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TO DEGRADE OR NOT TO DEGRADE: THE ROLE OF P300/CBP-ASSOCIATED FACTOR
(PCAF) IN CIITA STABILITY AND UBIQUITINATION

by

JEANNE K. BROOKS

Under the Direction of Dr. Susanna F. Greer

ABSTRACT

The ubiquitin-proteasome pathway plays vital roles in multiple cellular processes including protein turnover and transcription regulation. The fate of a ubiquitinated protein is determined by the number of ubiquitin molecules added and the site to which they are added. Monoubiquitinated proteins are stabilized and often activated, while polyubiquitinated proteins are rapidly targeted for degradation. Major histocompatibility complex class II (MHC II) molecules are a vital part of the immune response and are responsible for presenting antigens to CD4⁺ T cells. The class II transactivator (CIITA) is the master regulator of MHC II transcription and has been shown to have increased transactivity when monoubiquitinated. The focus of this thesis is on the impact of ubiquitination on CIITA stability and MHC II gene expression through the identification of an E3 ligase that targets and ubiquitinates CIITA.

INDEX WORDS: MHC II, pCAF, Ubiquitination, CIITA, Adaptive immunity

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JEANNE K. BROOKS

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

Master of Science

in the College of Arts and Sciences

Georgia State University

2009

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Jeanne K. Brooks
2009

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(PCAF) IN CIITA STABILITY AND UBIQUITINATION

by

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College of Arts and Sciences
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August 2009

DEDICATION

Once upon a time, in a land far, far away there was a little girl. This little girl would spend hours asking her father questions; questions about everything from why the sky is blue to what made her look like him. He patiently answered all of her questions to the best of his knowledge and always told her that one day she would grow up to be a scientist. She eventually began her college career to be an elementary school teacher, far from the scientist dreams her father had for her. One day she started her first biology class, and as that class went on she realized how fascinating and amazing the world could be through the eyes of a scientist. She thought back to what her father had been saying for so many years, and finally decided that maybe he was right. She changed her major and the rest is history.

This thesis is dedicated to the memory of my father who never got to see me become his “little scientist” as he always dreamed. His guidance, patience, and support of my learning and development throughout my childhood have made me the curious and questioning individual that I am today. Without his support and influence on my life I would not be the individual that I am. While my father was the biggest influence throughout my childhood and my journey towards science I would not be where I am today without the support of many family members and friends; especially my husband who has put up with many moods and stressful times, and has been my source of strength, reason, and at times even sanity.

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Also, my lab mates, how long the days would be without all of you there to share the joys, frustrations, and sometimes downright insanity of work in a laboratory. We have had some rough times, and some good ones and hopefully we've learned a little bit of science while we were at it. You are all so willing to help, and have helped me really learn and grow during our time together. I look forward to our future, not as fellow students, but as colleagues and friends.

My mom Denise, for always being there when I needed an ear to talk to and a little bit of encouragement; and of course my siblings, Andy, Adam, and Jessica, all of which have listened to my frustrations, and offered their version of encouragement, my husband Dustin for his unwavering support and encouragement throughout this. And finally my grandparents, Max and Betty, who still "don't have a clue what I do" but have supported me both materially and through their love and encouragement throughout my college career.

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CHAPTER 1.

INTRODUCTION

Innate Immunity

The immune system of vertebrates is made of two distinct branches, innate immunity and adaptive immunity (Medzhitov and Janeway 1998; Janeway, Travers et al. 2005). While innate immunity is evolutionarily ancient and found in most multicellular organisms, adaptive immunity is only found in higher order vertebrates. Both branches contain however specific cells and tissues that perform distinct roles in host defense against pathogens (Medzhitov and Janeway 1998; Janeway and Medzhitov 2002; Janeway, Travers et al. 2005). The innate branch of the immune system is the first line of defense against infection and is vital in controlling infections until and if an adaptive response is mounted. In addition, innate immunity also plays critical roles in activating the adaptive immune response (Medzhitov and Janeway 1998; Janeway and Medzhitov 2002; Janeway, Travers et al. 2005). The innate immune system initiates immediate inflammatory responses to infection, in which macrophages, neutrophils, basophils, eosinophils, and mast cells act rapidly in order to control infection (Janeway and Medzhitov 2002; Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). If a pathogen eludes these responses, the adaptive immune response is activated by the presentation of antigens from pathogens that have infiltrated secondary lymphatic tissue (Janeway and Medzhitov 2002; Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005).

While the innate immune system is a fundamental component of host defense, it lacks two key features that make the adaptive immune response critical for survival; specificity for the pathogens that it encounters, and immunological memory of these pathogens (Medzhitov and Janeway 1998; Janeway 2001; Janeway and Medzhitov 2002; Drozina, Kohoutek et al. 2005;

Janeway, Travers et al. 2005). The importance of these key factors, the ability to respond quickly to antigens, and the ability to provide long term protection from pathogens makes the adaptive immune response absolutely critical for survival (Farber, Acuto et al. 1997; Janeway 2001).

Adaptive Immunity

Once activated by pathogen, the adaptive immune response relies on specific effector cells which allow for precise pathogen recognition through a repertoire of receptors (Farber, Acuto et al. 1997). While the innate immune system also contains receptors effective at recognizing pathogens, these receptors are germline encoded, and are most effective at distinguishing between self and non-self (Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). The effector cells of the adaptive immune system, B-cell and T-cells, are collectively called lymphocytes (Medzhitov and Janeway 1998; Janeway and Medzhitov 2002; Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). Lymphocytes provide specific pathogen recognition through their receptors, which are generated through a series of genetic rearrangements to allow for the generation of receptors capable of recognizing any antigen presented to the host (Janeway and Medzhitov 2002; Janeway, Travers et al. 2005). Antigen presenting cells play a major role in the adaptive immune response, by presenting antigens to lymphocyte receptors and thus activating the adaptive immune response (Abbas and Janeway 2000; Janeway 2001; Janeway, Travers et al. 2005). Following the activation of a B or T cell, these cells clonally divide and generate a vast number of daughter cells, each of which contains the specific receptor needed to eliminate the pathogen (Janeway, Travers et al. 2005).

B cell receptors can occur either as cell bound receptors or as secreted antibodies (Janeway, Travers et al. 2005). Both receptor forms serve distinct functions in the adaptive

immune response to pathogens. Antibodies serve two functions to recognize and bind specifically to antigenic molecules, and to recruit additional immune cells to elicit specific effector functions at the site of infection (Janeway, Travers et al. 2005). The cell bound B cell receptor is able to recognize a wide variety of antigenic macromolecules and chemicals which allows the B cell to internalize, process, and present antigenic peptides to T cells (Abbas and Janeway 2000; Janeway 2001; Janeway, Travers et al. 2005). T cell receptors occur only in a cell bound form, a unique feature of the T cell receptor is that it does not bind to antigens directly, but rather antigens must be presented to the T cell receptor (Abbas and Janeway 2000; Janeway, Travers et al. 2005). An additional feature of T cells that makes them distinct from B cells is that there are several different types of T cells, each serving a unique function in the adaptive immune response.

T Cells

There are several distinct types of T cells that have been well defined with specific known effector functions (Janeway, Travers et al. 2005). Natural killer T cells are considered part of the innate immune system, but are an important link between the innate and adaptive immune responses (Janeway, Travers et al. 2005). These cells do not have antigen specific receptors but recognize abnormal cells, and following this recognition, can release cytokines and lytic granules to kill the abnormal cell (Janeway, Travers et al. 2005). Regulatory T cells or T_{regs} serve to suppress autoreactive T cells and thus play a large part in preventing autoimmune diseases. Accordingly, the loss of T_{regs} has been shown to be linked to a higher instance of autoimmune diseases including rheumatoid arthritis and multiple sclerosis (Abbas and Janeway 2000; Marzo, Kinnear et al. 2000; Janeway, Travers et al. 2005; Swanberg, Lidman et al. 2005). Cytotoxic CD8 killer T cells recognize and destroy cells that are infected with cytosolic antigens,

specifically viral or tumor antigens (Abbas and Janeway 2000; Janeway, Travers et al. 2005).

These cells play a fundamental role in controlling infection, because they attempt to prevent the spread of infection by killing cells that are abnormal (Abbas and Janeway 2000; Janeway, Travers et al. 2005)

The remaining subset of T cells are the helper $CD4^+$ T cells which are further divided into two classes: T_H1 and T_H2 helper cells. Both classes of $CD4^+$ T are responsible for activating additional cells of the immune system through the secretion of specific cytokines (Abbas and Janeway 2000; Janeway and Medzhitov 2002). $CD4^+$ T_H1 cells are activated by intracellular antigens or antigens that have been ingested by phagocytic cells. The T cells then release interferon- γ to activate the phagocytic cells to kill the ingested pathogen. In addition $CD4^+$ T_H1 cells can also activate B cells to stimulate endocytosis of pathogens (Abbas and Janeway 2000; Janeway, Travers et al. 2005). $CD4^+$ T_H2 helper cells are specific in activating B cells and stimulating antibody production against antigens (Abbas and Janeway 2000; Janeway, Travers et al. 2005). $CD4^+$ T_H2 helper cells do not recognize antigens directly but rather recognize major histocompatibility complex II (MHC II) molecules that process and present antigens on both antigen presenting cells and nucleated cells activated during an inflammatory response (Medzhitov and Janeway 1998; Abbas and Janeway 2000; Janeway 2001; Janeway, Travers et al. 2005). Following activation of both $CD8^+$ and $CD4^+$ T cells, memory T cells will be created, these cells are a fundamental part of the adaptive immune response and are where the important immunological memory of the adaptive immune response is derived (Abbas and Janeway 2000; Janeway, Travers et al. 2005). These memory cells remain in the system after an infection is cleared and upon re-infection of the host are able to mount a rapid and specific immune response to the antigen (Abbas and Janeway 2000; Janeway, Travers et al. 2005). This same principle of

memory also applies to B cells (Janeway, Travers et al. 2005). This interaction between T cells and MHC molecules is crucial for the activation and maintenance of the adaptive immune response.

Major Histocompatibility Complex

As stated previously T cells do not recognize antigens directly, instead antigenic peptides are presented to T cells by MHC molecules (Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). Cells infected with a pathogen will display these molecules on their surface to alert T cells that an infectious agent is present (Abbas and Janeway 2000; Janeway, Travers et al. 2005). There are two classes of MHC molecules, MHC I and MHC II; while similar, each class performs a specific function in the adaptive immune response (Medzhitov and Janeway 1998; Janeway and Medzhitov 2002; Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). The MHC complexes vary in the type of cell that presents them, in the antigen they can present and the type of effector cell that they are able to activate (Janeway 2001; Janeway, Travers et al. 2005). MHC I molecules are responsible for presenting exogenous peptides from the cytosol, such as viral or tumor antigens, to CD8 T cells, and are therefore expressed on all nucleated cells (Abbas and Janeway 2000; Janeway, Travers et al. 2005). MHC II molecules are responsible for presenting exogenous peptides from intracellular compartments to CD4⁺ T cells, and are expressed constitutively on the antigen presenting cells of the immune system: B cells, dendritic cells, and macrophages (Abbas and Janeway 2000; LeibundGut-Landmann, Waldburger et al. 2004; Janeway, Travers et al. 2005). Expression of MHC II molecules on other nucleated cells types can be induced by the inflammatory cytokine interferon- γ , and this inducible expression plays a major role in altering T cell response to infections (LeibundGut-Landmann, Waldburger et al. 2004; Janeway, Travers et al. 2005).

Variations of MHC II

MHC II molecules are heterodimeric, transmembrane glycoproteins which are constitutively expressed on the surface of antigen presenting cells and their expression is further inducible by interferon- γ on all nucleated cells (Waldburger, Masternak et al. 2000; Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). The genes that encode MHC II complexes are located on chromosome six, and are extremely variable (Drozina, Kohoutek et al. 2005; Janeway, Travers et al. 2005). This variation results in different MHC II isotypes that can be expressed on the surface of cells. The main isotypes of MHC II are the HLA-DP, HLA-DQ, and HLA-DR; two additional isotypes, HLA-DM and HLA-DO are considered non-classical and are not expressed on the surface of cells but, are instead involved in the cytoplasmic loading of peptides (Waldburger, Masternak et al. 2000; Drozina, Kohoutek et al. 2005). Together these different MHC II isotypes are able to present different peptides to T cells, which in turn further increases the diversity of peptides that the immune system can recognize (Waldburger, Masternak et al. 2000; Drozina, Kohoutek et al. 2005).

Activation by MHC

Both classes of MHC molecules activate the adaptive immune response. MHC I presentation activates CD8⁺ T cells to kill the infected cell which is presenting the exogenous peptide (Abbas and Janeway 2000; Janeway, Travers et al. 2005). MHC II presentation activates CD4⁺ T cells, which leads to further activation of the adaptive immune response, specifically macrophage phagocytosis and B cell antibody production (Abbas and Janeway 2000; Janeway, Travers et al. 2005). The expression of MHC II genes is critical for the initiation, maintenance, and termination of all responses to extracellular pathogens by the adaptive immune response,

therefore the expression and activation of these genes is of specific importance and is tightly regulated (Abbas and Janeway 2000; Janeway 2001; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005).

MHC II Expression

MHC II molecules present antigens to CD4⁺ T cells play a major role in the regulation of the adaptive immune response, and are therefore highly regulated. Dysregulation of MHC II expression has dire results because of the vital role that CD4⁺ T cells play in activating the adaptive immune response. Deficiencies in MHC II molecules result in a rare but fatal disease called Bare Lymphocyte Syndrome (BLS) (Reith and Mach 2001; Drozina, Kohoutek et al. 2005). BLS is characterized by the lack of constitutive and inducible MHC II expression (Drozina, Kohoutek et al. 2005; Otten, Leibundgut-Landmann et al. 2006). In comparison, over expression of MHC II is associated with the majority of autoimmune diseases, including diabetes, arthritis and multiple sclerosis (Swanberg, Lidman et al. 2005). MHC II molecules also play a role in the immune response to tumors, as tumors often down regulate MHC II expression to evade the immune system (Guy, Krajewski et al. 1986; Wang 2003).

MHC II expression, both constitutive and inducible, is therefore tightly regulated, and this regulation occurs primarily at the level of transcription (Waldburger, Masternak et al. 2000). Multiple proteins orchestrate the transcriptional regulation of MHC II molecules (Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Schnappauf, Hake et al. 2003). The proximal promoter region of MHC II genes is a highly conserved region that contains several cis-acting regulatory elements, the S, X, X2, and Y boxes (Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005; Zika and Ting 2005). These elements bind to and recruit specific regulatory proteins to the promoter that orchestrate the

transcription of MHC II genes. Regulatory factor X, (RFX), is a complex of three subunits (RFX5, RFX-AP, RFX-ANK), which binds to the S and X boxes (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005; Zika and Ting 2005). Nuclear factor Y, (NFY), a complex of three subunits (NFYA, NFYB, and NFYC) binds to the Y box (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005; Zika and Ting 2005). Finally the cyclic adenosine monophosphate, (c-AMP), responsive element binds at the X2 box (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005; Zika and Ting 2005).

Each of these factors is ubiquitously expressed and absolutely required for MHC II expression; however their expression and interactions with the proximal promoter regions of MHC II genes are insufficient for activation of MHC II transcription (Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005). These factors, in addition to other basal transcription factors, combine at the proximal promoter region to form a binding platform termed the enhanceosome, which recruits the master regulator of MHC II, the class II transactivator (CIITA) (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Harton, Zika et al. 2001; Jabrane-Ferrat, Nekrep et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004; Muhlethaler-Mottet, Krawczyk et al. 2004; Drozina, Kohoutek et al. 2005; Zika and Ting 2005; Otten, Leibundgut-Landmann et al. 2006). CIITA is absolutely critical for the expression of MHC II genes. Although other factors are expressed and bind constitutively to the MHC II promoter, transcription is halted until CIITA is expressed and binds to the MHC II promoter, thus making it the master regulator of MHC II genes (Waldburger, Masternak et al. 2000;

Jabrane-Ferrat, Nekrep et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004; Drozina, Kohoutek et al. 2005).

Following the recruitment of CIITA to the enhanceosome, CIITA binds to all 7 subunits of the enhanceosome complex. Chromatin remodeling enzymes are also recruited to the promoter to “open” chromatin structure and allow transcription to occur (Harton, Zika et al. 2001; Drozina, Kohoutek et al. 2005). Two histone acetyltransferases, (HATs), CREB binding protein (CBP/p300), and p300/CBP-associated factor, (pCAF), have been shown to be recruited to and to remodel the chromatin structure of the MHC II promoter both prior to and in the presence of CIITA (Harton, Zika et al. 2001; Zika and Ting 2005). In addition CIITA has been shown to possess intrinsic HAT activity (Harton, Zika et al. 2001). Once the chromatin structure is opened, CIITA promotes transcription elongation of MHC II genes through interactions with positive transcription elongation factor b, (pTefb), and RNA polymerase II, (pol II), (Drozina, Kohoutek et al. 2005; Kohoutek, Blazek et al. 2006). The final step in MHC II gene activation is the recruitment of cyclin-dependent kinases, (CDKs), to the promoter by CIITA to phosphorylate the C-terminal domain of RNA pol II, all of which increases the rate of transcription elongation (Drozina, Kohoutek et al. 2005) (Figure 1).

CIITA Regulation

CIITA is a non-DNA binding protein which binds to the enhanceosome complex on the MHC II promoter to activate transcription of MHC II genes (Jabrane-Ferrat, Fontes et al. 1996; Jabrane-Ferrat, Nekrep et al. 2003; Drozina, Kohoutek et al. 2005). CIITA is 1130 amino acids in length, and can be divided into multiple functional domains. The first of these domains is the activation domain (AD); located in the N-terminal region, (amino acids 30-61), of CIITA which is responsible for CIITA’s ability to interact with other proteins of the enhanceosome (Chin, Li

et al. 1997; Kretsovali, Agaloti et al. 1998; Camacho-Carvajal, Klingler et al. 2004; Drozina, Kohoutek et al. 2005). The second domain is a proline/serine/threonine (P/S/T) domain, (amino acids 163 to 322), which is involved in CIITA's ability to interact with basal transcription factors to initiate MHC II transcription (Chin, Li et al. 1997; Camacho-Carvajal, Klingler et al. 2004; Drozina, Kohoutek et al. 2005). The P/S/T domain has also been shown to contain a degron sequence, (amino acids 275-305), which contains proteolytic signal sequences that destabilize CIITA and lead to its degradation (Schnappauf, Hake et al. 2003; Drozina, Kohoutek et al. 2005). The GTP-binding domain (GBD), (amino acids 421-461), and four leucine-rich repeat domains (LRR), have been shown to be required for the localization of CIITA into the nucleus which is critical for its function in the transactivation of MHC II genes (Chin, Li et al. 1997; Linhoff, Harton et al. 2001; Camacho-Carvajal, Klingler et al. 2004; Drozina, Kohoutek et al. 2005). (Figure 2)

Post-translational modifications to CIITA also play a major role in controlling its functions. CIITA is post-translationally modified in several different ways, including phosphorylation, acetylation, and ubiquitination (Harton, Zika et al. 2001; Greer, Harton et al. 2004; Drozina, Kohoutek et al. 2005). These modifications precisely regulate CIITA's location, function and stability inside the cell. Phosphorylation and acetylation have been shown to increase CIITA activity at the MHC II promoter (Masternak, Muhlethaler-Mottet et al. 2000; Spilianakis, Papamatheakis et al. 2000; Harton, Zika et al. 2001; Tosi, Jabrane-Ferrat et al. 2002; Jabrane-Ferrat, Nekrep et al. 2003; Sisk, Nickerson et al. 2003; Wright and Ting 2006). Both pCAF and CBP/p300 have been shown to be responsible for acetylation of CIITA, and it has been suggested that acetylation may also assist in the ubiquitination of CIITA (Spilianakis, Papamatheakis et al. 2000; Greer, Zika et al. 2003).

Ubiquitination has also been shown to be a vital post-translational modification of CIITA, and it has been well demonstrated that monoubiquitination of CIITA increases its stability and activation at the MHC II promoter (Greer, Zika et al. 2003). However the mechanisms of how CIITA is stabilized and maintained at the MHC II promoter throughout an immune response remain unclear. Understanding how post-translational modifications of CIITA regulate its stability and maintenance at the promoter will help to clarify the molecular mechanisms that govern MHC II expression and thus the adaptive immune response.

Ubiquitin-Proteasome System

Ubiquitination plays an important role in both the degradation and the stabilization of proteins, and therefore is a tightly regulated process. Ubiquitin molecules are conjugated to lysine residues in the substrate through a series of enzymatic reactions which allow the formation of multi-ubiquitin chains, consisting of mono, di, tri, and poly species of ubiquitinated target proteins (Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006). Ubiquitination reactions rely on three types of enzymes; termed E1, E2, and E3 (Scheffner, Nuber et al. 1995; Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006). The first step in the ubiquitination cascade involves ATP hydrolysis and is catalyzed by E1, the ubiquitin activating enzyme, which activates the carboxyl terminal end of ubiquitin (Scheffner, Nuber et al. 1995; Pickart 2004; Taylor and Jobin 2005; Sun 2006). In the next reaction, one of several E2, (ubiquitin conjugating enzymes), transfers activated ubiquitin from E1 to the active site of E2 forming an E2-ubiquitin thiol ester intermediate (Scheffner, Nuber et al. 1995; Ciechanover, Orian et al. 2000; Pickart 2004; Taylor and Jobin 2005; Sun 2006). In the majority of cases the ubiquitin-proteasome pathway then utilizes an E3 ubiquitin ligase enzyme to catalyze the transfer of ubiquitin from E2 to a lysine residue on the substrate protein (Scheffner, Nuber et al.

1995; Ciechanover, Orian et al. 2000; Pickart 2004; Taylor and Jobin 2005; Sun 2006; Arama, Bader et al. 2007). In a few cases, ubiquitin may also be transferred directly from E2 to the protein substrate (Scheffner, Nuber et al. 1995; Ciechanover, Orian et al. 2000; Pickart 2004; Taylor and Jobin 2005; Sun 2006; Arama, Bader et al. 2007).

The ubiquitin cascade has a high level of specificity and regulation due to its hierarchical structure; there is only one known E1 enzyme which recognizes E2 enzymes and there are a limited number of E2 enzymes (Scheffner, Nuber et al. 1995; Hersko and Ciechanover 1998; Ciechanover, Orian et al. 2000; Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006; Arama, Bader et al. 2007). Hundreds of E3 ligases have been identified, and each E3 ligase has a limited number of substrate proteins that it is capable of interacting with (Scheffner, Nuber et al. 1995; Hersko and Ciechanover 1998; Ciechanover, Orian et al. 2000; Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006; Arama, Bader et al. 2007). E3 ligases are capable of recognizing not only E2 enzymes, but also the protein substrate that they are interacting with (Scheffner, Nuber et al. 1995; Hersko and Ciechanover 1998; Ciechanover, Orian et al. 2000; Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006; Arama, Bader et al. 2007) (Figure 3). The addition of the first ubiquitin to the substrate by the E2 or E3 ligase results in monoubiquitination of the protein substrate (Adams 2003; Taylor and Jobin 2005). Subsequent addition of ubiquitin to a lysine residue of the first ubiquitin leads to di, tri, and poly-ubiquitination (Adams 2003; Taylor and Jobin 2005). Following the formation of a monoubiquitinated protein substrate, proteins are stabilized in a monoubiquitinated state or polyubiquitin chains are formed through the subsequent reactions through the E1-E2-E3 cascade (Scheffner, Nuber et al. 1995; Ciechanover, Orian et al. 2000; Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006).

Recent work has indicated that the formation of polyubiquitin chains also occurs by an E3 transferring a preformed ubiquitin chain to the protein substrate (Li, Tu et al. 2007). In some instances an E4 enzyme, the ubiquitin chain assembly factor, is required to promote the degradation of certain substrates (Hatakeyama and Nakayama 2003; Kaneko, Hatakeyama et al. 2003; Kaneko-Oshikawa, Nakagawa et al. 2005). These reports make clear that there remains a great deal to be characterized regarding both the mechanism of ubiquitination and the enzymes involved.

It is well accepted that the fate of the protein is determined by both the number of ubiquitin molecules and the site to which they are added (Taylor and Jobin 2005; Sun 2006). Ubiquitination can occur in two unique ways, via a lysine 48 linked chain on the protein substrate, or via a lysine 63 linked ubiquitin chain (Taylor and Jobin 2005; Sun 2006). A lysine 48 linked ubiquitin chain targets the protein substrate for degradation by the 26S proteasome (Taylor and Jobin 2005; Sun 2006). Proteins that are polyubiquitinated via a lysine 63 chain have been shown to be involved in new cellular functions (Taylor and Jobin 2005; Sun 2006). (Figure 4) Proteins that are monoubiquitinated, by the addition of a single ubiquitin molecule to one or more lysine residues of the substrate protein, have been demonstrated to be involved in cellular trafficking, histone modifications, DNA repair, and transcriptional activation (Brooks, Li et al. 2004; Taylor and Jobin 2005; Sun 2006). Addition of di- and tri- species of ubiquitin has no known biological function, while polyubiquitinated proteins are targeted to the 26S proteasome for degradation (Taylor and Jobin 2005; Sun 2006). In order for a polyubiquitinated protein to be degraded, the 19S ATPase subunits of the lid of the 26S proteasome bind to and recognize the poly-ubiquitin chain, which is then cleaved from the protein and free ubiquitin molecules are released and are reactivated by E1 (Adams 2003; Taylor and Jobin 2005; Hegde

and Upadhy 2006; Sun 2006). The protein substrate is denatured and shuttled into the 20S proteolytic core of the proteasome, where the protein is cleaved into small peptides (Adams 2003; Taylor and Jobin 2005; Hegde and Upadhy 2006; Sun 2006) (Figure 5).

The Effect of Ubiquitination on Transcription

While ubiquitination is traditionally thought of as a tag which targets proteins for degradation by the 26S proteasome, transcriptional regulation by ubiquitin has also been demonstrated. Recently, mono-ubiquitination of transcriptional activators in yeast has been implicated in promoting transcription by protecting transcription factors from degradation (Archer, Burdine et al. 2008). Ubiquitination has also been implicated in playing an important role in both the stability and activation of CIITA (Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003). CIITA has been shown to have increased promoter association in the presence of monoubiquitin, and to have an increase in MHC II transactivation (Greer, Zika et al. 2003).

pCAF and Its Role in Ubiquitination

pCAF is traditionally known as a chromatin remodeling enzyme and as a histone acetyltransferase. pCAF has been shown to be not only recruited to the MHC II promoter by CIITA, but to activate transcription at the MHC II promoter (Drozina, Kohoutek et al. 2005; Wright and Ting 2006). pCAF has also recently been shown to have intrinsic E3 properties (Linares, Kiernan et al. 2007). pCAF's intrinsic E3 properties are responsible for controlling the expression of human double minute 2, (Hdm2); which is a protein that controls p53 stability (Linares, Kiernan et al. 2007). The ability of pCAF to ubiquitinate Hdm2 was also shown to be to some extent dependent upon its HAT domain (Linares, Kiernan et al. 2007). It has been demonstrated that pCAF has two ubiquitination domains, one that is responsible for auto-

ubiquitination and the other responsible for the ubiquitination of Hdm2 (Linares, Kiernan et al. 2007). (Figure 6)

Previous studies have demonstrated that pCAF must be localized to the promoter for transcription of MHC II genes to occur, that pCAF cooperates with CIITA at the MHC II promoter, and that the interaction of pCAF and CIITA is not dependent upon pCAF's HAT domain (Harton, Zika et al. 2001). Studies have further shown that CIITA's ubiquitination status is increased in the presence of pCAF (Greer, Zika et al. 2003). The ubiquitin ligase for CIITA is unknown and pCAFs potential role in regulating CIITA ubiquitination remains to be explored. We hypothesize that pCAF is playing a novel role as an E3 ligase for CIITA in addition to its traditional role as a HAT. In this thesis we show pCAF has a profound effect on CIITA ubiquitination and stability. Ubiquitination is increased in the presence of WT pCAF, but is decreased in the presence of an E3 ligase mutant pCAF. In addition, we demonstrate that knocking down pCAF results in a decrease of CIITA binding at the MCH II promoter. Further CIITA has an extended half life and is stabilized in the presence of WT pCAF, but a shortened half life and is destabilized CIITA in the presence of a pCAF E3 ligase mutant. Taken together, these results demonstrate that pCAF plays a vital role in the stability of CIITA, and thus the expression of MHC II genes. The identification of an E3 ligase responsible for the ubiquitination of CIITA will further our understanding of the principles that govern the expression of CIITA and of MHC II genes. These studies will enhance our ability to develop therapeutic approaches to autoimmune disease and tumors through the manipulation of the adaptive immune system.

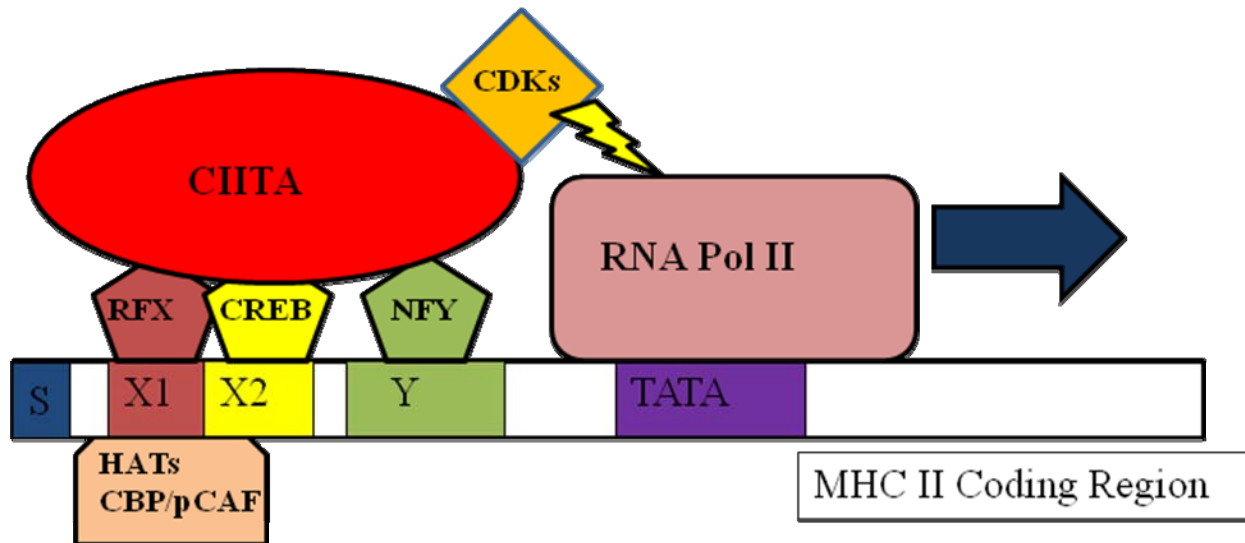


Figure 1: CIITA Enhanceosome Complex. The CIITA enhanceosome complex at the proximal promoter of MHC II orchestrates the transcription of MHC II molecules. CIITA binds to several transcription factors, RFX, NFY, and CREB, which in turn recognize cis-acting regions, W, X1, X2, and Y, of the MHC II promoter. Together this enhanceosome complex allows RNA polymerase to initiate transcription of MHC II genes.

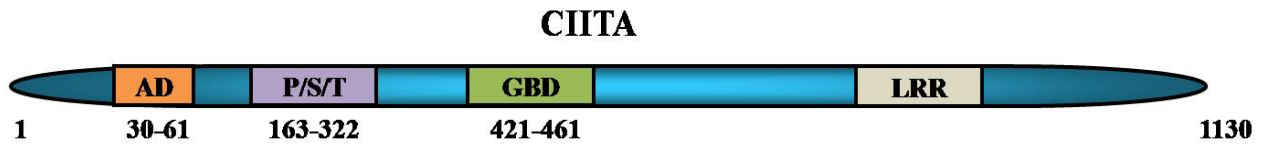


Figure 2: Functional Domains of CIITA. CIITA contains four functional domains: the N-terminal activation domain (AD), a proline/serine/threonine rich (P/S/T) domain, a GTP-binding domain (GDB), and a C-terminal leucine rich region (LRR).

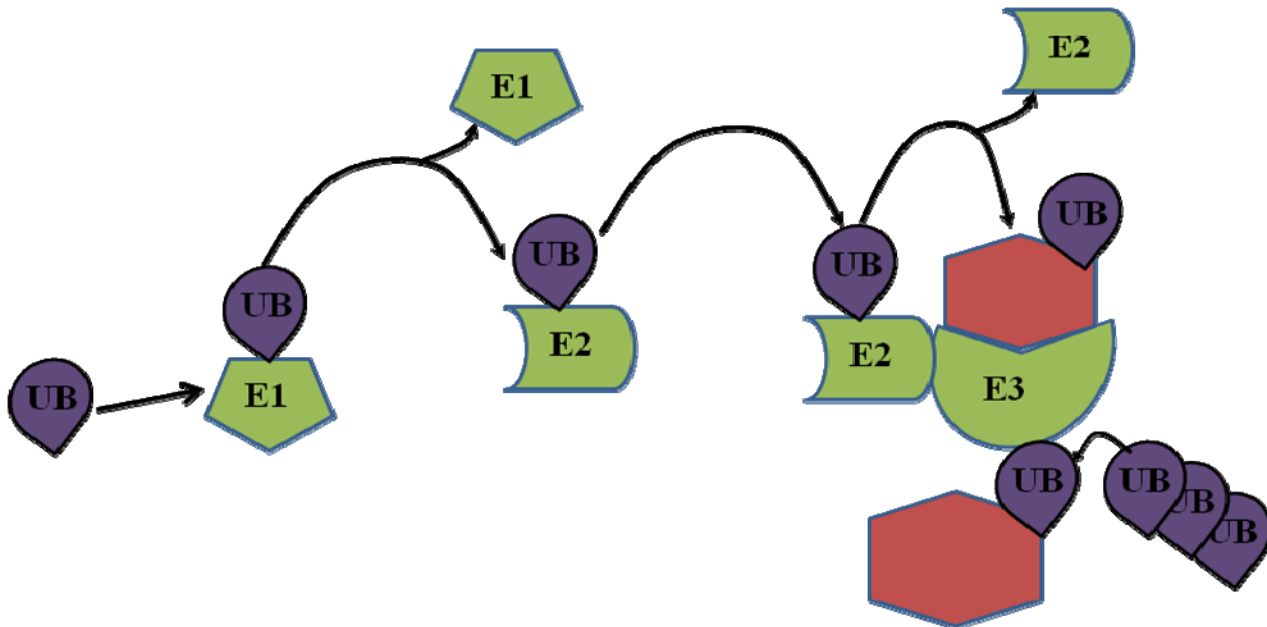


Figure 3: The Ubiquitin-Proteasome Pathway Enzyme Cascade. E1 (ubiquitin activating enzyme) activates the C-terminal end of an ubiquitin molecule. E2 (ubiquitin conjugating enzyme) then transfers the ubiquitin molecule to the E2 active site. E3 (ubiquitin ligase enzyme) then transfers the ubiquitin molecule from E2 to a lysine residue on the substrate protein.

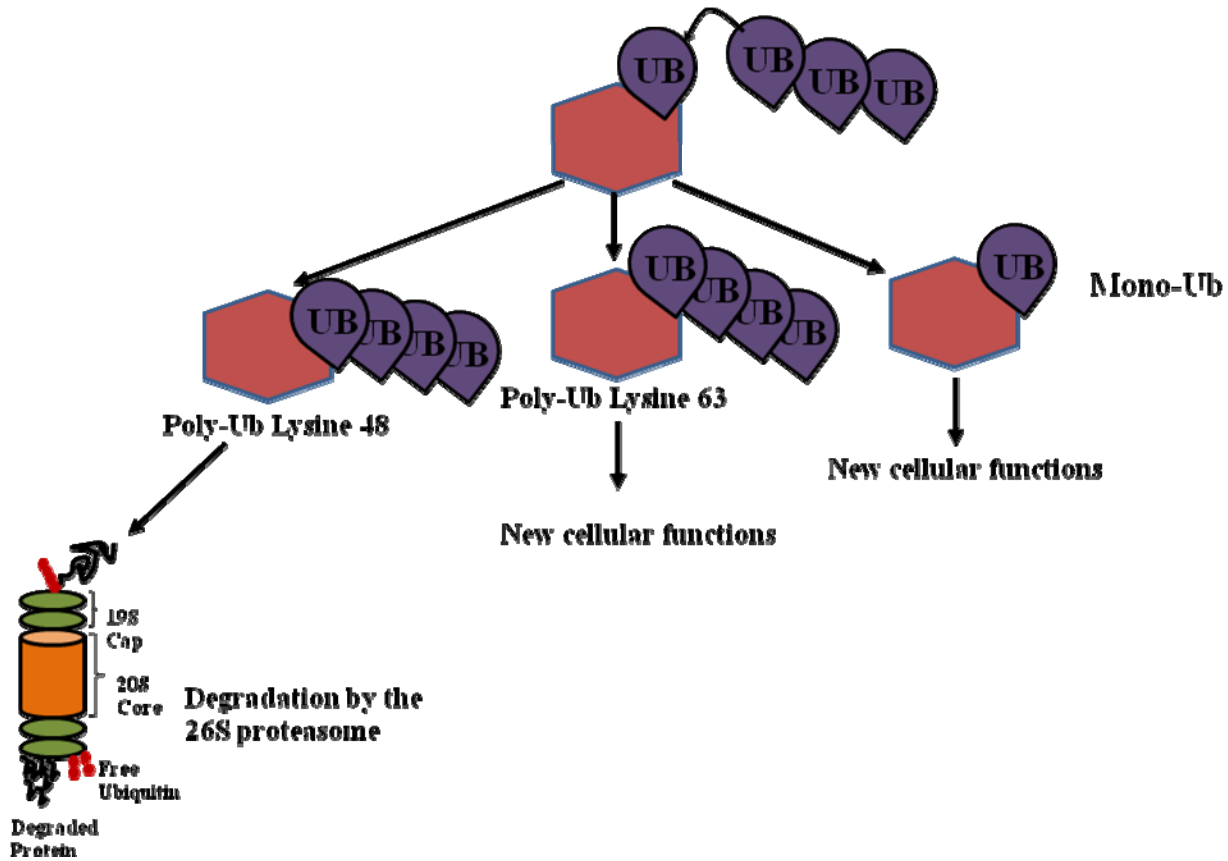


Figure 4: Ubiquitination Patterns. The fate of a protein substrate is determined by the ubiquitination pattern of protein substrates by the E3 ligase. Poly-ubiquitination at lysine 48 leads to degradation by the 26S proteasome, poly-ubiquitination at lysine 63 and mono-ubiquitination lead to new cellular functions for the protein substrate.

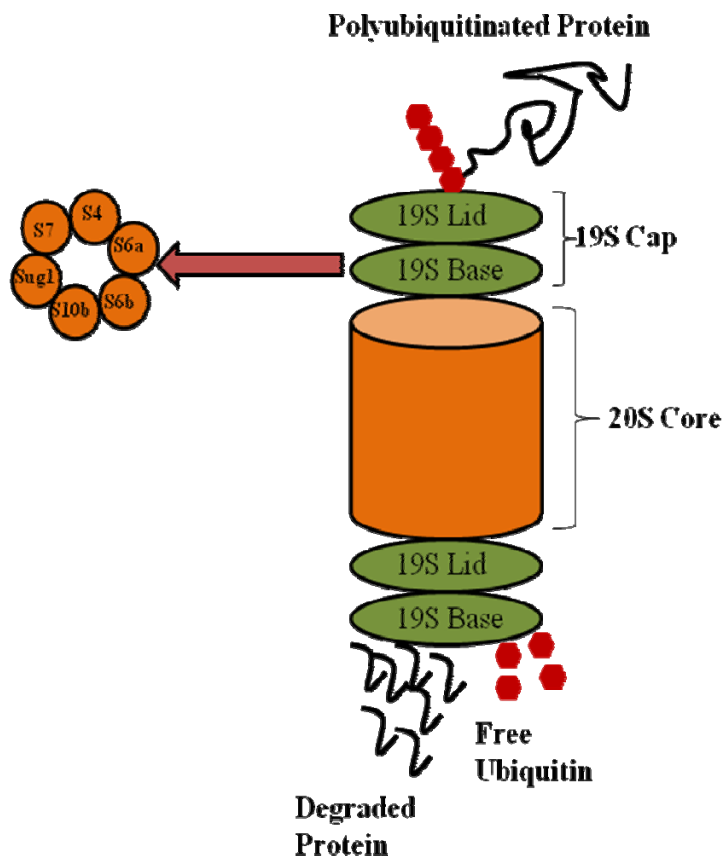


Figure 5: The 26S Proteasome. The 26S proteasome is composed of a 19S regulator cap and a 20S proteolytic core. The 19S regulator is composed of a lid and a base; the lid is required for the proteolytic activity of the proteasome and is made of eight non-ATPase subunits. The 19S base contains six ATPases: S4, S6a, S6b, S7, Sug1, and S10b; in addition to three non-ATPase subunits. The 20S core of the proteasome contains the catalytic function and is made of four stacked rings which consist of 1-7 α , 1-7 α' , 1-7 β , and 1-7 β' subunits. The 19S ATPases recognize and unfold polyubiquitinated proteins. Degraded proteins are shuttled into the 20S proteolytic core, where they are cleaved into peptides. The peptides and free ubiquitin are then released and recycled.

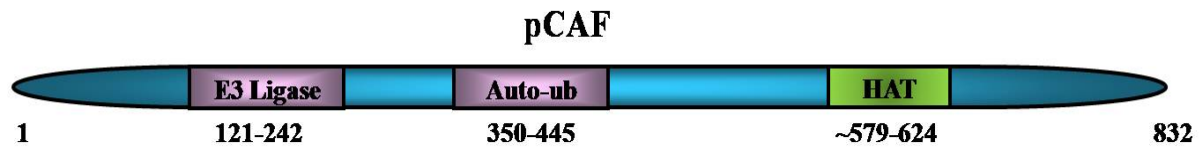


Figure 6: Functional Domains of pCAF. pCAF contains three functional domains: an E3 ligase domain, an auto ubiquitination domain, and a histone acetyltransferase domain.

CHAPTER 2.

MATERIALS AND METHODS

Maintenance of Cell Line

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

Plasmids and Reagents

HLA-DRA Luciferase and flag tagged CIITA were previously described (Cressman, Chin et al. 1999; Cressman, O'Connor et al. 2001; Greer, Zika et al. 2003). Flag tagged pCAF constructs, WT pCAF, and E3 ligase mutant pCAF (Δ E3) were a generous gift from Dr. O. Coux, and Dr. M. Benkirane (Linares, Kiernan et al. 2007). Monoclonal anti-CIITA was obtained from Rockland (Gilberstville, PA), anti-ubiquitin was obtained from Biomol (Plymouth Meeting, PA), and anti-pCAF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-flag was obtained from Sigma-Aldrich (Saint Louis, MO). Small interfering RNA (siRNA) for pCAF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), control siRNA was obtained from Qiagen (Valencia, CA).

Transient Transfection and Luciferase Reporter Assays

HeLa cells were plated in 6 well tissue culture plates at a density of 5×10^5 . Following adhesion, cells were transfected with 100ng *HLA-DRA*, 3.33ng Renilla, 25ng pcDNA or 25ng CIITA, and where indicated 100ng of WT pCAF, and Δ E3 pCAF. Transfection was carried out using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours post transfection cells were harvested and lysed in 1 X Passive Lysis Buffer

(Promega, Madison, WI). Following lysis luciferase assays were performed following the manufacturer's protocol.

Knockdown Luciferase Reporter Assay

HeLa cells were plated in 6 well tissue culture plates at a density of 5×10^4 . Following adhesion cells were transfected with 1.0 μ g of siRNA, diluted in buffer ECR (Qiagen), using the RNAi transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected with a pCAF specific pooled siRNA (Santa Cruz) or with control scrambled sequence siRNA (Qiagen). Thirty hours after siRNA treatment cells were transfected with 100ng *HLA-DR*, 3.33ng Renilla, 25ng pcDNA or 25ng CIITA, and where indicated 100ng of WT pCAF. Twenty-four hours post transfection cells were lysed as above, and luciferase assays were performed.

Half Life Assays

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following cell adhesion cells were transfected with 5 μ g of flag-CIITA, and where indicated 3 μ g of either flag-WT pCAF or flag- Δ E3 pCAF, using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours post transfection cells were treated with cycloheximide (Sigma, Saint Louis, MO) at the indicated time points to inhibit protein biosynthesis. Cells were also treated with proteasome inhibitor MG132 (EMD Biosciences, San Diego, CA) as indicated. Cells were then harvested and lysed in 1% NP-40 (1 M Tris [pH 8.0], 1 M KCl, 10% NP-40, 0.5 M EDTA, 5 M NaCl, 1 M DTT, distilled water [dH₂O]). Lysates were centrifuged, normalized for protein concentration, and denatured with Leammli buffer (Bio-Rad, Hercules, CA), boiled and separated by SDS polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose and immunoblotted with anti-flag (Sigma-Aldrich).

Knockdown Half Life Assays

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following adhesion cells were transfected as indicated with 1.0 μ g of siRNA, diluted in buffer ECR (Qiagen), using the RNAi transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected with a pCAF specific pooled siRNA (Santa Cruz) and with a control scrambled sequence siRNA (Qiagen). Thirty hours after siRNA treatment cells were transfected with 5 μ g of flag-CIITA. Half life assays were then performed as described above.

Densitometry

Developed immunoblots were analyzed using MultiGauge software (Version 3.1, Fujifilm). Quantification mode was used to select the area of the band to be measured; a background area was also selected to be subtracted from the bands measured. Half life readings were normalized to the zero hour time point. Ubiquitination readings were normalized to CIITA expression.

RNA Expression

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following attachment cells were transfected with 3 μ g of flag-WT pCAF and/or flag- Δ E3 pCAF, where indicated, using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Cells were stimulated with 500 U/ml IFN- γ . Twenty-four hours following stimulation cells were harvested, washed with PBS, and centrifuged. Total RNA was prepared from the samples with 1ml of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was resuspended in 60 μ l DEPC water (Qiagen) and then stored at -80°C .

Omniscript reverse transcription kit (Qiagen) was used to make 20 μ l of cDNA from 1 μ g of RNA. Specific antisense primers (Sigma, Saint Louis, MO) were used for reverse

transcription (RT). PCR was done in an eppendorf microcycler, according to the manufacturer's protocol (Qiagen). Real-time PCR was done with an ABI prism 7900 (Applied Biosystems, Foster City, CA). MHC II probe was labeled 5' with 6-carboxyfluorescein (FAM) reporter dye and 3' with *N,N,N,N*-tetramethyl-6-carboxyrhodamine (TAMRA) quencher dye. Primer and probe sequences are as follows: MHC II sense sequence, 5'-AA GCCAACCTGGAAATCA-3'; antisense sequence, 5'-GGCTGTTCGTGAGCACAGTT-3'; probe sequence, 5'-6 FAM-CTCCGATCACCAATGTACCTCCAGA-TAMRA-3'. Housekeeping gene GAPDH RNA was used to normalize values. The GAPDH primers and probe used were previously described (Medhurst, Harrison et al. 2000). Values presented are from real time PCR reactions and were calculated based on standard curves generated for each gene, and were analyzed using the SDS 2.0 program.

Knockdown RNA Expression

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following attachment cells were transfected with 1.0 μ g of siRNA, diluted in buffer ECR (Qiagen), using the RNAi transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected with a pCAF specific pooled siRNA (Santa Cruz) or with control scrambled sequence siRNA (Qiagen). Thirty hours after siRNA treatment cells were stimulated with 500 U/ml IFN- γ . Cells were harvested and 10% of the total cell volume was lysed in 1% NP-40 (1 M Tris [pH 8.0], 1 M KCl, 10% NP-40, 0.5 M EDTA, 5 M NaCl, 1 M DTT, distilled water [dH₂O]), with protease inhibitors and were analyzed by western blot for pCAF knockdown as above. The remaining fraction of cells was subjected to RNA extraction as described above.

Chromatin Immunoprecipitation

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following adhesion cells were transfected with 1.0 μ g of siRNA, diluted in buffer ECR (Qiagen), using the RNAi transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were transfected with a pCAF specific pooled siRNA (Santa Cruz) or with a control scrambled sequence siRNA (Qiagen). Thirty hours after siRNA treatment cells were stimulated with 500 U/ml IFN- γ . 18 hours after stimulation cells were harvested 10% of the total cell volume was lysed in 1% NP-40 (1 M Tris [pH 8.0], 1 M KCl, 10% NP-40, 0.5 M EDTA, 5 M NaCl, 1 M DTT, distilled water [dH₂O]), with protease inhibitor and analyzed by Western blotting for pCAF knockdown using anti-pCAF Santa Cruz Biotechnology (Santa Cruz, CA). The remaining fraction of cells were subjected to a ChIP assay. Briefly, cells were cross-linked 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 (1%SDS, 10mM EDTA, 50mM Tris [pH 8], dH₂O) with protease inhibitor for 20 minutes on ice and were sonicated to generate sheared DNA an average of 500 to 750 base pairs in length. Samples were precleared with salmon sperm coated agarose beads (Upstate), and half of the lysate was immunoprecipitated with 5 μ g of polyclonal anti-CIITA (Rockland) at 4°C. The second half of the lysate was immunoprecipitated with 5 μ g of an isotype control antibody (Upstate). After the overnight incubation 60 μ l of salmon sperm coated agarose beads were added to the samples and immunoprecipitated for an additional 2 hours. Then samples were washed for 5 min at 4°C with the following buffers: low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.0], 150 mM NaCl, dH₂O), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris [pH 8.0], 500 mM NaCl, dH₂O), LiCl

buffer (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8.0], dH₂O), and 1 X Tris-EDTA buffer and were then eluted with SDS elution buffer (1% SDS, 0.1 M NaHCO₃, dH₂O). After elutions, cross-links were reversed overnight with 5M NaCl at 65°C and the immunoprecipitated DNA was isolated using a phenolchloroform-isoamyl alcohol mix (Invitrogen) as per the manufacturer's instructions. The isolated DNA was analyzed via real time PCR using primers spanning the W-X-Y box of the MHC II *HLA-DRA* promoter. MHC II promoter primers and probe sequences are as follows: MHC II probe 5'-6 FAMCTGGACCCTTTGCAAGAACCCTTCCC-TAMRA-3'; sense primer, 5'-TCCAATGAACGGAGTATCTTGTG T-3'; and antisense primer, 5'-TGAGATGACGCATCTGTTGCT-3'. Values were calculated based on the standard curves generated.

Co-immunoprecipitation

HeLa cells were plated in 10cm tissue culture plates at a density of 8×10^5 . Following cell adhesion cells were transfected with 5µg of myc-CIITA and where indicated 3µg of flag-WT pCAF, using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours after transfection cells were harvested and lysed in 1% NP-40 (1 M Tris [pH 8.0], 1 M KCl, 10% NP-40, 0.5 M EDTA, 5 M NaCl, 1 M DTT, distilled water [dH₂O]). Lysates were centrifuged, normalized for protein concentration, and precleared with 50µl mouse IgG beads (Sigma-Aldrich, St. Louis, MO). Lysates were then immunoprecipitated with 50µl anti-myc agarose beads (Sigma-Aldrich). Immune complexes were then denatured with Leammli buffer Bio-Rad, Hercules, CA), boiled and separated by SDS- polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose and immunoblotted for ubiquitin with

anti-ubiquitin (Biomol). Equal loading was confirmed with immunoblotting with anti-flag (Sigma Aldrich) and anti-myc (Sigma Aldrich).

CHAPTER 3.

RESULTS

MHC II molecules present exogenous peptides to CD4⁺ T cells and play a major role in the regulation of the adaptive immune response. Deficiencies in MHC II molecules result in a rare fatal disease called Bare Lymphocyte Syndrome (BLS) (Reith and Mach 2001; Drozina, Kohoutek et al. 2005). The expression of MHC II molecules is controlled by the master regulator CIITA (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Harton, Zika et al. 2001; Jabrane-Ferrat, Nekrep et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004; Muhlethaler-Mottet, Krawczyk et al. 2004; Drozina, Kohoutek et al. 2005; Zika and Ting 2005; Otten, Leibundgut-Landmann et al. 2006). Post-translational modifications to CIITA, including ubiquitination, are critical to its function, but remain to be well characterized.

pCAF Drives CIITA Transactivity

pCAF is traditionally known for its HAT activities, directed both toward chromatin remodeling and as a histone acetyltransferase or multiple transcription factors and co-activators. pCAF has recently been shown to have intrinsic E3 ligase properties in addition to its known HAT activity (Linares, Kiernan et al. 2007). pCAF is recruited to the MHC II promoter, and is known to participate in MHC II transcription (Drozina, Kohoutek et al. 2005; Wright and Ting 2006). CIITA is the master regulator of MHC II gene expression as CIITA expression and binding at the MHC II promoter is critical for the expression of MHC II genes (Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004; Drozina, Kohoutek et al. 2005). To first determine the effect of pCAF on CIITA transactivation, luciferase reporter assays were performed with HeLa cells transfected with 100ng of MHC II *HLA-DRA*-Luc reporter construct, 3.33ng of Renilla, 25ng of CIITA, and

100ng of pCAF (Figure 7). *HLA-DRA* expression in the presence and absence of CIITA and pCAF are indicated in Figure 7A. In the presence of overexpressed CIITA and pCAF, CIITA transactivation at the MHC II promoter *HLA-DRA* promoter is increased 6 fold over cells expressing only CIITA.

CIITA Is Stabilized by the Presence of WT pCAF

CIITA transactivation is regulated by multiple post translational modifications including monoubiquitination which has been shown to enhance CIITA binding to the MHC II promoter (Greer, Zika et al. 2003). While monoubiquitination has been well demonstrated to stabilize CIITA binding and activity at the MHC II promoter, the mechanisms that govern CIITA ubiquitination remain unclear (Greer, Zika et al. 2003; Drozina, Kohoutek et al. 2006). As CIITA transactivation was enhanced by pCAF, we next sought to determine the effect of pCAF on CIITA stability. To determine CIITA stability in the presence of overexpressed pCAF, we performed half life assays in HeLa cells. HeLa cells were transfected with flag-CIITA and flag-pCAF as indicated (Figure 7B) and were treated with cycloheximide to inhibit protein biosynthesis. As a control one sample was treated with a proteasome inhibitor MG132, as indicated, which resulted in the accumulation of CIITA. Cells were harvested at indicated times points and lysates subjected to SDS-gel electrophoresis and immunoblotting with anti-flag antibodies to determine CIITA half life in the absence (Figure 7B, top panel) and in the presence of overexpressed pCAF (Figure 7B, bottom panel). Immunoblots were further analyzed using densitometry to indicate changes in CIITA half life (Figure 7C). CIITA transactivation is enhanced and CIITA half life is extended in the presence of pCAF; these data indicate that pCAF is playing a positive role in the regulation of CIITA activity.

Knockdown of pCAF Leads to a Decrease in CIITA Transactivity

Monoubiquitination has previously been shown to increase CIITA transactivity at the MHC II promoter (Greer, Zika et al. 2003). pCAF has been shown to be recruited to the MHC II promoter by CIITA, and to activate transcription at the MHC II promoter (Drozina, Kohoutek et al. 2005; Wright and Ting 2006). pCAF has also recently been shown to have intrinsic E3 ligase properties (Linares, Kiernan et al. 2007). Because we saw an increase in CIITA transactivity and a stabilization of CIITA expression in the presence of expressed pCAF, we next sought to determine if reducing pCAF expression would also affect CIITA transactivity. To investigate the effects of reducing pCAF expression in the regulation of CIITA transactivation, we utilized control or pCAF specific siRNA duplexes to modulate expression of endogenous pCAF in HeLa cells (Figure 8A) and assayed CIITA activity with luciferase assays. Luciferase assays to determine CIITA transactivation demonstrated a significant decrease in CIITA transactivity in cells transfected with pCAF siRNA compared to that of control siRNA (Figure 8B). Importantly CIITA activity is reconstituted in pCAF knockdown cells transfected with WT pCAF, demonstrating the specificity of the pCAF siRNA construct (Figure 8B).

Knockdown of pCAF Decreases the Expression of MHC II

We have demonstrated that expressed pCAF increases both the transactivation and the stability of CIITA and that in the presence of reduced pCAF expression, CIITA transactivation is decreased. We next sought to investigate the effects of decreased levels of pCAF on the expression of MHC II genes. To investigate effects of pCAF on MHC II gene expression, we used control or pCAF specific siRNA duplexes to modulate pCAF expression, and then assayed MHC II gene expression (Figure 8C). The message level of MHC II is significantly decreased in

the absence of pCAF, indicating that the ability of pCAF to modulate CIITA regulation plays a major role MHC II expression.

Knockdown of pCAF Leads to a Reduction of CIITA Binding at the MHC II Promoter

How CIITA is recruited to and stabilized at the MHC II promoter through the duration of an immune response remains largely unknown, though monoubiquitination has been shown to increase CIITA stability at the MHC II promoter (Greer, Zika et al. 2003; Drozina, Kohoutek et al. 2006). As changing levels of endogenous pCAF reduced both CIITA transactivation and MHC II gene expression, we were interested in the effects of pCAF knockdown on the ability of CIITA to stably bind to the MHC II proximal promoter. To determine pCAF's role in recruiting and/or stabilizing CIITA at the MHC II promoter, we utilized control or pCAF specific siRNA duplexes to specifically knock down pCAF expression in HeLa cells and then performed ChIP experiments to determine the level of CIITA binding at the MHC II promoter (Figure 8D). ChIP experiments demonstrate an almost total lack of CIITA binding at the MHC II promoter in the absence of pCAF (Figure 8D).

CIITA Ubiquitination Is Increased in the Presence of pCAF

Mono-ubiquitination has been shown to increase the transactivity of CIITA (Greer, Zika et al. 2003). As expressed pCAF stabilizes CIITA and pCAF knockdown dramatically decreases CIITA binding to the MHC II proximal promoter, we next sought to determine pCAF's role in CIITA ubiquitination. To determine if pCAF regulates the ubiquitination status of CIITA, we performed co-immunoprecipitation (IP) experiments to assay CIITA ubiquitination in the presence of WT pCAF. HeLa cells were transfected with myc-CIITA and flag-pCAF. Polyclonal antibody was used to isolate CIITA associated immune complexes. Immune complexes were then subjected to immunoblotting for endogenous ubiquitin. CIITA

ubiquitination was enhanced in the presence of expressed pCAF (Figure 9A, top panel). Equal transfection of CIITA and pCAF was confirmed by immunoblot analysis of lysates (Figure 9A, lower panels). The immunoblots were analyzed using densitometry software (Figure 9B).

Increased CIITA ubiquitination is seen in the presence of overexpressed pCAF.

MHC II Expression Is Dependent on pCAF E3 Ligase Activity

We have shown that pCAF increases the transactivation, stability and ubiquitination of CIITA; we have further shown that a knockdown of pCAF leads to a decrease in MHC II expression. We next sought to determine if the effect of pCAF on MHC II gene expression is due to pCAF's E3 ligase activity. To investigate effects of the E3 ligase domain of pCAF on MHC II gene expression, we assayed MHC II message levels in HeLa cells which were transfected WT pCAF or Δ E3 pCAF as indicated (Figure 10). The message level of MHC II is significantly decreased in the presence of Δ E3 pCAF, indicating that pCAF E3 ligase activity to modulates CIITA activation and MHC II expression.

CIITA Is Destabilized in the Presence of Δ E3 pCAF

As pCAF's E3 ligase activity is important for MHC II gene expression, we next sought to determine if the E3 ligase domain of pCAF is important for stable CIITA expression. To determine if CIITA is destabilized in the presence of Δ E3 pCAF, we performed half life assays in HeLa cells. HeLa cells were transfected with flag-CIITA and flag- Δ E3 pCAF as indicated and were then treated with cycloheximide to inhibit protein biosynthesis (Figure 11). Cells were harvested at indicated times points and lysates subjected to SDS gel electrophoresis and immunoblotting with anti-flag (Figure 11A). To quantitate CIITA expression, the immunoblots were analyzed using densitometry software (Figure 11B). The half life of CIITA is significantly

decreased in the presence of $\Delta E3$ pCAF, indicating that the ability of pCAF to modulate CIITA monoubiquitination plays a major role in regulating CIITA activity, and thus MHC II expression.

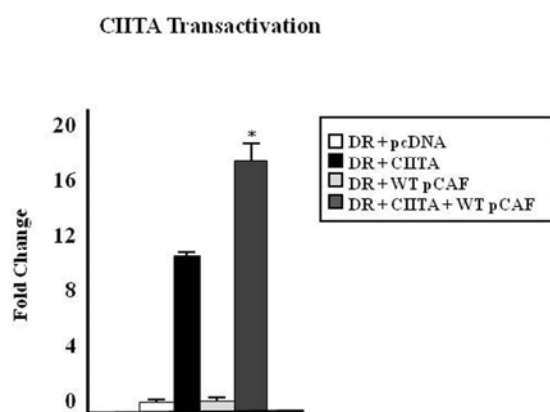
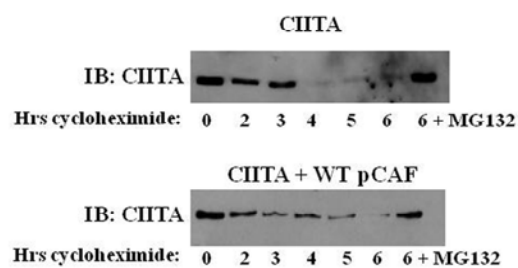
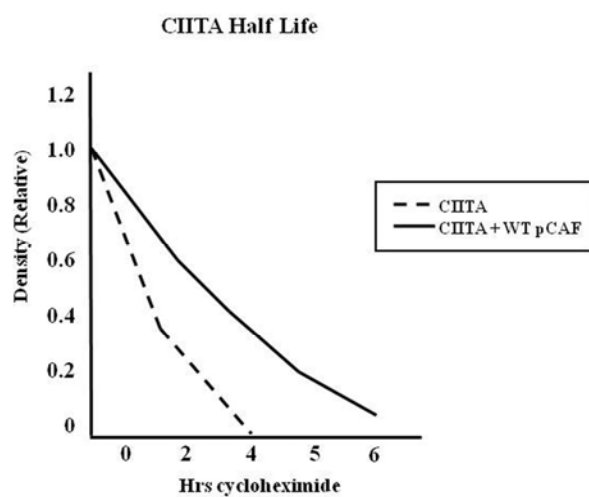
A**B****C**

Figure 7: Transactivation and Stabilization of CIITA. (A) Enhanced CIITA Transactivation in the presence of pCAF. HeLa cells were transfected with 100ng of MHC II *HLA-DRA*-Luc reporter construct, 3.33ng of Renilla, 25ng of CIITA, and 100ng of pCAF. Luciferase assays were performed in triplicate in three independent experiments and data are presented as fold increase in the luciferase activity. Results are standardized to Renilla values and represent the mean \pm S.D. * $P < .05$. **(B) Stabilization of CIITA by pCAF.** HeLa cells were transfected with 5 μ g of flag-CIITA and 3 μ g of flag-pCAF as indicated. Twenty-four hours post transfection HeLa cells were treated with cycloheximide to inhibit protein synthesis. Positive control sample seven was treated with proteasome inhibitor MG132. Cells were harvested at indicated time points, and analyzed via Western blotting for flag-CIITA. **(C) Densitometry of the Western blots in B.** Relative density is plotted and to demonstrate CIITA stabilization in the presence of expressed WT pCAF. Results in B and C are representative data of three experiments.

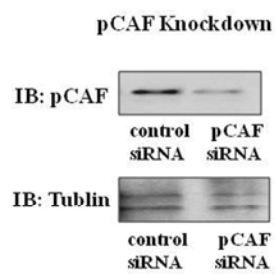
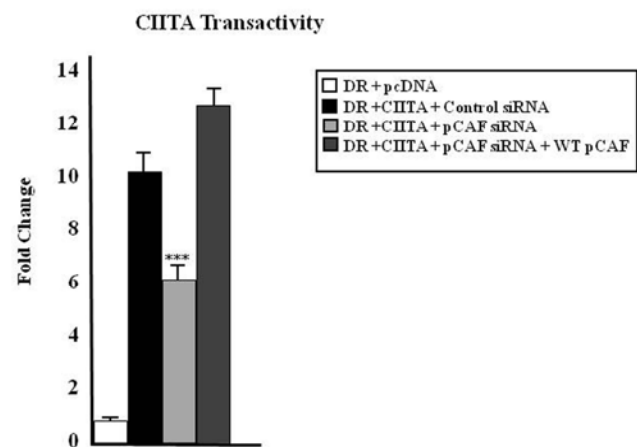
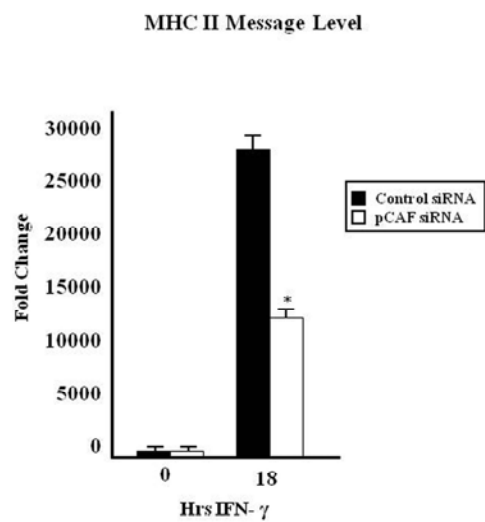
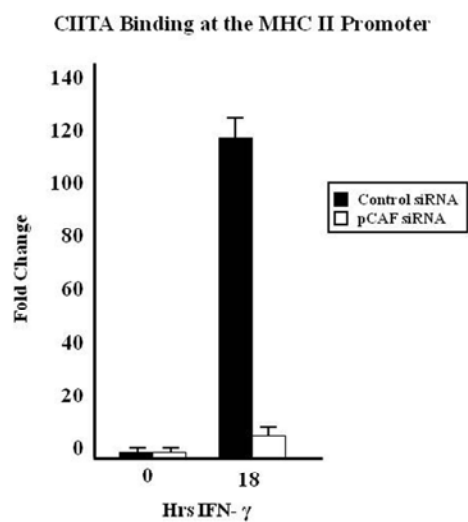
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Figure 8: pCAF Knockdown Decreases CIITA Transactivation and Promoter Binding (A) **pCAF siRNA specifically decreases pCAF protein expression.** HeLa cells were transfected with control or pCAF specific siRNA duplexes, harvested and subjected to Western blot analysis using anti-pCAF mAb (top blot). Specificity of pCAF siRNA is shown by subjecting lysates to Western blot analysis using anti-tubulin mAb (bottom blot). Data presented are representative data of three experiments. (B) **CIITA transactivity is decreased in the absence of pCAF.** HeLa cells were transfected with MHC II *HLA-DRA*-Luc reporter construct (DR), Renilla, CIITA, pcDNA and WT pCAF and were treated with control or pCAF specific siRNA duplexes. Reduced expression of pCAF, (compare black and light gray bars), results in decreased CIITA transactivity while the addition of WT pCAF, (compare black and dark gray bars), restores CIITA transactivity. Luciferase assays were performed in triplicate in two independent experiments, and data are presented as fold increase in the luciferase activity. Results presented are standardized to Renilla values and represent the mean \pm SEM. *** $P < .0005$ versus control siRNA. (C) **pCAF knockdown decreases MHC II gene expression.** Gene expression assays were carried out in control or pCAF siRNA transfected HeLa cells stimulated with IFN- γ . Levels of MHC II mRNA were measured by real-time PCR and were normalized to GAPDH mRNA. Data presented are results of three independent experiments and represent the mean \pm SEM. * $P < .05$ versus control siRNA. (D) **pCAF knockdown decreases CIITA binding at the MHC II promoter.** ChIP assays were carried out in control or pCAF siRNA transfected HeLa cells stimulated with IFN- γ . Lysates were immunoprecipitated with control or endogenous CIITA antibody. Associated DNA was analyzed via real-time PCR using primers spanning the MHC II promoter. Values were normalized to the total amount of MHC II promoter DNA added to the reaction (input values). Data are presented as fold increase in the MHC II promoter DNA relative to unstimulated IP samples \pm S.D. Results are representative data of three independent experiments.

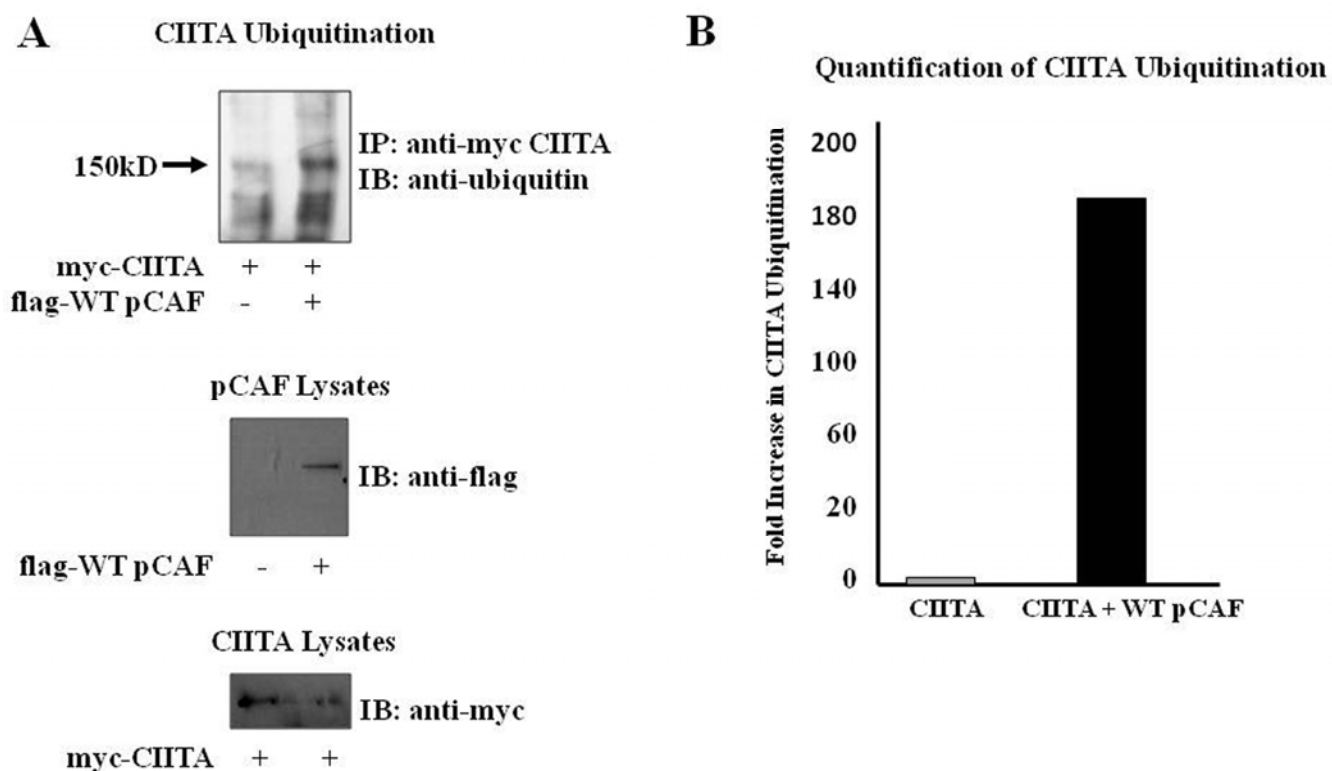


Figure 9: CIITA Ubiquitination Is Enhanced by pCAF. (A) Myc-CIITA was co-transfected without or with WT pCAF in HeLa cells, CIITA was immunoprecipitated and immune complexes were separated by SDS gel electrophoresis and then blotted with anti-ubiquitin antibody. Lysates for myc-CIITA and flag-pCAF are shown. An increase is seen in the ubiquitination of CIITA in the presence of WT pCAF, upper blot. (B) CIITA ubiquitination results were analyzed using densitometry. The relative density of CIITA ubiquitination was normalized to the level of CIITA expressed. Data shown is representative of three independent experiments.

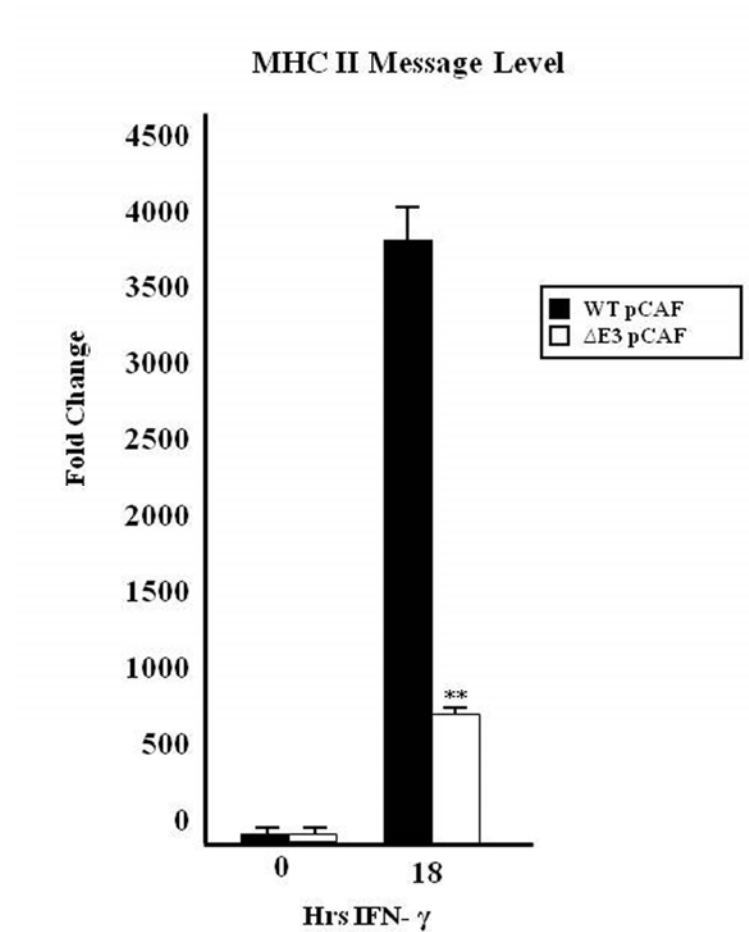


Figure 10: pCAF E3 Ligase Activity Affects MHC II Gene Expression. Gene expression assays were carried out in HeLa cells transfected with 5 μ g of flag-WT pCAF or flag- Δ E3 pCAF and stimulated with IFN- γ . Levels of MHC II mRNA were measured by real-time PCR and were normalized to GAPDH mRNA. Data presented are results of three independent experiments and represent the mean \pm SEM. ** P<.005 versus WT pCAF.

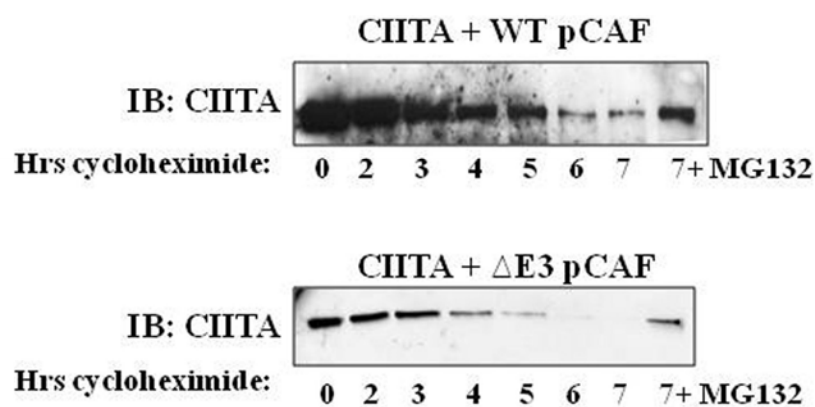
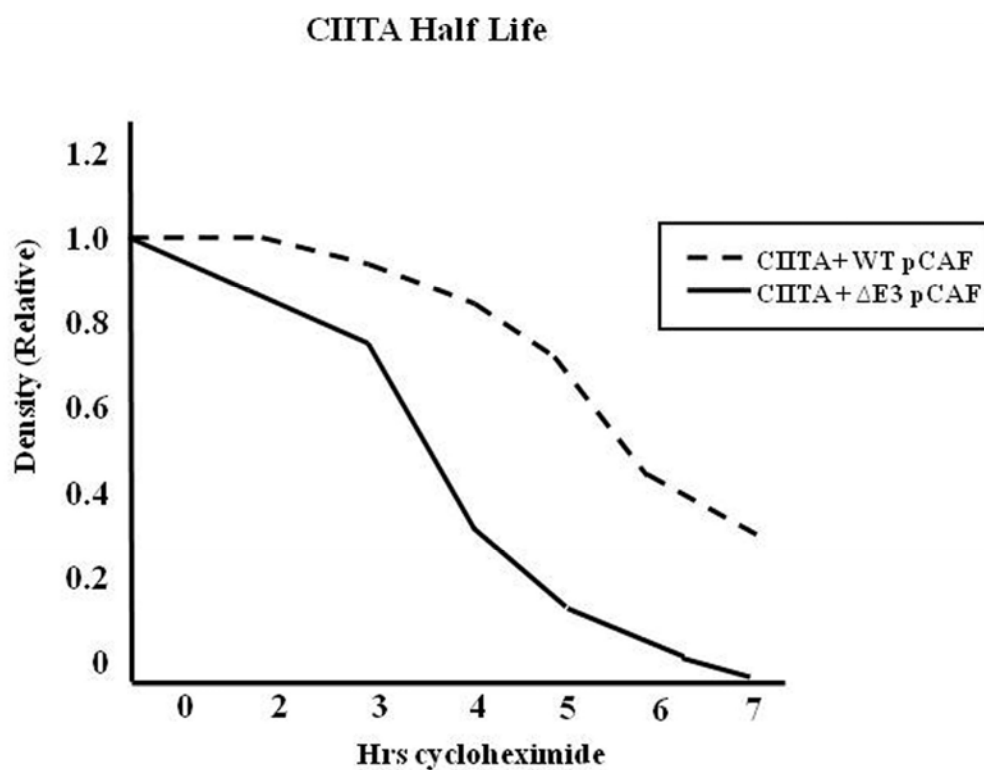
A**B**

Figure 11: Destabilization of CIITA by pCAF. (A) HeLa cells were transfected with 5 μ g of flag-CIITA and 3 μ g of flag-WT pCAF or flag- Δ E3 pCAF. Cells were then treated with

cycloheximide to inhibit protein synthesis and sample seven was treated with MG132 to inhibit proteasome activity. Cells were harvested at indicated time points and analyzed via Western blotting for CIITA. (B) Densitometry was performed on the Western blots in A. Relative density is plotted and shows that CIITA stability is reduced in the presence of $\Delta E3$ pCAF as compared to WT pCAF, resulting in a decreased half life. Data are representative of three independent experiments.

CHAPTER 4.

DISCUSSION

MHC II complexes are responsible for presenting exogenous antigens to CD4⁺ T cells and thus stimulating an adaptive immune response (Abbas and Janeway 2000; LeibundGut-Landmann, Waldburger et al. 2004; Janeway, Travers et al. 2005). MHC II complexes are constitutively expressed on antigen presenting cells including B cells, dendritic cells, and macrophages and are inducibly expressed on all nucleated cells by the inflammatory cytokine interferon- γ . Both constitutive and inducible expression of MHC II plays a key role in altering T cell response to infections (Abbas and Janeway 2000; LeibundGut-Landmann, Waldburger et al. 2004; Janeway, Travers et al. 2005). Deficiencies in MHC II expression result in the fatal immune disease Bare Lymphocyte Syndrome (BLS), while over expression of MHC II complexes is associated with the majority of autoimmune diseases, including diabetes, arthritis, and multiple sclerosis (LeibundGut-Landmann, Waldburger et al. 2004; Drozina, Kohoutek et al. 2005; Swanberg, Lidman et al. 2005; Otten, Leibundgut-Landmann et al. 2006). MHC II expression is therefore tightly regulated at the level of transcription through the regulated expression of CIITA, the master regulator of MHC II gene expression (Waldburger, Masternak et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003; Schnappauf, Hake et al. 2003).

Following stimulation with inflammatory cytokines including INF- γ , activation of the JAK-STAT signal transduction cascade promotes the inducible expression of CIITA (Morris, Beresford et al. 2002; Pattenden, Klose et al. 2002; Ni, Karaskov et al. 2005). Once CIITA is expressed, its activities within the cell are tightly regulated. Post-translational modifications, such as phosphorylation, acetylation, and ubiquitination of CIITA have been shown to regulate its function (Harton, Zika et al. 2001; Greer, Harton et al. 2004; Drozina, Kohoutek et al. 2005;

Drozina, Kohoutek et al. 2006). Several studies have indicated that the acetylation and phosphorylation of CIITA aid in its ubiquitination (Drozina, Kohoutek et al. 2005; Drozina, Kohoutek et al. 2006). Ubiquitination has been demonstrated to be a critical regulating post-translational modification of CIITA, as monoubiquitination of CIITA increases CIITA stability and transactivation at the MHC II promoter (Greer, Zika et al. 2003; Drozina, Kohoutek et al. 2006).

The stable expression and promoter binding of CIITA is vital for an effective immune response as MHC II genes are not expressed until the master regulator CIITA binds to the MHC II enhanceosome complex to initiate transcription (Jabrane-Ferrat, Fontes et al. 1996; Waldburger, Masternak et al. 2000; Harton, Zika et al. 2001; Jabrane-Ferrat, Nekrep et al. 2003; LeibundGut-Landmann, Waldburger et al. 2004; Muhlethaler-Mottet, Krawczyk et al. 2004; Drozina, Kohoutek et al. 2005; Zika and Ting 2005; Otten, Leibundgut-Landmann et al. 2006). Monoubiquitination has been previously shown to play be a post translational modification capable of stabilizing proteins, specifically transcription factors, while polyubiquitination leads to protein and transcription factor degradation (Greer, Zika et al. 2003; Pickart 2004; Taylor and Jobin 2005; Drozina, Kohoutek et al. 2006; Sun 2006). While the mechanisms that govern the expression of CIITA have been described, how CIITA is stabilized and maintained at the MHC II promoter throughout an immune response remains unclear. Understanding how post-translational modifications to CIITA regulate CIITA stability and maintenance at the MCH II promoter will clarify the molecular mechanisms governing MHC II expression and initiation, maintenance and termination of the adaptive immune response.

In order to understand the mechanisms regulating stable binding of CIITA at the MHC II promoter, the E3 ubiquitin ligase for CIITA must be identified and its mechanism of action,

monoubiquitination versus polyubiquitination, characterized. The identification of the E3 ubiquitin ligase for CIITA has several important implications. The aberrant expression of MHC II genes has been connected to many diseases, from BLS and tumor growth, to autoimmune disease (Reith and Mach 2001; Drozina, Kohoutek et al. 2005; Otten, Leibundgut-Landmann et al. 2006). Monoubiquitination regulates the stability and function of CIITA; determination of the ligase responsible for CIITA monoubiquitination will enable the ability to alter the ubiquitination status of CIITA and may lead to new therapies targeting conditions which result from CIITA misregulation.

pCAF is known as a chromatin remodeling enzyme and as a histone acetyltransferase. pCAF has previously been demonstrated to be recruited to and activate transcription of the MHC II promoter, but the effects of pCAF on MHC II transcription were attributed to its HAT activities (Drozina, Kohoutek et al. 2005; Wright and Ting 2006). It has recently been demonstrated that pCAF has an intrinsic ubiquitination domain that plays a role in the ubiquitination and stability of critical cell cycle proteins (Linares, Kiernan et al. 2007). Our research is novel as it indicates that pCAF, in addition to its role as a HAT, contributes to the regulation of CIITA transactivation via its E3 ligase activity, and that it can affect both the ubiquitination pattern and stability of CIITA.

To determine if pCAF affects the stability and transactivation of CIITA, we performed luciferase assays, a knockdown of endogenous pCAF, a ChIP, and a gene expression assay. We show that pCAF drives CIITA transactivity and that the expression of WT pCAF increases CIITA stability. We next showed that a knockdown of endogenous pCAF leads to a decrease in CIITA transactivity, a decrease in MHC II RNA expression, and to a substantial decrease in CIITA binding at the MHC II promoter. We showed that the effects of a pCAF knockdown are

specific and can be reversed through the over expression of WT pCAF. Together these results argue that pCAF is playing an important role in the transactivation and stabilization of CIITA.

Based on the above observations we next sought to determine if pCAF affects the ubiquitination status of CIITA. pCAF is known to function as a HAT, but recent studies have shown that it also contains an E3 ubiquitin ligase domain (Linares, Kiernan et al. 2007). In addition pCAF has been shown to affect the ubiquitination status of the critical cell cycle protein, p53 (Linares, Kiernan et al. 2007). We first showed that in the presence of WT pCAF, CIITA ubiquitination is increased. We further demonstrate that expression of MHC II is increased in the presence of WT pCAF, but decreased in the presence of Δ E3 pCAF, indicating pCAF ubiquitination results in stable CIITA promoter binding. Finally we demonstrate that in the presence of Δ E3 pCAF, CIITA is destabilized. In summary these data indicate pCAF is regulating the monoubiquitination status of CITIA through its E3 ubiquitin ligase domain.

Ubiquitination plays a role in both the degradation and the stabilization of proteins. Ubiquitin molecules are conjugated to lysine residues, and are able to form both mono and multi-ubiquitin chains on target proteins (Adams 2003; Pickart 2004; Taylor and Jobin 2005; Sun 2006). The fate of a protein is determined by both the number of ubiquitin molecules added and the site to which they are added (Taylor and Jobin 2005; Sun 2006). Polyubiquitinated proteins via a lysine 48 chain are targeted to the 26S proteasome for degradation (Taylor and Jobin 2005; Sun 2006). Proteins that are polyubiquitinated via a lysine 63 chain have been shown to be involved in new cellular functions (Taylor and Jobin 2005; Sun 2006). Proteins that are monoubiquitinated have been demonstrated to be involved in cellular trafficking, histone modifications, DNA repair, and transcriptional activation (Taylor and Jobin 2005; Sun 2006). Our data indicates that CIITA is monoubiquitinated by pCAF: CIITA transactivation and

stability are increased in the presence of expressed pCAF, while a knockdown of pCAF decreases CIITA transactivity, stability, and binding at the MHC II promoter and ultimately leads to a decrease in MHC II gene expression.

pCAF was first demonstrated to be recruited to the MHC II promoter to function as a HAT. pCAF has been shown to acetylate CIITA, which enhances CIITA transactivation (Spilianakis, Papamatheakis et al. 2000; Zika and Ting 2005). This acetylation has been hypothesized to aid in the ubiquitination of CIITA (Drozina, Kohoutek et al. 2005). pCAF has also been demonstrated to play a role in remodeling the chromatin structure at the MHC II promoter through the acetylation of histones (Spilianakis, Papamatheakis et al. 2000; Drozina, Kohoutek et al. 2005; Zika and Ting 2005). Several recent studies have indicated that pCAF may be playing an additional role at the MHC II promoter (Harton, Zika et al. 2001; Drozina, Kohoutek et al. 2005). One study demonstrated that pCAF's interaction with CIITA is independent of its HAT domain, and that pCAF increases CIITA transactivity independent of its HAT domain (Harton, Zika et al. 2001). Another study implicated pCAF in playing a role in the ubiquitination of CIITA by demonstrating that CIITA's ubiquitination pattern is increased in the presence of pCAF (Greer, Zika et al. 2003). Together these data support our novel observations that pCAF is the E3 ligase for CIITA.

Furthering our understanding of the regulation of CIITA through the identification of the E3 ubiquitin ligase for CIITA has profound implications for therapeutic treatments for conditions resulting from the aberrant expression of MHC II molecules. The potential to control the stability and transactivation of CIITA through regulating its ubiquitination status may allow researchers to manipulate the immune systems of patients with tumors, BLS, and autoimmune diseases. Our study increases knowledge of the molecular events occurring at the MHC II

promoter and demonstrates a novel role for pCAF at the MHC II promoter as the E3 ligase for CIITA. Ubiquitination is however a complex process that is highly regulated; additional studies are needed to elucidate the ubiquitination site of CIITA and the manner in which pCAF ubiquitinates CIITA and to further our understanding of how ubiquitination regulates the expression of MHC II genes.

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