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EARLY EPIGENETIC REGULATION OF THE
ADAPTIVE IMMUNE RESPONSE GENE CIITA

by

NINAD MEHTA

Under the Direction of Susanna F Greer, PhD

ABSTRACT

The precise regulation of Major Histocompatibility class II (MHC-II) genes plays an important role in the control of the adaptive immune response. MHC-II genes are expressed constitutively in only a few cell types, but their expression can be induced by the inflammatory response cytokine interferon gamma (INF-γ). The regulation of MHC-II is controlled by a Master Regulator, the class II transactivator (CIITA). Multiple studies have shown that CIITA regulated expression of MHC-II is controlled and induced by INF-γ. It has been also shown that a functional CIITA gene is necessary for the expression of MHC-II genes. CIITA is thus a general regulator of both constitutive and inducible MHC-II expression. Although much is known about the transcription factors necessary for CIITA expression, there is little information as to the epigenetic modifications and the requisite enzymes needed to provide these transcription factors access to DNA. Previous studies in the Greer lab have shown that increased levels of acetylation of histones H3 upon INF-γ stimulation, as does tri-methylation of H3K4 upon prolonged cytokine stimulation. Similar observations were made at early time points post IFN-γ stimulation, where there is an

instantaneous increase in the levels of H3K18ac and H3K4me3. In contrast to this, the levels of silencing modifications begin to drop with in the first 20 minutes of IFN-γ stimulation. The binding of STAT1 reaches its peak at about 60 minutes and the first transcripts for the protein start to appear as early as 40 minutes post the cytokines stimulation. Our study is the first to link the rapidly occurring epigenetic changes at the CIITA promoter pIV to EZH2

INDEX WORDS: Transcriptional regulation, Epigenetics, Histone modifications, Histone remodeling enzymes, Major histocompatibility complex class II, Class II transactivator.

EARLY EPIGENETIC REGULATION OF THE ADAPTIVE IMMUNE RESPONSE GENE CIITA

by

NINAD MEHTA

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

Masters of Science

College of Arts and Sciences

Georgia State University

2010

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2010

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TABLE OF CONTENTS

ACKNOWLEDGMENT iv

TABLE OF CONTENTS v

LIST OF FIGURES vii

LIST OF ABBREVIATIONS vii

1. INTRODUCTION 1

1.1 Histones and Nucleosome Structure 2

1.2 Epigenetics and Histone Modifications 3

1.3 Histone Acetylation 3

1.4 Histone Methylation 6

1.5 Transcriptional Regulation of the Adaptive Immune Response 8

1. 6 Transcriptional Regulation of the Class II Transactivator 10

2. METHODS AND MATERIAL 15

2.1 Cell Lines 15

2.2 Antibodies 15

2.3 Primer & Probe 15

2.4 siRNA constructs and transient transfections 16

2.5 Chromatin immunoprecipitations (ChIPs) 16

2.6 Chromatin immunoprecipitation in EZH2 knockdowns 17

2.7 Dual Crosslinking Chromatin immunoprecipitations (Dual ChIPs) 18

2.8 RNA transcript levels 18

3. RESULTS 19

3.1 Rapid epigenetic changes occur at CIITA pIV following IFN-γ stimulation 20

3.2 CIITA pIV is rapidly activated for transcription by the inflammatory cytokine IFN-γ. 23

3.3 Increases in CIITA pIV transcription correlate with decreased promoter binding of the regulatory histone methyltransferase EZH2. 26

3.4 Loss of EZH2 leads to an aberrant CIITA expression. 29

4. DISCUSSION 31

5. REFERENCE 35

LIST OF FIGURE

Figure 1. Histone post-translational modifications 4

Figure 2. MHC-II expression cascade 11

Figure 3. CIITA pIV 13

Figure 4. Histone 3 Lysine 18 (H3K18ac) acetylation at CIITA promoter IV 20

Figure 5. Histone 3 Lysine 4 (H3K4me3) trimethylation at CIITA promoter IV 21

Figure 6. Histone 3 Lysine 9 (H3K9me3) trimethylation at CIITA promoter IV 24

Figure 7. Histone 3 Lysine 27 (H3K27me3) trimethylation at CIITA promoter IV 25

Figure 8. STAT1 binding at CIITA promoter IV 27

Figure 9. CIITA message levels post IFN-γ stimulation 28

Figure 10. EZH2 binding at CIITA promoter IV 30

Figure 11. CIITA message levels post IFN-γ stimulation in cells treated with EZH2 siRNA and All Star siRNA. 32

LIST OF ABBREVIATIONS

acH3	Acetylated histone H3
CIITA	Class II transactivator
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
COMPASS	Complex of proteins associated with SET1
CREB	cAMP response element binding protein
EZH2	Enhancer of zeste homolog 2
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMTase	Histone methyltransferase
H3K4me3	Histone H3 trimethylated at lysine 4
H3K9ac	Histone H3 acetylated at lysine 9
H3K9me2	Histone H3 dimethylated at lysine 9
H3K18ac	Histone H3 acetylated at lysine 18
H3K36me3	Histone H3 trimethylated at lysine 36
H3R17	Histone H3 dimethylated at arginine 17
IFN-γ	Interferon gamma
IRE	Interferon responsive element
IRF1	Interferon regulatory factor 1

K	Lysine
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NFY	Nuclear Factor Y
R	Arginine
RFX	Regulatory Factor X
RPMI	Roswell Park Memorial Institute
SAGA	Spt-Ada-Gcn5-acetyltransferase
siRNA	Short interfering RNA
STAT	Signal transducer and activator of transcription

1. INTRODUCTION

DNA in eukaryotic cells is organized into highly condensed chromatin which is further divided between an open structure termed euchromatin and a closed structure termed heterochromatin (Wu and Grunstein, 2000). In order to form higher order chromatic structures, eukaryotic DNA is initially wrapped around histone proteins (Luger et al., 1997b; Richmond and Davey, 2003) which are small (~15-17kDa), basic, and highly conserved (Lewin, 2004). Once DNA is wrapped around histones, the basic repeating subunit of chromatin, termed the nucleosome, is formed (Berger, 2002a; Dorigo et al., 2004; Luger et al., 1997a; Rohs et al., 2009). Nucleosomes themselves further assemble into higher-order structures, which are stabilized by the linker histone H1 (Widom, 1998).

Changes in higher order DNA packaging occur as genes are activated and inactivated. At the most basic level of transcriptional control is the regulated switch between euchromatin and heterochromatin as genes are turned on and off (Demetriou et al., 2009; Masternak et al., 1998). At the heart of regulated gene expression is the tightly controlled interaction between DNA and histone proteins. Binding of DNA to histones is thought to be primarily regulated by epigenetic modifications to histone tails which govern, at least in part, the ability of DNA and histones to interact. The mechanisms underlying epigenetic modifications, and the epigenetic code that contributes to the status of open versus closed chromatin, remains an area of intense investigation.

One striking effect of epigenetic regulation is exemplified by the observation that, though the genetic content of yeast and mammalian cells is strikingly similar, the organisms themselves not. Vast phenotypic differences between organisms such as these

are due in large part to epigenetic differences in gene regulation, which in turn control which proteins are expressed and when. Understanding the epigenetic 'code', i.e., how and when epigenetic modifications are added to histones, and how these modifications change histone and DNA interactions, will have a critical impact on our understanding of transcriptional regulation of gene expression. Increased knowledge of epigenetic regulation of cellular activities will play an important role in our appreciation of cell differentiation, of developmental events, of the immune response, and of a host of other cellular activates.

1.1 Histones and Nucleosome Structure

Histones are alkaline proteins, and are thus basic in nature (Chakrabarti et al., 2003; Clayton et al., 2006; Jenuwein and Allis, 2001b). Positively charged histones can interact with negatively charged DNA in order to control DNA condensation and accessibility. Histones are a group of highly conserved proteins which are divided into five major types: histone H1/H5, H2A, H2B, H3, and H4 (Redon et al., 2002; Tanaka et al., 2004). The five types of histones are further divided into two super-classes: core histones and linker histones. The core histones consist of H2A, H2B, H3, and H4, while histones H1 and H5 are linker histones (Redon et al., 2002). The nucleosome consists of two copies of each of the core histones and is capable of packaging approximately 147 base pairs of DNA wrapped around histones in a 1.65 left-handed super-helical turn (Luger et al., 1997b). The nucleosome core consists of two H2A-H2B dimers and an H3–H4 tetramer. Histone H1 binds to the DNA that is entering and exiting the nucleosome structure and acts as a stabilizing member of the nucleosome complex (Kouzarides, 2007). A fifth protein, histone H1 and H5 also aids the coil formation of dinucleosomes (Widom, 1998). These

dinucleosomes further fold over each other to form 30 nm chromatin fibers and the 30 nm fibers condense to form chromatin. Chromatin itself is found in two forms: heterochromatin which is inactive, highly condensed, and closed for transcription, and euchromatin which is active and transcriptionally accessible (Lewin, 2004).

1.2 Epigenetics and Histone Modifications

A number of related but distinct forces impact interactions between histones and DNA. The dipole generated due to the alpha-helices found in histones H2B, H3, and H4 leads to an accumulation of positive charges at points of interaction between negatively charged phosphate groups on DNA (Palau et al., 1980). Hydrogen bonds are formed between the DNA backbone and the amide groups present in histones while non-polar and salt bridges form between the sugar bases and the side chains of basic amino acids (Ward et al., 2009). In addition to these regulatory chemical interactions are epigenetic regulatory mechanisms which significantly impact interactions between DNA and histones. The term 'epigenetics' literally means 'outside the conventional genetics' and, similar to DNA, epigenetic interactions are thought to be regulated by a code, commonly referred to as the 'histone code' (Demetriou et al., 2009). Histones control transcription in part via regulatory epigenetic modifications which occur on histone tails (Jenuwein and Allis, 2001b; Turner, 2002; Zhang and Reinberg, 2001). These N-terminal, post-translational modifications to histone tails include acetylation, methylation, phosphorylation, and ubiquitination (among others) and together these modifications play a crucial role in establishing the histone code **(Figure 1)** (Bird, 2002; Jaenisch and Bird, 2003).

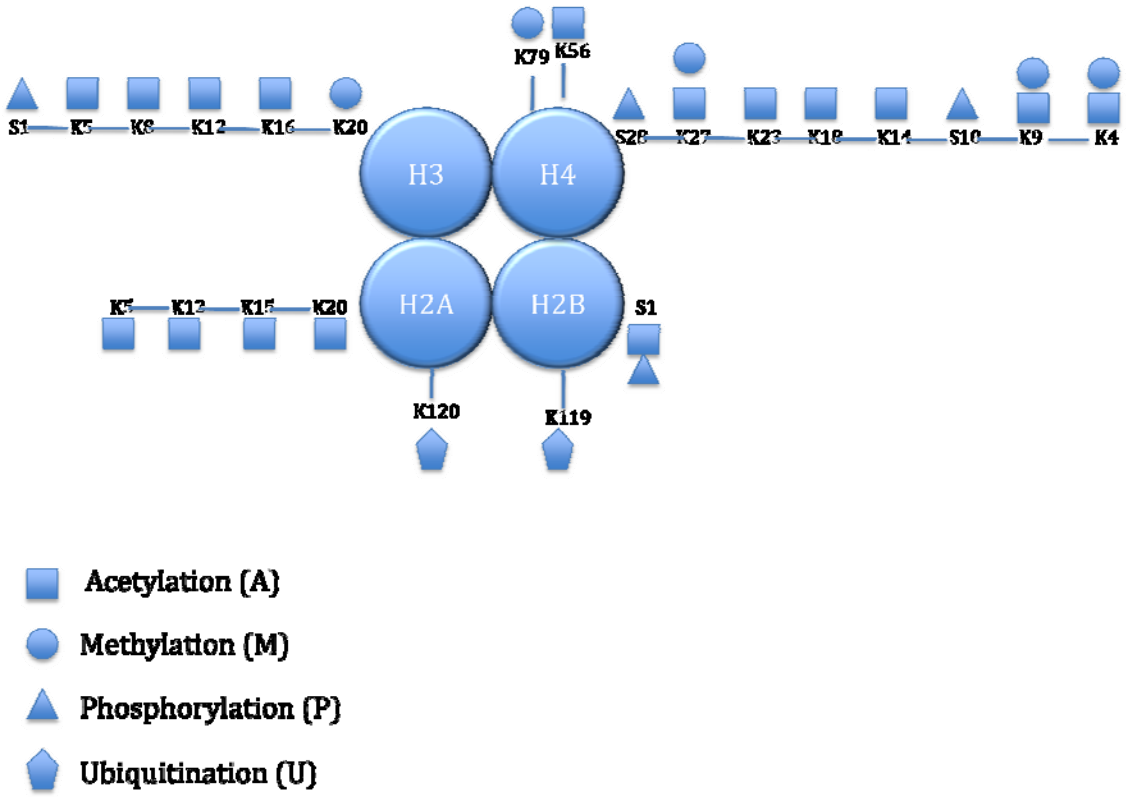

Figure 1. Histone post-translational modifications

The N-terminal tail regions of histone proteins are regions for post-translational modifications. Potential and proposed locations of post-translational modifications on the histone tails are shown.

1.3 Histone Acetylation

(HATs) has been associated with transcriptional activation, and is the most well studied of all histone modifications (Croce et al., 2003; Ma et al., 2001). Acetylation loosens the interaction between histones and DNA by neutralizing the positive charge on the amino acid lysine (Chen et al., 1999; Martin et al., 2006; Steger et al., 1998b). Histone acetyltransferases bind histones and add acetyl groups to the lysine residues of histone tails (Berger, 2002a; Daujat et al., 2002; Harton et al., 2001). The reversal of this modification, i.e. the removal of the acetyl group, is catalyzed by enzymes called histone deacetylases (HDACs) (Carrozza et al., 2003; Chuang et al., 2009). The source of the acetyl group for the acetylation of histones for HATs is Acetyl-Coenzyme A; when HDACs remove the acetyl group, it is subsequently transferred to a Coenzyme A molecule (Clayton et al., 2006; Lusser et al., 2001). As acetylation is a reversible process, removal of the acetyl group leads to an increase in the positive charge of histones and leads to compacted chromatin structure and decreased transcription (Schiltz et al., 1999; Shahbazian and Grunstein, 2007; Steger et al., 1998a).

Histone acetyltransferases (HATs) responsible for the addition of acetyl groups to the N-terminal tail of histones are recruited by DNA-binding transcription factors (Agalioti et al., 2002; Berger, 2007a; Chan and La Thangue, 2001a; Daujat et al., 2002; Harton et al., 2001) resulting in acetylation of histones at promoter sites (Bannister and Kouzarides, 1996). There are a variety of well known HATs, including general control of amino-acid synthesis 5 (GCN5), CREB binding protein (CBP), p300, and p300/CBP-associated factor (pCAF)(Hamamori et al., 1999; Krumm et al., 1998). In addition, there are a number of less

characterized proteins that have been found to have HAT activity, and the ability to acetylate various lysine residues in histone proteins (Bannister and Kouzarides, 1996; Chan and La Thangue, 2001b; Ogryzko et al., 1996; Roth et al., 2001b).

1.4 Histone Methylation

Methylation, like acetylation, is another epigenetic modification common to histones. Methylation occurs at arginine (R) and lysine (K) resides of histones and methyl groups are added to target histone residues by histone methyltransferases (HMTases) (Bauer et al., 2002a; Berger, 2002b; Berger, 2007b; Daujat et al., 2002). Methylation of arginine residues has been linked to activation of transcription (Bauer et al., 2002b; Daujat et al., 2002), but is more commonly associated with transcriptional silencing (Bauer et al., 2002b; Jenuwein and Allis, 2001b). However, the mechanisms by which methylation contributes to modifications to chromatin structure are not understood.

Histone methyltransferases are organized into two major groups of proteins. The first group contains both the SET-domain and the non-SET-domain lysine methyltransferases (Nishioka et al., 2002) which attach methyl groups to the e-nitrogen of lysine residues, and are responsible for mono-, di- and tri- methylation (Cheng and Zhang, 2007). The second group of enzymes contains the protein arginine methyltransferases (PRMTs) (Dalvai and Bystricky, 2010), which attach methyl groups to arginine residues (Bannister and Kouzarides, 2004; Dalvai and Bystricky, 2010). Recent studies have identified a variety of histone demethylases including Lysine-Specific Demethylases 1(LSD1) (Cheng and Zhang, 2007) and Jumonji C (JmjC)-domain-containing proteins, each of which are capable of removing methyl groups from lysine residues (Fodor et al., 2006;

Klose et al., 2006; Lee et al., 2007a; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). Deimination is the process of removing methyl-groups from the arginine moiety on histone H3 and H4 (Cuthbert et al., 2004). Peptidylarginine deminase 4(PAD4) controls the level of arginine methylation in cells by converting the modified arginine to citrulline (Wang et al., 2004). While the exact mechanism by which histone methylation controls transcription is poorly understood, cross-talk between histone methylation modifications and other histone modifying enzymes has been observed (Muller and Verrijzer, 2009). Histone H3K4 hypermethylation inhibits H3K9 methylation and is associated with the demethylation of H3K27. As hyper, or tri-methylation of lysine 27 is associated with a closed chromatin structure, H3K4 tri-methylation results in decreased H3K27 methylation, a more open chromatin conformation, and increased transcription (Agger et al., 2007; Lee et al., 2007b; Wang et al., 2001). Conversely, methylation of H3K36 reestablishes closed chromatin conformation by recruiting histone deacetylases, resulting in a loss of histone acetylation and decreased transcription (Carrozza et al., 2005; Joshi and Struhl, 2005; Lee and Shilatifard, 2007).

Additional known histone modifications include sumoylation, ADP-ribosylation, ubiquitination, and phosphorylation (Berger, 2002a). Sumoylation is the attachment of the polypeptide SUMO to the histone and has been associated with gene silencing (Freiman and Tjian, 2003; Shilatifard, 2006). ADP-ribosylation is a modification which results in the addition of ADP and ribose moieties and is mainly found to be associated with histones during DNA replication (Berger, 2002b). Histone ubiquitination has been associated with gene transcription, regulation, and silencing (Adamski et al., 2004b; Berger, 2002b; Gu et

al., 2008; Hochstrasser, 1996). Finally, phosphorylation of histones has been commonly been associated with active transcription (Berger, 2002b).

In comparison to sumoylation, ribosylation, ubiquitination, and phosphorylation, the histone modifications of acetylation and methylation are better understood in terms of their kinetics, their structures, and their profound effects on chromatin dynamics (Jones and Baylin, 2002). In addition, changes in histone acetylation and methylation have been associated with various diseases including cancer (Cooper and Foster, 2009) and autoimmune disorders(Brooks et al., 2010; Richardson, 2007; Strickland and Richardson, 2008). For example, epigenetic imprinting patterns change in prostate cancer (Cooper and Foster, 2009) where observations of losses in histone methylation imprints and gains in histone acetylation imprints are common (Cooper and Foster, 2009; Eberharter and Becker, 2002).

1.5 Transcriptional Regulation of the Adaptive Immune Response

A functional immune system is required for the survival of all jawed vertebrates. The human immune system is divided into two parts; innate and adaptive. The innate immune system initiates a non-specific immune response against invading pathogens (Finlay and Falkow, 1997; Sospedra and Martin, 2005) as compared to the adaptive immune system which initiates an antigen specific response(van den Elsen et al., 2004). Receptor specific cells of the adaptive immune system are collectively known as lymphocytes (Van Furth, 1969; van Furth et al., 1966) of which there are two types termed B cells and T cells whose rapid activation and inactivation are necessary to initiate, maintain, and eventually terminate an adaptive immune response (Sainte-Marie, 2010).

Many immune response genes are tightly regulated at the level of transcription in order to maintain control over adaptive immunity (Basta et al., 1987b; Parham, 2005; Sospedra and Martin, 2005; Wang, 2003). One such important group of highly regulated genes is the major histocompatibility complex, MHC (Mach et al., 1996; Wright and Ting, 2006a).

MHC is an important gene family, the protein product of which is expressed on the cell surface of all jawed vertebrates (Gelin et al., 2009). MHC molecules play crucial roles in the presentation of self and non-self antigenic peptides to T cells during an adaptive immune response and are divided into three subgroups: MHC class I, MHC class II and MHC class III (Apanius et al., 1997; Gelin et al., 2009). MHC- I is constitutively expressed on all nucleated cells and presents antigen fragments to cytotoxic T cells via interactions with the CD8 co-receptor (Apanius et al., 1997; Greenberg, 1991). MHC II is constitutively expressed on antigen presenting cells, and is inducibly expressed on nucleated cells in response to pro-inflammatory cytokines (Jabrane-Ferrat et al., 2003; Wright and Ting, 2006b). MHC class II proteins present antigens to T cells by binding to CD4 molecules on T helper cells (Guy et al., 1986; Wright and Ting, 2006b). MHC-III proteins compose a large group of diverse proteins, which include complement proteins, and are involved in immune responses driven by inflammatory cytokines (Basta et al., 1987a; Parham, 2005).

The precise regulation of MHC-II genes plays an important role in the control of adaptive immune responses, and thus limits infection, autoimmune disease and tumor growth(Adamski et al., 2004b; Handunnetthi et al., 2010). MHC-II genes are expressed constitutively in only a few cell types, but their expression can be markedly induced by the inflammatory response cytokine interferon gamma (IFN-γ) (Basta et al., 1987a; Parham,

2005). 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 et al., 2004). The proximal promoter of MHC-II *HLA-DRA* genes consists of conserved cis acting elements and an X and a Y box (Gomez et al., 2005; Zika and Ting, 2005). CIITA is recruited to the MHC-II promoter through interactions with constitutively expressed transcription factors that when combined on the promoter form the MHC-II enhanceosome complex (Masternak et al., 2000). The enhancesome complex consists of regulatory factor X (RFX) (Boss and Jensen, 2003b), nuclear factor Y (NF-Y) (Boss and Jensen, 2003b; van den Elsen et al., 2004) and cAMP response element binding protein (CREB) (Cox and Goding, 1992). In addition, HATS (CBP/p300, pCAF, and SRC-1) and two HMTases (CARM1 and EZH2) interact with the MHC-II promoter and with CIITA (Fontes et al., 1999b; Kretsovali et al., 1998; Zika et al., 2005; Zika and Ting, 2005). 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 **(Figure 2)** (Boss and Jensen, 2003a; Collins et al., 1984; Radosevich et al., 2007).

1. 6 Transcriptional Regulation of the Class II Transactivator

The expression of CIITA correlates with MHC-II expression: cells that lack CIITA expression do not express MHC-II and expression of CIITA drives expression of MHC-II in all nucleated cells (Adamski et al., 2004a; Gerloni and Zanetti, 2005; Guy et al., 1986; Kaufman et al., 1984). Four distinct promoters regulate expression of CIITA in a highly regulated and cell type specific manner (Herrero et al., 2002). CIITA promoter I drives

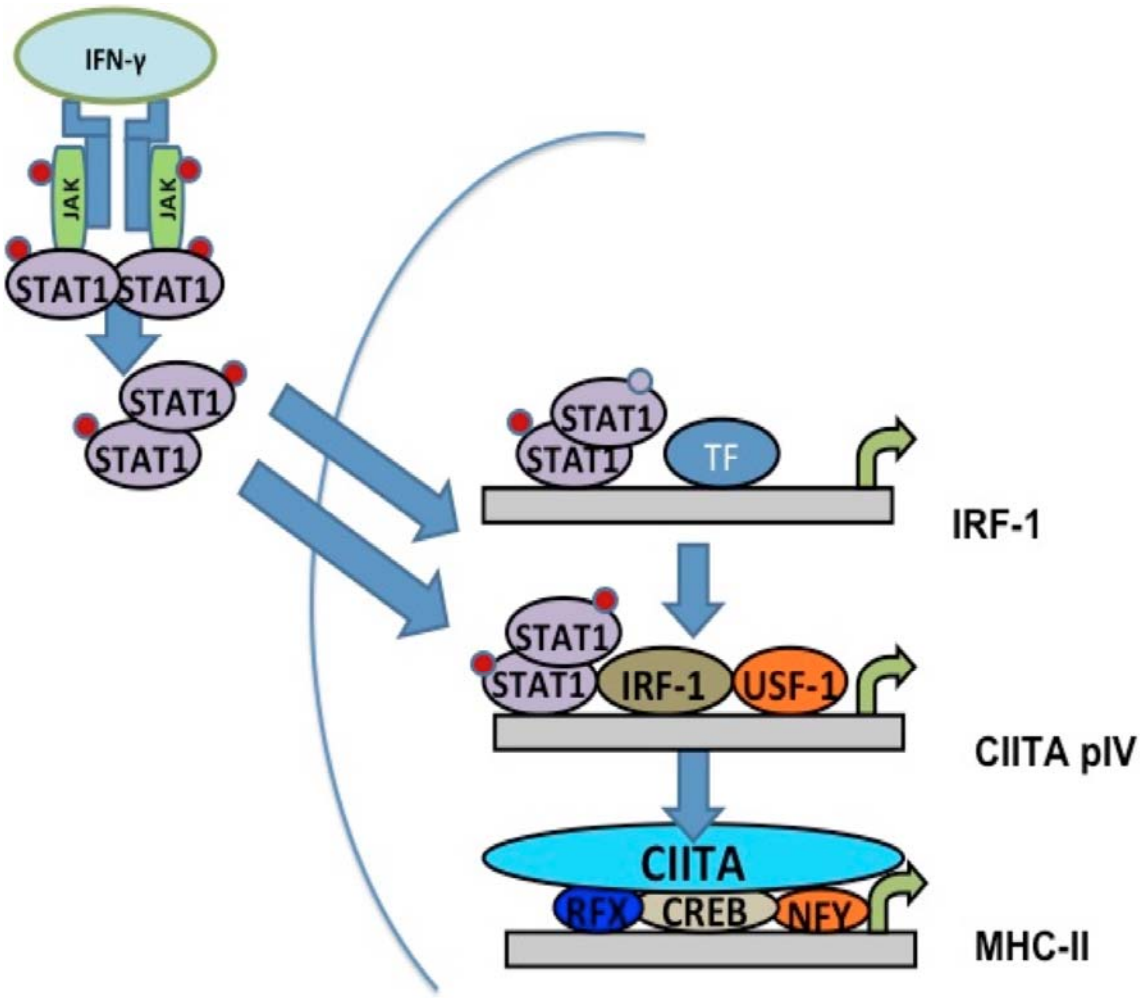

Figure 2. MHC-II expression cascade

Transcriptional regulation of MHC-II expression is controlled by a Master Regulator, the class II transacvitor (CIITA). IFN-γ induction of CIITA expression occurs via the JAK-STAT pathway. The IFN-γ receptor activates the Janus tyrosine kinases,JAK1 and JAK2, leading to phosphorylation of Signal Transduction and Activator of Transcription 1 (STAT1). Upon phosphorylation, STAT1 homodimerizes and translocates to the nucleus where it drives transcription from both CIITA and interferon regulatory factor 1 (IRF-1) promoters. STAT1, IRF-1 and Upstream stimulatory factor 1 (USF-1), bind to CIITA pIV, which leads to the transcription of CIITA. CIITA acts as a master regulator for the expression of MHC-II.

CIITA expression in dendritic cells, CIITA promoter III is responsible for CIITA expression in B cells, and CIITA promoter IV is responsible for the inducible expression of CIITA and is regulated by IFN-γ (Masternak et al., 2000; Muhlethaler-Mottet et al., 1997). To date, the function of CIITA promoter II remains unknown. CIITA pIV is composed of three *cis*-acting elements: an IFN-γ activated sequence (GAS), an E-box, and an IFN response element (IRE) site. IFN-γ stimulation leads to the activation of the JAK/STAT pathway by triggering the phosphorylation of signal transducer and activator of transcription 1 (STAT1) homo-dimers which then translocate to the cell nucleus and regulate the expression of multiple genes (Muhlethaler-Mottet et al., 2004; Muhlethaler-Mottet et al., 1997). STAT1 phosphorylation drives the expression of interferon regulatory factor 1 (IRF1). STAT1 and IRF1 are recruited along with the ubiquitous factor 1 (USF-1) to CIITA pIV. STAT1 binds to the GAS element, USF-1 localizes to the E-box, and IRF1 is recruited at the IRE element (Muhlethaler-Mottet et al., 2004). Binding of these regulatory elements to CIITA pIV leads to the expression of CIITA transcripts, which have previously been observed two hours post IFN-γ stimulation **(Figure 3)** (Moreno et al., 1999; Morris et al., 2002b; Morris et al., 2000b).

Despite the requirement for CIITA expression in MHC-II transcription, strikingly few studies have investigated regulation of the early remodeling events occurring at CIITA pIV and no thorough investigation has occurred as to the early regulatory epigenetic modifications to CIITA pIV. Increases in the levels of acetylation of histones H3 and H4 has been demonstrated to occur upon IFN-γ stimulation (Agalioti et al., 2002; Morris et al., 2002b), and increased tri-methylation of histone H3K4 has been observed upon prolonged

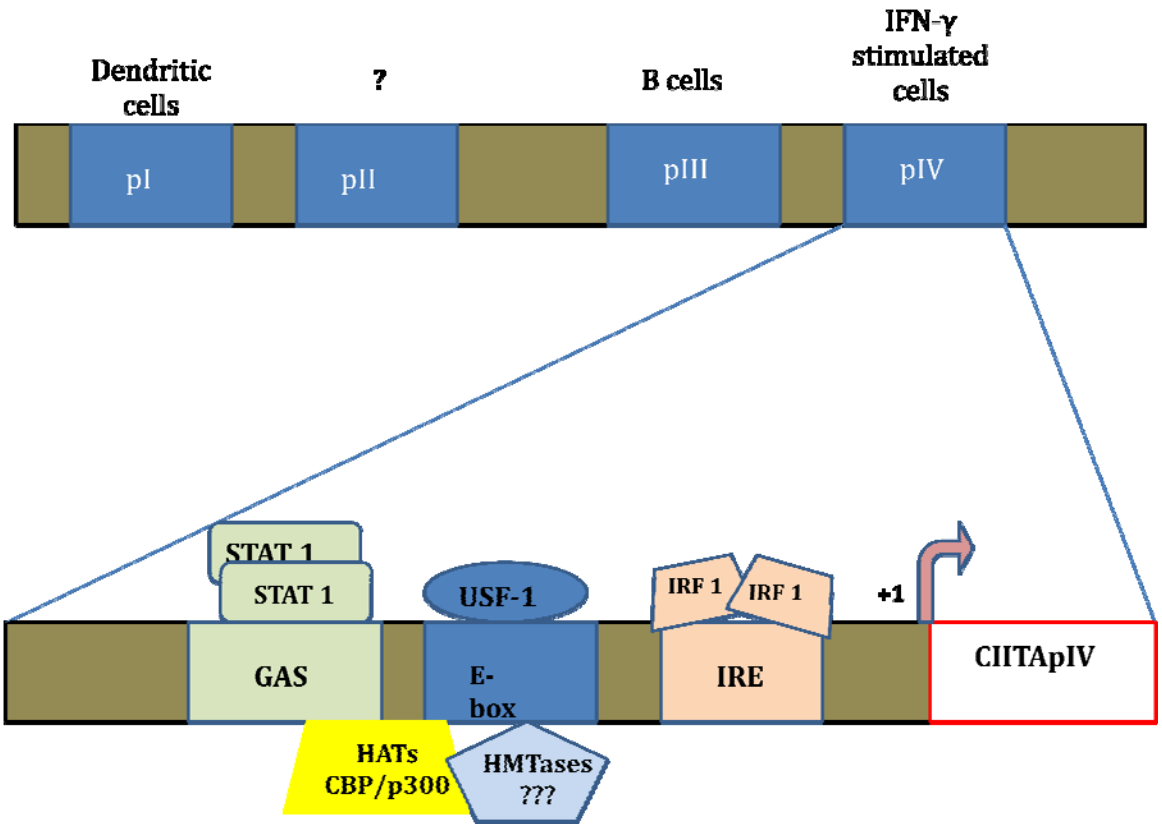

Figure 3. CIITA pIV

Dendritic cells express CIITA through promoter pI while the B-cells use promoter pIII to express CIITA. The role of pII is not known. pIV is the IFN-γ inducible promoter for CIITA. pIV consists of three *cis*-acting elements: an IFN-γ activated sequence (GAS) that binds STAT1 homodimers, an E-box that binds USF-1 and an IFN-response element (IRE) site that binds IRF-1.

cytokine stimulation (Ni et al., 2005). The literature unto this time has shown only two histone modifying enzymes to be recruited to CIITA pIV: the HAT CBP/p300 and the ATP dependent chromatin remodeler BRG1 that binds prior to STAT1 recruitment (Ni et al., 2005; Pattenden et al., 2002).

Much remains to be investigated as to the dynamic chromatin structure that regulates the rapid expression of CIITA upon IFN-γ stimulation. Histone modification studies carried out at CIITA pIV traditionally assay two distinct conditions: unstimulated cells and then a significantly later time period of 4, 18 or 48 hours after IFN-γ (Blanck, 2002; Koues et al., 2009a; Koues et al., 2010; van den Elsen et al., 2000). In the case of CIITA pIV expression, these time points might are potentially less relevant when considering dynamic and regulatory epigenetic modifications (Morris et al., 2002a; Morris et al., 2000b). Previous observations of rapid induction of CIITA message levels from CIITA PIV demonstrate that this CIITA promoter is likely in an open conformation within two hours of IFN-γ stimulation(Morris et al., 2002a); thus, an important area of study is the epigenetic events occurring before these early CIITA transcripts appear.

Finally, the study of CIITA and its transcription control is of great importance. As the master regulator of MHC-II genes (Masternak et al., 2000), aberrant expression of CIITA leads to inappropriate and dysregulated expression of MHC-II proteins (Fontes et al., 1999a; Masternak et al., 2003; 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 all known autoimmune diseases while suppression of MHC-II

expression leads to tumor development and immune deficiency syndromes (Cooper and Foster, 2009)

2. METHODS AND MATERIALS

2.1 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.

2.2 Antibodies:

Histone Modification Antibodies–H3K27me3, H3k9me3 and H3K18ac were from Active Motif (Carisbad, CA) and H3K4me3 antibodies were from Abcam (San Francisco, CA). Antibodies recognizing the enzyme EZH2 were from Millipore (Billerica, MA).

2. 3 Primers and Probes:

All primers and probes were ordered from Sigma-Aldrich (St. Louis, MO).

Primers And Probe	Sequence
GAPDH(ChIP)Forward	AATGAATGGGCAGCCGTTA
GAPDH(ChIP)Reverse	TAGCCTCGCTCCACCTGACT
GAPDH(ChIP) Probe	CCTGCCGGTGACTAACCCTGCGCTCCT
CIITApIV(ChIP) Forward	CAGTTGGGATGCCACTTCTGA
CIITApIV(ChIP) Reverse	TGGAGCAACCAAGCACCTACT
CIITApIV(ChIP) Probe	AAGCACGTGGTGGCC
GAPDH(mRNA)Forward	GGAAGCTCACTGGCATGGC

GAPDH(mRNA)Reverse	TAGACGGCAGGTCAGGTCCA
GAPDH(mRNA) Probe	CCCCACTGCCAACGTGTCAGTG
CIITApIV(ChIP) Forward	GGGAGAGGCCACCAGCAG
CIITApIV(ChIP) Reverse	CGGCTACCACATCCAAGG
CIITApIV(ChIP) Probe	CAAATTACCCACTCCGACCG

2.4 siRNA constructs and transient transfections:

Short interfering RNA (siRNA) were used for the transient knockdown of EZH2. All Star scrambled siRNA was used as the negative control siRNA and this and designed siRNA constructs were obtained from Qiagen (Valencia, CA). HeLa cells were transfected with EZH2 siRNA and were treated with 500 U/ml of IFN-γ from Peprotech (Rocky Hill, NJ). The siRNA transfection were carried out using the RNAiFect Transfection reagent and the protocol provided by Qiagen (Valencia, CA). A separate set of HeLa cellswere transfected with the scrambled siRNA. Cells were lysed in NP40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5 M NaCl, 1M DTT, dH_20) supplemented with Compete EDTA-free protease inhibitor from Roche (Florence, SC) and knock down efficiency was specificity was assessed by western blotting.

2.5 Chromatin immunoprecipitations (ChIPs):

HeLa cells were stimulated with 500 U/ml IFN-γ. Formaldehyde cross-linking was performed with 1% formaldehyde for 10 minutes at room temperature. 0.125 M glycine was added for 5 minutes at room temperature to stop the 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. The samples were sonicated at constant pulse to generate DNA fragments of 500-800 bp. The sonicated samples were run in 1% agarose gels and stained with Ethidium BromideEtBr to check for 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, 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 3 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 IP'd DNA was isolated using phenol:chloroform:isoamyl alcohol mix (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using primers spanning the GAPDH promoter and CIITA pIV. Values graphed were calculated based on standard curves generated.

2.6 Chromatin immunoprecipitation in EZH2 knockdowns*:*

HeLa cells were transfected with EZH2 specific siRNA (Qiagen, CA) or control siRNA (Qiagen). Cells were treated with 5mM sodium butyrate (Millipore, MA) and 500U/ml IFN-γ as indicated and 10% of the total cell volume was lysed with 1% Nonidet P-40 (NP40) buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH_2O) with

protease inhibitor and analyzed by western blot for EZH2 knockdown. The remaining fraction of cells was used to perform ChIP assays as indicated above.

2.7 Dual Crosslinking Chromatin immunoprecipitations (Dual ChIPs):

HeLa cells were stimulated with 500 U/ml IFN-γ at indicated time intervals. Cells were harvested and washed with Phosphate Saline Buffer (PBS) and 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 pH7.4 was added for 5 minutes at room temperature to stop the cross-linking. Cells were rinsed with PBS and were resuspended in 10ml of PBS. Formaldehyde cross-linking was performed with 1% formaldehyde for 10 minutes at room temperature, followed by the addition of 0.125 M for 5 minutes at room temperature to stop further cross linking. The remaining steps of the dual ChIP protocol were done as described in the ChIP protocol above.

2.8 RNA transcript levels

HeLa cells were plated at a density of 8×10^5 cells/10 cm plate and were stimulated with 500 U/ml IFN-γ (Peprotec, NJ) at experiment specific time intervals. Cells were harvested and total RNA was prepared using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. Omniscript reverse transcription kit (Qiagen, CA) was used to generate cDNA using gene-specific antisense primers for CIITA pIV mRNA and GAPDH mRNA. cDNA was quantified via real-time PCR using gene specific primers and probes specific for CIITA pIV and GAPDH . Real-time PCR values were generated based on standard curves generated for each gene and are presented as fold changes over the levels of GAPDH message levels. For the EZH2 knock down studies, RNA extraction protocol

remained the same as mentioned above. The extracted RNA was converted into cDNA using gene specific reverse primers for CIITA pIV mRNA, EZH2 mRNA, and GAPDH mRNA. cDNA was quantified via real-time PCR using gene specific primers and probes specific for CIITA pIV, EZH2, and GAPDH . Real-time PCR values were generated based on standard curves generated for each gene and are presented as fold changes over the levels of GAPDH message levels

3. RESULTS

3.1 Rapid epigenetic changes occur at CIITA pIV 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 typically occurs on lysine residues and is often targeted to lysine (K) residue 18 of histones H3 and H4 (Gorisch et al., 2005; Jenuwein and Allis, 2001a; Roth et al., 2001a). Prior epigenetic studies of IFN-γ inducible CIITA pIV have documented weak to moderate acetylation of histones H3 and H4 in the absence of cytokine induction (Morris et al., 2002b; Wright and Ting, 2006a). This basal acetylation of CIITA pIV allows binding of ubiquitiously expressed USF-1 to CIITA pIV. Following IFN-γ stimulation, CIITA pIV is available for further transcription factor binding; STAT-1 and IRF-1 can bind, recruit the basal transcriptional machinery, and together with USF-1 can drive rapid and robust transcription of CIITA. Binding of STAT-1 and IRF-1 correlates with dramatic changes in CIITA pIV acetylation; of these changes, we and others have previously demonstrated marked increases in H3K18 acetylation following IFN-γ stimulation (Beresford and Boss, 2001b; Truax et al., 2010).

As rapid CIITA expression is required in the initial stages of an adaptive immune response, we sought to determine whether CIITA pIV undergoes rapid epigenetic remodeling in the presence inflammatory cytokine. To evaluate H3K18 acetylation levels immediately following IFN-γ stimulation, HeLa cells were stimulated with IFN-γ, immunoprecipitated with antibodies specific to H3K18 acetylation, and analyzed by real-time PCR with primers spanning the CIITA pIV proximal promoter. ChIP assays showed elevated levels of H3K18 upon IFN-γ stimulation that were significantly increased within 20 minutes of stimulation and remain significantly elevated through 120 minutes of IFN-γ stimulation **(Figure 4, left panel).** Of note, although H3K18 acetylation is immediately increased by inflammatory cytokine at CIITA pIV, maximal levels of acetylation are not reached until 18h following IFN-γ stimulation **(Figure 4, right panel)**.

We and others have previously reported that increases in histone H3 acetylation frequently overlap spatially and temporally with increases in an additional activating epigenetic modification at histone H3: lysine 4 trimethylation (H3K4me3) (Koues et al., 2009b; Wu et al., 2008). In order to determine if regulatory patterns and changes occur in regards to H3K4me3, chromatin immunoprecipitation experiments were performed to determine levels of H3K4me3 at CIITA pIV at time points immediately following IFN-γ stimulation. 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 **(Figure 5)**.

Converse to gains in lysine 18 acetylation and lysine 4 hypermethylation, a loss of histone 3 lysine 9 hypermethylation (H3K9me3) is associated with open chromatin

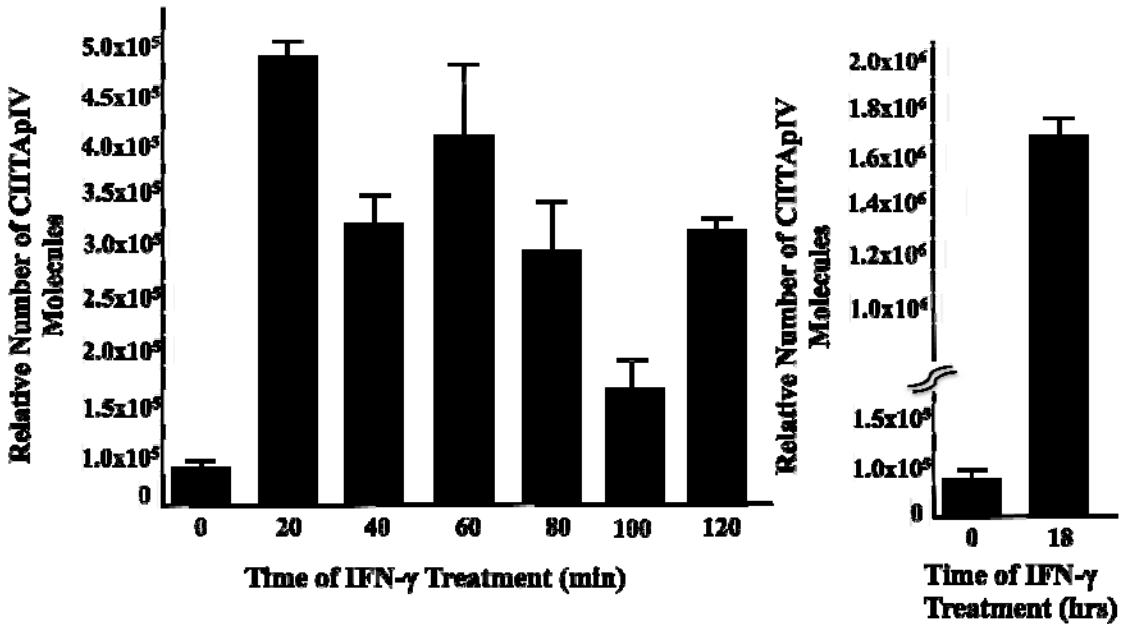

Figure 4. Histone 3 Lysine 18 (H3K18ac) acetylation at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody to H3K18ac. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from an irrelevant control antibody IP (the average relative control value was 1200). Values 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 separate cell lysate preps with each sample analyzed in triplicate.

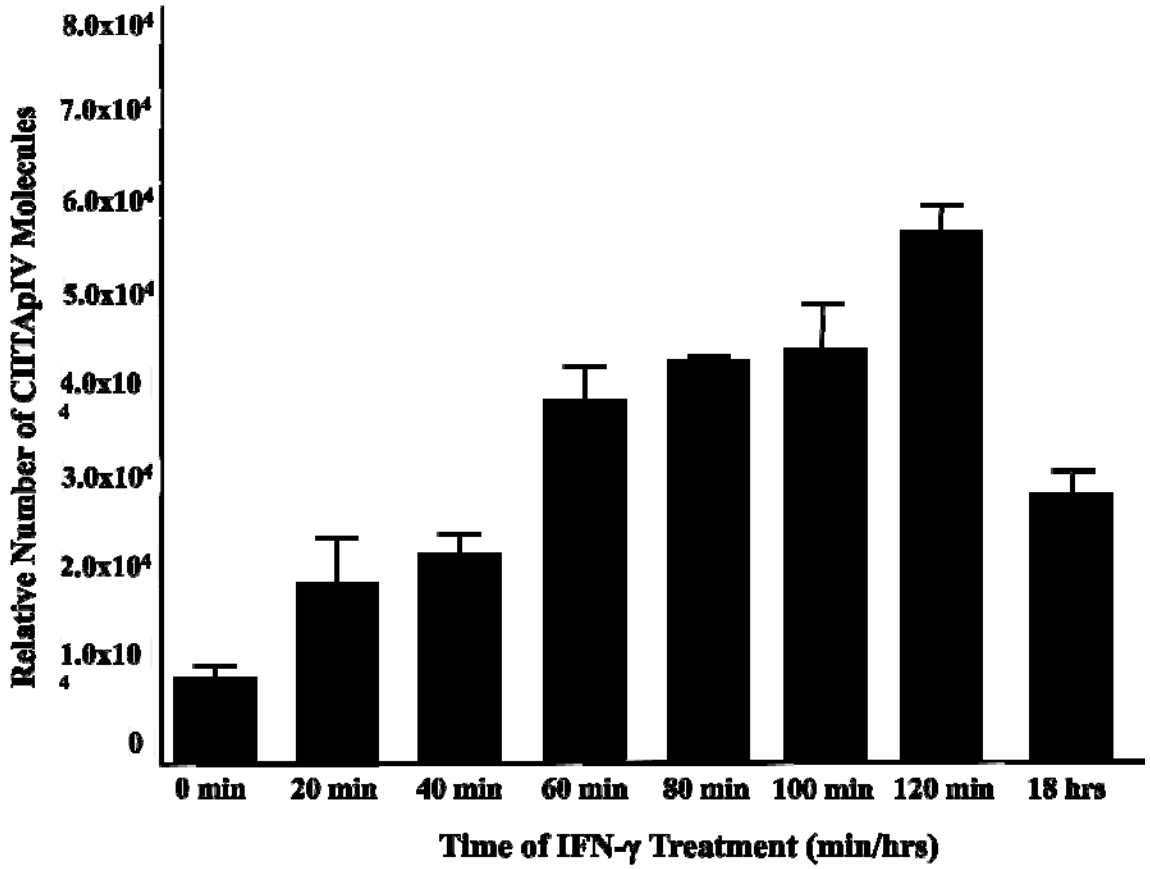

Figure 5. Histone 3 Lysine 4 (H3K4me3) trimethylation at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody to H3K4me3. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from irrelevant control antibody IP (the average relative control value was 1734). Values 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 a single cell lysate preps with each sample analyzed in triplicate.

structure and is thus an indication of active transcription (Agalioti et al., 2002). To determine levels of H3K9me3 at CIITA pIV at early time points following IFN-γ stimulation, HeLa cells were stimulated with IFN-γ, subjected to IP with antibody against endogenous trimethylated H3K9, and analyzed by real-time PCR with primers spanning the CIITA pIV proximal promoter. ChIP assays showed significant decreases in H3K9me3 at CIITA pIV within 20 minutes of IFN-γ stimulation **(Figure 6)**. 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.

Previous studies suggest that the addition of activating methylation events H3K4 we observed in Figure 5 frequently correlate with the depletion of silencing hypermethylation events on histone H3 lysine 27 (H3K27me3) (Ng et al., 2003). To characterize changes in the levels of H3K27me3 at CIITA pIV, we expanded our epigenetic map of this promoter by performing ChIP assays in IFN-γ stimulated HeLa cells. Stimulated HeLa cells were immunoprecipitated with antibody to endogenous H3K27me3 and isolated DNA was analyzed by real time PCR using primers spanning CIITA pIV. In **Figure 7** we observe a gradual decline in H3K27me3 that reached significant levels following 80 minutes of IFN-γ stimulation.

3.2 CIITA pIV is rapidly activated for transcription by the inflammatory cytokine IFN-γ.

Following IFN-γ treatment, dimerization of the 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 et al., 1994). STAT-1 binds the GAS

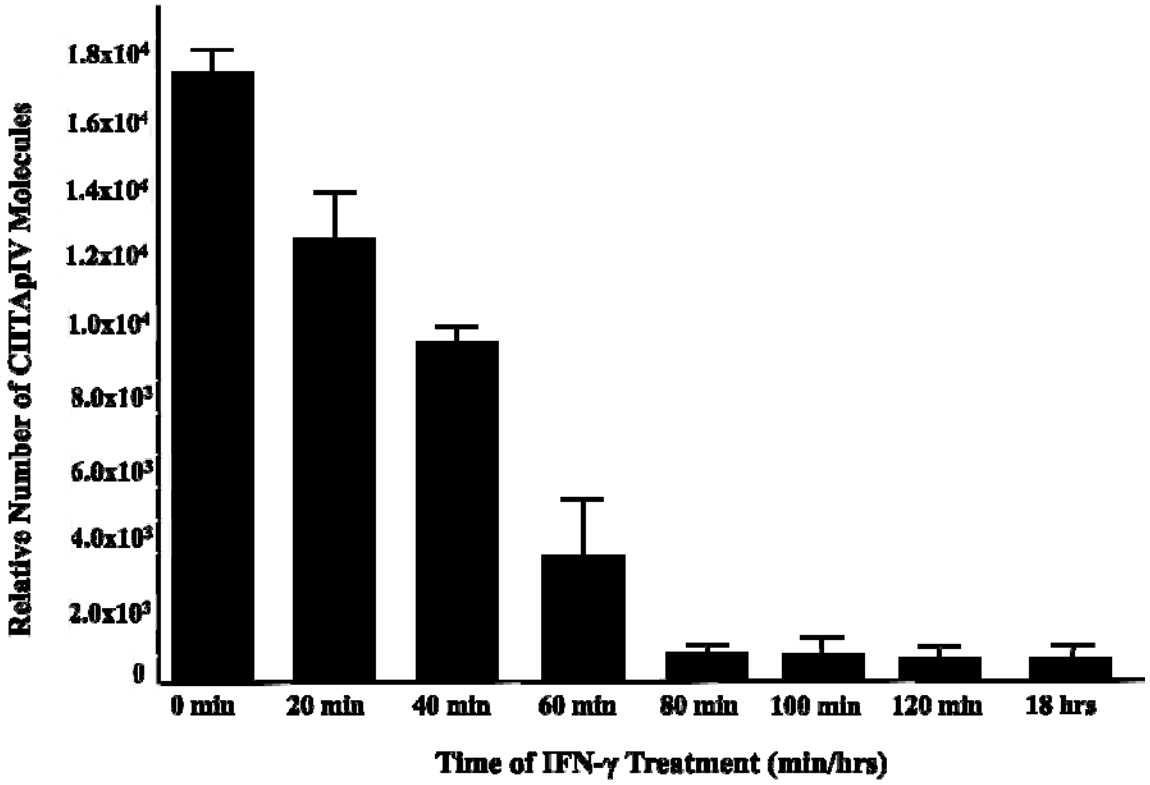

Figure 6. Histone 3 Lysine 9 (H3K9me3) trimethylation at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody to H3K9me3. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from irrelevant control antibody IP (the average relative control value was 598). Values 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 separate cell lysate preps with each sample analyzed in triplicate.

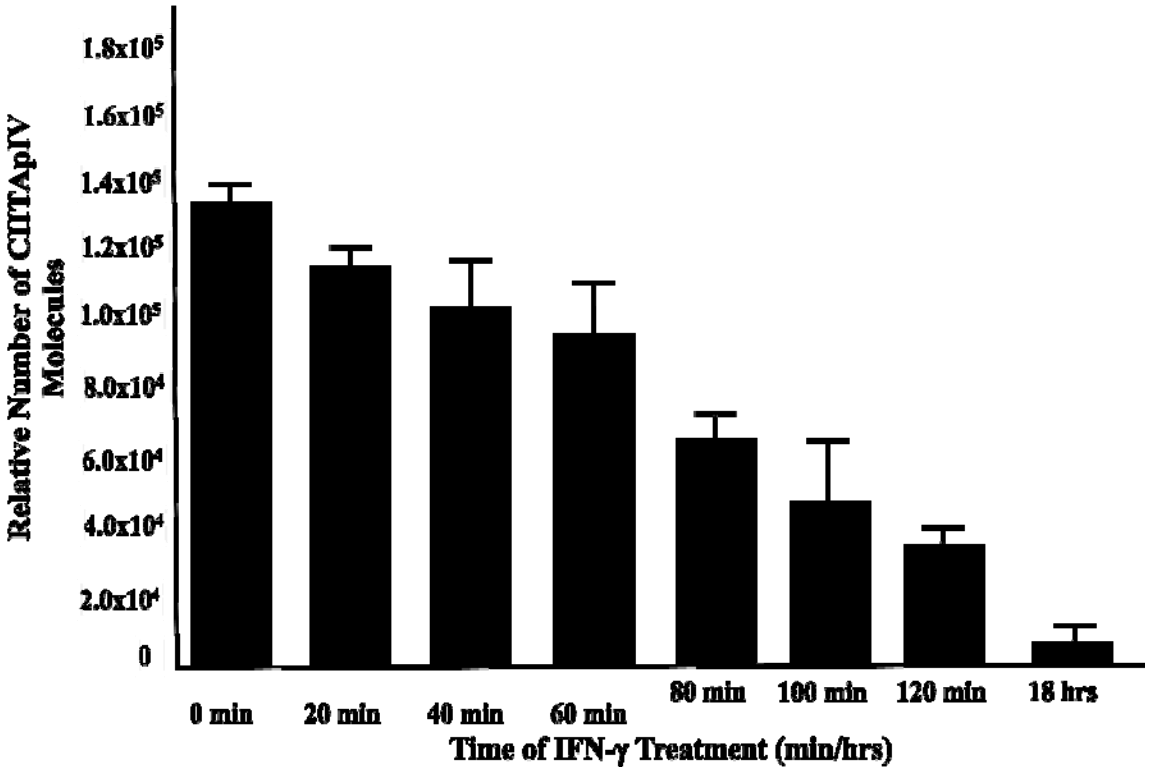

Figure 7. Histone 3 Lysine 27 (H3K27me3) trimethylation at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody to H3K27me3. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning that IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from irrelevant control antibody IP (the average relative control value was 234). Values 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 a single cell lysate preps with each sample analyzed in triplicate.

element of CIITA pIV and, along with USF-1, IRFs, and the basal transcriptional machinery, assembles the transcriptional initiation complex necessary to promote transcription from CIITA pIV (Chang et al., 1994; Morris et al., 2002a; Muhlethaler-Mottet et al., 1998). As binding of STAT1 to CIITA pIV only occurs if the promoter is in an open conformation, we addressed the effectiveness of early activating epigenetic modifications to CIITA pIV by performing dual cross-linking ChIP assays to determine the levels of STAT-1, bound to CIITA pIV following IFN-γ stimulation. ChIP mapping assays demonstrated significant STAT-1 localization to CIITA pIV within 20 minutes of IFN-γ stimulation **(Figure 8)**. These data provide further evidence that rapid changes in CIITA pIV chromatin structure are effectively opening the promoter and allowing assembly of initiation complexes on the proximal promoter of CIITA pIV.

Based on the above observations, significant activating epigenetic events occur at CIITA pIV within the first 20 minutes of INF-g stimulation. To address the effectiveness of these modifications in activating expression of CIITA from pIV, we assayed CIITA pIV transcript levels in HeLa cells stimulated with IFN-γ for various time points. In contrast to previous studies demonstrating delayed CIITA mRNA expression (Boss and Jensen, 2003b; Morris et al., 2002b), real-time PCR results in **Figure 9** indicate CIITA transcript levels increase to detectable levels as early as 40 minutes following IFN-γ stimulation.

3.3 Increases in CIITA pIV 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

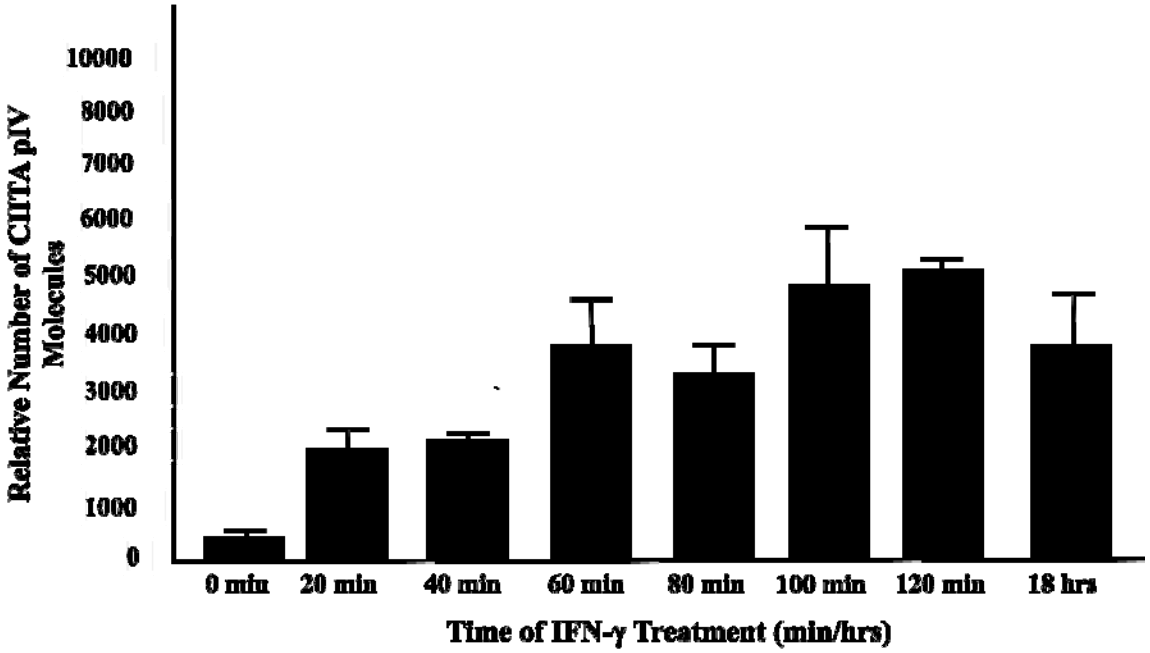

Figure 8. STAT1 binding at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody toSTAT1. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from irrelevant control antibody IP (the average relative control value was 432). Values 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 two separate cell lysate preps with each sample analyzed in triplicate.

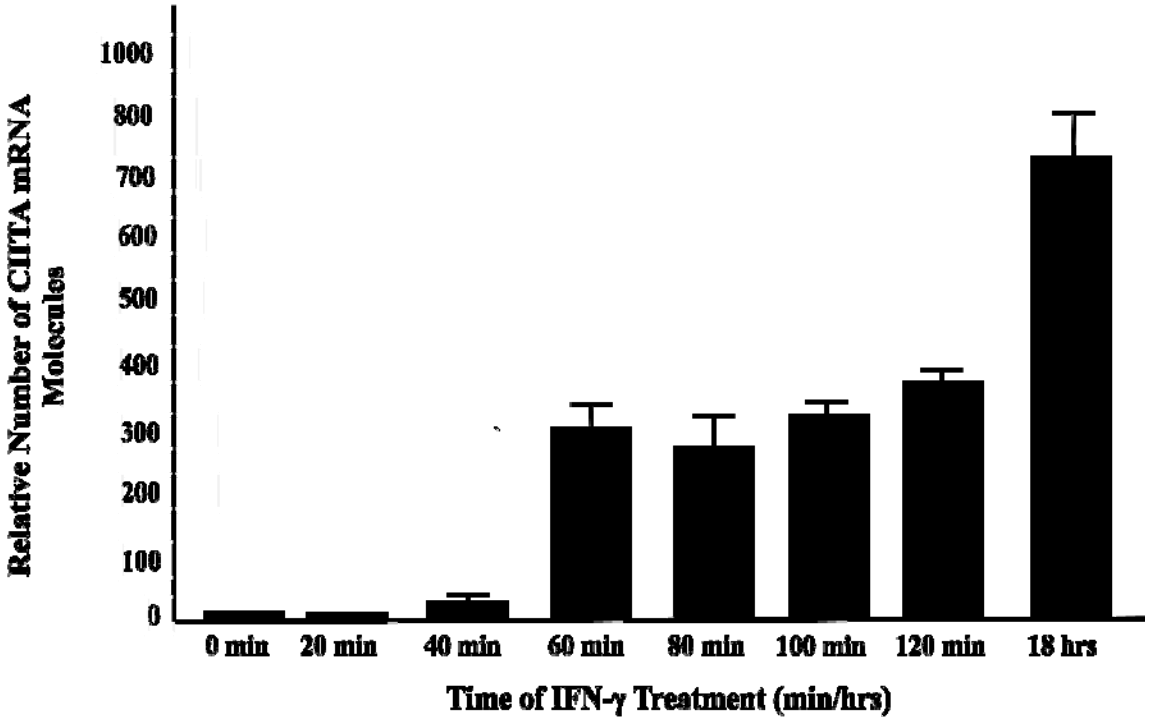

Figure 9. CIITA message levels post IFN-γ stimulation

CIITA and GAPDH mRNAs were quantitated by real time RT-PCR performed on mRNA isolated from HeLa cells following treatment with IFN-γ for the time period indicated. The relative value represents the value of CIITA mRNA and was determined after normalization to GAPDH mRNA.

complexes and histone modifying enzymes. Histone methyltransferases (HMTs) are chromatin remodeling enzymes capable of adding one, two or three methyl groups to lysine residues and one or two methyl groups to arginine residues on histones

H1, H2A, H3 and H4 (Bannister and Kouzarides, 2004; Schubeler et al., 2004) and can activate (H3K4) or suppress (H3K27) transcriptional processes. 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 has previously been shown to interact with CIITA (Morris et al., 2000a; Wright and Ting, 2006b). In order to determine if the loss of H3K27me3 observed at CIITA pIV was consistent with a rapid loss of EZH2 binding to the promoter, we performed dual crosslinking ChIP assays by immunoprecipitating endogenous EZH2 from IFN-γ stimulated HeLa cells. Data in **Figure 10** demonstrates an abundance of EZH2 at CIITA pIV 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 correlates with the previously observed decreases in H3K27me3 and increases in activating modifications, promoter availability and CIITA transcript levels. Based on this observed we tired to determine the role of EZH2 in controlling the expression of CIITA.

3.4 Loss of EZH2 leads to an aberrant CIITA expression.

As mentioned earlier, EZH2 is a key epigenetic regulator. EZH2 expression was knocked down by the use of siRNA. The efficiency of the siRNA was determined using western blotting and by quantifying the EZH2 mRNA present in the cell. There was about a 60% reduction case of the mRNA level of EZH2 in Ezh2 siRNA transfected cell when

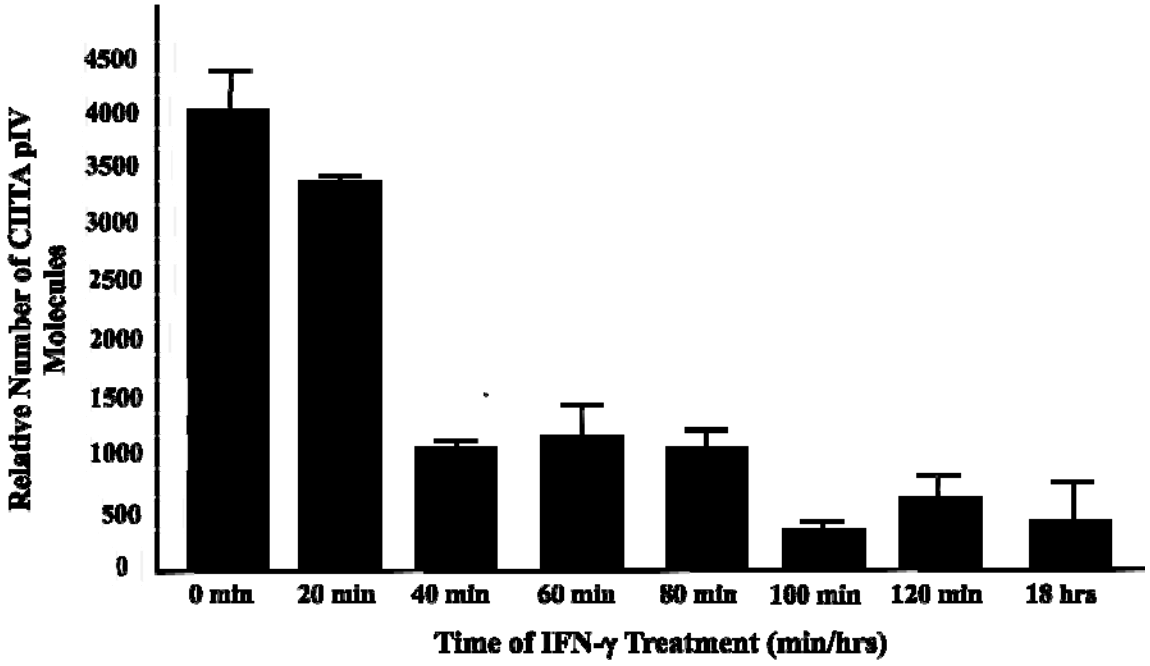

Figure 10. EZH2 binding at CIITA promoter IV

HeLa cells were stimulated with IFN-γ, harvested, crosslinked with formaldehyde, and cell were lysed and sonicated to generate fragments of approximately 750bp. Lysates were immunoprecipitated (IP) with antibody to EZH2. Associated DNA was isolated and analyzed via Real-time PCR using primers spanning the IRF-1 binding site at CIITA PIV. Real-time PCR values were determined by subtracting values obtained from irrelevant control antibody IP (the average relative control value was 127). Values 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 a three cell lysate preps with each sample analyzed in triplicate.

compared to cells that were transfected with a scrambled siRNA **(Figure 11)**. In case of the EZH2 siRNA transfected cell was observed that the CIITA mRNA is present in unstimulated cells. EZH2 siRNA transfected cells were treated for 20 minutes of IFN-γ showed a rise in the levels of CIITA mRNA transcript. In contrast to this the cell that were transfected with the scrambled siRNA showed no CIITA message in unstimulated cells and also the cells that were stimulated with IFN-γ for 20 minutes **(Figure 11)**.

4. DISCUSSION

Our study is the first to describe the early epigenetic events occurring at CIITA pIV. As the master regulator of MHC-II genes, levels of CIITA regulate MHC-II expression and are therefore tightly regulated (Adamski et al., 2004a; Gerloni and Zanetti, 2005; Guy et al., 1986; Kaufman et al., 1984) as over expression of MHC-II leads to the development of autoimmune disorders and low MHC-II expression is correlated with increased incidence of cancer and immunodeficiency disorders (Brooks et al., 2010; Parham, 2005; Schulz and Hatina, 2006; Simon and Lange, 2008). CIITA plays a crucial role in maintaining control over the expression of MHC- II expression (Beresford and Boss, 2001a) and previous studies from our lab and others have shown that epigenetic events play an important role in controlling CIITA expression (Boss and Jensen, 2003a; Greer et al., 2003; Koues et al., 2009b; Morimoto et al., 2004). We observe here that in rapid succession following IFN-γ stimulation, regulatory epigenetic events begin to occur at CIITA pIV, the primary IFN-γ inducible CIITA promoter of CIITA genes. We first observed that H3K18 acetylation levels begin to rise post IFN-γ stimulation. The increase in the H3K18 acetylation modification is

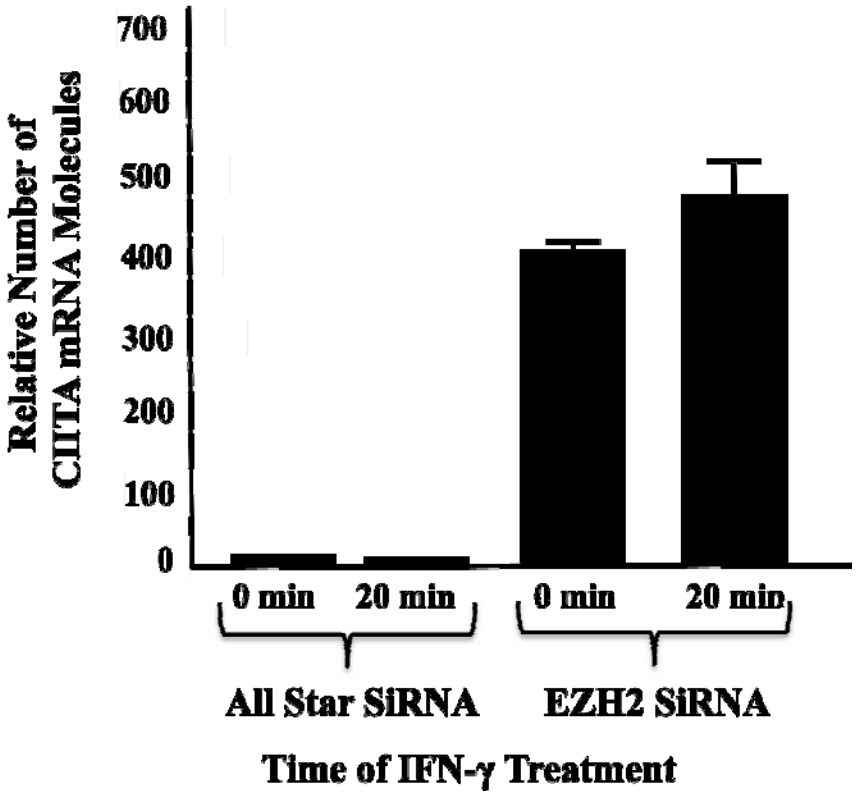

Figure 11. CIITA message levels post IFN-γ stimulation in cells treated with EZH2 siRNA and All Star siRNA.

CIITA and GAPDH mRNAs were quantitated by real time RT-PCR performed on mRNA isolated from HeLa cells that were transfected with the respective siRNA. Following the siRNA transfections, the cells were treatment with IFN-γ for the time period indicated. The relative value represents the value of CIITA mRNA and was determined after normalization to GAPDH mRNA.

non-linear and likely indicates an immediate, but non-maximal loosening of the chromatin template following IFN-γ stimulation. We next observed steady increases in the activating addition of methyl groups to histone H3 lysine 4. The levels of H3K4me3 significantly increase throughout the 120 minute time course indicating a dependence on accumulation of epigenetic marks in addition to H3K18 acetylation for maximal methylation of lysine 4.

In contrast to gains in K18 acetylation and loses in K4 methylation is the silencing modification of H3K9me3, which is lost at CIITA pIV upon IFN-γ stimulation. Levels of H3K9me3 slowly and significantly decline over the 120 minute IFN-γ time course and remain at baseline for the duration of IFN-γ stimulation. A similar trend is observed in the case of H3K27me3. Loss of the inhibitory H3K27me3 modification occurs upon IFN-γ stimulation, with the decline in K27me3 mirroring gains in H3K4me3, and suggesting competitive targeting of lysines 27 and 4 in epigenetic regulation at CIITA pIV.

Rapid changes in regulatory epigenetic modifications to histone H3 at CIITA pIV following IFN-γ stimulation are indicative that chromatin is quickly opened and transcription rapidly occurs following exposure of cells to inflammatory cytokine. This assumption was confirmed by observations that STAT-1 quickly binds CIITA pIV and drives expression of CIITA transcripts. STAT1 binding to the GAS sequence is one of the first trans acting events occurring at CIITA pIV (Morris et al., 2002a) and STAT-1 binding and CIITA expression correlate with the rise in the levels of H3K18ac and H3K4me3, and with the drops in the levels of H3K9me3 and H3K27me3.

It is important to note that levels of STAT-1 binding to CIITA pIV do not significantly change after 60 minutes of IFN-γ stimulation, indicating that beyond this point, CIITA pIV

maintains an open chromatin structure and active levels of transcription, despite greatly enhanced levels of H3K18 acetylation at later timepoints. Observations that levels of H3K18ac do not peak until after 120 minutes of IFN-γ stimulation are in contrast to the rapid decrease in suppressing levels of H3K9me3 and H3K27me3, and suggest that the removal of methyl groups from CIITA pIV may play important roles in the initial control of CIITA activation. Similar observations that strong binding of the HMT EZH2 to CIITA pIV rapidly declines following IFN-γ stimulation add further support to the regulatory functions histone methylation at CIITA pIV.

Relevant to our study are observations that changes in chromatin structure (Jones and Baylin, 2002; Jones and Laird, 1999) and tissue specific over expression of EZH2 are associated with various types of cancers (Cooper and Foster, 2009; Jones and Laird, 1999; Schulz and Hatina, 2006; Simon and Lange, 2008). As EZH2 can collaborate with DNA methyltransferases (DNMT), and histone deacetylases (Jackson et al., 2002; Margueron et al., 2008; Simon and Lange, 2008), it is likely that improper methylation at CpG islands concurs with aberrant histone methylation and leads to faulty gene expression patterns in many cancers. Our study is the first to link the regulation of rapidly occurring epigenetic changes to EZH2 and has strong implications for the regulation of not only CIITA, but for many other inducible genes. If EZH2 is truly a 'master regulator' of epigenetic modifications to histones, then the development of drugs targeting EZH2 will be of significant future interest.

In regards to the epigenetic regulation of CIITA, our study is the first report implicating EZH2 in regulating the initial chromatin remodeling events at cytokine-

inducible promoters. Knowledge that CIITA pIV is rapidly released from surrounding chromatin following IFN-γ stimulation strongly suggest that EZH2 is predominantly involved in initial chromatin remodeling in response to stimuli, A full understanding of the contributions of EZH2 to the epigenetic regulation of CIITA expression requires further study into the molecular interactions occurring at this promoter.

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