine tumor models have demonstrated that $CD4^+$ T cells are required for an effective anti-tumor immune response (Greenberg, Cheever et al. 1981; Fujiwara, Fukuzawa et al. 1984; Hock, Dorsch et al. 1991; Lauritzsen, Weiss et al. 1994). Loss of MHC II expression has also been associated with decreased numbers of tumor infiltrating T cells and with increased aggressiveness of colorectal and laryngeal carcinomas (Garrido and Ruiz-Cabello 1991; Warabi, Kitagawa et al. 2000). These observations suggest that $CD4^+$ T cells are able to respond to tumor antigens presented via MHC II to induce an effective immune response and emphasize the importance of understanding the transcriptional regulation of MHC II genes in cancer cells.

Tight regulation of MHC II transcription and expression is necessary for proper initiation, stabilization, and termination of adaptive immune responses. MHC II genes are regulated by a

multi-protein enhanceosome complex that binds the W-X-Y region of the MHC II promoter, assembly of which is stabilized by the Class II transactivator, CIITA (Boss 1997; Ting and Trowsdale 2002). Although CIITA does not directly bind the MHC II promoter, its association with the pre-assembled enhanceosome complex is required for MHC II expression and serves to coordinate steps leading to transcriptional initiation (Fontes, Jiang et al. 1997; Mahanta, Scholl et al. 1997). CIITA recruits to the MHC II proximal promoter components of the basal transcriptional machinery, histone acetyltransferases (HATs), histone deacetylases (HDACs), chromatin remodeling complexes, and the kinases that phosphorylate RNA pol II (Fontes, Kanazawa et al. 1999; Beresford and Boss 2001; Zika, Greer et al. 2003; Drozina, Kohoutek et al. 2005). CIITA transcription is tightly regulated with multiple promoters regulating transcription of CIITA in a tissue specific manner (Muhlethaler-Mottet, Otten et al. 1997; Pai, Askew et al. 2002). Promoter I drives expression of CIITA in dendritic cells (Landmann, Muhlethaler-Mottet et al. 2001), the function of promoter II is unknown, and promoter III drives constitutive CIITA expression in B cells (Lennon, Ottone et al. 1997). Transcription of CIITA in non-antigen presenting cells is induced by the inflammatory cytokine interferon gamma (IFN-γ) and is primarily achieved by coordinate binding of multiple transcription factors to the IFN-γ inducible promoter, CIITA promoter IV (CIITApIV) (Piskurich, Gilbert et al. 2006). Transcriptional activation of CIITA-pIV by IFN-γ requires the assembly of the following transcription factors: interferon regulatory factor 1 (IRF-1), signal transducer and activator of transcription 1 (STAT-1), and ubiquitous factor 1 (USF-1) to conserved DNA sequences on CIITApIV. STAT-1 directly binds ubiquitously expressed USF-1 at the E-box of the IFN-γ activated sequence (GAS). STAT-1 also initiates transcription from the IRF-1 promoter; once IRF-1 is expressed, it subsequently binds the IFN response element (IRE) site at CIITApIV (Piskurich, Linhoff et al. 1999).

Previous studies from our lab and others indicate that epigenetic modifications to chromatin play important roles in regulating MHC II and CIITApIV transcription (Truax, Koues et al. ; Koues, Dudley et al. 2008). In unstimulated cells, the MHC II proximal promoter exhibits low basal acetylation which allows for binding of the ubiquitously expressed components of the enhanceosome complex. Following cytokine stimulation, acetylation of histones H3 and H4 significantly increases, allowing for recruitment of CIITA, the basal transcription machinery and initiation of MHC II transcription (Beresford and Boss 2001; Wright and Ting 2006). CIITA-pIV is also regulated by proximal promoter epigenetic modifications and is characterized as a bivalent promoter with both activating and repressing chromatin marks. In unstimulated cells, CIITApIV exhibits elevated trimethylation of histone H3 lysine 27 (H3K27me3) and low acetylation of histones H3 and H4. In the presence of IFN-γ, changes in higher order chromatin structure are followed by increases in acetylation of histones H3 and H4 (Agalioti, Chen et al. 2002; Morris, Beresford et al. 2002), increased trimethylation of histone H3 lysine 4 (H3K4me3) (Ni, Karaskov et al. 2005), and a significant and rapid decrease in H3K27me3 (Morris, Beresford et al. 2002; Holling, van Eggermond et al. 2006; Holling, Bergevoet et al. 2007). The histone methyltransferase (HMTase) largely responsible for the addition of methyl groups to H3K27 is the Enhancer of Zeste Homolog 2 (EZH2) (Cao, Wang et al. 2002; Cao and Zhang 2004), the catalytic subunit of the Polycomb repressive complex 2 (PRC2) which is involved in maintaining epigenetic memory and transcriptional silencing (Cao, Wang et al. 2002; Kuzmichev, Nishioka et al. 2002). While EZH2 has previously been demonstrated to bind CIITApIV in malignant uveal melanoma cells, mechanistic roles for EZH2 in regulating CIITA and MHC II expression remain to be elucidated. Indeed, although studies have well demonstrated EZH2 is frequently

over expressed in a wide variety of cancers including tumors of the prostate and breast, mechanistic links of EZH2 to cancer progression remain areas of intense investigation.

Decreased expression of MHC II has been previously described in breast tumors (Garrido and Ruiz-Cabello 1991; Dadmarz, Sgagias et al. 1995). Variants of the MDA MB 435 human breast cancer cell line differ in their metastatic ability (Zhang, Fidler et al. 1991) and have recently been classified as members of the HER-2 over expressing subtype of breast carcinoma (Holen and Coleman 2010). Previous work has demonstrated that among these variant lines, 435 Brain 1 cells exhibit poor metastatic ability while 435 Lung 2 cells exhibit high metastatic ability (Zhang, Fidler et al. 1991; Shi, Vinyals et al. 2006). In this study, we analyzed MHC II and CIITA expression patterns in variants of MDA MB 435. Initial studies determined a loss of cell surface MHC II in the MDA MB 435 variants which correlated with increased metastatic potential, decreased CIITA expression, and suppression of CIITApIV. We provide evidence that in the MDA MB 435 variants, CIITApIV maintains a closed chromatin conformation in the presence or absence of IFN-γ stimulation. Elevated levels of EZH2 at CIITApIV and the resulting increases in CIITApIV H3K27me3 leave the proximal promoter inaccessible for transcription factor binding or transcription initiation. Decreased expression of EZH2 restores expression of CIITA and MHC II in unstimulated cells, with substantial impact on MHC II expression in each of the MDA MB 435 variants. These observations suggest EZH2 is a pivotal regulator of CIITApIV silencing and further implicate this histone methyltransferase as a candidate target for use in activating immune responses against tumors.

5.3 RESULTS:

Differential expression of MHC II by variants of MDA MB 435 human breast carcinoma correlates with decreased MHC II mRNA levels.

Down regulation of cell surface expression of MHC II molecules is frequently noted on tumor cells. Low level expression of MHC II impairs immune recognition of tumors and allows undetected tumor growth and tumor metastasis (Gobin, Peijnenburg et al. 1997; van den Elsen, Holling et al. 2004). Flow cytometry was used to determine cell surface expression of MHC II molecules (versus mouse IgG2a κ isotype control antibody, **(Supplementary Figure 5.9)** in three variants of MDA MB 435 human breast cancer cells **(Figure 5.1 A)**, in HeLa cells **(Figure 5.1 B)** and in immortalized, but non-tumorigenic, epithelial breast MCF 10A cells **(Figure 5.1 C).** Figure 1A and C demonstrates that HeLa cells and MCF-10A cells respond similarly to IFN-γ stimulation by increasing cell surface expression of MHC II molecules. Levels of MHC II expression in MDA MB 435 cells were similar to HeLa cells upon 24 hours of IFN-γ stimulation **(Figure 5.1 A)** while MHC II expression was reduced in poorly metastatic 435-Brain 1 cells 24 hours post IFN-γ. Further reductions in cell surface expression of MHC II are seen in highly metastatic 435-Lung 2 cells similarly stimulated with IFN-γ **(Figure 5.1 A)**. These results are in agreement with our findings that MHC II mRNA levels are decreased in poorly metastatic 435-Brain 1 and almost eliminated in highly metastatic 435-Lung 2 cancer cells **(Figure 5.1 E)**. Additionally, we have determined that MHC II mRNA levels in breast cancer cells **(Figure 5.1 E)** showed a significant 100 fold reduction in the MHC message levels while comparing to our control HeLa cells **(Figure 5.1 D)**.

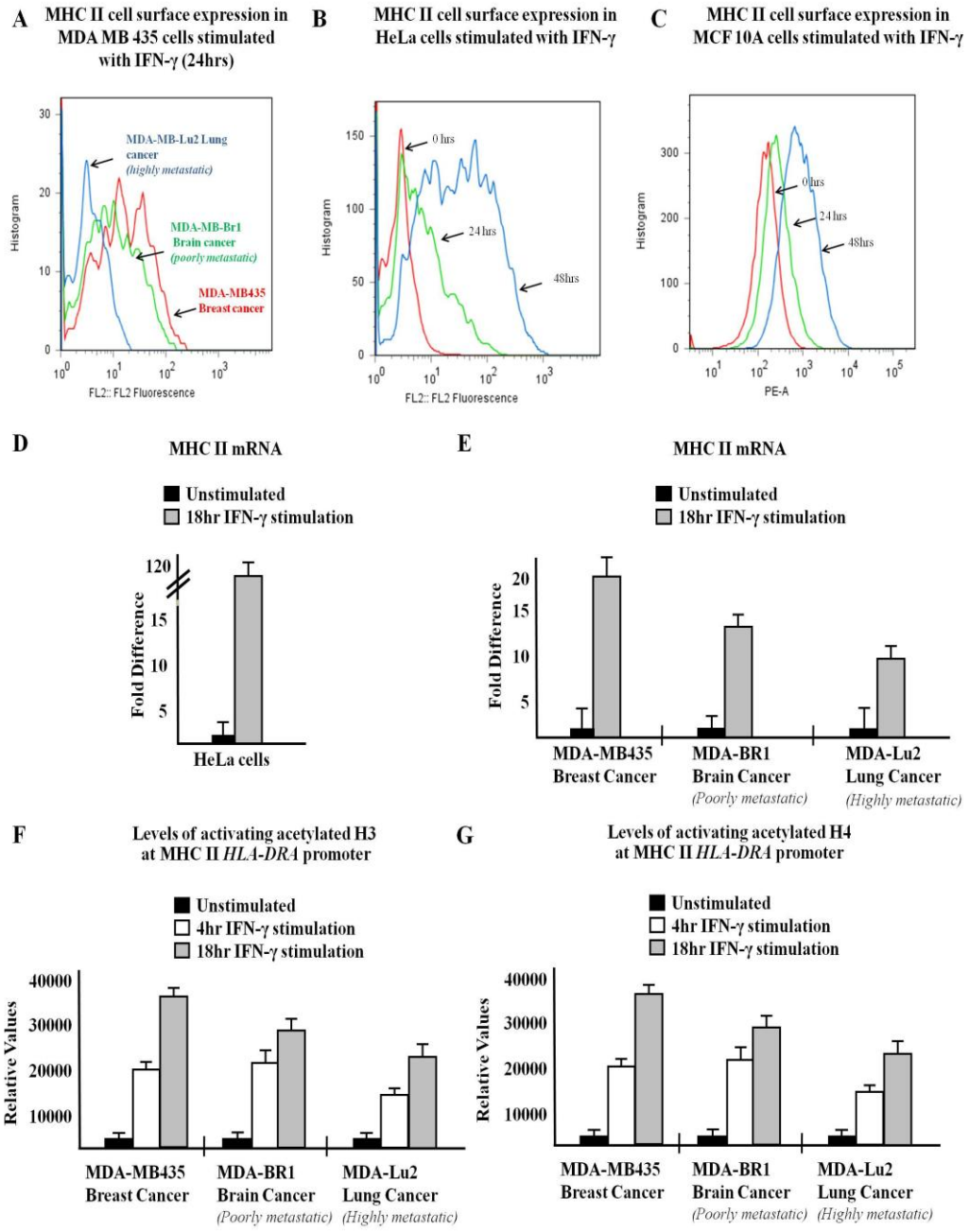

Figure 5.1 MHC II cell surface expression and mRNA expression is decreased in MDA MB 435 cells.

(A-C) Cell surface expression of MHC II on MDA MB 435 cells (A) on HeLa cells (B) and on non metastatic breast MCF 10A cells (C). Cells were stimulated with IFN-γ for 24 hours, trypsinized, washed, and incubated with PE-labeled anti-human HLA-DR antibody. Following anti-

body incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto. Results shown are representative of three independent experiments.

(D-E) Reduced expression of endogenous MHC II mRNA in breast cancer cells (E) compared to HeLa cells (D). Cells were left unstimulated or were stimulated with IFN-γ for 0 to 18 hours and levels of mRNA were measured by real-time PCR and were normalized to GAPDH mRNA. Results shown represent the mean ± SD of four independent experiments.

(F-G) Levels of acetylated H3 (F) and acetylated H4 (G) at the MHC II *HLA-DRA* proximal promoter. ChIP assays were carried out in cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous acetylated H3 (F) or antibody to endogenous acetylated H4 (G) and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as the relative increase in MHC II DNA relative to unstimulated samples. Control IP values for (E) and (F) were 950 ± 200. Values for acetylated H3 (E) and acetylated H4 (F) IPs represent mean ± SEM of three independent experiments.

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The above observations demonstrate significant reductions in expression of MHC II by MDA MB 435 breast cancer cell lines that correlate with increasing metastatic capability. As MHC II cell surface expression is indispensable for T cell interaction and the presentation of tumor derived peptides to helper T cells (Garrido and Ruiz-Cabello 1991; Warabi, Kitagawa et al. 2000), the highly metastatic 435-Lung 2 breast cancer cells may down regulate MHC II genes to avoid immune recognition.

Histone H3 and histone H4 are acetylated at the activated MHC II proximal promoter in MDA MB 435 cells.

Transcription occurs within the highly ordered context of chromatin and an open chromatin structure is required for transcription initiation. To determine the availability of the MHC II promoter for transcription factors binding, we assayed levels of acetylation on histone H3 and H4 in MDA MB 435 cells. Metastatic variants of MDA MB 435 cells were left untreated or were stimulated with IFN-γ for 0-18 hrs and were subjected to chromatin immunoprecipitation (ChIP) analyses. Lysates were immunoprecipitated with antibody against acetylated H3 **(Figure 5.1 F)** or acetylated H4 **(Figure 5.1 G)** and analyzed via real time PCR. Following IFN-γ stimulation, levels of acetylated H3 and H4 significantly increase in all MDA MB 435 variants, indicating open MHC II promoter regions. Previous studies demonstrated that histone H3 lysine 18 (K18) is strongly acetylated at activated MHC II promoters (Rybtsova, Leimgruber et al. 2007). ChIP experiments were next performed to determine levels of MHC II promoter acetylation on H3K18 in MDA MB 435 variants. Cells were stimulated with IFN-γ as indicated, were sub-

jected to ChIP analyses with endogenous antibody against acetylated H3K18, and were analyzed by real time PCR with primers and probe spanning the MHC II proximal promoter. Endogenous levels of H3K18 acetylation were rapidly elevated with cytokine stimulation and no significant changes in H3K18 acetylation were observed between different metastatic variants of MDA MB 435 cells **(Figure 5.2 A)** or in comparison to HeLa cells **(supplementary Figure 5.10 A).** The histone acetyltransferase (HAT) CREB binding protein (CBP/p300) interacts with CIITA (Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999) and is known to both acetylated H3 and H4 at the MHC II proximal promoter and to enhance binding of CIITA to the MHC II enhanceosome complex (Chan and La Thangue 2001). ChIP analysis indicated recruitment of CBP to the MHC II proximal promoter upon cytokine stimulation in each of the variants of MDA MB 435 cells **(Figure 5.2 B)** and a similar level of CBP was observed at the MHC II proximal promoter in HeLa cells **(supplementary Figure 5.10 B)**. These data indicate neither histone acetylation of the MHC II proximal promoter nor the recruitment of the HAT CBP are significantly affected in the MDA MB 435 cells.

Silencing epigenetic marks decrease at the activated MHC II proximal promoter in MDA MB 435 cells.

As demonstrated above, although MDA MB 435 cells lose the ability to inducibly express MHC II on a cell surface as their metastatic ability increases; however, the MHC II proximal promoter is inducibly acetylated in these cells.

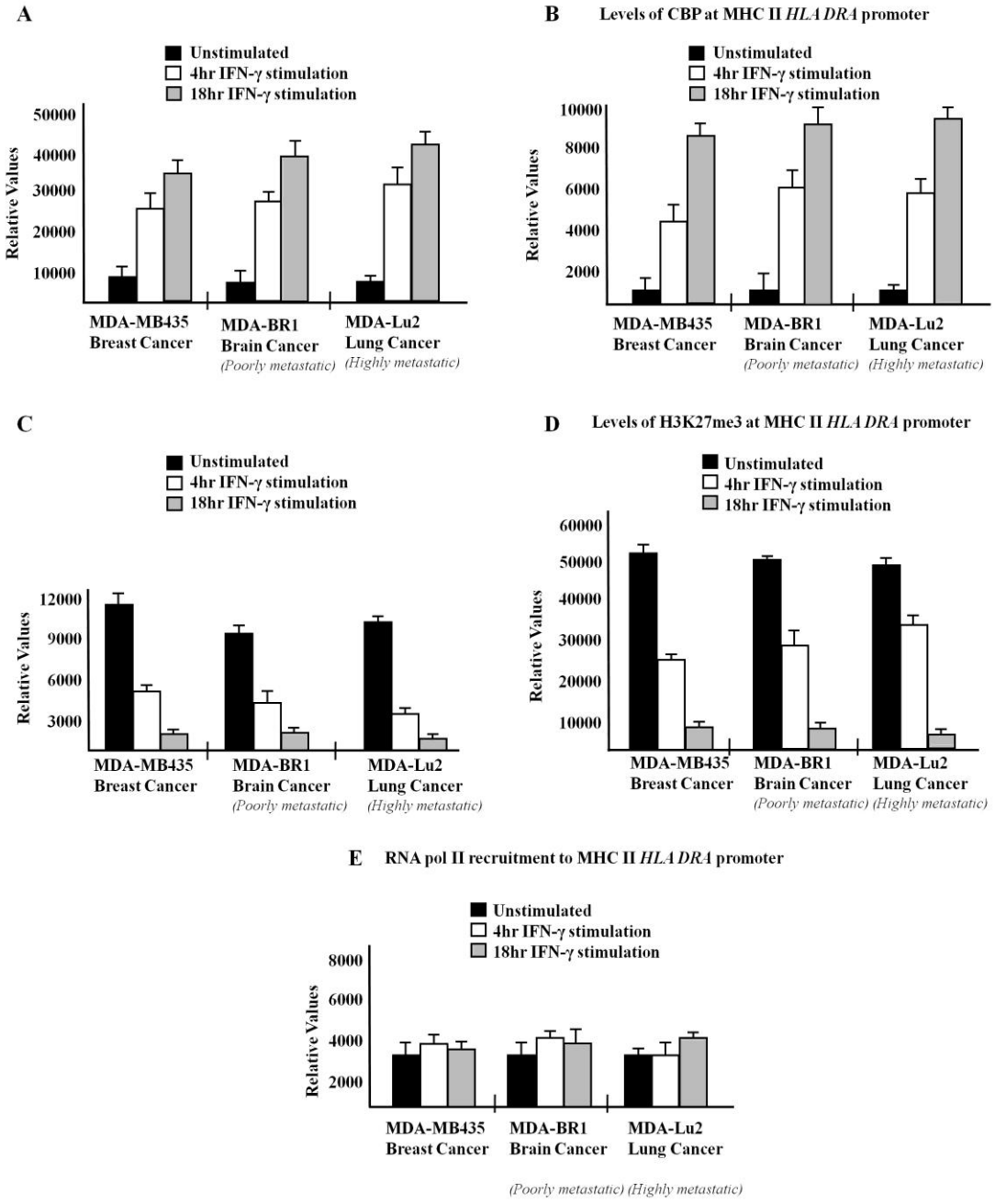


Figure 5.2 Despite expected patterns of epigenetic modifications and enzyme recruitment at MHC II *HLA-DRA*, RNA pol II fails to inducibly bind the proximal promoter region.

Levels of acetylated H3K18 (A), CBP (B), H3K9me3 (C), H3K27me3 (D) and RNA pol II (E) at the MHC II *HLA-DRA* proximal promoter. ChIP assays were carried out in MDA MB 435, MDA MB 435 Brain 1 and MDA MB 435 Lung 2 cells stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody or antibody against indicated endogenous protein and as-

sociated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as a relative increase in MHC II DNA relative to unstimulated samples. Control IP values were 800 ± 200 (A-B), 900 ± 150 (C-D), and 1000 ± 100 (E). Values represent mean ± SEM of three independent experiments.

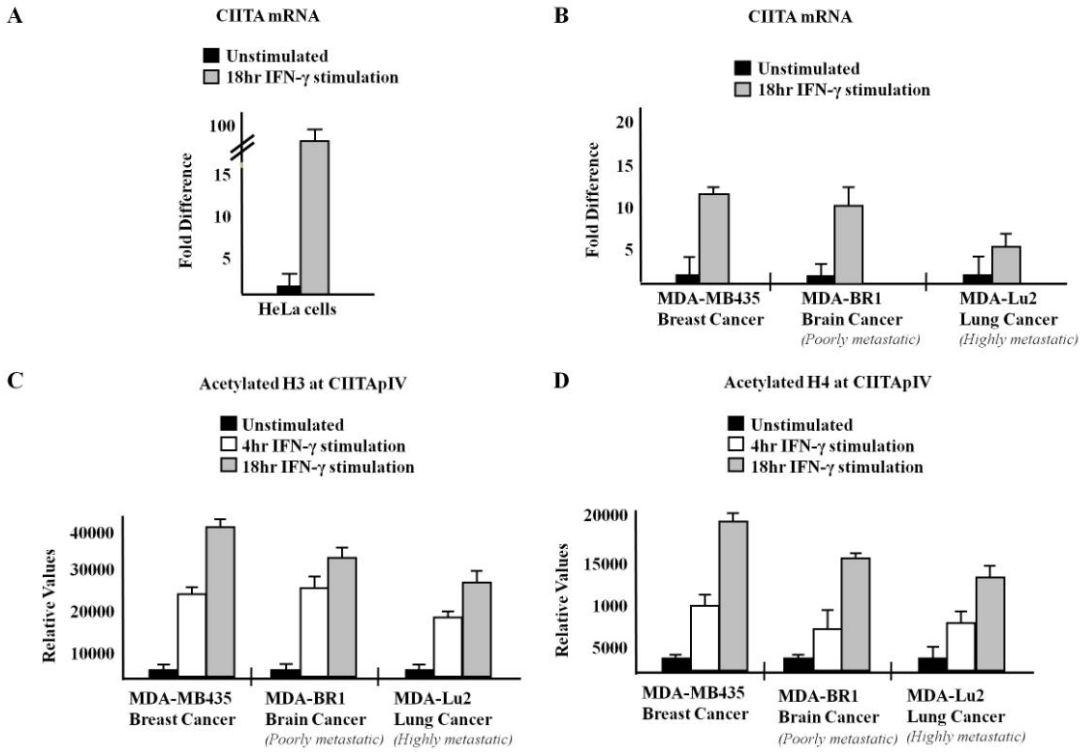


Figure 5.3 Significantly decreased levels of CIITA mRNA in MDA MB 435 breast cancer cells in the presence of lower levels of promoter acetylation (A).

(A-B). HeLa (A) and MDA MB 435, MDA MB 435 Brain 1, MDA MB 435 Lung 2 cells (B) were treated with IFN-γ for 0 to 18 hours. Levels of mRNA were measured by real-time PCR and normalized to GAPDH mRNA. Real-time PCR was performed in triplicate and results represent the mean ± SD of four independent experiments.

(C-D) Levels of acetylated H3 (C) and acetylated H4 (D) at CIITApIV in breast cancer cells 435 Lung 2. ChIP assays were carried out in MDA MB 435, MDA MB 435 Brain 1 and MDA MB 435 Lung 2 cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous acetylated H3 (C) or antibody to endogenous acetylated H4 (D) and associated DNA was isolated and analyzed via real-time PCR using primers spanning the GAS-IRF-E box of CIITApIV. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as a relative increase in CIITApIV DNA relative to unstimulated samples. Control IP values for (C) and (D) were 600 ± 150. Values for control IPs and acetylated H3 (C) acetylated H4 (D) IPs represent mean ± SEM of three independent experiments.

To determine parallel patterns of histone methylation at the MHC II proximal promoter, HeLa cells and MDA MB 435 cell variants were stimulated with IFN-γ and ChIP analysis was performed to determine levels of trimethylation at histone H3 lysine 9 (H3K9me3) and histone H3 lysine 27 (H3K27me3). Levels of H3K9me3 and H3K27me3 were significantly reduced at the MHC II proximal promoter within 4hrs of cytokine stimulation and are eliminated 18 hrs post IFN-γ stimulation in MDA MB 435 cells **(Figure 5.2 C-D).** Similar levels of H3K9me3 **(supplementary Figure 5.10 C)** and H3K27me3 **(supplementary Figure 5.10 D)** were observed at the MHC II proximal promoter in HeLa cells stimulated with IFN-γ for 0-18hrs. These data indicate aberrant histone methylation at the MHC II proximal promoter is not responsible for suppressed cell surface expression of MHC II.

RNA polymerase II fails to inducibly bind the MHC II promoter in MDA MB 435 cells.

Following cytokine stimulation, levels of acetylation increase and levels of methylation levels decrease at the MHC II promoter in MDA MB 435 cells, indicating MHC II promoter associated chromatin is open and available for transcription. To determine assembly of the basal transcription machinery at the MHC II proximal promoter, we investigated promoter recruitment of RNA polymerase II (RNA pol II) in MDA MB 435 cells. Cells were stimulated with IFN-γ for 0-18hrs and subjected to ChIP analyses with antibodies against endogenous RNA pol II. In each of the variants of MDA MB 435 cells, RNA pol II was constitutively bound to the MHC II proximal promoter, but no additional binding occurred in response to IFN-γ stimulation **(Figure 5.2 E).** By comparison, ChIP analyses in HeLa cells indicate robust inducible recruitment of

RNA pol II to the MHC II proximal promoter **(supplementary Figure 5.10 E).** Thus, although the MHC II promoter is epigenetically available, RNA pol II is not inducibly recruited to the open MHC II promoter.

Despite suppression of CIITA mRNA in MDA MB 435 variants, histone H3 and histone H4 are inducibly acetylated at CIITApIV

CIITA expression and subsequent binding to the MHC II proximal promoter is essential for transcriptional activation of MHC II genes. To determine relative expression levels of CIITA mRNA in the MDA MB 435 variants, cells were stimulated with IFN-γ for 0-18hrs, subjected to Trizol extraction, and analyzed via real time PCR using primers specific for CIITA. Levels of CIITA mRNA in each of the MDA MB 435 cell line variants was suppressed and decreased as metastatic ability increases **(Figure 5.3 B)**. Additionally, when comparing with CIITA mRNA levels in HeLa cells **(Figure 5.3 A)** MDA MB 435 breast cancer cells demonstrate about 80 fold decrease. We and others have previously demonstrated that epigenetic modifications play important roles in the transcriptional regulation of CIITA (Truax, Koues et al. ; Koues, Dudley et al. 2008) and in the silencing of MHC II genes in tumors (van den Elsen, van der Stoep et al. 2000; Holling, Schooten et al. 2004; Satoh, Toyota et al. 2004) (Magner, Kazim et al. 2000; Holtz, Choi et al. 2003; Kanaseki, Ikeda et al. 2003; Holling, Bergevoet et al. 2007). To determine global acetylation levels of histone tails at CIITApIV in the MDA MB 435 variants, ChIP assays utilizing antibodies against endogenous acetylated H3 and acetylated H4 were performed. Real time PCR analysis indicated levels of acetylated histone H3 **(Figure 5.3 C)** and acetylated histone H4 **(Figure 5.3 D)** increase at CIITApIV upon stimulation with IFN-γ. By comparison, in

HeLa cells levels of inducible acetylation on histone 3 and histone 4 at CIITApIV are more robust than the levels observed in each of the MDA MB 435 cell variants **(Supplementary Figure 5.11 A and C).** To analyze levels of acetylated H3K18 and association of the HAT CBP at CIITApIV in the MDA MB 435 variants, cells were left untreated or were stimulated with IFN-γ for 0-18 hrs, subjected to IP with antibody to endogenous acetylated H3K18 **(Figure 5.4 A)** or CBP **(Figure 5.4 B)**, and analyzed via real time PCR with primers and probe spanning the CIITApIV proximal promoter. In each of the MDA MB 435 variants, levels of acetylated H3K18 and CBP binding to CIITApIV increased upon cytokine stimulation. Similar to the above observations of global acetylation, levels of inducible acetylation on histone H3K18 and levels of CBP binding, are reduced at CIITApIV in the MDA MB 435 variants compared to HeLa cells **(supplementary Figure 5.12 A and B).**

CIITApIV is specifically and inducibly hypermethylated at CIITApIV in MDA MB 435 cell variants

To determine levels of histone H3 methylation in MDA MB 435 cell variants, ChIP experiments utilizing antibodies against endogenous trimethylated (me3) histone H3 lysine 9 (K9) and lysine 27 (K27) were performed. Real time PCR analysis indicated elevated basal levels of H3K9me3 at CIITApIV in MDA MB 435 cell variants that significantly decrease upon stimulation with IFN-γ in the MDA MB 435 variants **(Figure 5.4 C)** and in HeLa cells **(supplementary Figure 5.12 C)**. Elevated basal levels of H3K27me3 were observed at CIITApIV in MDA MB 435 cell variants; however following the IFN-γ stimulation, levels of H3K27me3 significantly increase with increasing metastatic potential of the MDA MB 435 cell variants **(Figure 5.4 D)**.

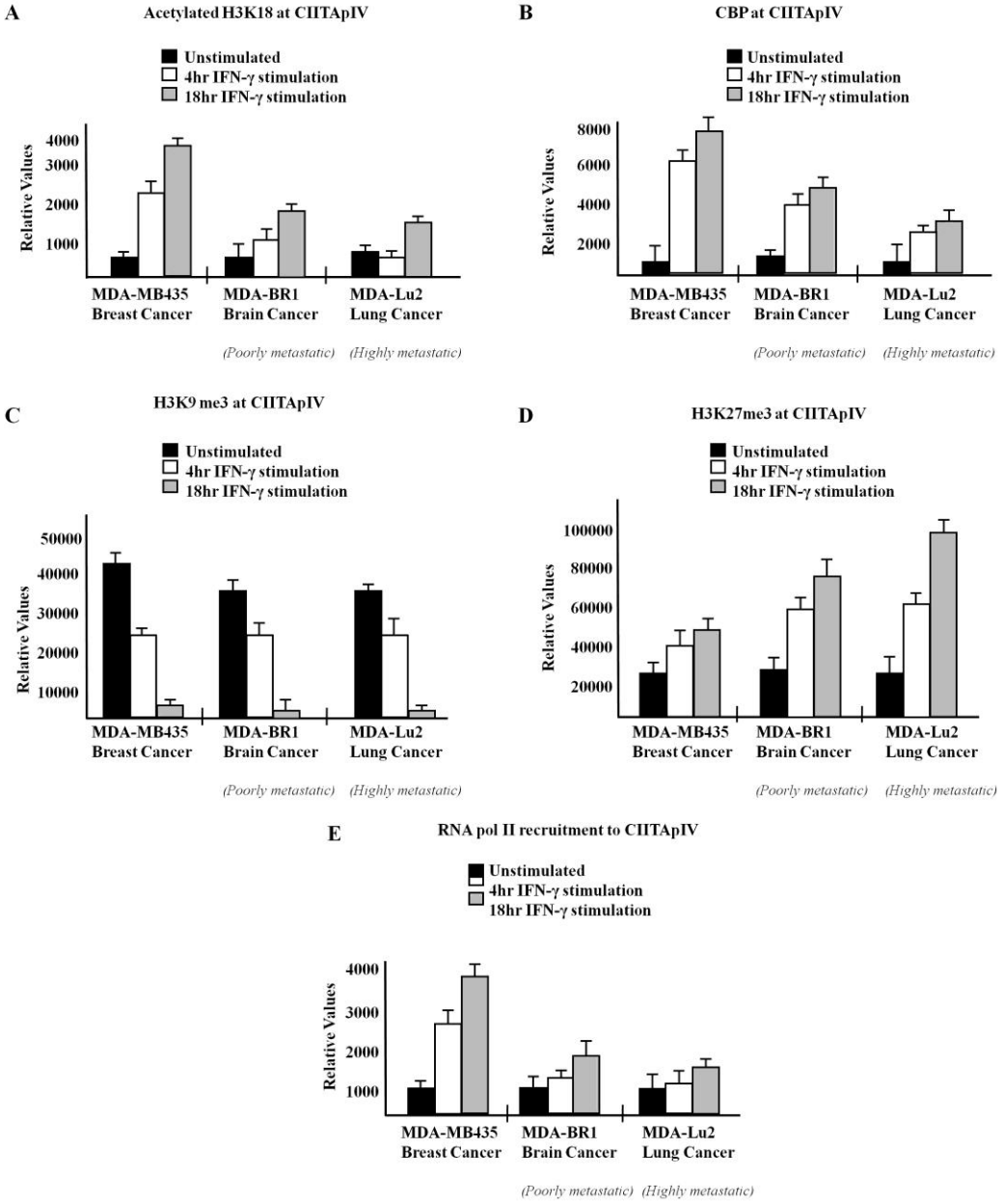


Figure 5.4 Decreased CIITApIV levels of H3K18 acetylation and decreased CPB and RNA Pol II recruitment correlates with significantly elevated levels of H3K27me3 in metastatic cells.

(A-E) Levels of acetylated H3K18, CBP, H3K9me3, H3K27me3 or RNA pol II at CIITApIV. ChIP assays were carried out in MDA MB 435, MDA MB 435 Brain 1 and MDA MB 435 Lung 2 cells stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody or antibody to indicated endogenous proteins and associated DNA was isolated and analyzed via real-time PCR using primers spanning the IRF-E-GAS box of the CIITApIV proximal promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction

(input). Input values represent 5% of the total cell lysate. IP values are presented as a relative increase in CIITA isoform IV relative to unstimulated samples. Control IP values were 800 ± 200 (A); 750 ± 150 (B); 1000 ± 100 (C-D); and 600 ± 120 (E). Values represent mean ± SEM of three to five independent experiments.

The inducible hypermethylation of lysine 27observed at CIITApIV is cell line specific as ChIP assays performed in HeLa cells demonstrate elevated levels of H3K27me3 at CIITApIV that decrease upon IFN-γ stimulation **(Supplementary Figure 5.12 D)**. To determine if CIITApIV hypermethylation is sequence specific, ChIP experiments were performed to detect endogenous levels of H3K27me3, H3K9me3, and H3K18ac at the GAPDH promoter **(supplementary Figure 5.13)**. These data demonstrate low levels of methylation and high levels of acetylation at the GAPDH promoter that are unchanged by IFN-γ stimulation and do not significantly differ between the MDA MB 435 cell variants. Gains in H3K27methylation at CIITApIV in the MDA MB 435 cell variants are not an indicative of increases in levels of histone H3 or histone H4 as ChIP assays demonstrate no significant changes in the level of histone H3 **(supplementary Figure 5.11 B)** and histone H4 **(supplementary Figure 5.11 D)** in the MDA MB 435 cell variants. In sum these data indicate the elevated levels of inducible H3K27me3 at CIITApIV are responsible for the suppressed levels of CIITA mRNA in the MDA MB 435 cell variants.

IFN-γ inducible recruitment of RNA pol II, STAT-1, and IRF-1 to CIITApIV is diminished in MDA MB 435 cell variants

An open chromatin confirmation is required for the initiation of transcription. Data in Fig. 4D indicate that the CIITApIV proximal promoter is methylated and in a closed confirmation in MDA MB 435 cell variants. To further confirm the status of chromatin at CIITApIV in these cells, promoter recruitment of RNA pol II was analyzed by ChIP assay. Cells were stimulated with IFN-γ for 0-18hrs and were subjected to immunoprecipitation with antibody against

endogenous RNA pol II. In each of the variants of MDA MB 435 cells, RNA pol II was constitutively bound to CIITApIV, but only in the MDA-MB3435 Breast line was further significant binding induced by IFN-γ stimulation **(Figure 5.4 E).** In the MDA-BR1 Brain and MDA-Lu2 Lung lines, no additional significant RNA pol II binding occurred in response to IFN-γ stimulation **(Figure 5.4 E).** By comparison, ChIP analyses in HeLa cells demonstrate robust inducible recruitment of RNA pol II to CIITApIV **(supplementary Figure 5.12 E).**

The transcription factors STAT-1 and IRF-1 are required for CIITApIV transcription in response to IFN-γ stimulation (Piskurich, Linhoff et al. 1999). Western blot analyses were performed to determine if the lack of CIITA mRNA in MDA MB 435 cell variants was due to reduced expression of STAT-1 and IRF-1. Levels of STAT-1 **(Figure 5.5 A)** and IRF-1 **(Figure 5.5 B)** remain unchanged in the three variants of MDA MB 435 cells indicating both STAT-1 and IRF-1 are expressed and available for binding CIITApIV. Next, we performed ChIP assays to investigate binding of STAT-1 and IRF-1 to CIITApIV in each of the MDA MB 435 cell variants and HeLa cells. Cells were stimulated with IFN-γ for 0-18hrs and subjected to immunoprecipitation with antibody against endogenous STAT-1 or endogenous IRF-1. ChIP data indicate low levels of STAT-1 IRF-1 are recruited to CIITApIV in each of the MDA MB 435 cell variants with minimal enhancement of binding upon IFN-γ stimulation **(Figure 5.5 C-D).** In addition MDA MB 435 breast cancer cells showed lower levels of STAT-1 **(Figure 5.5 C)** and IRF-1 **(Figure 5.5 D)** at CIITApIV in comparison to control HeLa cells **(Figure 5.5 C and D upper panel).** In sum, hypermethylation of CIITApIV results in significantly reduced recruitment of STAT-1 and IRF-1 and prevents inducible binding RNA pol II in response to cytokine stimulation.

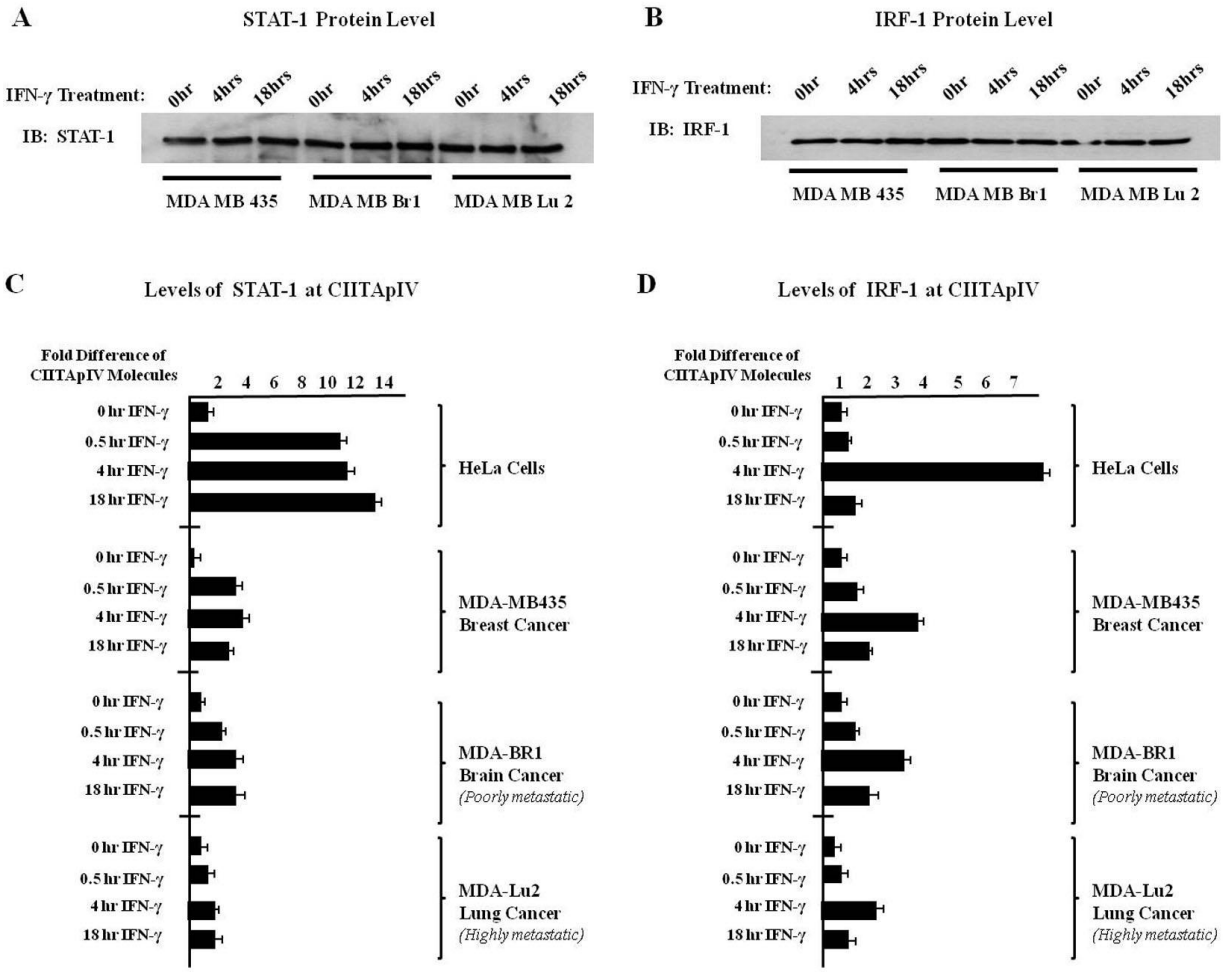


Figure 5.5 Although expressed, recruitment of STAT-1 and IRF-1 to CIITApIV decreases in metastatic breast cancer cells.

(A-B) Expression of STAT-1 (A) and IRF-1 in parental MDA MB 435 (left), in 435 Brain 1 (middle), and in 435 Lung 2 cells (right). MDA MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cells were left untreated or stimulated with IFN-γ as indicated. Lysates were subjected to IB for STAT-1 (A) or IRF-1 (B). Results reported are data representative of two independent experiments. (C-D) ChIP assays were carried out in MDA MB 435, MDA MB 435 Brain 1 and MDA MB 435 Lung 2 and HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were immunoprecipitated (IP) with control antibody, with antibody to endogenous STAT-1 (C) or IRF-1 (D), and associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITA isoform IV proximal promoter. IP values are presented as relative increase in CIITApIV promoter DNA relative to unstimulated STAT-1(C) and IRF-1(D) IP samples. Control IP values for (C) and (D) were 0.8 ± 0.4. Values for control IPs, STAT-1 and IRF-1 IPs represent mean ± SEM of three independent experiments.

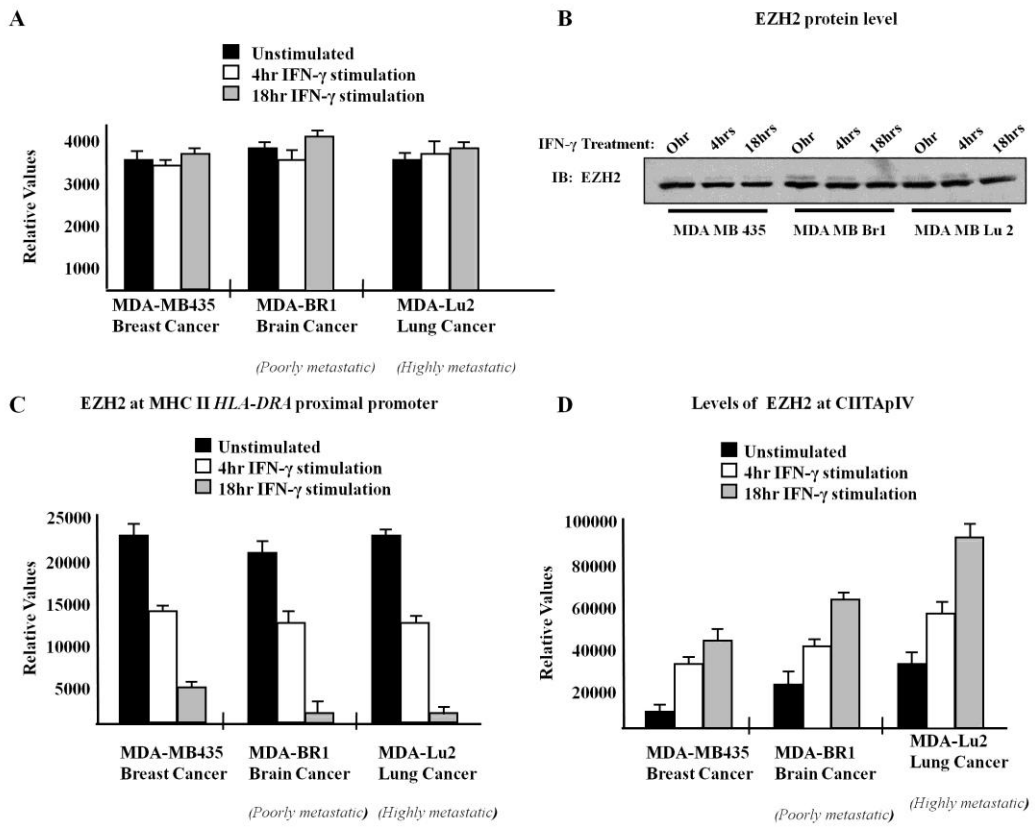


Figure 5.6 In MDA MB cells, IFN-γ stimulation results in specific and significantly increased binding of EZH2 at CIITApIV.

(A) EZH2 mRNA expression. MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cells were stimulated with IFN-γ for 0 to 18 hours. Cell lysates were analyzed for levels of EZH2 mRNA by real-time PCR and were normalized to GAPDH mRNA. Real-time PCR was performed in triplicate and results represent the mean ± SD of three independent experiments. (B) EZH2 protein expression. MDA MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cells were stimulated with IFN-γ, harvested and subjected to Western Blot analysis of endogenous EZH2. Results shown are representative data of three independent experiments. (C -D) Levels of EZH2 at MHC II (C) and CIITApIV (D) proximal promoters post cytokine stimulation. Double crosslinking ChIP assays were carried out in cells stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody and antibody to endogenous EZH2 and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC-II *HLA-DRA* (C) and primers spanning IRF-E-GAS box of CIITApIV (D) promoters. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as relative increase in the MHC II (C) and CIITA (D) promoter DNA relative to unstimulated samples. Control IP values for (C and D) were 1500 ± 350. Values for control and EZH2 IPs represent mean ± SEM of three independent experiments.

Lack of transcription factor and RNA pol II binding at CIITApIV leads to significantly reduced expression of CIITA and MHC II in the poorly metastatic 435 Brain 1 and highly metastatic 435 Lung 2 cells.

Binding of the histone methyltransferase EZH2 to CIITApIV is significantly and specifically increased in the MDA MB 435 cell variants

Histone methyltransferases (HMTs) are chromatin remodeling enzymes that add one, two, or three methyl groups to lysine residues on histones (Bannister and Kouzarides 2004). We have recently demonstrated the HMT enhancer of zeste homolog 2 (EZH2), a known regulator H3K9me3 and H3K27me3 (Humphreys, Lyle et al. 2000; Cao, Wang et al. 2002), to be a critical regulator of IFN-γ inducible transcription from CIITApIV (Mehta, Truax et al. 2011). Initial analyses confirm that each of the MDA MB 435 variants express similar levels of EZH2 mRNA **(Figure 5.6 A)** and EZH2 protein **(Figure 5.6 B).** To determine if EZH2 aberrantly binds CIITApIV in the MDA MB 435 cell variants, ChIP assays were performed. Cells were stimulated with IFN-γ for 0-18hrs and were subjected to immunoprecipitation with antibody against endogenous EZH2. Chromatin immunoprecipitations show that EZH2 binds the MHC II promoter **(Figure 5.6 C)** and CIITApIV **(Figure 5.6 D)** in unstimulated cells (note differences in Y axis). Four hours post cytokine stimulation, EZH2 occupancy decreases at the MHC II proximal and reaches baseline binding 18hrs following cytokine stimulation **(Figure 5.6 C)**. Similar patterns of EZH2 binding to the MHC II proximal promoter were observed in HeLa cells stimulated with IFN-γ for 0-18hrs **(supplementary Figure 5.14 A**). Striking differences in EZH2 binding pat-

terns were observed at CIITApIV in the MDA MB 435 variants. In unstimulated cells, EZH2 binds to CIITApIV at levels similar to that of the MHC II proximal promoter. However, upon cytokine stimulation, EZH2 binding increases in each variant of MDA MB 435 cells at both four and 18 hrs post IFN-γ stimulation **(Figure 5.6 D)**. By comparison, in HeLa cells, patterns of EZH2 binding to CIITApIV (**supplementary Figure 5.14 B)** are similar to binding of EZH2 at the MHC II proximal promoter **(supplementary Figure 5.14 A).**

Knockdown of EZH2 significantly reduces H3K27me3 at CIITApIV in the MDA MB 435 variants

To investigate roles for EZH2 in the suppression of CIITApIV in the MDA MB 435 variants, we utilized siRNA duplexes to specifically knock down endogenous expression of EZH2 and then performed ChIP assays to detect levels of H3K27me3 at CIITApIV. siRNA mediated knockdown of EZH2 resulted in a >90% decrease in endogenous EZH2 protein expression but did not impact endogenous tubulin expression **(Figure 5.7 A)**. To further determine efficiency of the siRNA duplexes, EZH2 mRNA levels were tested in each of the MDA MB 435 variants **(Figure 5.7 B).** Cells treated with EZH2 specific siRNA (black bars) showed a 90% reduction in EZH2 mRNA levels when compared to cells treated with control siRNA (white bars), indicating EZH2 siRNA specifically and efficiently decreases EZH2 in the presence or absence of cytokine stimulation.

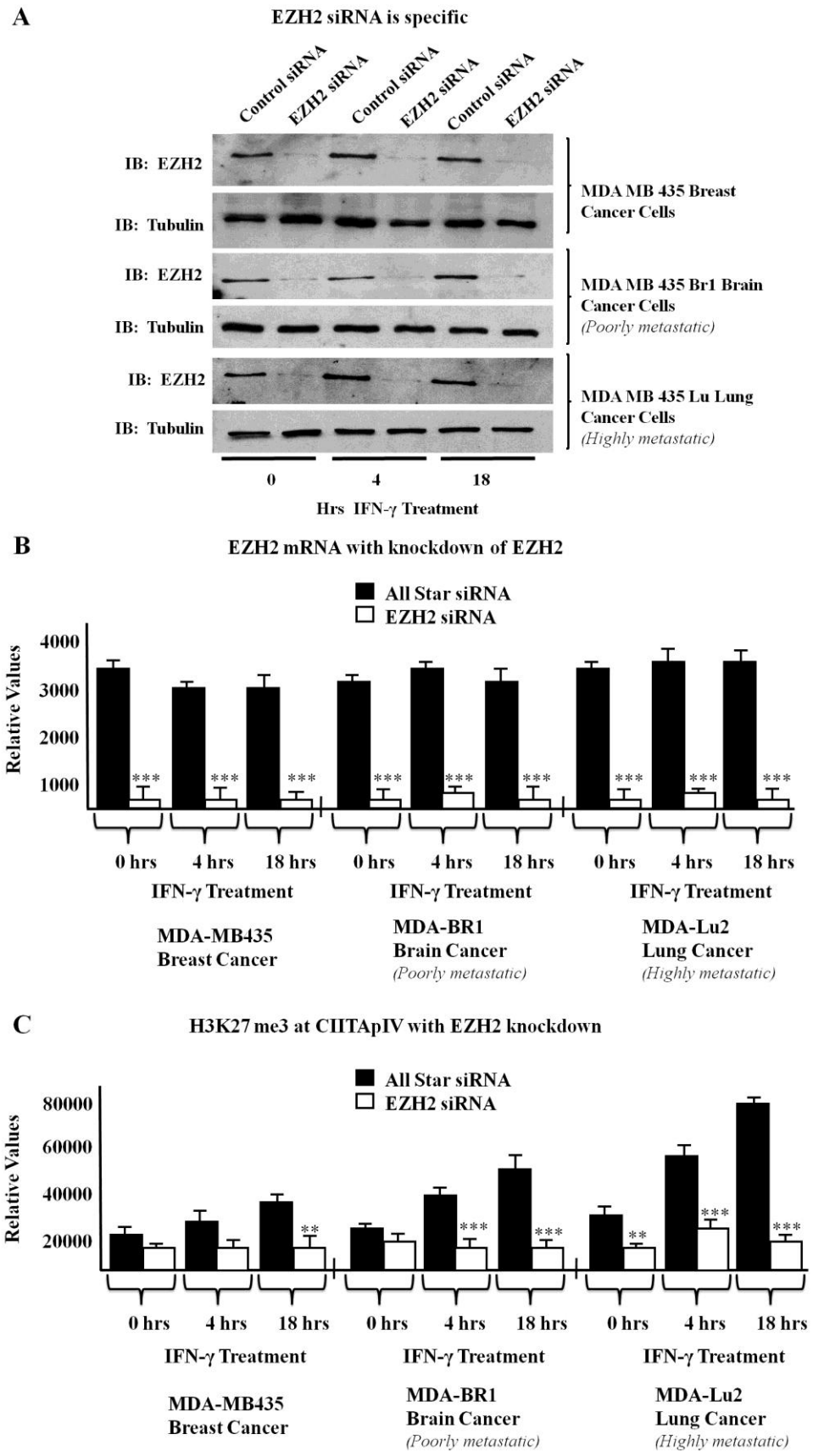
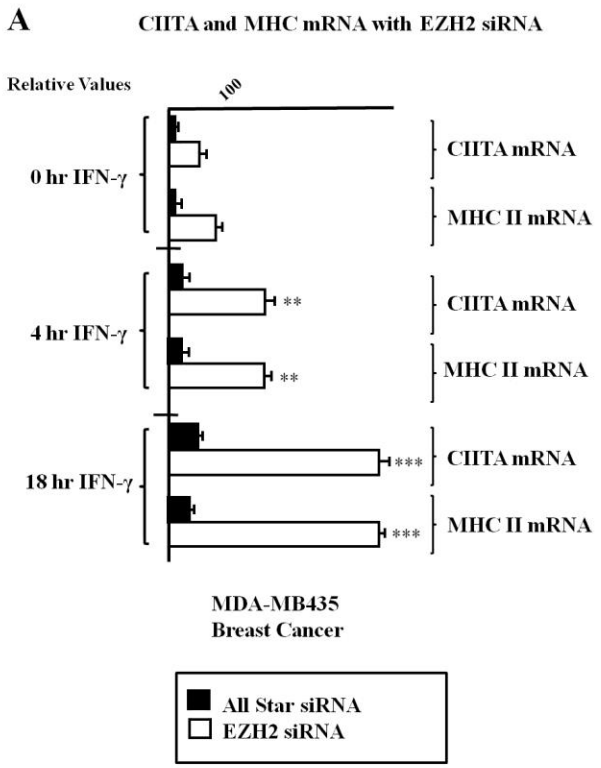
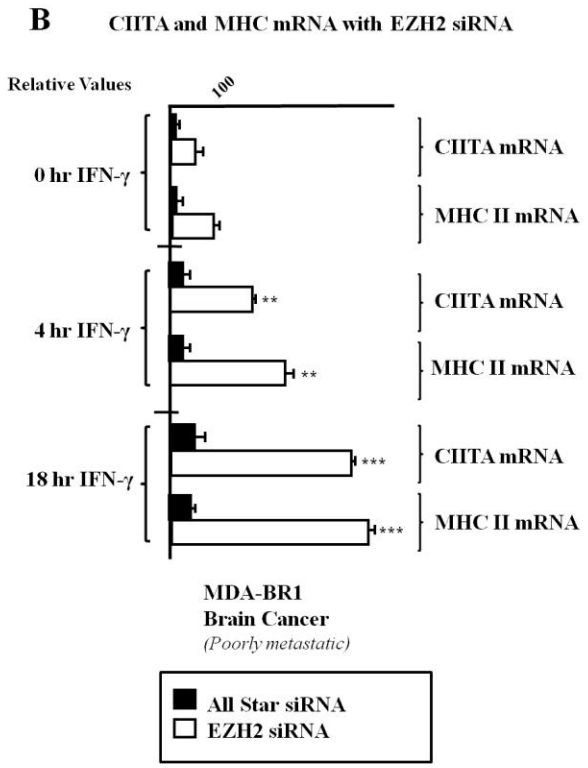

A
EZH2 siRNA is specific
Control siRNA
EZH2 siRNA
Control siRNA
EZH2 siRNA
Control siRNA
EZH2 siRNA
IB: EZH2
IB: Tubulin
MDA MB 435 Breast Cancer Cells
IB: EZH2
IB: Tubulin
MDA MB 435 Br1 Brain Cancer Cells
(Poorly metastatic)
IB: EZH2
IB: Tubulin
MDA MB 435 Lu Lung Cancer Cells
(Highly metastatic)
0
4
18
Hrs IFN-γ Treatment
B
EZH2 mRNA with knockdown of EZH2
All Star siRNA
EZH2 siRNA
Relative Values
4000
3000
2000
1000
***
0 hrs
4 hrs
18 hrs
IFN-γ Treatment
MDA-MB435
Breast Cancer
MDA-BR1
Brain Cancer
(Poorly metastatic)
MDA-Lu2
Lung Cancer
(Highly metastatic)
C
H3K27 me3 at CIITApIV with EZH2 knockdown
All Star siRNA
EZH2 siRNA
Relative Values
80000
60000
40000
20000
**
***
0 hrs
4 hrs
18 hrs
IFN-γ Treatment
MDA-MB435
Breast Cancer
MDA-BR1
Brain Cancer
(Poorly metastatic)
MDA-Lu2
Lung Cancer
(Highly metastatic)

Figure 5.7 EZH2 knockdown decreases CIITApIV histone H3K27 trimethylation.

(A-B) EZH2 knockdown is efficient and specific. MDA MB 435, MDA MB 435 Brain 1, and MDA MB Lung 2 cells were transfected with either EZH2 specific or control siRNA. 10% of the lysates were subjected to IB for endogenous EZH2 or tubulin. Results reported are representative data of three independent experiments. The remaining lysates were subjected to EZH2 mRNA isolation, quantization by real-time PCR, and normalization to GAPDH mRNA as above. Real-time PCR was performed in triplicate and results represent the mean ± SD of three independent experiments.

(C) Levels of trimethylated H3K27 in breast cancer cells. ChIP assays were carried out in MDA MB 435, MDA MB 435 Brain 1, and MDA MB Lung 2 cells transfected with either EZH2 or control siRNA and stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody or antibody to endogenous H3K27me3 and associated DNA was isolated and analyzed via real-time PCR using primers spanning the IRF-E-GAS box of the CIITApIV promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as relative increase in CIITA cDNA relative to unstimulated samples. Control IP values were 950 ± 300. Values represent mean ± SEM of three independent experiments.


A
CIITA and MHC mRNA with EZH2 siRNA
Relative Values
100
0 hr IFN-γ
4 hr IFN-γ
18 hr IFN-γ
CIITA mRNA
MHC II mRNA
**
***
MDA-MB435
Breast Cancer
All Star siRNA
EZH2 siRNA


B
CIITA and MHC mRNA with EZH2 siRNA
Relative Values
100
0 hr IFN-γ
4 hr IFN-γ
18 hr IFN-γ
CIITA mRNA
MHC II mRNA
**
***
MDA-BR1
Brain Cancer
(Poorly metastatic)
All Star siRNA
EZH2 siRNA

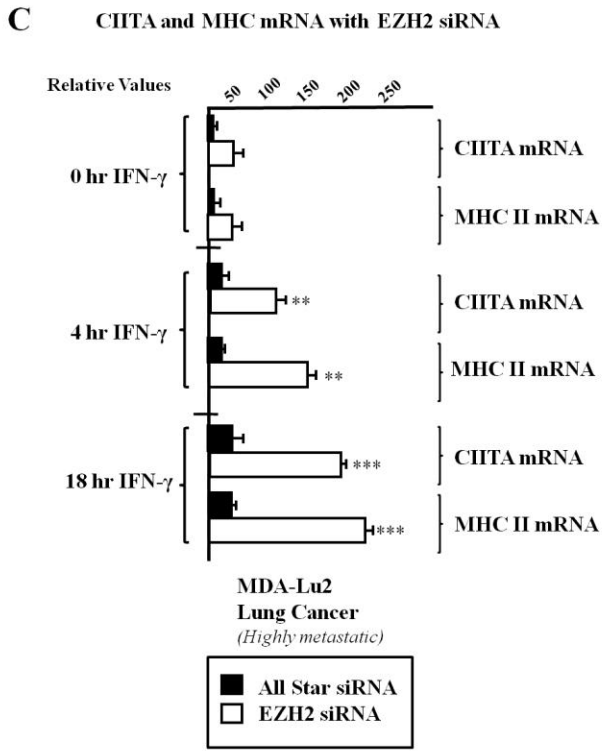

C
CIITA and MHC mRNA with EZH2 siRNA
Relative Values
50
100
150
200
250
0 hr IFN-γ
4 hr IFN-γ
18 hr IFN-γ
CIITA mRNA
MHC II mRNA
**
***
MDA-Lu2
Lung Cancer
(Highly metastatic)
All Star siRNA
EZH2 siRNA

Figure 5.8 EZH2 knockdown significantly increases mRNA levels of CIITA and MHC II in MDA MB 435 (A), MDA MB 435 Br-1 (B), and MDA MB 435 Lu 2 cells (C).

MDA MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cells were transfected with either EZH2 specific or with control siRNA and were stimulated with IFN-γ for 0 to 18 hours. Levels of MHC II and CIITA mRNA were measured by real-time PCR and were normalized to GAPDH mRNA. Real-time PCR was performed in triplicate and results represent the mean ± SD of three independent experiments. **.**

To determine levels of H3K27me3 at CIITApIV in the EZH2 siRNA treated MDA MB 435 cell variants, ChIP assays were performed. As seen previously at CIITApIV in the MDA MB 435 variants, in cells treated with control siRNA, levels of H3K27me3 increase at CIITApIV upon IFN-γ stimulation **(Figure 5.7 C, black bars)**.

When specific siRNA was used to knockdown EZH2, significant decreases in CIITApIV H3K27me3 were observed in each of the MDA MB 435 variants upon IFN-γ treatment **(Figure 5.7C, white bars)**. These data suggest EZH2 is responsible for the elevated levels of H3K27me3 in the MDA MB 435 variants.

Knocking down EZH2 restores suppressed levels of CIITA and MHC II mRNA in each of the MDA MB 435 variants

To determine if decreased expression of EZH2 and the resulting decrease in CIITApIV H3K27me3 regulates activation of CIITA and MHC II genes in the MDA MB 435 variants, mRNA experiments were performed. Cells were transfected with EZH2 specific or control siR-NA as indicated and were stimulated with IFN-γ for 0-18 hrs. Following stimulation cells were lysed and CIITA and MHC II mRNA levels were quantified via real time PCR. Both CIITA and MHC II mRNA levels are significantly elevated in cells treated with EZH2 specific siRNA **(Figure 5.8 A-C, white bars)** when compared to cells treated with control siRNA **(Figure 5.8 A-C,**

black bars). Knocking down EZH2 restored CIITA and MHC II message levels in each of the MDA MB 435 variants, both with and without IFN-γ stimulation.

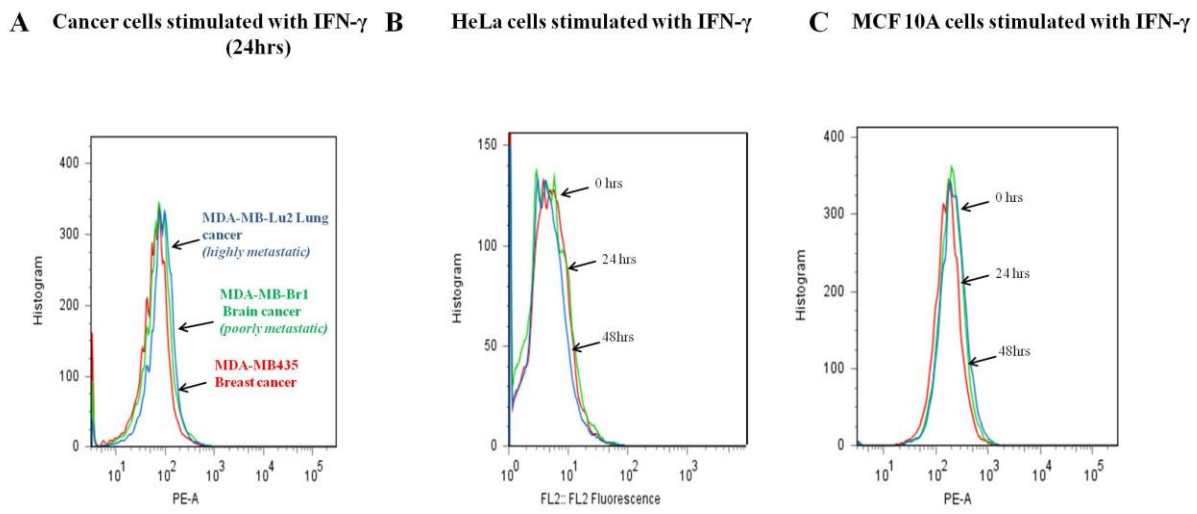


Figure 5.9 (Supplementary Figure 1) Control cell surface staining for variants of MDA MB 435 breast cancer cells (A) HeLa (B) and MCF 10A (C).

(A-C) MDA MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cell (A) HeLa cells (B) and MCF 10A cells (C) were stimulated with IFN-γ as indicated. Post stimulation cells were trypsinized, washed, and incubated with PE-labeled mouse control IgG. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto. Results shown are representative of three independent experiments.

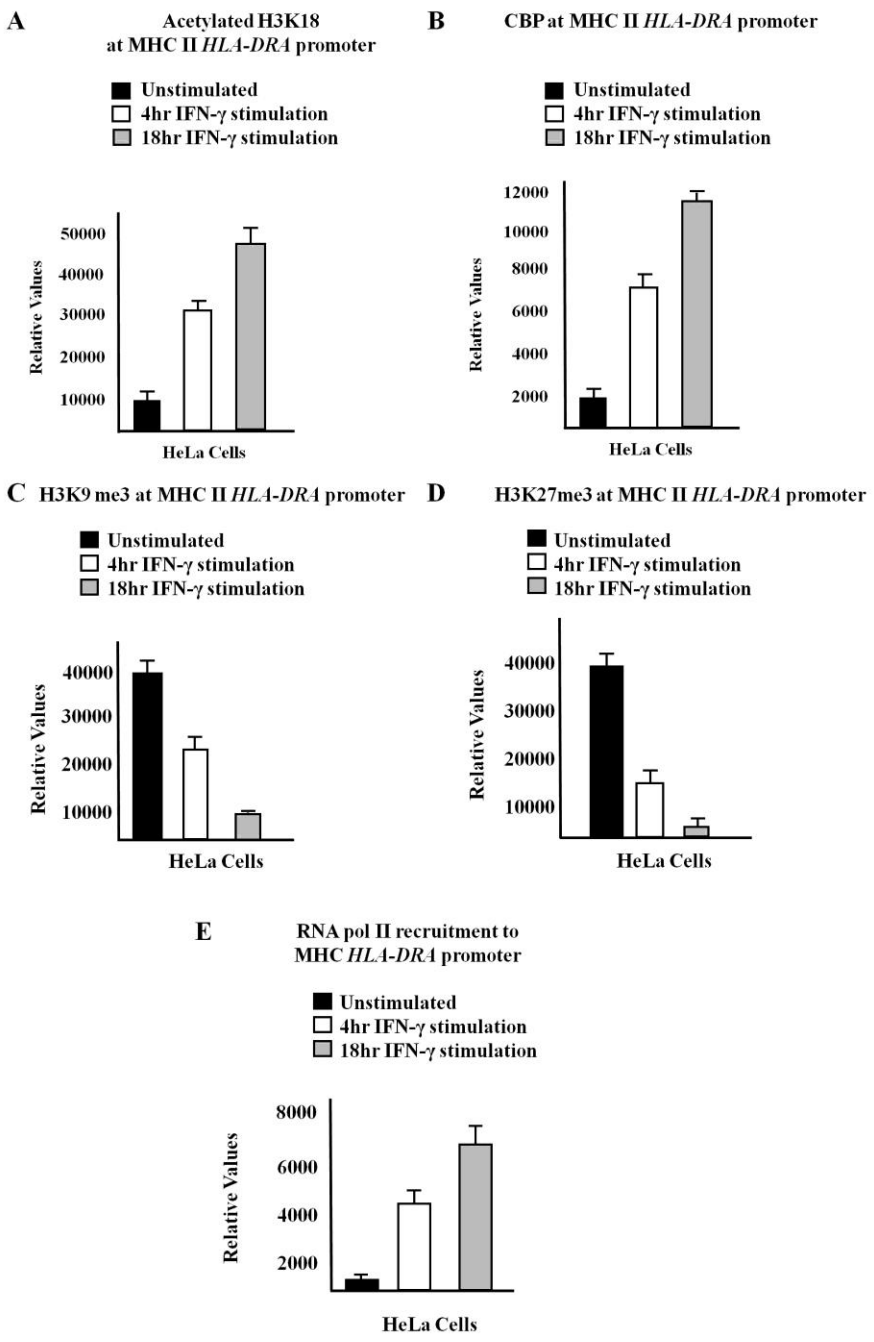


Figure 5.10 (Supplementary Figure 2) IFN-γ induced levels of acetylated H3K18, CBP, H3K9me3, H3K27me3 and RNA pol II at MHC II *HLA-DRA* in HeLa cells.

(A-E) Levels of H3K18 acetylation (A), CBP (B) trimethylated H3K9 (C), trimethylated H3K27 me3 (D), and RNA pol II (E) at MHC II. ChIP assays were carried out in HeLa cells stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody or antibody to indicated endogenous proteins and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of promoter DNA added to the reaction (input). Input values

represent 5% of the total cell lysate. IP values are presented as a relative increase in MHC II DNA relative to unstimulated samples. Control IP values were 550 ± 100 (A-B); were 450 ± 100 (C-D); and 850 ± 150 (E). Values represent mean ± SEM of two independent experiments.

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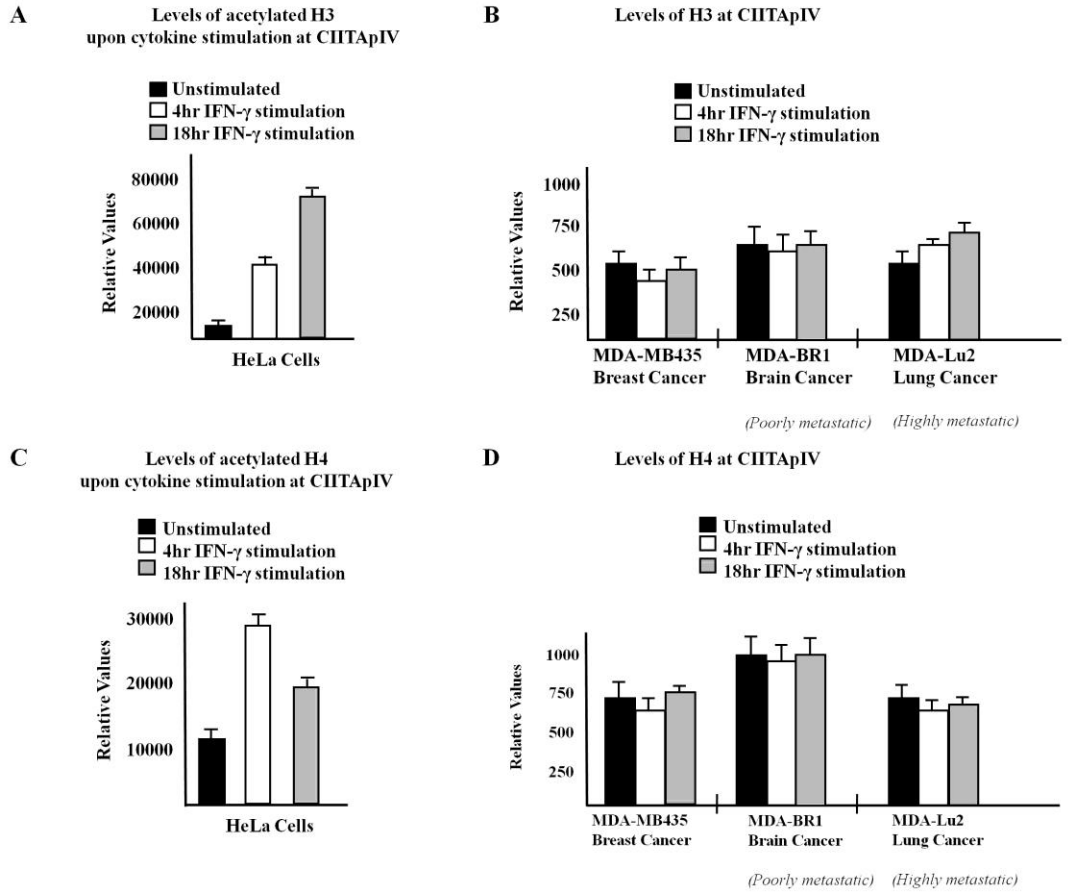

Figure 5.11 (Supplementary Figure 3) Levels of acetylated H3 and H4 increase in HeLa cells stimulated with IFN-γ and levels of histone H3 and H4 are unchanged in MDA MB 435 cells.

(A and C) Levels of acetylated H3 (A) and acetylated H4 (C) at CIITApIV increase upon IFN-γ stimulation in HeLa cells. Cells were stimulated as indicated. Following stimulation lysates were subjected to ChIP analyses and IP with control antibody or antibody against endogenous acetylated H3 (A) or acetylated H4 (C). DNA was analyzed via real time PCR with primers and probes spanning CIITApIV proximal promoter. IP values are graphed as increase over unstimulated samples. Control IP values were 780 ± 200. Values represent mean ± SEM of two independent experiments. (B and D) Constant levels of H3 (B) and H4 (D) at CIITApIV in MDA MB 435 cells. ChIP analyses were performed in MDA MB 435, MDA MB 435 Brain 1, and MDA MB 435 Lung 2 cells stimulated with IFN-γ for 0 to 18 hours. Lysates were IP with control antibody or antibody to endogenous histone 3 (B) or endogenous histone H4 (D) and associated DNA was isolated and analyzed via real-time PCR using primers for the CIITApIV promoter. Real-time PCR values were normalized as above and control IP values for (B and D) were 600 ± 100. Values represent mean ± SEM of two independent experiments.

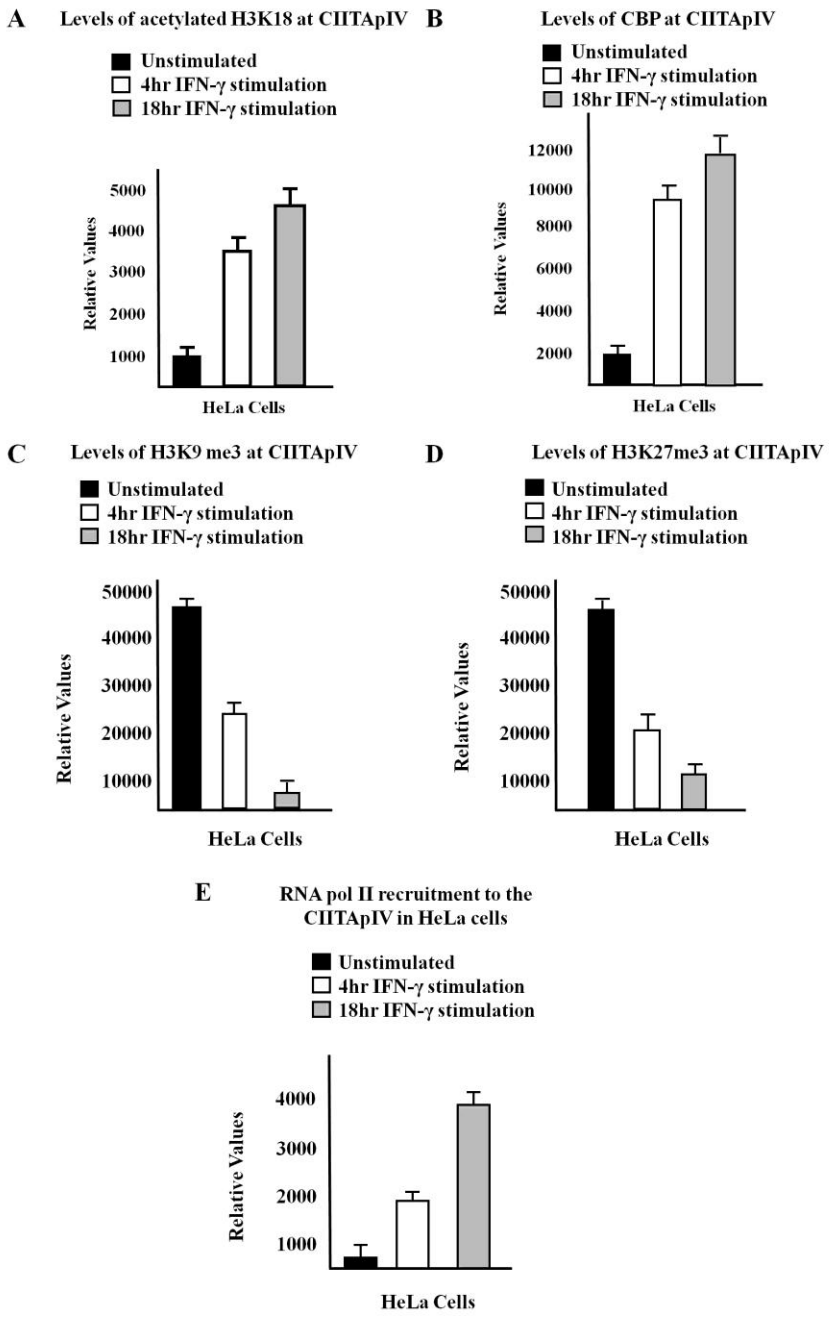

Figure 5.12 (Supplementary Figure 4) Levels of H3K18 acetylation, CBP, H3K9me3, H3K27me3, and RNA pol II at CIITApIV in HeLa cells.

(A-E) Levels of H3K18 acetylation (A), CBP (B) trimethylated H3K9 (C), trimethylated H3K27 (D), and RNA pol II (E) at CIITApIV in HeLa cells. HeLa cells were stimulated with IFN-γ for 0-18hrs and subjected to ChIP assay as in supplementary Fig. 2 above. Lysates were IP with iso-type control antibodies or antibodies against indicated endogenous proteins. DNA was analyzed via real time PCR as described in supplementary Fig. 2 using primers specific for CIITApIV.

Data are presented as relative increase in CIITA relative to unstimulated samples. Isotype control values were 540 ± 200 (A-B); 950 ± 200 (C-D) and 800± 200 (E). Values represent mean ± SEM of two independent experiments.

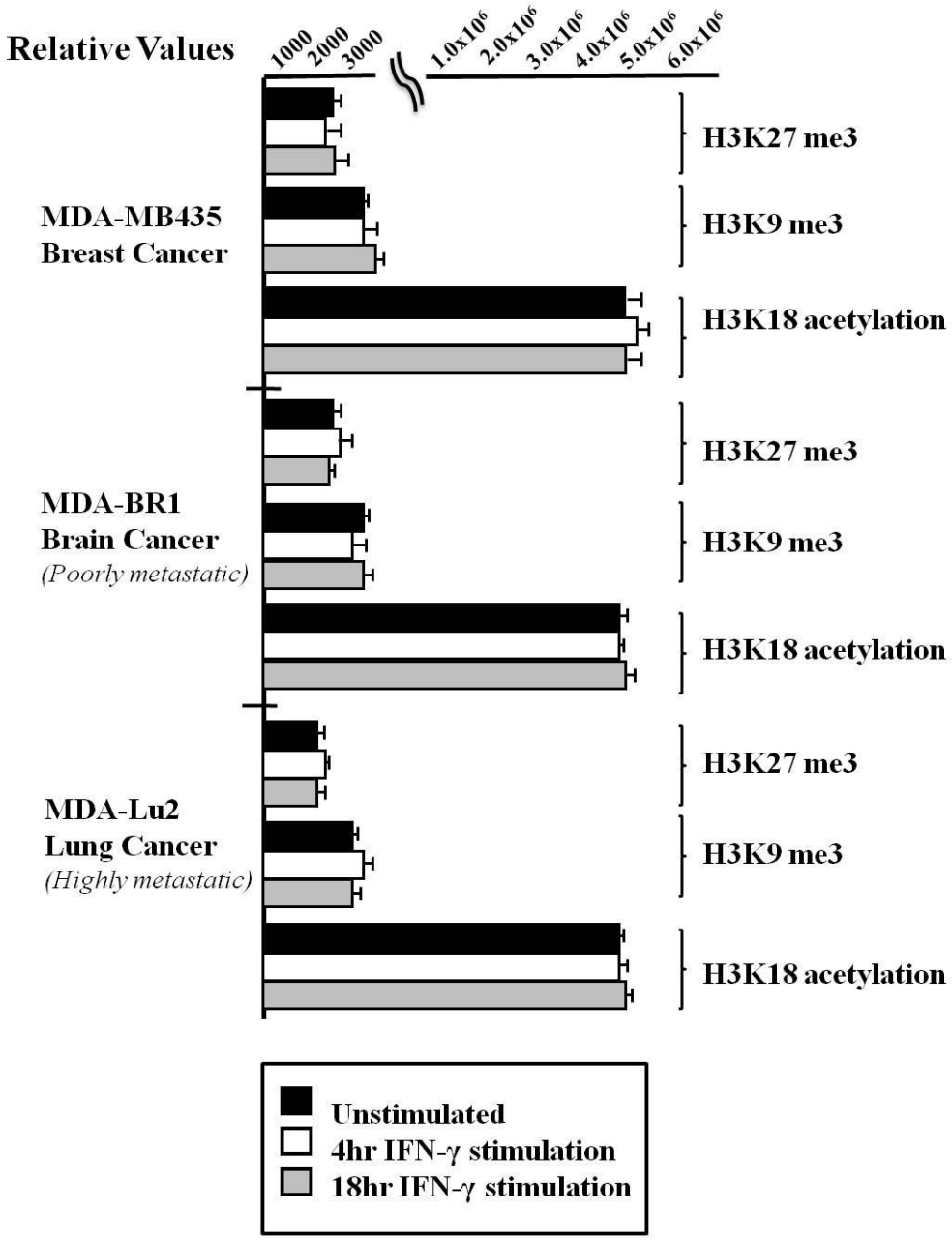
Relative Values
1000
2000
3000
1.0x10^6
2.0x10^6
3.0x10^6
4.0x10^6
5.0x10^6
6.0x10^6
MDA-MB435
Breast Cancer
H3K27 me3
H3K9 me3
H3K18 acetylation
MDA-BR1
Brain Cancer
(Poorly metastatic)
H3K27 me3
H3K9 me3
H3K18 acetylation
MDA-Lu2
Lung Cancer
(Highly metastatic)
H3K27 me3
H3K9 me3
H3K18 acetylation
Unstimulated
4hr IFN-γ stimulation
18hr IFN-γ stimulation

Figure 5.13 (Supplementary Figure 5) Levels of H3K27me3, H3K9 me3, and H3K18 acetylation at the GAPDH proximal promoter are unaffected by cytokine in MDA MB 435 (top), MDA MB 435 Brain 1 (middle), and MDA MB 435 Lung 2 cells (bottom).

Cells were stimulated as indicated and were subjected to ChIP assay as above. Lysates were IP with isotype control antibodies or antibodies against indicated endogenous. Associated DNA was isolated and analyzed via real time PCR as described in supplementary Fig.2 using primers specific for the GAPDH proximal promoter. Data are presented as relative increase in GAPDH DNA relative to unstimulated samples. Isotype control values were 700 ± 200. Values represent mean ± SEM of two independent experiments.

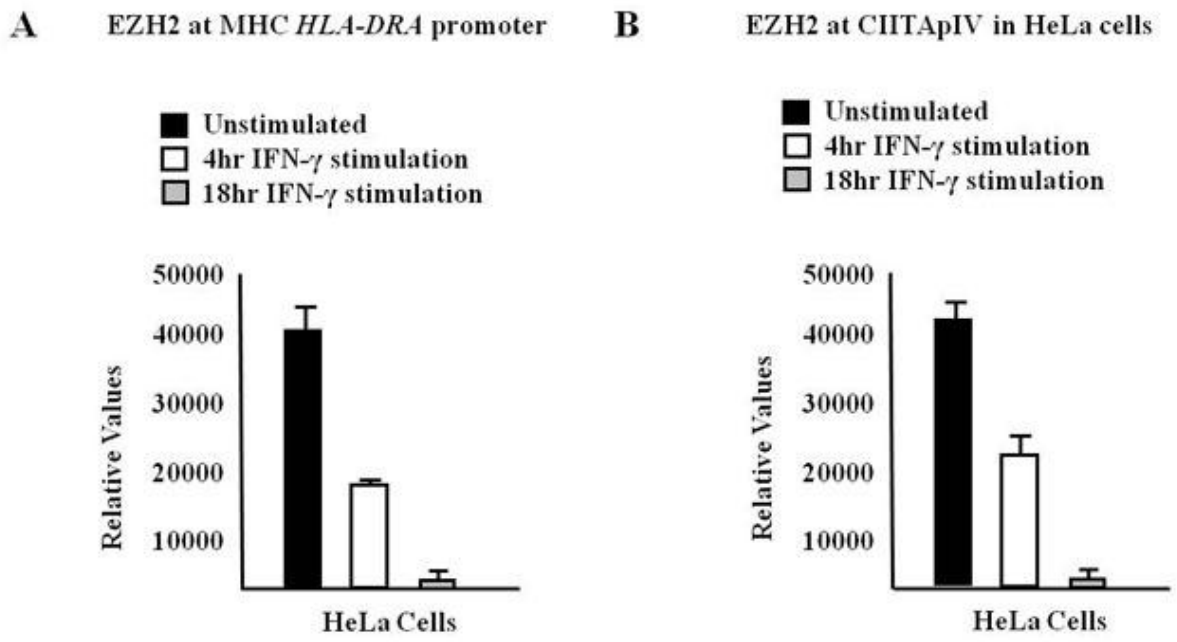

Figure 5.14 (Supplementary Figure 6) Levels of EZH2 decrease at MHC II and CIITApIV post cytokine stimulation.

(A-B) Levels of EZH2 at MHC II (A) and CITApIV (B) decrease with cytokine stimulation in HeLa cells. HeLa cells were stimulated with IFN-γ as indicated. Lysates were IP with isotype control antibody or with antibody against endogenous EZH2. Associated DNA was analyzed via real time PCR using primers for MHC II (A) or for the CIITApIV (B) proximal promoter. Graphed values were normalized to unstimulated samples. Control IP values for A were 500 ± 150 (A) and 950 ± 250 (B). Values represent mean ± SEM of two independent experiments.

5.4 DISCUSSION

Our study reveals a mechanism by which the histone methyltransferase EZH2 promotes suppression of transactivation from CIITApIV and cell surface expression of MHC II. In variants of the breast cancer cell line MDA MB 435, metastatic potential negatively correlates with expression of MHC II and with active transcription of the MHC II master regulator, CIITA. We found that in these cells, transactivation from CIITApIV was actively suppressed by increased binding of the histone methyltransferase EZH2 and by increased H3K27me3 in the presence of the inflammatory cytokine IFN-γ. These findings support our recent observation that EZH2 is a master regulator of CIITA and, that by exerting critical control over CIITApIV H3K27me3, EZH2 regulates transactivation from CIITApIV (Mehta, Truax et al. 2011). Together, these findings indicate therapies targeting EZH2 expression in breast cancer may directly impact the expression of CIITA by tumor cells resulting in increased cell surface expression of MHC II and increased tumor recognition by the adaptive immune response.

Tumors which best avoid immune recognition are an increased risk for metastasis and tumor related mortality. Tumors of the breast are no exception to this rule with the most common sites for breast cancer metastasis being the lung, liver, bone, brain, and auxiliary lymph nodes (Price 1990). To achieve metastatic ability, tumor cells specifically alter immune gene expression patterns in order to escape host immune surveillance. Multiple tumor types, including colorectal and gastric tumors (Satoh, Toyota et al. 2004), trophoblastic tumors (Morris, Spangler et al. 2000), T cell leukemias, and developmental tumors (van den Elsen, Gobin et al. 2001), accomplish specific decreases in MHC II expression by silencing IFN-γ inducible CIITA-

pIV (Croce, De Ambrosis et al. 2003). Additional studies indicate that many established tumor cells lack expression of cell surface MHC II (de Waard-Siebinga, Kool et al. 1995), further implicating MHC cell surface expression as an important factor in predicting tumor metastasis and patient prognosis. Decreased expression of MHC II has been observed in metastatic breast tumors and in multiple breast cancer cell lines, indicating that suppression of MHC II may have causative, rather than correlative, relationships with immune evasion and with metastatic potential in breast tumors (Ruiz-Cabello, Klein et al. 1991; Gorelik, Kim et al. 1993; Dadmarz, Sgagias et al. 1995; Maiorana, Cesinaro et al. 1995; Walter, Lingnau et al. 2000; Sotiriadou, Perez et al. 2001). The ramifications of our study to tumors of the breast are clear and support previous findings regarding suppression of CIITA in breast cancer (Shi, Vinyals et al. 2006): breast tumors may increase metastatic properties through epigenetically targeting suppression of cell surface expression of MHC II.

We show here that silencing of MHC II molecules in metastatic breast cancer cell lines is associated with dysregulated epigenetic modifications at the proximal promoter of CIITApIV. Significant decreases in CIITA transcript levels in cells stimulated with IFN-γ resulted in both decreased MHC II transcripts and in decreased cell surface expression of MHC II. Expression of MHC II genes correlates metastatic ability of the MDA MB 435 variants resulting in baseline expression of MHC II in the highly metastatic MDA MB 435 Lu2 cells. The impairment in CIITA transcript levels correlates with elevated H3K27me3 at CIITApIV, while neither H3K9me3 nor H3 or H4 acetylation demonstrate alterations in the MBD MB 435 variants. These observations underline the dominance of H3K27me3 in regulating the transcriptional status of CIITApIV chromatin. The lack of transactivation from CIITApIV is not due to defective IFN-γ signaling as

requisite CIITApIV transcription factors STAT-1 and IRF-1 are expressed in the nucleus (Shi, Vinyals et al. 2006) but exhibit limited capability to bind CIITApIV in the presence of IFN-γ stimulation.

As the catalytic subunit of PRC2, EZH2 adds three methyl groups to lysine 27 resulting in chromatin condensation. Converse to decreased MHC II expression in tumors is the overexpression of EZH2 which is observed in diverse tumors types including prostate, breast and bladder cancers and has been linked to enhanced tumor cell proliferation (Raman, Mongan et al. 2005; Bachmann, Halvorsen et al. 2006; Collett, Eide et al. 2006). Reports indicate elevated expression of EZH2 correlates with metastatic tumor growth and with clinically aggressive behaviors in prostate and breast cancer (Xiao 2011). Elevated levels of EZH2 have been recognized as a negative prognostic marker for a number of breast and prostate cancer patients (Varambally, Dhanasekaran et al. 2002; Kleer, Cao et al. 2003) and are associated with poor outcomes to tamoxifen therapy in advanced breast cancer patients (Reijm, Jansen et al.). In sum, accumulating evidence indicates EZH2 may be a primary culprit in metastatic tumor growth and, as such, is currently studied as a target for therapeutic intervention. We demonstrate here a significant association between high levels of EZH2 and H3K27me3 at CIITApIV and the metastatic ability of human breast cancer cells. Perhaps most striking is the observation that decreased expression of EZH2 resulted in constitutive expression of CIITApIV and MHC II transcripts in unstimulated cells. These results provide increased evidence for EZH2 as a target for anti-tumor immunotherapies and provide additional mechanistic links to roles for EZH2 in tumor cell metastasis.

Our results support a hypothesis that hypermethylation of histone tails by EZH2 represses CIITApIV gene expression in metastatic variants of MDA MB 435 cells. It is noteworthy that the master regulator CIITA is in turn controlled by its own master regulator, EZH2. One possibility is that CIITA silencing, however critical, may be the nonspecific result of increased expression of EZH2. Alternatively, and in agreement with previous findings that overexpression of CIITA in MDA MB 435 variants blocks lung metastasis (Yan, Shen et al. 2006), our data suggests EZH2 is a pivotal and specific contributor to CIITApIV silencing, potentially be recruiting DNA methyltransferases to CIITApIV. Indeed, EZH2 has been demonstrated to interact with DNA methyltransferases and EZH2 binding has been shown to be required for DNA methylation of EZH2 target promoters (Vire, Brenner et al. 2006). Indeed, previous studies on MDA MB 435 variants demonstrate only partial methylation of CIITApIV (Shi, Vinyals et al. 2006). Suppression of MHC II on tumor cells is critical for tumor cell survival, or at a minimum, for avoiding immunosurveillance. While the mechanisms responsible for the pronounced effects of overexpression of EZH2 on tumor metastasis remain largely unknown, it seems likely to be targeted suppression, rather than coincidence, that results in elevated EZH2 binding and silencing CIITA-pIV in metastatic breast cancer cells.

5.5 MATERIALS AND METHODS

Cells

435-Lung 2 and 435-Brain 1 variants of the estrogen independent MDA MB 435 human breast cancer cell line were a gift from Dr. Janet Price (Shi, Vinyals et al. 2006). MDA MB 435 variants were maintained using modified Eagle (MEM) medium (Mediatech Inc., Herndon, VA) supplemented with 5% fetal bovine serum (FBS) with the exception of MDA MB 435 Brain 1 which were supplemented with 10% FBS, 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide. MCF 10A (non tumorigenic human epithelial) and HeLa (human epithelial) cells were purchased from ATCC (Manassas, VA). MCF 10A cells were maintained in Mammary Epithelial Cell Basal medium (MEBM) with the following supplements: 0.5 ml hydrocortisone, bovine pituitary extract (BPE); 2 ml gentamicin sulfate amphotericin-B (GA-1000); 0.5 ml human recombined epidermal growth factor in buffered BSA saline solution (rhEGF); and 0.5 ml human recombinant insulin. HeLa cells were maintained in high glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 5mM L-glutamine, and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies

Antibodies recognizing IRF-1 and STAT-1 were purchased from Santa Cruz (Santa Cruz, CA). Antibodies recognizing Histone H3, acetylated histone H3, histone H4, acetylated histone

H4, and rabbit and mouse immunoglobulin G (IgG) isotype control antibodies were purchased from Millipore (Lake Placid, NY). Antibodies recognizing Histone 3 (trimethyl K27), histone 3 (trimethyl K9), histone 3 (acetyl K18), and EZH2 antibodies were purchased from Abcam (Cambridge, MA). Antibody recognizing EZH2 was purchased from Millipore (Lake Placid, NY); antibody recognizing Tubulin was purchased from Santa Cruz (Santa Cruz, CA); HRP conjugated mouse antibody was purchased from Promega (Madison, WI); and HRP conjugated rabbit antibody was purchased from Pierce (Rockland, IL).

Flow cytometry for MHC II cell surface expression

Cells were plated at a density of 7 x10^5, stimulated with IFN-γ (500U/ml) as indicated, and were trypsinized, washed with PBS and resuspended in 100 µl of incubation buffer (0.5% bovine serum albumin in PBS). 10µg Phycoerythrin (PE)-labeled anti-human *HLA-DR* (clone L243, Biolegend, San Diego, CA) or PE mouse IgG2a κ isotype control antibody (Biolegend) was added and the cell suspension was rotated at 4°C for 45 minutes. Following antibody incubation, cells were washed with PBS and fixed with 2% paraformaldehyde. MHC II cell surface expression was measured by FACS-Canto (Becton Dickinson, San Jose, CA) and analyzed using FlowJo. All samples were analyzed using 10,000 events per sample.

siRNA Constructs and Transient Transfections

Short interfering RNA (siRNA) duplexes predesigned against human EZH2 (Qiagen) were used for transient knockdown of EZH2. Cells were transfected with 1ug of EZH2 specific

siRNA or All Star scrambled sequence control (Qiagen) using RNAiFect transfection reagent (Qiagen) according to the manufacturer's instructions and were treated with IFN-γ (500U/ml) as indicated. Cells were lysed in NP-40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP-40, 0.5M EDTA, 5M NaCl, 1M DTT, dH_2O) with EDTA free protease inhibitor (Roche) and analyzed by western blot for knockdown efficiency and specificity.

RNA Expression

Cells were plated at a density of $8x10^5$ cells and were stimulated as indicated with IFN-γ (500U/ml). Cells were harvested and were subjected to RNA extraction with 1ml of Trizol reagent (Invitrogen) as previously described (Truax, Koues et al. ; Bhat, Turner et al. 2008). An Omniscript Reverse Transcription Kit (Qiagen) was used to generate cDNA from extracted RNA. Isolated DNA was analyzed by Real-time PCR on an ABI prism 7900 (Applied Biosystems) using primers and probes for CIITA, MHC II and GAPDH (Truax, Koues et al. ; Bhat, Turner et al. 2008). Primers sequences for EZH2 were as follows: EZH2 sense TTCATGCAACACCCAACACT**,** EZH2 antisene GAGAGCAGCAGCAAACTCCT and EZH2 probe FAM-TTACCAGCATTTGGAGGGAG -TAMRA. Values generated from Real-time PCR reactions were calculated on the basis of standard curves generated.

RNA expression in siRNA treated cells

Cells were plated at a density of $8x10^5$ cells, transfected with EZH2 specific or control siRNA, and twenty four hours later were stimulated as indicated with IFN-γ (500U/ml). Cells

were harvested and 10% of the cell volume was lysed with 1% Nonidet P-40 lysis buffer and subjected SDS PAGE. RNA was extracted and analyzed from the remaining fraction of cell volume as above.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). Briefly, cells were plated at a cell density of $3x10^6$, stimulated with IFN-γ as indicated (500U/ml), and were crosslinked with 1% formaldehyde for 10 minutes at room temperature. Crosslinking was stopped by the addition of 0.125M glycine for five minutes at room temperature. Cells were lysed in SDS lysis buffer and were sonicated at constant pulse to generate an average of 500-750bp sheared DNA. Sonicated lysates were precleared with salmon-sperm coated agarose beads (Upstate) and half of the lysate was immunoprecipitated with 5µg of indicated antibody overnight at 4°C. The remaining half of the lysate was immunoprecipitated with isotype control antibody (Upstate). Following a two hour immunoprecipitation with 50µl of salmon-sperm coated agarose beads, samples were washed for 3 minutes at 4°C with each of the following buffers: low salt buffer, high salt buffer, LiCl, and 1xTE and were eluted with SDS elution buffer. Following elution, cross-links were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using previously published primers spanning the W-X-Y box of the MHC-II *HLA-DRA* promoter and the GAS-IRF-E box of CIITApIV (Truax, Koues et

al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). Values generated from Real-time PCR reactions were calculated based on standard curves generated.

Dual crosslinking Chromatin Immunoprecipitation (dChIP) Assay

Cells were plated at a density of 2.5×10^6 cells and were stimulated as indicated with 500 U/ml IFN-γ. Cells were harvested and washed with PBS, resuspended in 10 ml of PBS (pH 8.0) containing 1mM $MgCl_2$ and 1μM of Disuccinimidyl Glutarate (DSG) and were incubated at room temperature for 45 minutes. Following crosslinking, 0.1 mM Tris pH 7.4 was added for five minutes at room temperature to block further cross-linking. Cells were washed in PBS, re-suspended in 10ml of PBS and Formaldehyde cross-linking was performed as described above for the ChIP assay.

Chromatin Immunoprecipitation with siRNA

ChIP with siRNA assays were performed as previously described (Truax, Koues et al. ; Koues, Dudley et al. 2008). Briefly, cells were plated at a density of $8x10^5$, transfected with 1μg EZH2 specific or control siRNA (Qiagen), and stimulated as indicated with IFN-γ (500U/ml). 10% of the cell volume was lysed with 1% Nonidet P-40 lysis buffer and was analyzed by western blot for knockdown efficiency and specificity. The remaining cell volume was crosslinked, lysed in SDS lysis buffer, and subjected to sonication and the above described ChIP assay.

5.6 ACKNOWLEDGMENTS

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The authors wish to thank Dr. Janet Price for MDA MB 435 cell lines.

6 CHAPTER VI: CONCLUSIONS:

The process of gene expression is utilized by all living organisms, including eukaryotes, prokaryotes, and viruses to generate macromolecules for life. There are several steps in which gene expression can be regulated including transcription, translation, and posttranslational modifications. Genes are organized as very long strands of DNA which carry genetic information and are tightly packed into nuclei (Ogbourne and Antalis 1998). DNA is wrapped around histone proteins which are then condensed into chromatin (Luger, Mader et al. 1997; Richmond and Davey 2003). Genes can be constitutively silenced or expressed, or belong to a group of inducible genes which are expressed as needed (Nakayama, Rice et al. 2001; Agalioti, Chen et al. 2002; Jacobs and Khorasanizadeh 2002; Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). To allow for these diverse modes of gene expression, chromatin is dynamic, allowing for rapid changes in gene expression in response to various stimuli. Both silenced and active chromatin structures are associated with specific histone modifications. These covalent modifications include acetylation, methylation, phosphorylation, sumoylation, and ubiquitination; each of which plays important roles in gene expression by determining the level of accessibility of chromatic DNA. Transcriptionally silenced heterochromatin is frequently methylated on histone H3 at lysines 9 and 27 (Nakayama, Rice et al. 2001; Jacobs and Khorasanizadeh 2002; Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). Euchromatin is active chromatin and is heavily acetylated on lysine residues on histones H3 and H4 (Eberharter and Becker 2002; Gorisch, Wachsmuth et al. 2005). The addition and removal of histone modifications is mediated by a group of histone modifying enzymes termed histone acetyltransferases, histone deactetylases,

histone methyltransferases, and histone demethylases. The study of posttranslational modifications of histone tails has given rise to a “histone code” which, together with the “DNA code,” determines the availability of DNA for gene expression.

The ubiquitin proteasome system (UPS), largely known for its ability to degrade polyubiquitinated proteins, has recently emerged as a novel player in transcriptional regulation of various genes (Ciechanover 1994; Hochstrasser 1996; Kinyamu, Chen et al. 2005). The 19S subunit of the 26S proteasome contains six ATPases which assemble into a hexameric ring to associate with the 20S proteasome. Full roles for 19S ATPases in proteasome mediated degradation remain unclear, but their roles in unfolding of ubiquitinated proteins directed towards the 20S core are critical for protein degradation (Coux, Tanaka et al. 1996; Glickman, Rubin et al. 1999). Initial observations that the 20S core and 19S regulatory particle also exist as independent complexes in both nuclear and cytoplasmic components led to studies of independent contributions of these components to cellular activity (Brooks, Fuertes et al. 2000). These efforts resulted in observations that the 19S ATPases could separate from the 19S proteasome and associate with activation domains of transcription factors and with inducible promoters (Swaffield, Melcher et al. 1995; Rubin, Coux et al. 1996; Gonzalez, Delahodde et al. 2002; Sun, Johnston et al. 2002). Intense efforts have identified independent roles for 19S ATPases in regulating transcriptional activity of multiple yeast genes (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Morris, Kaiser et al. 2003; Lee, Ezhkova et al. 2005; Sulahian, Johnston et al. 2006). Studies in mammalian systems have lagged behind those in yeast, however interactions of the 19S proteasome with the tumor suppressor p53, the HIV- promoter and coding region, the retinoic acid receptor, the glucocorticoid receptor, and the viral transcription factor

E1A have driven further development of the field (Ferdous, Kodadek et al. 2002; Kinyamu, Chen et al. 2005; Rasti, Grand et al. 2006; Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007). Despite these advances, the mechanism by which 19S ATPases regulates mammalian transcription remains unknown.

In order to understand functional involvement of the 19S proteasome in gene expression, we have investigated the role of the 19S ATPase S6a in regulating transcriptional initiation of CIITA. We choose CIITA for further investigation because, as the master regulator of MHC class II genes, CIITA is a critical and inducible gene (Wang 2003). We and others have previously demonstrated interactions between the UPS and mono and polyubiquitinated CIITA (Bhat, Truax et al. ; Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003) and a dependence of CIITA activation on 19S ATPase activity (Bhat, Truax et al. ; Truax, Koues et al. ; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). We initially observed that proteasome inhibition has a diminishing effect on both CIITA transactivity and on expression of MHC II genes. Studies by Bhat et al, demonstrated roles for the 19S ATPase Sug1 in regulating transcriptional initiation of MHC II genes and showed that in the absence of Sug1, levels of CIITA recruited to MHC II proximal promoter are significantly reduced (Bhat, Turner et al. 2008). Studies by Koues et al implicated novel roles for the 19S ATPase Sug1 in epigenetic regulation of transcription of MHC II genes (Koues, Dudley et al. 2008; Koues, Dudley et al. 2009). However, the full role of the 19S ATPases in regulating transcription of CIITA and MHC II genes remains to be investigated.

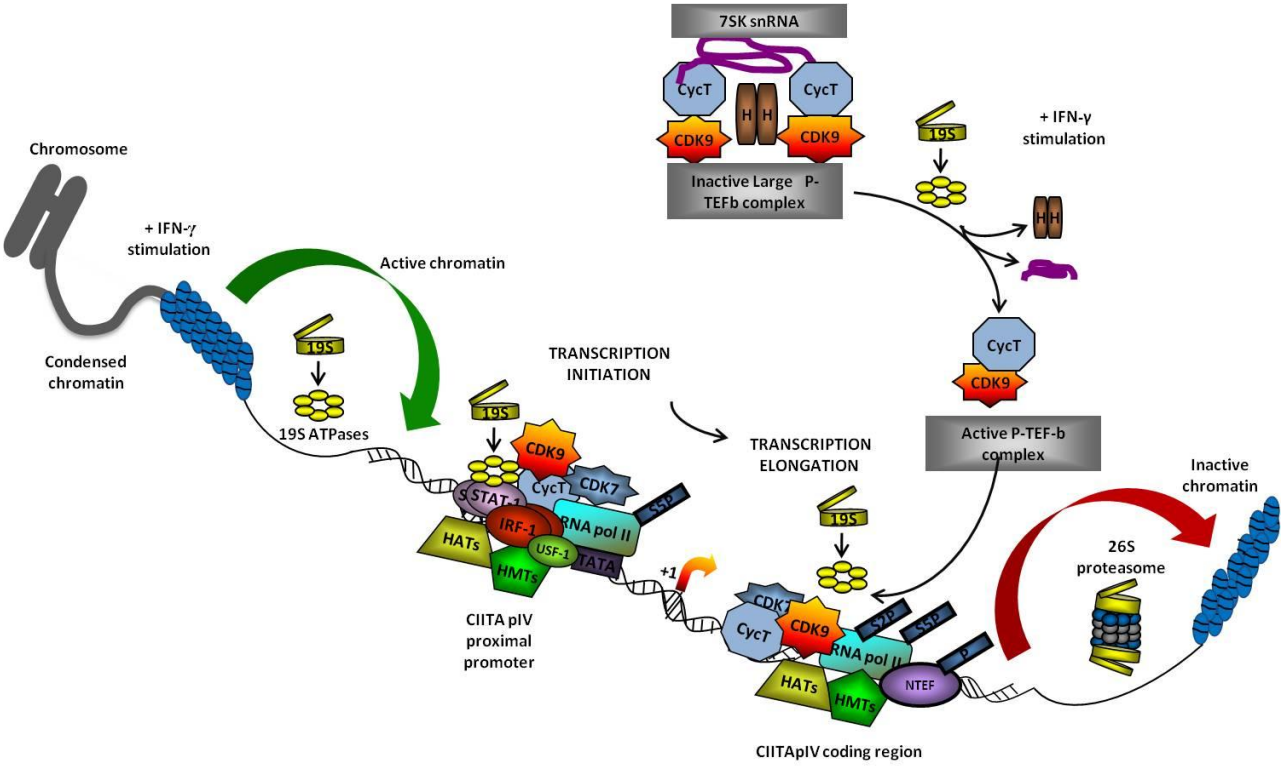

Figure 6.1 Transcription regulation of CIITA genes.

IFN-γ stimulation activates JAK STAT signaling pathway and results in the recruitment of gene specific transcription factors and histone modifying enzymes, which, enabled by the actions of 19S ATPases, are crucial in mediating transcriptional initiation of CIITA genes. Cytokine stimulation is aided by subunits of the proteasome in promoting the switch from an inactive to active form of P-TEF-b. Activated P-TEF-b is recruited to the CIITA promoter and phosphorylates serine 5 on the CTD of RNA polymerase II to initiate mRNA synthesis. Shortly after the transcription start site, the polymerase pauses; the release of the polymerase is mediated by a series of phosphorylation events involving serine 2 on the RNA pol II CTD and NTEF which initiates productive elongation, chromatin modifications, and termination.

S6a is an important, and largely unstudied, 19S ATPase known for binding to polyubiquitin chains on proteins targeted for degradation (Lam, Lawson et al. 2002). Following IFN-γ stimulation, our studies show S6a rapidly binds to the proximal promoters of both MHC II *HLA-DRA* and CIITApIV. Decreased expression of S6a results in significant decreases in the message levels of both MHC II and CIITA. Previous studies have documented that expression of CIITA-pIV following IFN-γ stimulation depends on the recruitment of the transcription factors STAT-1 and IRF-1 to CIITA proximal promoter. Indeed, we observed S6a interactions with STAT-1, and significant reductions in STAT-1 and IRF-1 CIITApIV binding in the absence of S6a. S6a knockdown decreases acetylation of histone 3, lysine 18 and of histone 4, lysine 8 at CIITApIV, further indicating this 19S ATPase plays critical roles in chromatin remodeling, in recruitment of transcription factors, and in assembly of the preinitiation complex required for efficient transcription of CIITA genes **(Figure 6.1)**. As the 19S ATPase S6a recognizes ubiquitinated proteins, we were unsurprised by our observations that decreased expression of S6a negatively impacts degradation by the 26S proteasome as evidenced by increases in p53 half life. However, blocking proteasome activity with the proteasome inhibitor MG132 does not affect levels of acetylation at CIITApIV, suggesting S6a mediated effects on acetylation and recruitment of transcription factors to CIITApIV are proteasome independent. The 19S ATPase S6b also binds inducibly to CIITApIV and has similar effects on chromatin structure as does S6a. Thus far, our studies demonstrate the 19S ATPase S6a plays important roles in transcriptional initiation of CIITA and MHC II genes.

In regards to transcriptional elongation, we have mapped robust binding of S6a to the CIITA coding region, providing novel evidence that 19S ATPases may play roles in elongation.

In data not shown, we observe that all six of the 19S ATPases bind the CIITApIV proximal and coding regions following IFN-γ stimulation. Occupancy of the 19S ATPase S6a has also been observed at the coding region of HIV-1 (Lassot, Latreille et al. 2007), where all the 19S ATPases have shown enriched binding to the coding region, but where levels of S6a binding were significantly higher. Following successful assembly of preinitiation complexes, RNA pol II initiates synthesis of messenger RNA in a process mediated by phosphorylation at serine 5 on CTD repeats of the polymerase (Dahmus 1996; McCracken, Fong et al. 1997; Hirose, Tacke et al. 1999; Hirose and Manley 2000). In higher eukaryotes, polymerase frequently pauses after synthesis of approximately 50 bases of RNA due to actions of negative elongation factor (NELF) and 5,6-dichloro 1-B- ribofuranosylbenzimidazole (DRB) sensitivity inducing factor (DSIF) (Saunders, Core et al. 2006). RNA pol II pausing is overcome by recruitment of CDK9 which phosphorylates NELF and DSIF as well as serine 2 at on RNA pol II CTD repeats. These phosphorylations initiate a cascade of events including productive elongation, mRNA synthesis, transcriptional termination, and chromatin modifications **(Figure 6.1)** (Mancebo, Lee et al. 1997; Zhu, Pe'ery et al. 1997; Darzacq, Shav-Tal et al. 2007). To determine if S6a plays roles in maintaining transcription, we knocked down S6a expression using S6a specific siRNA and observe that in the absence of S6a, levels of RNA pol II and the cyclin T1component of P-TEF-b are reduced at both the CIITA proximal promoter and coding region. In addition, co-immunoprecipitations studies indicate S6a associates with the Hexim-1 and CDK9 components of the P-TEF-b complex **(Figure 6.1)**. Proteasome inhibition via MG132 treatment does not significantly impact levels of RNA pol II at either the CIITA proximal promoter or coding region, indicating effects of S6a siRNA are degradation independent. These observations agree with yeast studies using genome wide ChIP analyses which demonstrated that there are hundreds of genes that differentially asso-

ciate with either the 19S proteasome or 20S proteasome, indicating 19S and 20S subunits of the 26S proteasome have independent functions in regulating different genes (Sikder, Johnston et al. 2006). As the status of chromatin plays critical roles in regulating transcription elongation, future investigations will address roles of the 19S ATPases in maintaining acetylation levels on histone H3 and histone H4 as well as methylation on histone H3 at lysine 36 on actively transcribing genes (Krogan, Kim et al. 2003; Liu, Kaplan et al. 2005; Pokholok, Harbison et al. 2005; Rao, Shibata et al. 2005).

Our studies have shown that one way 19S ATPases mediate transcription regulation of CIITA genes is via affects on both chromatin structure and on recruitment of various transcription factors and coactivators. To more fully address the dynamics of epigenetic events at CIITA genes, we have analyzed the chromatin structure of CIITApIV following cytokine stimulation. As CIITA is a master regulator for critical inflammatory MHC II genes, rapid alterations in chromatin structure of CIITApIV are necessary for rapid induction of adaptive immune responses. We have previously observed that acetylation levels on histone H3 lysine 18 rise 18 hours post IFN-γ stimulation (Truax, Koues et al. ; Koues, Dudley et al. 2008), however the increase was nonlinear, indicating rapid loosening of CIITApIV chromatin. In contrast to activating modifications are the silencing modifications of trimethylation on histone H3 lysine 9 and lysine 27, which are significantly reduced within the initial two hours of cytokine stimulation. The rapid loss of these silencing modifications from CIITApIV is opposite from the trend of activating methylation at CIITApIV histone H3 lysine 4. Together, these observations suggest the changes in histone modifications observed at CIITApIV occur very rapidly following cytokine stimulation allowing for STAT-1 to bind to CIITApIV 20 minutes following IFN-γ treatment.

STAT-1 binding to CIITApIV also correlates with robust and rapid increases in CIITA transcripts. Epigenetic modifications to CIITApIV following IFN-γ stimulation demonstrate a balance between rapidly decreasing silencing modifications and a slower gain of activating modifications. Of note are our observations that activating modifications do not reach maximum levels until 120 minutes post cytokine stimulation while silencing modifications are significantly reduced in a much more rapid fashion. These data indicate removal of methyl groups from lysine 27 of histone H3 at CIITApIV is the dominant histone modification in the transcriptional control of CIITA genes. This observation correlates with our observation that CIITApIV binding of the histone methyltransferase EZH2 declines rapidly following IFN-γ stimulation, suggesting EZH2 is a master regulator of CIITA genes. As described below, we have gone on to demonstrate that EZH2 plays significant roles in suppressing transactivation of CIITA and cell surface expression of MHC II, adding support to our observations of the critical roles played by EZH2 as an important regulator of the accessibility of CIITA genes.

Metastatic tumors have altered their expression of multiple genes in order to escape host immune surveillance. Recent studies indicate that MHC II cell surface expression is an important factor in predicting tumor metastasis (Brocker, Suter et al. 1984; Concha, Esteban et al. 1991; de Waard-Siebinga, Kool et al. 1995). Multiple tumor types including uveal melanoma (de Waard-Siebinga, Kool et al. 1995), T cell leukemias (van den Elsen, Gobin et al. 2001), and colorectal, gastric (Satoh, Toyota et al. 2004), and trophoblastic tumors (Morris, Spangler et al. 2000) avoid the host immune response through repressing cell surface expression of MHC II. Decreased expression of MHC II has also been observed in breast cancer, including our own study of MDA MB 435 cells. Our studies tested three variants of MDA MB 435 breast cancer cells, and dem-

onstrate that the metastatic ability of these variants negatively correlates with the cell surface expression of MHC II and with transcription of CIITA. We show that in MDA MB 435 cells CIITApIV is suppressed by increased binding of the HMT EZH2 which correlates with elevated levels of the suppressing modification trimethylation on histone H3 lysine 27, even in the presence of sustained cytokine stimulation. Our studies indicate metastatic tumors may target epigenetic modifications of CIITA genes in order to suppress expression of MHC II and avoid the immune response.

Of note are our observations that the metastatic variant of MDA MB 435 cells has substantially decreased surface expression of MHC II, which correlates with low transcript levels of MHC II and CIITA following IFN-γ stimulation. Aberrant expression of CIITA is driven by elevated levels of trimethylation on histone H3 lysine 27 at CIITApIV. Conversely, neither the levels of trimethylation on histone H3 lysine 9 nor the levels of acetylation on histone H3 or histone H4 are impacted. These observations highlight the importance and dominance of the silencing modification of trimethylation at H3 lysine 27 in regulating expression of CIITA genes. As a consequence of the inaccessibility of CIITApIV DNA, we observe significantly reduced binding of the transcription factors STAT-1 and IRF-1, as well as RNA pol II to the proximal promoter of CIITApIV. In direct opposition to the closed status of CIITApIV in the metastatic variant of MDA MB 435 is the epigenetic state of MHC II which shows no abnormalities of any tested histone modifications. In these cells, MHC II genes are open and available following cytokine stimulation; therefore, it is the lack of the master regulator CIITA that drives the absence of MHC II expression in these IFN-γ stimulated cells.

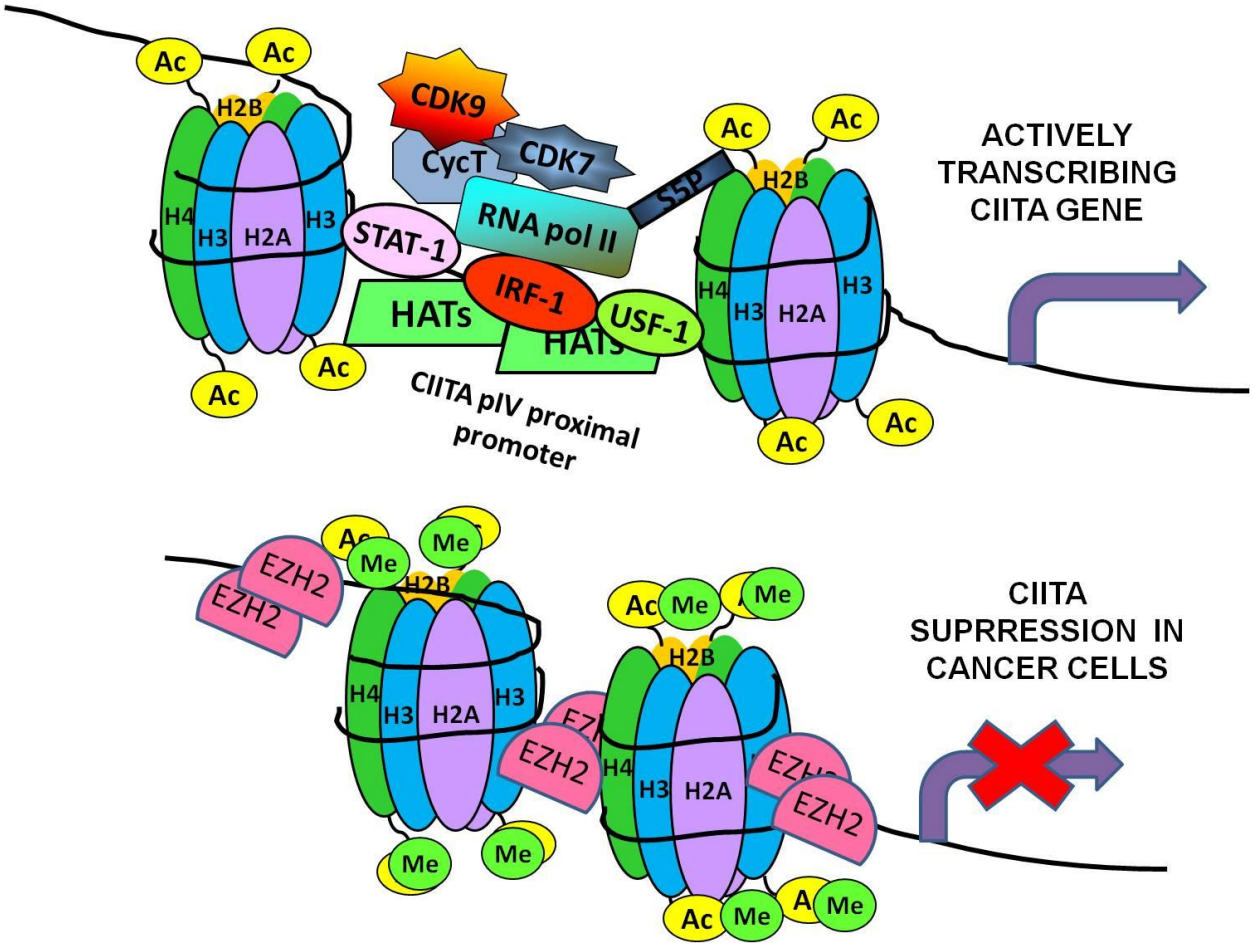

Figure 6.2 Transcriptional regulation of CIITA genes by the histone methyltransferase EZH2.

Metastatic breast cancer cells suppress CIITA expression to avoid recognition by the host immune system. Elevated CIITApIV binding of the histone methyltransferase EZH2 results in hypermethylation of the CIITApIV proximal promoter on histone H3 lysine 27 and in inaccessibility of the CIITA promoter.

Significantly, knocking down EZH2 restores suppressed expression of CIITA and MHC II in these cells with and without IFN-γ stimulation, suggesting that EZH2 is the critical regulator of CIITA gene silencing. In sum, our data shows that CIITA, the master regulator of MHC II genes, is suppressed in metastatic variants of MDA MB 435 cells. Our observations indicate hypermethylation of histone tails by the histone methyltransferase EZH2 drives significant down regulation of CIITApIV **(Figure 6.2)**.

It is noteworthy that the master regulator CIITA is controlled by its own master regulator, EZH2. Future research will determine if elevated levels of EZH2 are observed at additional genes that are silenced in metastatic tumors and important regulators of tumor immune surveillance. The mechanisms by which EZH2 silences CIITApIV remain to be fully investigated and include the possibility that EZH2 recruits DNA methyltransferases to CIITApIV, as suppression of CIITA may result from a combination of silencing modifications to histone tails and DNA methylation.

To conclude, we showed expression of CIITA in response to cytokine stimulation is highly regulated at the level of transcription. We have added substantial evidence to demonstrate "the proteasome is not just degrading anymore", as we show subunits of the 19S proteasome play important role in transcriptional regulation of mammalian genes. The 19S ATPase S6a is an important player in these transcriptional processes as it regulates expression of CIITA at the levels of transcriptional initiation and elongation **(Figure 6.1)**. In addition our study is the first to describe the early epigenetic events that occur at CIITApIV. Our data indicate CIITA coding DNA is ra-

pidly opened and available for transcription factor binding following cytokine stimulation. Moreover, we show the histone methyltransferase EZH2 is powerful contributor to silencing of CIITA genes. Based on our observations, EZH2 plays the role of "master regulator" of epigenetic modifications to CIITA associated histones **(Figure 6.2)**. Expression of CIITA and MHC II is significantly decreased in metastatic breast cancer cells MDA MB 435 due to high levels of trimethylation on histone H3 lysine 27 facilitated by elevated occupancy of EZH2. Knowledge that CIITApIV is controlled by EZH2 provides new insights into our understanding of how cancer cells avoid recognition by the immune system. Based on the work in this dissertation, we now have a better understanding of how transcription of CIITA and MHC II genes is regulated. These findings enhance our knowledge of the role of the 26S proteasome and epigenetic modifications in regulating CIITA activity and MHC II expression and provide novel targets for therapeutic strategies to manipulate MHC II in expression in autoimmune diseases and in tumor cells.

7 CHAPTER VII: REFERENCES

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