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Dynamic Regulation of the Class II Transactivator by Posttranslational Modifications

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DYNAMIC REGULATION OF THE CLASS II TRANSACTIVATOR BY
POSTTRANSLATIONAL MODIFICATIONS

by

JULIE E. MORGAN

Under the Direction of Susanna Fletcher Greer, PhD

ABSTRACT

The class II Transactivator (CIITA) is the master regulator for Major Histocompatibility Class II (MHC II) molecules. CIITA is dynamically regulated by a series of Posttranslational Modifications (PTMs). CIITA is responsible for initiating transcription of MHC II genes, thus allowing peptides derived from extracellular antigens to be presented to CD4⁺ T cells. CIITA's PTMs are necessary for regulation of CIITA's location, activity, and stability. Our work identifies the kinase complex ERK1/2 as being responsible for phosphorylating the previously identified regulatory site, serine (S) 280 on CIITA. Phosphorylation by ERK1/2 of CIITA S280

leads to increased levels of CIITA mono-ubiquitination and overall increases in MHC II activity. We further identify a novel ubiquitin modification on CIITA, lysine (K) 63 linked ubiquitination poly ubiquitination. Our data shows novel crosstalk between K63 ubiquitination and ERK1/2 phosphorylation. K63 ubiquitinated CIITA is concentrated to the cytoplasm, and upon phosphorylation by ERK1/2, CIITA translocates to the nucleus, thus demonstrating that CIITA's location and activity is regulated through PTM crosstalk. While ubiquitination has been shown to be a critical PTM in the regulation of CIITA, the enzyme(s) mediating this important modification remained to be elucidated. Previous reports implicating the histone acetyltransferase (HAT), pCAF as an ubiquitin E3 ligase were intriguing, as pCAF is also known to participate in the acetylation of both histones at the MHC II promoter and in acetylation of CIITA. We now identify novel roles for pCAF in the regulation of CIITA. We show pCAF acts as an E3 ligase, mediating mono, K63, and K48 linked ubiquitination of CIITA. We therefore demonstrate an additional substrate for the "dual acting" enzyme, pCAF. In sum, our observations identify enzymes involved in both the phosphorylation and ubiquitination of key residues of CIITA, which ultimately regulate CIITA activity. Together our observations contribute to knowledge of CIITA's growing network of PTMs and their role in regulating the adaptive immune response, and will allow for development of novel therapies to target dysregulated CIITA activity during adaptive immune responses.

INDEX WORDS: Major Histocompatibility class, II, Class II Transactivator, Posttranslational Modifications, Ubiquitination, Phosphorylation, ERK1/2, pCAF, Posttranslational Modification crosstalk

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POSTTRANSLATIONAL MODIFICATIONS

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JULIE E. MORGAN

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

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

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2015

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August 2015

DEDICATION

Being a naturally curious person, science seemed like the perfect profession for me. The first day I walked into the Greer lab, I began a journey like no other. This journey would take me through ups, downs, winding around and through a gamut of emotions. The life of a scientist seems exciting and full of discoveries; I quickly learned most of what fuels science are the many failures as compared to the successes. Being a scientist you deal with a lot of reading, thinking about and generating hypothesis, designing experiments to test your hypothesis, and for your hypothesis to yield far different results than what you predicted. Science requires an immense amount of time, effort, dedication, and sacrifice, which none would be possible without the support of family and friends.

Because of the afore mentioned, I would like to dedicate this dissertation to all of the people in my life whom have helped me get to this stage, whether big or small all of the efforts and support and love have culminated in me accomplishing this goal. First, I would like to thank with all my heart and love, my amazing husband John Morgan; without his love, guidance, and support, I would have never had the courage to begin this adventure; my parents Richard and Sharon Mauldin, whom have always stood by me and given me all the love, care, and encouragement a child needs, my brothers Jeff and Jeremy who made me tough and taught me to never give up. Lastly, I want to thank all of my wonderful friends new and old that have been there through the happy times, struggles, and successes. You have all made an impact on me, and share in this accomplishment!

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I would like to also thank my committee members Dr. Casonya Johnson and Dr. Zhi-Ren Liu for their valuable guidance and input through all the years of my research.

I spent many long hours away from home and family, my amazing lab mates certainly made the long and arduous days much better. During my time in the Greer Lab, I saw many people come and go; I would like to thank each and every one of my lab mates for making the lab a great place to work. I want to especially thank Dr. Agnieszka Truax, for always encouraging and believing in me. She has always been there to pick me up during my lowest of lows, and celebrate my highest of highs. When I started this journey, I never expected to leave this experience with life long friend. Also, I want to thank Nathaniel Boyd; not only is he a wonderful colleague, he is an amazing person and friend. Again, I never expected such a cherished friendship from this experience. Lastly, I would like to thank Ronald Shanderson for being the best mentee anyone could ever have! You made my graduate experience that much better. It has been an absolute pleasure to watch you develop into a fantastic scientist and I can't wait to see what the future holds for you.

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LIST OF ABBREVIATIONS:

Adenosine Triphosphate (ATP)

ATP dependent proteolysis factor 1 (APF-1)

Antigen Presenting Cells (APCs)

Arginine (R)

Bare Lymphocyte Syndrome (BLS)

cAMP Responsive Binding Element (CREB)

Class II Transactivator (CIITA)

CREB binding proteins (CBP)

Class II Transactivator Isoform III (CIITA IF3)

Co-immunoprecipitation (Co-IP)

Cyclin dependent kinase (CDK)

Deoxyribonucleic Acid (DNA)

Extracellular Signal regulated kinase 1/2 (ERK1/2)

Fetal Bovine Serum (FBS)

Forkhead box protein O4 (FOXO4)

Forhead box protein O1 (FOXO1)

Histone Acetyltransferase (HAT)

Histone Deacetylase (HDAC)

Human Leukocyte Antigen (HLA)

Histone Methyltransferase (HMT)

Immunoblot (IB)

Interferon- γ (IFN- γ)

Immunoprecipitate (IP)

Interleukin-1 receptor (IL-1R)

Interleukin-1 receptor associated kinase (IRAK)

Lysine (K)

Lysine 48 (K48)

Lysine 63 (K63)

Major Histocompatibility Complex (MHC)

Messenger RNA (mRNA)

Nuclear Factor Kappa B (NF- κ B)

Nuclear Factor-Y (NF-Y)

Phorbol-12-Myristate-13-acetate (PMA)

Phosphorylation (P)

Posttranslational Modifications (PTMs)

Proliferating cell nuclear antigen (PCNA)

Regulatory Factor X (RFX)

RNA Polymerase II (RNA Pol II)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Serine (S)

Serine 5 (Ser5)

Serine 2 (Ser2)

Severe Combined Immunodeficiency (SCID)

TNFR associated factor 6 (TRAF6)

Tumor necrosis factor receptor (TNFR)

Ubiquitin (Ub.)

Ubiquitin activating enzyme (E1)

Ubiquitin conjugating enzyme (E2)

Ubiquitin E3 ligase (E3)

Ultra Violent (UV)

1 CHAPTER 1: INTRODUCTION

1.1 POSTTRANSLATIONAL MODIFICATIONS:

Posttranslational Modifications (PTMs) are defined as the enzymatic processing of a polypeptide chain following translation from mRNA and are classified as either the addition of a chemical group such as a phosphate, or the addition of another protein moiety, such as an ubiquitin molecule. PTMs are key in the regulation of many cellular processes, including protein degradation (Geiss-Friedlander and Melchior 2007), regulatory processes, signaling (Morrison, Kinoshita et al. 2002), gene expression, intra-cellular trafficking, and protein-protein interactions (Hunter 2007). Over the last several decades, the human proteome has been shown to be increasingly more diverse than that of the human genome. The human genome encodes for an estimated 20,000 to 25,000 genes (International Human Genome Sequencing 2004). While human proteome is estimated to contain over 1 million proteins, this difference between genes and proteins can be attributed in part to PTMs (Jensen 2004). In addition many PTMs are reversible, allowing protein activity to be regulated in a more versatile way and therefore increasing the functional diversity of the modified protein and the overall proteome. PTMs can occur on many amino acids side chains or peptide linkages and are mediated by various enzymatic molecules. Over the past decade, PTMs have become an area of intense investigation as they have been implicated in a variety of diseases including heart disease (Smith and White 2014), cancer (Jin and Zangar 2009), neurodegenerative diseases (Karve and Cheema 2011),

and a host auto-immune disorders (Cohen 2000, Sollid and Jabri 2011, Dunne, Overbergh et al. 2012, Burska, Hunt et al. 2014, Ryan, Nissim et al. 2014, Mastrangelo, Colasanti et al. 2015).

Currently there are over 200 different types of known PTMs (Walsh 2006) the most abundant of which include ubiquitination, phosphorylation, and acetylation, each of will be a main focus of this dissertation. PTMs elicit various functional and activation changes on proteins, often via competition for the same residues within a single protein (Yang and Seto 2008). PTMs have unique abilities to work closely with each other to enhance their functions and as well as oppose each other mediating changes in overall protein function (Yang and Seto 2008, Wu, Kong et al. 2009), which is termed PTM crosstalk.

PTM crosstalk is generally classified into two different forms, positive or negative (Hunter 2007). Positive crosstalk, the initial PTM will trigger the addition or removal of a second PTM, or generate a recognition site for another protein. Negative crosstalk leads to direct competition for a specific residue or by concealing a site, causing another PTM unable to occur at that site (Hunter 2007, Venne, Kollipara et al. 2014). While single PTM events can regulate protein function, PTM crosstalk is another level in expanding the functionality of the human proteome. This dissertation will focus on PTMs and crosstalk that occurs in the aspect of regulating a critical adaptive immune molecule.

1.2 UBIQUITIN AND THE UBIQUITINATION PATHWAY:

Aron Ciechanover, Avram Hersko, and Irwin Rose first elucidated the process of ubiquitination and subsequently received the Nobel Prize in Chemistry in 2004. Ubiquitin (Ub) is a highly conserved 76 amino acid protein and is ubiquitously expressed in all eukaryotic cells either in free form or as a part of a complex. Ubiquitin was first described and named adenosine triphosphate (ATP) dependent proteolysis factor 1 (APF-1), which was discovered as a part of a complex (Ciechanover, Hod et al. 1978). In 1980 Wilkinson, with no knowledge of the previous findings showed a small protein which was identical as APF-1, and named it ub. (Wilkinson, Urban et al. 1980) Ubiquitination is a highly regulated PTM and is historically associated with protein degradation; however more recent evidence supports non-proteolytic roles for ubiquitination including roles in transcription regulation (Conaway, Brower et al. 2002, Greer, Zika et al. 2003, Greer, Harton et al. 2004, Bhat, Truax et al. 2010). The coordinated process of attaching an active Ub molecule to a substrate is known as ubiquitination (Adams 2003) which is catalyzed by three distinct and regulated enzymatic reactions. These reactions rely on an Ub activating enzyme (E1), an Ub conjugating enzyme (E2), and an Ub ligase (E3). To initiate ubiquitination, the E1 activates Ub in the presence of adenosine triphosphate (ATP) and transfers Ub to an E2 enzyme. Next, an E3 recognizes and binds both the target substrate and the Ub/E2 complex, allowing for Ub to be transferred to the target substrate (Ciechanover, Heller et al. 1980, Pickart 2001). The Ub molecule is attached to the substrate through an isopeptide bond, which links the C-terminus of Ub glycine 76 to the ϵ -amino group of a lysine (K) residue on the substrate (Ciechanover 1994, Pickart 2001, Wang, Deng et al. 2001). This three-step enzymatic process is the initiating reaction that occurs for all known downstream

ubiquitination reactions (Ciechanover 1994, Ciechanover 1994, Pickart 2001, Ciechanover 2010), but the cascade of events will have very different consequences based on the number of Ub moieties added and the topology of the ubiquitination reaction (**Figure 1.1**). In mammalian cells, while there is a single E1 enzyme, there are more than 30 known E2 enzymes, and greater than 650 known E3 ligases, each with differing substrate specificities (de Bie and Ciechanover 2011).

The sequential addition of Ub molecules to the internal lysine residues of Ub leads to the formation of poly-Ub chains. There are seven internal lysine (K) residues of Ub: K6, K11, K27, K29, K33, K48, and K63. Of these seven internal lysine residues, only K48 is known to signal and promote protein degradation by targeting substrates to the 26S proteasome (Holling, van der Stoep et al. 2002, Baton, Deruyffelaere et al. 2004, Wu, Kong et al. 2009, Elsen 2011). While ubiquitination is traditionally known to mediate protein degradation, recent studies have identified non-traditional roles for ubiquitination involving mono and poly-ubiquitination via K63 linkage (Mallery, Vandenberg et al. 2002, Brenkman, de Keizer et al. 2008) (**Figure 1.2**). These “non-traditional” poly-ubiquitination reactions have been implicated in the alteration of protein function, localization, and interactions with other proteins (Fang and Weissman 2004). One focus of this dissertation will be investigating K63 linked ubiquitination and the role it has in regulating the adaptive immune response.

Mono-ubiquitination of substrates has been linked to transactivation, DNA repair, alteration of histones, gene transcription, and protein localization (Zhu, Linhoff et al. 2000). There are many examples of the roles mono-ubiquitination has in regulating cellular processes.

One example, proliferating cell nuclear antigen (PCNA) requires mono-ubiquitination for DNA repair following UV damage, this mono-ubiquitination event is required for error-prone DNA repair (Sun and Chen 2004). Additionally, the transcription factor Foxhead box protein O4 (FOXO4), is multi mono-ubiquitinated at several lysine residues. These multi mono-ubiquitination events are signals which lead to increased nuclear localization, ultimately enhanced transcriptional activity (Brenkman, de Keizer et al. 2008). Lastly, the Greer lab has also identified a role in multi mono-ubiquitination in the regulation of the Class II Transactivator (CIITA) where, CIITA's transactivity is increased following multi mono-ubiquitination (Drozina, Kohoutek et al. 2006, Bhat, Truax et al. 2010).

K63 poly-ubiquitination has been shown to be important in many cellular functions including signal transduction and gene transcription (Wang, Deng et al. 2001, Fang and Weissman 2004). K63 Ub also has previously been linked to endocytosis (Lauwers, Jacob et al. 2009), protein trafficking (Debald, Schildberg et al. 2013), and DNA damage repair (Wang, Gao et al. 2012) and protein-protein interactions (Mallery, Vandenberg et al. 2002, Hayakawa 2012, Emmerich, Ordureau et al. 2013, Ori, Kato et al. 2013). K63 ubiquitination of TNFR-associated factor 6 (TRAF6) links IL-1R signaling to the activation of NF- κ B (Habelhah 2010). TRAF6 modification by K63 ubiquitination allows for additional proteins to bind directly to the K63 ubiquitin chain resulting in additional downstream effects including phosphorylation and nuclear translocation (Balkhi, Fitzgerald et al. 2008, Hayakawa 2012, Emmerich, Ordureau et al. 2013, Ori, Kato et al. 2013, Zhou, Shen et al. 2013). IRAK1 is also modified via K63 ubiquitination, resulting in multiple protein-protein interactions and downstream

phosphorylation events in the NF- κ B pathway (Balkhi, Fitzgerald et al. 2008). In both cases, K63 ubiquitination bring two protein complexes together to act as a scaffold.

Less is known of the functional topology with the other lysine linkages (K6, K11, K27, K29, and K33) and further structural analysis is needed to address these questions (Woelk 2007). Because of the lack of knowledge in this area, this is likely an area of focus in years to come.

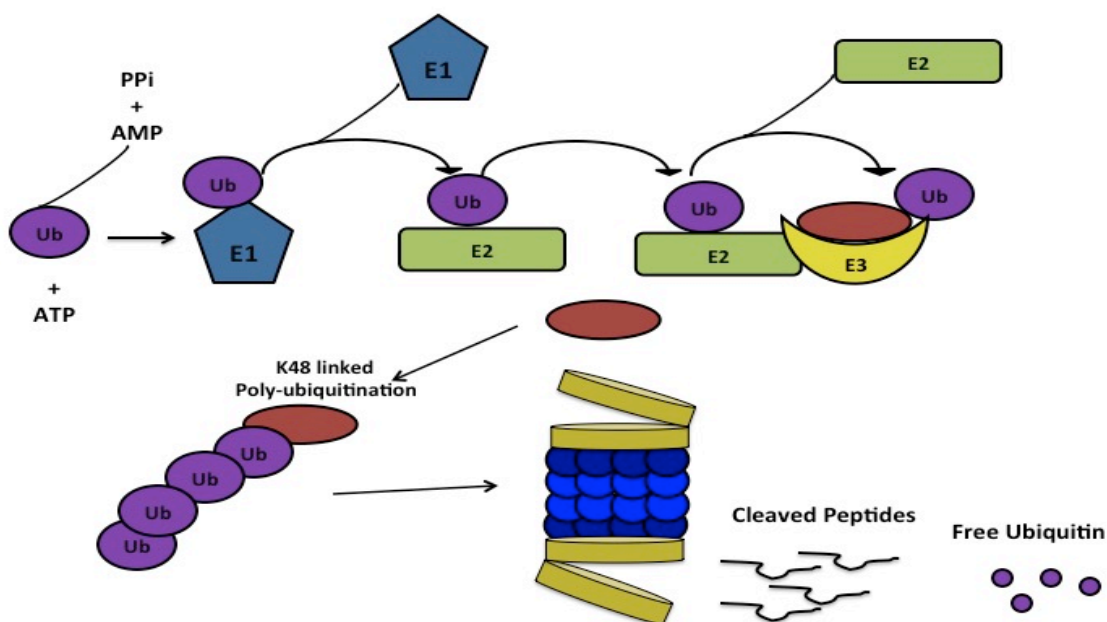


Figure 1.1: The Enzymatic cascade of ubiquitination:

A molecule of Ubiquitin (Ub) is dependent upon activation by ATP. Once activated by ATP, Ub is covalently linked to an E1 activating enzyme. Activated Ub is transferred from an E1 to and E2 conjugating enzyme. Ub can either then be directly or with the assistance of and E3 ubiquitin ligase added to a lysine residue on the target protein. If the targeted protein is marked for degradation multiple Ub molecules are added via the 48th lysine of ubiquitin. When at least 4 Ub molecules have been added via K48, the protein is then recognized by the 26S proteasome and degraded. The role of the 19S proteasome is to recruit poly-ubiquitinated proteins. The 19S cleaves the Ub, unfolds the protein, and direct proteins into the 20S catalytic core. The 20S catalytic is responsible for cleaving proteins into peptides (Ciechanover and Schwartz 1994, Coux, Tanaka et al. 1996, Strickland, Hakala et al. 2000).

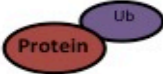
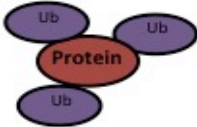
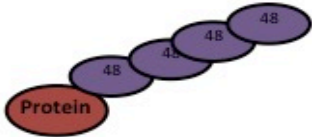

Ubiquitin Linkages		Mono-ubiquitination: involved Transcription regulation
		Multiple mono-ubiquitination: Transcription regulation
		K48 linked poly-ubiquitination: Tagging proteins for destruction via the 26S proteasome
		K63 linked poly-ubiquitination: Protein-Protein interactions, DNA repair, signal transduction, cellular trafficking

Figure 1.2: Ubiquitin linkage classifications regulate various cellular processes.

Ubiquitin (Ub) can be attached to a lysine residue on proteins either as a single moiety (mono-ubiquitination) or as linear or branched poly-ubiquitin chain. There are different topologies of poly-ubiquitination and each confers a different effect on the targeted protein they are attached to. Generally speaking proteins that are mono-ubiquitinated are not degraded via the 26S proteasome. Mono-ubiquitinated proteins have important roles in vesicle trafficking, assist in transcription regulation, and in DNA repair. Poly-ubiquitination through K48 linked signals proteins for degradation via the 26S proteasome, while K63 linked signals in DNA repair, cellular trafficking, and protein-protein interactions (Finley, Ciechanover et al. 2004, Kim, Kim et al. 2007, Balkhi, Fitzgerald et al. 2008, Hayakawa 2012, Wang, Gao et al. 2012, Ori, Kato et al. 2013)

1.3 PHOSPHORYLATION:

Phosphorylation is the addition of a phosphate (PO_4^{3-}) group on a protein (Levene and Alsborg 1906) and is considered to be a reversible PTM responsible for the regulation of various protein activities (Burnett and Kennedy 1954). Phosphorylation is a reversible enzymatic reaction regulated by two different enzymes (Barford, Das et al. 1998, Chang and Stewart 1998), kinases and phosphatases. Kinases catalyze the addition of the phosphate group and phosphatases catalyze the removal of a phosphate group (Burnett and Kennedy 1954) (**Figure 1.3**). Phosphorylation typically occurs on serine (S), threonine (T), and tyrosine (Y) residues within eukaryotic systems. Phosphorylation is key in protein activity and is often one of the main PTMs in regulating the function of a protein, which is often through conformational changes (Burnett and Kennedy 1954). Thus, phosphorylation can either lead to the activation or deactivation of a protein. Additionally, phosphorylation enables a protein to recruit other proteins through the use of conserved domain structures that recognize and are able to bind phosphomotifs (Barford, Das et al. 1998, Johnson 2001, Johnson and Lewis 2001, Johnson 2009).

Currently there are more than 1000 kinases and 500 phosphatases that have been discovered and classified (Cohen 2000). Phosphorylation has a wide breadth of roles in cellular processes in both prokaryotic and eukaryotic organisms (Cozzone 1988, Stock, Ninfa et al. 1989, Barford, Das et al. 1998, Chang and Stewart 1998). Phosphorylation regulates many cellular processes and signaling pathways such as: transcription, cell cycle, cell differentiation, intracellular communication, and immunological functions (Johnson 2009). It is estimated that approximately one-half of the cellular

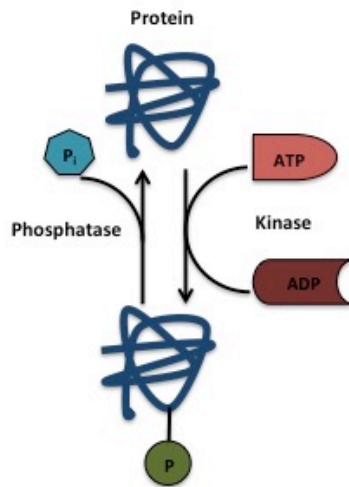


Figure 1.3: Reversible enzymatic phosphorylation process.

The process of phosphorylation is a reversible posttranslational modification that regulates protein function. Kinases add a phosphate group (PO_4^{3-}) and phosphatases reverse protein phosphorylation through hydrolyzation of the phosphate group. Phosphorylation occurs specifically on serine (S), threonine, (T), and tyrosine (Y) residues often causes conformational changes within a targeted protein.

proteins in a eukaryotic system are phosphorylated around 30% of all cellular proteins are phosphorylated (Cohen 2000). Many diseases such as cancer and autoimmune disorders are due to hyper phosphorylation (Cohen 2001, Gong, Liu et al. 2006, Ma, Trinh et al. 2013), erroneous dephosphorylation (Gong, Liu et al. 2006), or mutations within the kinases or phosphatases themselves (Lahiry, Torkamani et al. 2010). Phosphorylation can occur on a single site, or multiple sites within the protein (Cohen 2000). Because of the immense amount of investigation in this area, irregular phosphorylation has been recognized as a cause in human disease (Cohen 2001). While the process is well understood, and many of the enzymes involved have been identified and well characterized, it is likely that phosphorylation will continue to be an exciting area of investigation. Because so many diseases have been linked in some way to phosphorylation or dysregulated phosphorylation, development of small inhibitor molecules or drugs targeting specific kinases or phosphatases will continue as future treatment options.

1.4 LYSINE ACETYLATION:

Lysine acetylation is another PTM that is considered to be reversible process and is regulated by two distinct enzymes: Histone acetyl transferases (HAT) and histone deacetylases (HDAC) (Sadoul, Boyault et al. 2008). The process of acetylation is the addition of an acetyl group (CH_3CO) relying on acetyl-coenzyme A, which acts as the acetyl group donor onto a lysine (K) residue (Sadoul, Boyault et al. 2008) (**Figure 1.4**). Protein acetylation is a common PTM found in cellular biology and is known to regulate transcription factors, effector proteins, molecular chaperones, and various cytoskeletal proteins (Glozak, Sengupta et al. 2005). HATs facilitate acetylation and HDACs mediate deacetylation, resulting in activation/deactivation of

transcription factors (Legube and Trouche 2003). These opposing enzymatic reactions are necessary in regulating transcription factors (Legube and Trouche 2003).

The process of acetylation has been an intense area of investigation for a number of years, and cannot only be targeted to histones, but also in the regulation of many other proteins important for a variety of cellular processes. There are a vast number of examples as to the importance of lysine acetylation in the regulation of cellular process. One example is the tumor suppressor P53, which is critical in many signal transduction pathways. P53 is important for control of the cell cycle under stress conditions, and acetylation is absolutely vital for this action. If acetylation is completely abolished the role of P53 to regulate cell growth is no longer intact leading to uncontrolled cell proliferation (Sadoul, Boyault et al. 2008), which is one of the hallmarks of cancer. Acetylation or deacetylation has been associated with a variety of diseases including various cancer types and autoimmune disorders (Tang, Zhao et al. 2008). More recently HDAC inhibitors have been a focused for the use in treatment in many diseases (West and Johnstone 2014). As more acetylated proteins are identified in the proteome, a greater understanding of acetylation states and how they associate to diseases continues to grow. Investigating lysine acetylation will continue to be heavily explored as the specific small inhibitor molecules and drugs are a large focus in the treatment of a variety of diseases such as cancer.

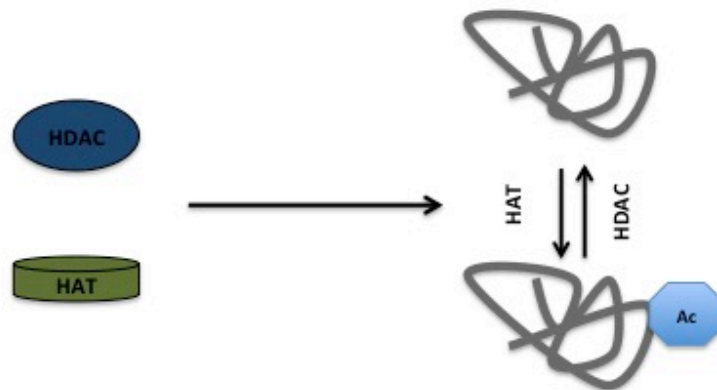


Figure 1.4: Reversible enzymatic process of acetylation.

Lysine (K) Acetylation is a reversible posttranslational modification involved in regulating various protein functions, in particular regulating transcription factors. Histone acetyl transferases (HATs) using the molecule Acetyl coenzyme A, adds an acetyl (CH_3CO) group and histone deacetylases (HDACs) catalyze the removal of the acetyl group.

1.5 THE IMMUNE SYSTEM:

Every day we are exposed to an unsterile pathogen filled world where our immune systems protect us from infection. The immune system is composed of innate and adaptive immune responses, with both responses collaborating to provide efficient and effective immune protection. Innate immunity is an immediate but non-specific response which provides the first line of defense against pathogens attack (Janeway and Medzhitov 2002). Adaptive immunity has evolved in higher order vertebrates and provides specific, but delayed immunity. An advantage of adaptive immunity is its ability to develop memory against previously seen pathogens. The features of specificity and long-term memory are what set the innate and adaptive immune responses apart (Janeway 2001, Chaplin 2010).

1.5 INNATE IMMUNITY:

The innate immune system is the non-specific arm of immunity and is the first line of defense (Janeway and Medzhitov 2002). The innate immune system is a fast acting, non-specific response to invading pathogens and is composed of various cell types working to defend the host when microorganisms breach the first line of defense, the skin and epithelial linings of internal organs. Effector cells and antimicrobial compounds play roles in neutralizing invading pathogens based on cell surface markers commonly seen on pathogenic surfaces (Janeway 2001, Janeway and Medzhitov 2002, Medzhitov 2007). The cells involved in the innate immune response recognize and respond to pathogens in a nonspecific way, and do not provide long lasting immunity. Innate immunity's role is to provide an immediate defense

against infection, and is present in all animal and plant life (Janeway, Travers et al. 2001, Travis 2009). The innate branch of the immune system is responsible for recruiting immune cells to sites of active infection through cytokine expression and for activating the complement cascade, which enables activation of additional effector cells, pathogen recognition, recognize bacteria, and removal of dead cells (Janeway, Travers et al. 2001, Chaplin 2010). Additionally, the innate system also is responsible for activating the adaptive immune response through the process of antigen presentation. The effector cells of the innate response include dendritic cells (DCs), natural killer cells (NKs), mast cells, basophils, and eosinophils. Together these cells identify the invading pathogen and prevent infection from spreading (Janeway, Travers et al. 2001) (Medzhitov 2007, Owen, Punt et al. 2013).

1.6 ADAPTIVE IMMUNITY:

The second arm of immunity, adaptive immunity is responsible for eliciting immune responses when the innate immune system has failed to contain an infection. Adaptive immunity is highly specific and is able to confer long-term memory, which sets it apart from innate immunity. The adaptive immune response takes longer to develop during a primary response; the upside of adaptive immunity is its ability to recognize a much larger repertoire of non-self and foreign antigens.

The adaptive immune response involves coordination between lymphocytes and antigen presenting cells (APCs). The production of lymphocytes occurs in the primary lymphoid organs (bone marrow and thymus), which upon development can circulate through the blood to the

secondary lymphoid organs (lymph nodes, tonsils, spleen, peyer's patches, and mucosa associated lymphoid tissues). B cells and T cells are classified as lymphocytes, which possess membrane bound receptors, termed the B cell receptor (BCR) and T cell receptor (TCR). These receptors are what lend to the specificity of both B and T cells (Smith-Garvin, Burns et al. 2010, Corcos, Osborn et al. 2011). These receptors are highly specific in the ability to recognize antigen. Once a B cells is activated, it may mature to become an antibody producing plasma cell, which will aid in the clearance of extracellular pathogens.

T cells are also involved in the clearance of infections; however they are able clear both extracellular and intracellular pathogens. T cells are further subdivided into three additional categories: cytotoxic $CD8^+$ T cels (T_c), $CD4^+$ T helper cells (T_h), and regulator T cells (T_{reg})(Wan 2010, Owen, Punt et al. 2013). $CD4$ and $CD8$ T cells both contain cell surface glycoproteins, in which these cells are name for(Wan 2010). There are sub categories for $CD4$ T cells, T helper ($Th1$) and T helper 2 ($Th2$) cells. $Th1$ cells primarily defend against intracellular bacteria, while $Th2$ cells defend against extracellular pathogens (Farber, Acuto et al. 1997, Gerloni and Zanetti 2005). Following activation, $CD4^+$ and $CD8^+$ T cells are able to a form memory cells, which persists after the infection is resolved in order to allow for a rapid expansion upon re-exposure of the same pathogen.

Both B and T cells are able to recognize antigens, the BCR can recognize free antigen and T cells typically only recognizes antigen when it is bound to the Major Histocompatibility complex (MHC) molecules (Gerloni and Zanetti 2005). To activate both lymphocytes, the BCR and TCR complexes have to recognize antigen, present this antigen to MHC molecules, and

receive an additional co-stimulatory signal (Janeway, Travers et al. 2001, Smith-Garvin, Burns et al. 2010, Corcos, Osborn et al. 2011). B cells bind intact antigens that are engulfed via receptor-mediated endocytosis (Corcos, Osborn et al. 2011, Owen, Punt et al. 2013). Once activated, B and T cells are able to induce signaling pathways that in turn activate transcription of genes that result in the proliferation and activation of these antigen specific B and T cells (Janeway 2001, Janeway, Travers et al. 2001, Flajnik and Kasahara 2010). These processes are what differentiate the adaptive vs. innate immune response.

1.7 MAJOR HISTOCOMPATIBILITY COMPLEX:

The Major Histocompatibility complex (MHC) is a set of cell surface glycoproteins that are encoded by a large gene family located on chromosome 6 (Geraghty, Inoko et al. 1999, Janeway, Travers et al. 2001). MHC molecules play important roles in recognizing ‘self’ versus ‘non-self’ (Janeway, Travers et al. 2001, Ting and Trowsdale 2002, Owen, Punt et al. 2013) and are therefore, a critical step in identifying and removing pathogens and tumors. The role of MHC molecules is to present antigen to a specific T cell types, thus activating the adaptive immune response. There are two known classifications of MHC molecules: MHC class I presents antigenic peptides derived from intracellular pathogens to CD8⁺ T cells (Penn 2002, Drozina, Kohoutek et al. 2005) while MHC class II presents antigenic peptides derived from extracellular origin pathogens to CD4⁺ T cells (Penn 2002, Drozina, Kohoutek et al. 2005). MHC I is expressed on all nucleated cells, while MHC II is constitutively expressed on antigen presenting cells (APCs) and can also be induced by the inflammatory cytokine interferon

gamma on all nucleated cells (Rojo, Saizawa et al. 1989, Drozina, Kohoutek et al. 2005, Gerloni and Zanetti 2005).

MHC class II is a critical component in the activation of the adaptive immune response and defects in MHC II expression are associated with two specific types of immunodeficiencies. Severe combined immunodeficiency syndrome (SCID) patients are highly susceptible to infection as they lack CD4⁺ T cells and cannot activate B cells, or provide CD4⁺T cells during CD8⁺ T cells responses (Reith and Mach 2001). Left untreated bare lymphocyte syndrome (BLS) results in death in infancy due to an inability fight infections, as CD4⁺ T cells cannot properly be activated (Reith and Mach 2001, Penn 2002, Owen, Punt et al. 2013). The over expression of MHC II molecules has further been linked to the development of all autoimmune diseases (Swanberg, Lidman et al. 2005, Jones, Fugger et al. 2006, Fernando, Stevens et al. 2008, Tsai and Santamaria 2013), while the suppression of MHC II is correlated with growth and tumor metastasis formation as a consequence of diminished immune surveillance (Guy, Krajewski et al. 1986, Mlecnik, Bindea et al. 2011, Truax, Thakkar et al. 2012, Osborn and Greer 2015). In sum, MHC II molecules are critical to the activation of the adaptive immune responses, dysregulation of the MHC II genes has significant implications, and thus the expression of MHC II is tightly regulated and is done so primarily at the level of transcription (Benoist and Mathis 1990, Drozina, Kohoutek et al. 2005).

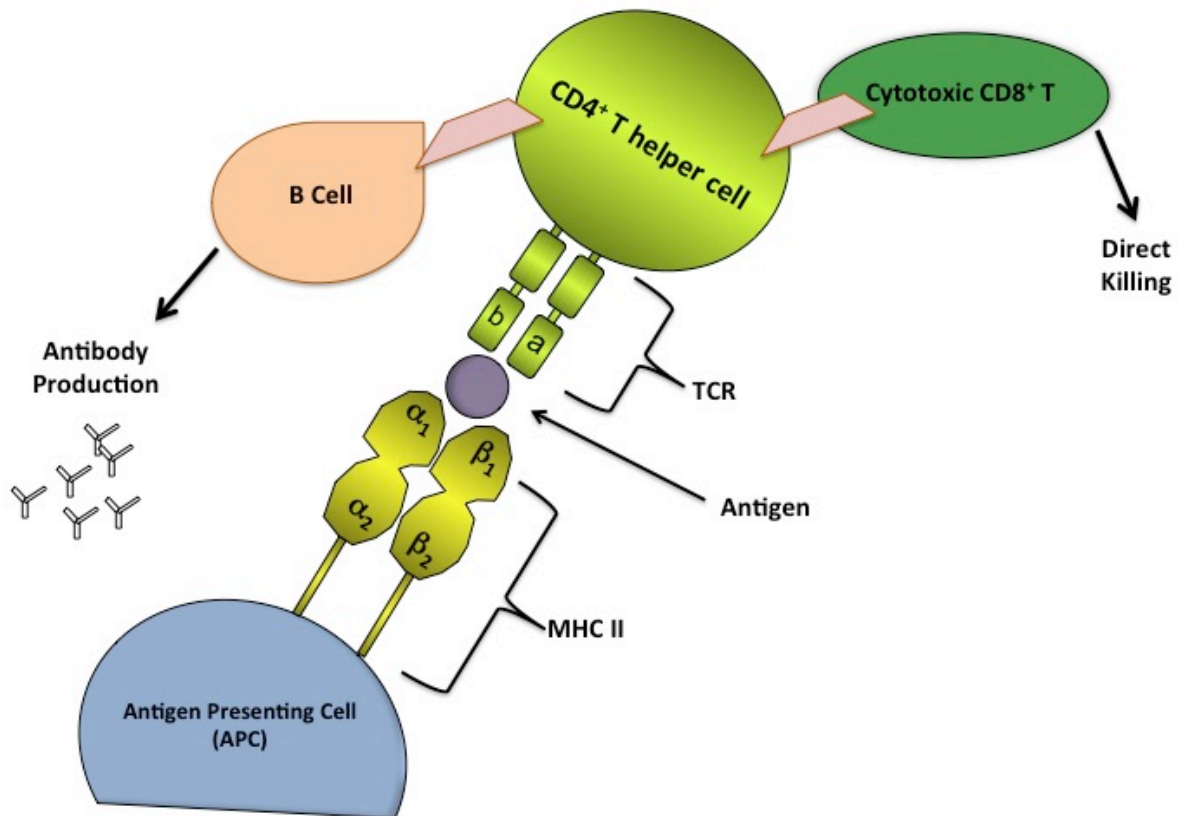


Figure 1.5: MHC II molecules are critical and necessary to activating the adaptive immune response, as they present extracellular antigens to CD4⁺ T cells.

MHC II molecules are cell surface glycoproteins that present antigens to CD4⁺ T cells which leads to T cell activation. MHC II is present on all antigen presenting cells (APCs) whose roles are to recognize, process and present antigenic peptides. Additionally, MHC II can be induced on all other cells types through the stimulation by the cytokine interferon gamma (IFN- γ). Upon CD4⁺ T cell activation, they are able to activate B cells to produce antigen specific antibodies and elicit an adaptive immune response. Activated CD4⁺ T cells also are able to activate cytotoxic CD8⁺ T cells, which mediate direct killing (Owen, Punt et al. 2013).

1.8 REGULATION OF MHC CLASS II:

MHC II molecules are cell surface glycoproteins that are constitutively expressed on the surface of antigen presenting cells such as macrophages, B cells, dendritic cells, and activated T cells (Cresswell and Howard 1997, Morris, Beresford et al. 2002). Additionally, MHC II expression can be induced in all other nucleated cells through inflammatory cytokines, with interferon gamma (IFN- γ) being the most stimulatory (Kaufman, Auffray et al. 1984). IFN- γ enables the induced expression of MHC II and promotes enhanced antigen presentation and induction of localized immune responses (Owen, Punt et al. 2013). IFN- γ is considered to be a type II interferon and activates MHC II transcription by binding to the type II IFN receptor where both constitutive and induced MHC II expression is tightly regulated at the level of transcription (Ting and Trowsdale 2002, Boss and Jensen 2003, Drozina, Kohoutek et al. 2006).

The MHC II gene is regulated through a cohesive interplay of many different transcription factors and histone remodeling enzymes that associate with a conserved regulatory region within MHC II genes (Ting and Trowsdale 2002, Boss and Jensen 2003, Drozina, Kohoutek et al. 2006). This regulatory region consists of three conserved sequences termed the X1, X2, and Y boxes (Ting and Trowsdale 2002, Drozina, Kohoutek et al. 2005, Burska, Hunt et al. 2014). These conserved sequences are recognized by ubiquitously expressed DNA binding factors. The X1 box is bound by Regulatory factor X (RFX), a trimeric protein consisting of RFX5, RFXANK, and RFXAP (Masternak, Barras et al. 1998, Nagarajan, Louis-Pence et al. 1999). The X2 box is bound by cAMP responsive element binding protein (CREB) (Moreno, Beresford et al. 1999). The Y box is bound by a trimeric protein, Nuclear factor-Y (NF-Y)

which itself of NF-YA, NF-YB, and NF-YC (Mantovani 1999, Drozina, Kohoutek et al. 2005). When these ubiquitously expressed co-factors are bound to their respective sequences; together they make up the enhanceosome complex (Masternak, Muhlethaler-Mottet et al. 2000). While assembly of the enhanceosome complex is a necessary step in MHC II gene regulation; alone enhanceosome assembly is insufficient for MHC II initiation (Masternak, Muhlethaler-Mottet et al. 2000). Once assembled, the enhanceosome is bound by the MHC II master regulator CIITA, binding of CIITA drives initiation of transcription of MHC II genes (Mach, Steimle et al. 1996, Masternak, Muhlethaler-Mottet et al. 2000, Boss and Jensen 2003). CIITA is a non-DNA binding protein, which binds the enhanceosome complex through interactions with RFX, CREB, NF-Y (Masternak, Muhlethaler-Mottet et al. 2000, Ting and Trowsdale 2002). Once bound to the enhanceosome, CIITA initiates transcription of MHC II genes by recruiting the basal transcriptional machinery including the TATA binding protein (TBP), TATA associated factors (TAFs) (Mahanta, Scholl et al. 1997), and histone modifying enzymes such as histone acetyl transferases (HATs) (Spilianakis, Papamatheakis et al. 2000, Wright and Ting 2006), histone deacetylases (HDACs) (Zika, Greer et al. 2003), and histone methyltransferases (HMTs) (Zika, Fauquier et al. 2005).

Together, these recruited enzymes alter MHC II promoter accessibility to DNA binding proteins. CIITA recruitment of HATs enhances acetylation levels at the MHC II proximal promoter leading to greater transcription efficiency (Sisk, Gourley et al. 2000). Association with HDACs results in chromatin remodeling (Zika, Greer et al. 2003), and recruitment of HMTs by CIITA increases chromatin accessibility (Zika, Fauquier et al. 2005). The role of CIITA in recruiting each of these associated co-factors is thus critical for the regulation of MHC II

transcription; coordination of the recruitment of chromatin remodeling enzymes and assembly of the enhanceosome earns CIITA the title of the “Master Regulator” of MHC II genes (**Figure 1.6**)

1.9 CIITA, THE MASTER REGULATOR OF MHC CLASS II:

CIITA is a non-DNA binding co-factor that is required for MHC II gene transcription (Masternak, Muhlethaler-Mottet et al. 2000, Boss and Jensen 2003, Drozina, Kohoutek et al. 2005). CIITA is not only indispensable for MHC II transcription, but CIITA also crucial in the transcription of other genes involved in the adaptive immune response, including cytokines IL-4 (Sisk, Gourley et al. 2000) and IL-10 (Yee, Chintalacharuvu et al. 2005) and the death receptor, Fas ligand (Gourley and Chang 2001). Because CIITA has roles in regulating many immune response genes and their expression, it is inescapable that CIITA is implicated in the development of disease. CIITA has been linked to the development of cancer (Yazawa, Kamma et al. 1999, Baton, Deruyffelaere et al. 2004, Truax, Thakkar et al. 2012, Osborn and Greer 2015), autoimmune disease (Stuve, Youssef et al. 2002, Burska, Hunt et al. 2014), Addison’s disease, (Skinningsrud, Husebye et al. 2008), and atherosclerosis (Buttice, Miller et al. 2006). The involvement of CIITA in the regulation of additional genes besides MHC II, which if dysregulated also lead to disease development, has fueled the area of intense investigation concentrated on understanding completely how CIITA is regulated.

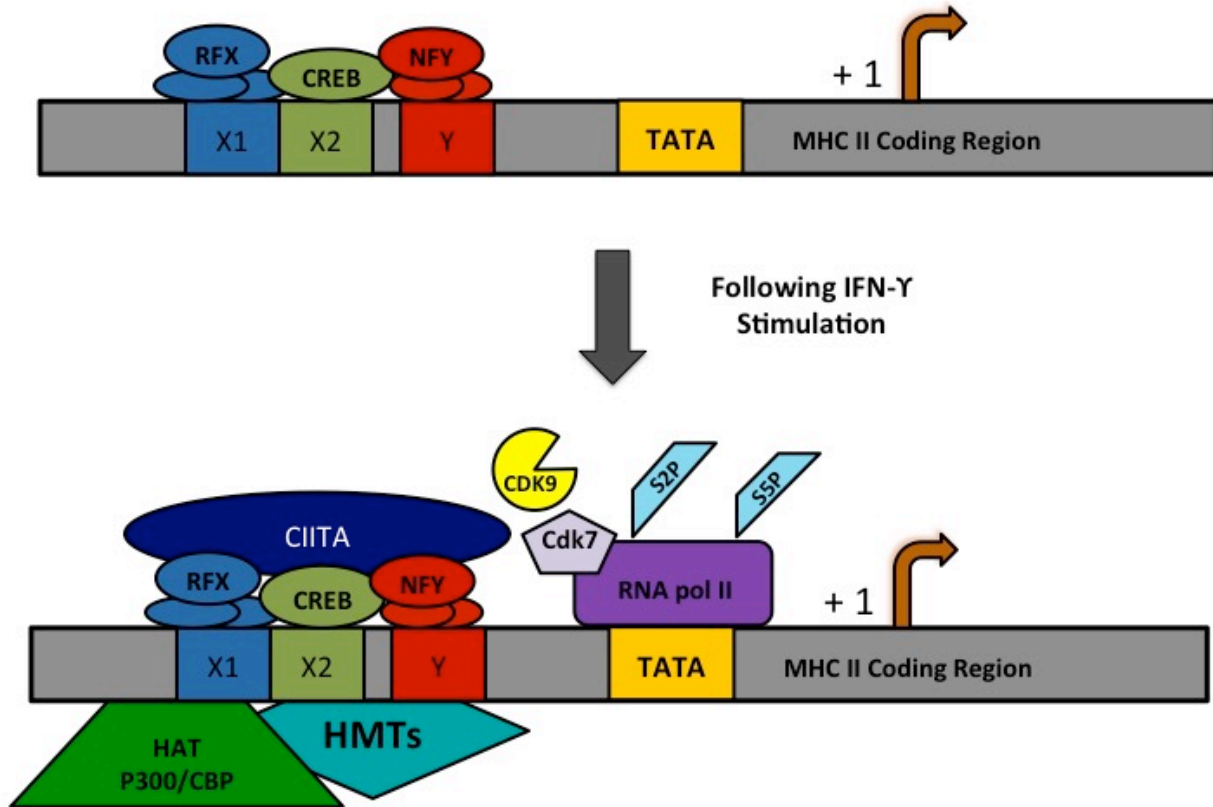


Figure 1.6: MHC II promoter region:

(Top) The MHC II proximal promoter consists of three conserved sequences: X1, X2, and Y boxes. The X1 box is bound by RFX, a trimeric protein complex consisting of RFX5, RFANK, and RFXAP. The X2 box is bound by cAMP response element binding protein (CREB), and the Y box is bound by the trimeric complex NF-Y. NF-Y is made up of NF-YA, NF-YB, and NF-YC. The culmination of these transcription factors binding to their respective boxes leads to the formation of the enhanceosome complex. The enhanceosome is necessary, but alone is insufficient to initiate MHC II transcription (Kretsovali, Agalioti et al. 1998, Fontes, Kanazawa et al. 1999, Fontes, Kanazawa et al. 1999, Greer, Zika et al. 2003, Zika, Fauquier et al. 2005). (Bottom) The enhanceosome creates a required interaction site for the binding of CIITA, which is absolutely necessary for MHC II transcription. CIITA recruits RNA polymerase II and all of the transcriptional machinery and histone modifying enzymes histone acetyl transferases (HATs), histone methyl transferases (HMTs), and histone deacetylases (HDACs) allowing for promoter accessibility (Kretsovali, Agalioti et al. 1998, Fontes, Kanazawa et al. 1999, Fontes, Kanazawa et al. 1999, Greer, Zika et al. 2003, Zika, Fauquier et al. 2005).

While CIITA is known as the master regulator for MHC II gene expression (Mach, Steimle et al. 1996), CIITA itself is also regulated at the level of transcription where it maintains tight regulation of MHC II (Ting and Trowsdale 2002). Four independent promoters regulate CIITA transcription in a cell specific manner: pI, pII, pIII, and pIV (Muhlethaler-Mottet, Otten et al. 1997). Each promoter transcribes unique first exons, which lie upstream of CIITA genes (Muhlethaler-Mottet, Otten et al. 1997). Due to these different transcriptional start sites; each promoter yields a different isoform of CIITA genes (Muhlethaler-Mottet, Otten et al. 1997); however each CIITA isotype has similar function (Muhlethaler-Mottet, Otten et al. 1997). The promoter I (pI) isotype is constitutively expressed in dendritic and macrophage cells (Muhlethaler-Mottet, Otten et al. 1997). The promoter II (pII) isotype is not well conserved amongst species and is considered to be inactive (Baton, Deruyffelaere et al. 2004). The promoter III (pIII) isotype is constitutively expressed in B cells, and is also enhanced through stimulation with IFN- γ in endothelial cells and fibroblasts (Piskurich, Linhoff et al. 1999). The promoter IV (pIV) isotype is expressed upon stimulation with IFN- γ and is the predominate isotype expressed in the IFN- γ stimulated cells (Piskurich, Linhoff et al. 1999). In all CIITA expressing cells, MHC II expression correlates with that of CIITA.

The focus of this dissertation will be on CIITA promoter III (pIII), which is constitutively expressed in B cells (Goodwin, Xi et al. 2001, Holling, van der Stoep et al. 2002, Drozina, Kohoutek et al. 2006, van den Elsen 2011). CIITA pIII contains six promoter elements each of which is necessary to initiate CIITA transcription in B cells (van den Elsen 2011). pIII consists of activation response elements (ARE 1) and (ARE 2) which bind acute myloid

leukemia (AML2) and transcription factor CREB-1, respectively (Holling, van der Stoep et al. 2002). Additional regulatory elements present on pIII include the E-twenty-Six/interferon gamma stimulate response element (Ets/ISRE) (van den Elsen 2011), which is bound by the transcription factors PU.1 and interferon regulatory factor 4 (IRF4) (van den Elsen 2011). Additional elements include the E-box; which interacts with transcription factor E47 (van den Elsen 2011) and the A and B sites which are bound by neurofibromin 1 (NF-1) and organic cation transporter 1 (Oct 1), respectively (Holling, van der Stoep et al. 2002) (**Figure 1.7**). When their appropriate binding partners bind each of the above elements, B-cell specific pIII is activated (Holling, van der Stoep et al. 2002, van den Elsen 2011). Thus, a coordinated effort of multiple transcription factors and co-factors is necessary to regulate CIITA pIII transcription. In addition to tight regulation at the transcription level, CIITA pIII is also regulated by a complex series of posttranslational modifications (Spilianakis, Papamatheakis et al. 2000, Greer, Zika et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008, Wu, Kong et al. 2009, Bhat, Truax et al. 2010), making CIITA one of the most dynamic and regulated proteins in the adaptive immune response.

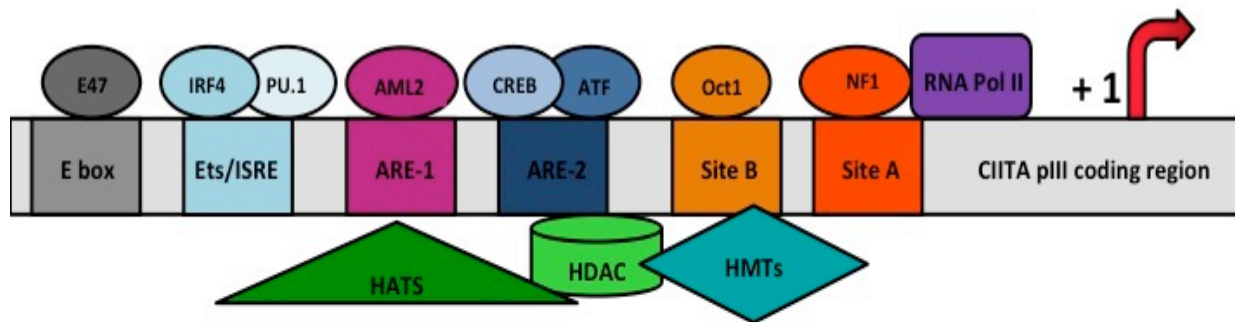


Figure 1.7: Schematic representation of CIITA promoter III proximal region:

The promoter region of CIITA pIII has six conserved sequences: E box, Ets/SRE, ARE-1, ARE-2, Site B, and Site A (Masternak, Muhlethaler-Mottet et al. 2000). Activation of pIII requires the transcription factor CREB-1 to bind to the activation element 2 (ARE-2) regions. PU.1/IRF4 bind the Ets/ISRE region of the promoter, E47 interacts with the E box, AML2 associates with the ARE-1 region, Oct1 associates with the Site B, and NF1 associates with site A (Masternak, Muhlethaler-Mottet et al. 2000)

The CIITA isoform III (IF3) protein is an 1130 amino acid non-DNA binding co-factor, which consists of four functional domains: an acidic activation domain (AAD) located at the N-terminus, a proline, serine, and threonine (P/S/T) domain, a GTP binding domain (GBD), and a leucine rich region (LRR) located at the C-terminus (Masternak, Muhlethaler-Mottet et al. 2000). CIITA also contains three nuclear localization signals (NLS) necessary for its nuclear translocation and activity (Cressman, O'Connor et al. 2001). The AAD is necessary for CIITA interaction with the enhanceosome complex (Chin, Li et al. 1997), the P/S/T domain is critical for initiation of MHC II transcription (Chin, Li et al. 1997, Camacho-Carvajal, Klingler et al. 2004), and both the GBD and LRR domains are required for nuclear localization (Chin, Li et al. 1997, Linhoff, Harton et al. 2001, Camacho-Carvajal, Klingler et al. 2004) (Tosi, Jabrane-Ferrat et al. 2002). The P/S/T region contains a proteolytic sequences site known as a degron (Drozina, Kohoutek et al. 2006, Bhat, Truax et al. 2010), a sequence that typically targets proteins for degradation (Drozina, Kohoutek et al. 2006, Bhat, Truax et al. 2010) (**Figure 1.8**).

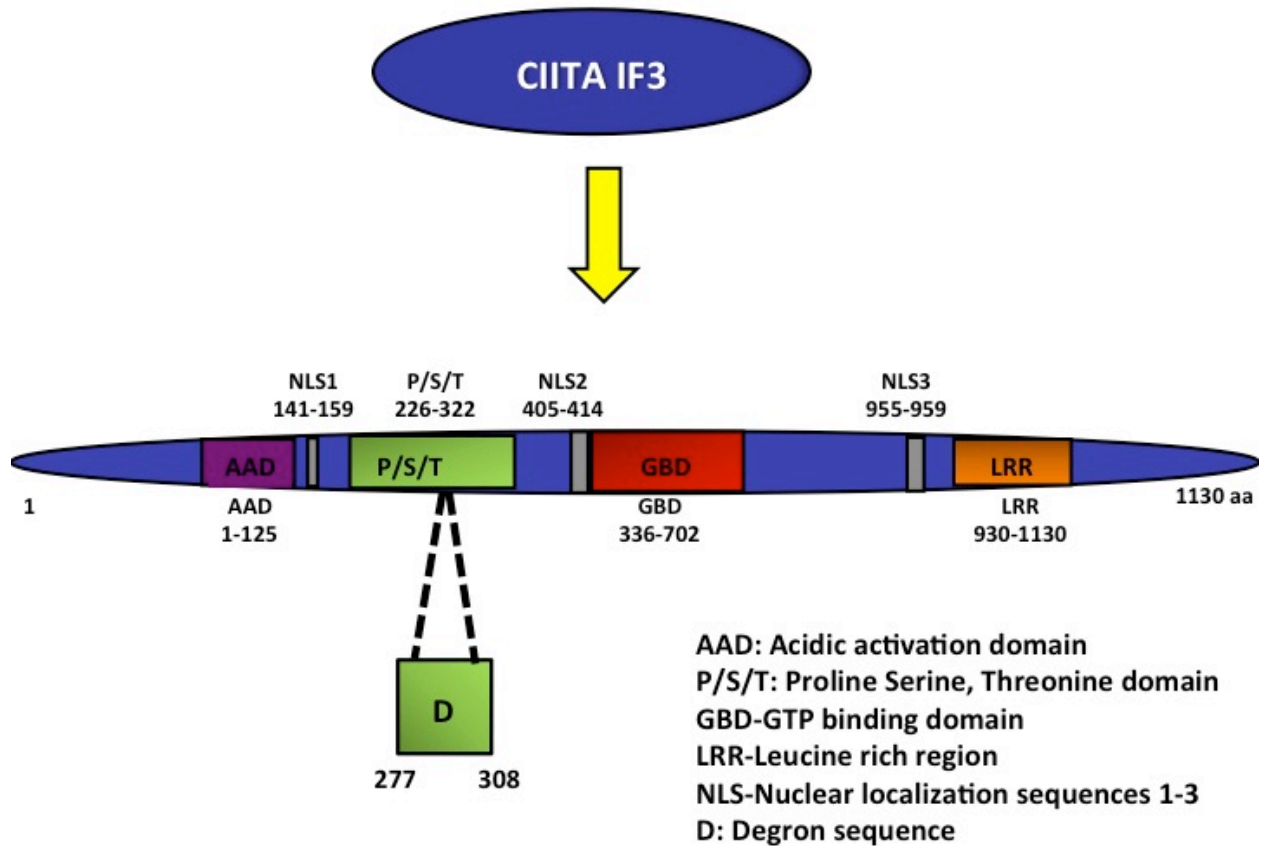


Figure 1.8: Schematic representations of domain structure CIITA proteins generated from PIII:

CIITA is a 1130 amino acid protein with four functional domains, the N-terminal transcriptional acidic activation domain (AAD) which is required for CIITA transactivity, a proline/serine/threonine rich (P/S/T) domain that is required for binding to the various factors and co-factors, the GTP binding domain (GBD), and the leucine rich repeat region, both of which are important for nuclear localization and oligomerization. CIITA also contains three nuclear localization sequences (NLS). These NLS sequences are required for the nuclear translocation of CIITA. Within the P/S/T domain, there is a proteolytic signal site, termed the degron (D). Degrons are specific sequences that target proteins for ubiquitination at proximal lysine residues for degradation. Figure is adapted from Ting J.T. and Trowsdale J., 2002.

1.10 POST-TRANSLATIONAL MODIFICATIONS OF CIITA:

While the transcription regulation of CIITA pIII is well-known, posttranslational modifications (PTMs) to CIITA and their role in CIITA regulation remains an area of active exploration. Because transcription and co-factors have highly specific binding sites at promoters, they are tightly regulated to allow for gene expression. As CIITA is a transcription factor, its regulation is achieved through various different levels of control including, genetic, transcriptional, and post-translational routes (Kodadek, Sikder et al. 2006); however, post-translational modifications are the most crucial. The genetic and transcriptional control of CIITA may be flawless and yield the correct protein, however, CIITA requires for its functionality to be heavily modified by many different types of post-translational modifications (Cressman, Chin et al. 1999, Spilianakis, Papamatheakis et al. 2000, Cressman, O'Connor et al. 2001, Sisk, Nickerson et al. 2003, Satoh, Toyota et al. 2004, Drozina, Kohoutek et al. 2006, Wu, Kong et al. 2009).

CIITA is modified through a variety of PTMs including, phosphorylation, ubiquitination, and lysine acetylation, each of which regulates CIITA in some capacity (Cressman, Chin et al. 1999, Spilianakis, Papamatheakis et al. 2000, Cressman, O'Connor et al. 2001, Sisk, Nickerson et al. 2003, Satoh, Toyota et al. 2004, Drozina, Kohoutek et al. 2006, Wu, Kong et al. 2009). **Table 1.1** shows known PTMs to CIITA and their roles in regulating CIITA (Wu, Kong et al. 2009).

Phosphorylation by the kinase PKA at serine 834 (S834) and serine 1050 (S1050), both lead to MHC II transcription inhibition (Li, Harton et al. 2001). Alternatively, PKA also phosphorylates serines 374 and 375 (S374, S375), which lie in the P/S/T and GBD domains, leading to an increase in MHC II transcription (Sisk, Nickerson et al. 2003). In this instance, the kinase PKA has differential phosphorylation abilities depending on the site of phosphorylation. Greer and colleagues also identified that within the P/S/T domain, there are three phosphorylation sites: serine 286, 288, and 293 (S286, S288, and S293), phosphorylation of these residues regulates CIITA nuclear localization but not its oligomerization (Greer, Harton et al. 2004). The kinase responsible for phosphorylating S286, S288, and S293 was later identified as ERK1/2 and cdc2 (Voong, Slater et al. 2008). Serine 280 (S280) that lies within the degran region within the P/S/T was shown by Bhat and colleagues to be phosphorylated (Bhat, Truax et al. 2010) and serine 280 phosphorylation was termed the “gate keeper”, as it was shown to be a critically important modification allowing for a tri mono-ubiquitination to occur and overall increased CIITA transactivity and MHC II transcription (Bhat, Truax et al. 2010). However, the kinase responsible remained to be identified. Additionally, serines 373 and 377 (S373 and S377) were shown by Xu and colleagues to be phosphorylated by the kinases GSK1 and CK1, which mediate collagen transcription without affecting MHC II expression (Xu, Harton et al. 2008). The above demonstrates that PTMs have different roles in regulating CIITA transactivity.

The histone acetyl transferases p300 and pCAF are responsible for acetylating CIITA at lysine 141 (K141) and lysine 144 (K144) located within the first nuclear localization signal at the N-terminus (Spilianakis, Papamatheakis et al. 2000). Acetylation at these sites causes CIITA

to be shuttled to the nucleus and an increase in MHC II expression (Spilianakis, Papamatheakis et al. 2000). The removal of acetyl groups by histone deacetylases (HDACs) also are involved in the regulation of CIITA activity. The HDAC, SIRT1 mediates the removal of acetyl groups from CIITA and increases CIITA transactivity (Kong, Fang et al. 2009, Wu, Kong et al. 2009, Wu, Kong et al. 2011). Conversely, HDAC2 mediated deacetylation of CIITA catalyzes the removal of acetyl groups and marks CIITA for degradation (Kong, Fang et al. 2009, Wu, Kong et al. 2009). Lastly, CIITA itself has been shown to possess intrinsic histone acetyl transferase activity, which lies at the N-terminus. As of current, there have been no known target proteins for such activity (Raval, Howcroft et al. 2001).

Ubiquitination has been an area of interest for CIITA regulation as more has been unlocked about the roles of ubiquitination in regulation of various protein processes. Mono-ubiquitination has been shown to increase CIITA transactivity (Greer, Zika et al. 2003) and it is known that once mono-ubiquitinated, CIITA's association with the enhanceosome complex increases at the MHC II promoter, ultimately leading to increased MHC class II transcription (Greer, Zika et al. 2003). Three sites within the P/S/T domain have further been identified as lysine 315 (K315), lysine (K330), and lysine (K333) (Bhat, Truax et al. 2010). Bhat and colleagues demonstrated when the triple mono-ubiquitination sites are mutated; CIITA stability and activity dramatically decrease (Bhat, Truax et al. 2010). Additionally, CIITA is K48 linked poly-ubiquitinated where it is recognized by the 26S proteasome and degraded (Schnappauf, Hake et al. 2003) (**Figure 1.9**). These studies provided depth to the role mono-ubiquitination and K48 linked poly-ubiquitination have on CIITA transactivation and it remains likely that there are additional sites of ubiquitination to be discovered as CIITA has 42 lysine residues and

which could be potential sites for ubiquitination. While several sites of ubiquitination have been identified, the E3 ligase that catalyzes these events has remained elusive. CIITA's PTM code has begun to be unraveled, as many sites of modifications, enzymes responsible, and roles in which these modifications have on overall location and activity of CIITA have been identified. However, the mechanisms of CIITA's recruitment stabilization at the MHC II promoter have yet to be identified.

1.11 SUMMARY:

The immune response is critical for the elimination of non-self and for survival in a non-sterile world. MHC II molecules are responsible for presenting extracellularly derived antigenic peptides to CD4⁺ T cells; thus, aberrant expression of MHC II results in disease and death. Over expression of MHC II is causative for autoimmune disorders, suppressed MHC II correlates with metastatic tumor growth, and loss of MHC II expression leads to the un-survivable immunodeficiencies including Bare Lymphocyte Syndrome (Zhu, Linhoff et al. 2000, Fang and Weissman 2004). The Master Regulator CIITA regulates the expression of MHC II; which in turn is regulated by a complex PTM "code". Multiple CIITA PTMs have been identified (Spilianakis, Papamatheakis et al. 2000, Greer, Harton et al. 2004, Wu, Kong et al. 2009, Bhat, Truax et al. 2010); however many of the mechanisms regulating these modifications, including the enzymes responsible, and the combinatorial results of their effects remain elusive. Insight gained into the complex regulation of the Master Regulator CIITA will impact our understanding and ability to regulate adaptive immune responses through manipulation of MHC

II expression and will be applicable to additional proteins regulated by a complex post-translational modification code.

The work contained within this dissertation will establish roles of PTMs in PTM cross talk and will increase our understanding of how PTMs work together to regulate protein function. For a number of years, the field of transcription regulation has focused on investigating and identifying DNA elements necessary for the control of genes. As the field progressed, sights were turned to histones and the modifications to histones which allow for regulation of chromatin structure (Lee, Smith et al. 2010). Histone modifications are diverse and mediate different responses, thus a great deal of attention has been given to understanding this process. Over the course of these investigations, the term “Histone Code” has been coined in an effort to understand how the vast PTMs to histones elicit the changes the changes in chromatin structure (Lee, Smith et al. 2010). Investigations into histone modifications have led to the compelling idea that PTM “code” is involved in regulating various cellular processes and recent investigations have focused on PTMs to non-histone proteins. Examples include p53, which has been well studied for its PTMs, which include acetylation, ubiquitination, and phosphorylation and has been suggested to be regulated by a potential PTM “code” (Yang and Seto 2008). Additionally, Forkhead-box O 1 (FoxO1) is another transcription factor and has been studied pertaining to its PTMs. FoxO1 is acetylated leading to phosphorylation, which allows for its retention in the cytoplasm (Matsuzaki, Daitoku et al. 2005, Walter, Clynes et al. 2008, Yang and Seto 2008). This phosphorylation also allows for the interaction of FoxO1 with its ubiquitin E3 ligase (van der Horst and Burgering 2007). It is thought that this series of events ultimately leads to ubiquitination (van der Horst and Burgering 2007). Studies of PTMs indicate multiple

PTMs across a protein may act in concert and may have combinatorial consequences (Sisk, Nickerson et al. 2003). While it is clear there is cross talk between PTMs on non-histone proteins, much remains to be known of PTMs and how they regulate each other. The work in this dissertation not only identifies mechanisms and enzymes involved in regulating CIITA's PTM network, but it also moves the field towards a better understanding of how these events govern each other and contribute to the PTM "code" for CIITA.

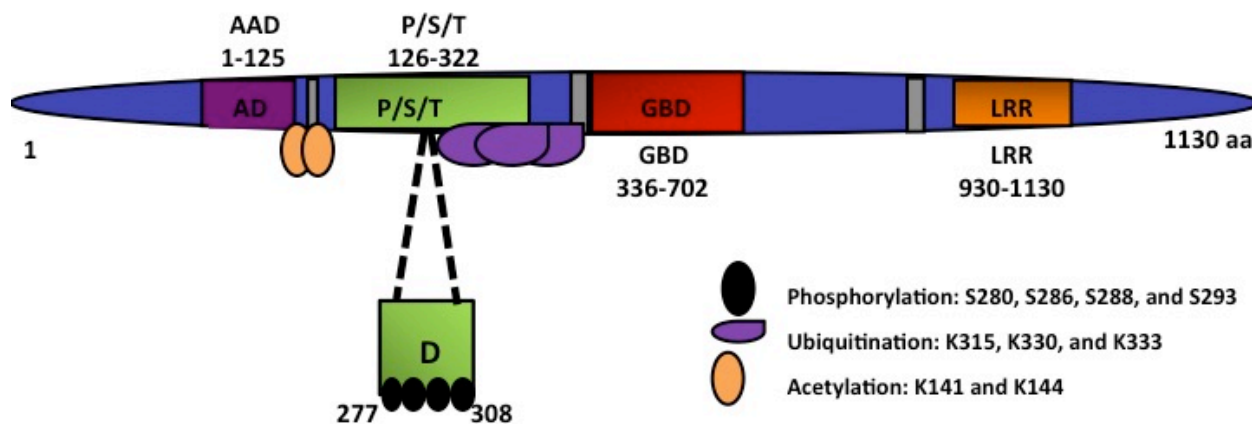


Figure 1.9: CIITA post-translational modifications:

CIITA isoform III is an 1130 amino acid protein, which contains four functional domains, many of which are modified by regulatory PTMs. CIITA is acetylated at lysine (K) 141 and 144 by histone acetyl transferase (HATs) including pCAF. Phosphorylation occurs within the degron sequence at serine (S280). Phosphorylation at S280 leads to mono-ubiquitination at lysine residues proximal to the degron sequence at K315, 330, and 333. Additional site of phosphorylation have previously been determined at S286, 288, and 293, which mediate suppression of CIITA transcription.

Table 1.1 Post-translation code of CIITA

Post-translational Modifications	Modification Sites	Modification Effects
Phosphorylation	S834, S1050	MHC II transcription inhibition
Phosphorylation	S286, S673, S942, S943, S944, S1128	Undetermined
Phosphorylation	S374, S375	Interaction with co-activator with p300 and oligomerization, and increased MHC I transcription
Phosphorylation	S373, S377	Inhibition of collagen transcription by interacting with co-repressor Sin3b
Phosphorylation	S286, S288, S293 S293	Inhibits MHC II transcription Nuclear transport Repression of CD36
Mono-Ubiquitination	K315, K330, K333	Increases MHC II transcription
Acetylation	K141, K144	Enhancement of nuclear localization of CIITA and increase in MHC II expression
Deacetylation	Undetermined	HDAC2 mediated deacetylation decreases CIITA stability and interaction with RFX5
Deacetylation	Undetermined	SIRT1 mediated deacetylated increases MHC II transcription

Note: This table is adapted from Wu X. et al., 2009

**2 CHAPTER II: The Class II Transactivator (CIITA) is regulated by
Posttranslational modification crosstalk between ERK1/2 phosphorylation, mono-
ubiquitination, and Lysine (K) 63 ubiquitination**

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2.1 ABSTRACT:

The class II transactivator (CIITA) is known as the master regulator for the Major Histocompatibility Class II (MHC II) molecules. CIITA is dynamically regulated through a series of intricate Posttranslational Modifications (PTMs). CIITA's role is to initiate transcription of MHC II genes, which are responsible for presenting extracellular antigen to CD4⁺ T cells. Here we identified ERK1/2 as the kinase responsible for phosphorylating the regulatory site, serine 280, which leads to increased levels of mono-ubiquitination and an overall increase in MHC II activity. Further, we identify that CIITA is also modified by lysine (K) 63 linked ubiquitination. K63 ubiquitinated CIITA is concentrated in the cytoplasm, and following activation of ERK1/2, CIITA phosphorylation occurs and K63 ubiquitinated CIITA translocates to the nucleus. CIITA ubiquitination and phosphorylation perfectly demonstrates how CIITA location and activity is regulated through PTM crosstalk. Identifying CIITA PTMs and understanding how they mediate CIITA regulation is necessary due to the critical role CIITA has in the initiation of the adaptive immune response.

2.2 INTRODUCTION:

Major Histocompatibility (MHC) class II molecules are cell surface glycoproteins which present extracellular antigenic peptides to CD4⁺T cells (Matheux and Villard 2004) in a process critical for activating adaptive immune responses (Glimcher and Kara 1992). MHC II expression is tightly regulated by transcriptional processes (Benoist and Mathis 1990), initiation of which require the class II transactivator (CIITA) to be recruited to the MHC II promoter

(Masternak, Muhlethaler-Mottet et al. 2000). CIITA is a non-DNA binding cofactor that binds to the components of an enhanceosome complex to initiate MHC II transcription (Masternak, Muhlethaler-Mottet et al. 2000). CIITA is therefore a master regulator of MHC class II transcription and is expressed from three separate promoters, pI, pIII, and pIV, each of which yields cell specific isoforms (Muhlethaler-Mottet, Otten et al. 1997). CIITA isoform I is primarily expressed in dendritic cells and macrophages, isoform III (IF3) is constitutively expressed in B cells and is also IFN- γ inducible (Piskurich, Linhoff et al. 1999, Piskurich, Gilbert et al. 2006). Isoform IV is expressed from all nucleated cells and is regulated through IFN- γ induction (Piskurich, Linhoff et al. 1999).

CIITA is tightly regulated through a series of various posttranslational modifications including acetylation, phosphorylation, and ubiquitination with IF3 in particular being heavily modified (Cressman, Chin et al. 1999, Spilianakis, Papamatheakis et al. 2000, Cressman, O'Connor et al. 2001, Sisk, Nickerson et al. 2003, Satoh, Toyota et al. 2004, Drozina, Kohoutek et al. 2006, Wu, Kong et al. 2009). Previous studies in our lab and others reveal phosphorylation to be an essential modification to direct CIITA nuclear localization, increased transactivation, and oligomerization (Cressman, Chin et al. 1999, Greer, Harton et al. 2004, Xu, Harton et al. 2008, Bhat, Truax et al. 2010). Several residues have been identified on CIITA IF3 as sites of phosphorylation, with serine (S) 280 in particular acting as a regulatory site. Once phosphorylated, mono-ubiquitination follows, leading to increased CIITA transactivity (Bhat, Truax et al. 2010), enhanced association with MHC II enhanceosome components, and increased MHC II transcription (Greer, Zika et al. 2003, Bhat, Truax et al. 2010). Additional phosphorylation at CIITA residues S286, 288, and 293 on IF3 by the kinase complex ERK1/2

leads to the down regulation of CIITA activity and overall nuclear export (Greer, Harton et al. 2004, Voong, Slater et al. 2008). CIITA IF3 poly-ubiquitination has been attributed to an overall decrease in CIITA activity due to degradation via the ubiquitin-proteasome pathway (Schnappauf, Hake et al. 2003). Table 1.1 summarizes the location and consequence of known PTMs CIITA IF3 and the enzymes involved.

Increasing evidence shows crosstalk between posttranslational modifications including phosphorylation and ubiquitination. These PTMs play roles in regulating protein location, interaction, and activity (Hunter 2007, Filtz, Vogel et al. 2014). Our previous observations of S280 phosphorylation regulating subsequent ubiquitination left the relationship between these two modifications and the enzymes involved unknown. Recent observations demonstrate CIITA IF1 has a similar regulatory phosphorylation site at S357, which resides within the CIITA degron and is phosphorylated by the kinase ERK1/2 (Drozina, Kohoutek et al. 2006). To gain better understanding of CIITA's posttranslational modification network and the intertwined roles of phosphorylation and ubiquitination in regulating CIITA activity, we investigated the effects of ERK1/2 phosphorylation at S280 and the impacts it would have on CIITA's ubiquitination landscape. We identified ERK1/2 as the kinase mediating phosphorylation of CIITA IF3 S280 leading to increased CIITA transactivation, increased levels of MHC II mRNA, and ultimately increased cell surface expression of MHC II. Moreover, we have linked ERK1/2 phosphorylation to increased CIITA mono-ubiquitination and to K63 poly-ubiquitination. These findings identified the kinase responsible for initiating the cascade of the posttranslational modifications and map the crosstalk between phosphorylation and ubiquitination that regulate this dynamic master regulator. We have identified a novel ubiquitin

K63 linkage on CIITA IF3 and further show that K63 ubiquitination is an important modification for changing the subcellular localization, and thus activity, of the MHC II master regulator.

2.3 MATERIALS AND METHODS:

Cell Culture: COS cells (monkey fibroblast) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco's modified Eagle (DMEM) medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 µg/ml of streptomycin and 2mM of L-glutamine. Cells were maintained at 37°C with 5% CO₂.

Plasmids: Flag-CIITA was described previously (Bhat, Truax et al. 2010). Myc-CIITA was kindly provided by Dr. Jenny Ting. Myc-S280A CIITA was generated using QuickChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specific primers with desired mutations were previously described (Bhat, Truax et al. 2010). Mutagenesis reactions were performed as per the manufacturer's protocol. Mutagenesis was confirmed by sequence analysis and expression analyzed by Western blot. Flag-ERK1 and Flag-ERK2 were kindly provided by Dr. Michael Weber (Vomastek, Iwanicki et al. 2008). HA-Mono ubiquitin (Ub.) and HA-poly Ub. were previously described (Bhat, Truax et al. 2010). The mono-ubiquitin construct contains lysine mutations to arginine; all seven internal lysines of ubiquitin are mutated (K6, 11, 27, 29, 33, 48, and, 63) thus inhibiting the formation of poly-ubiquitination. HA-K63 Ub. and HA-K48 Ub. were gifts from Dr. Ted Dawson (Addgene #17606 and #17605)(Lim, Chew et al. 2005), all other lysine residues within ubiquitin have been mutated to

arginine except K48 or K63 respectively, allowing only poly-ubiquitination to occur; ie where lysine residues form either K48 linked or K63 linked ubiquitination respectively. The *HLA-DRA* luciferase reporter construct was described previously (Bhat, Turner et al. 2008).

Co-Immunoprecipitations: COS cells were plated at a cell density of 8×10^5 cells/10 cm tissue culture plates. Cells were transfected with pCDNA, Myc-CIITA, Myc-S280A, Flag-ERK1, and Flag-ERK2 as indicated using GeneJuice (Merck Millipore, Darmstadt, Germany). 18h post transfection, cells were harvested and lysed with 1% NP40 supplemented with EDTA-free protease inhibitor (Roche) on ice. Cells were pre-cleared with mouse IgG (Sigma) and Protein G (Thermo Fisher) followed by immunoprecipitated with EZ view anti-c-Myc affinity gel beads (Sigma). Immune complexes were denatured and separated by SDS-PAGE gel electrophoresis. Gels were transferred and were individually immunoblotted with anti-Myc monoclonal antibodies (Abcam, Cambridge, MA). HRP conjugates were detected with HyGlo (Denville, Metuchen, NJ) to determine co-immunoprecipitation reactions. Protein content was normalized using the Nano Photometer P-class (Implen, Westlake Village, CA) for equal loading on non-immunoprecipitated whole cell lysates.

Transient transfections and Phosphorylation Assays: COS cells were plated at 5×10^4 cells/well density (70% confluency) in 6-well plates. Cells were transfected as indicated with either Flag-CIITA or Flag-S280A CIITA using GeneJuice according to manufacturer's instructions. 10 μ M of U0126 (Promega, Madison, WI) was added to indicated samples at time of transfections. 18h following transfections cells were harvested and lysed as previously described. Indicated samples were treated with λ -phosphatase (40 units/ μ L) lysed at 30°C for 1

hour. Lysates were normalized for protein concentration, and were separated on 5% SDS-PAGE gels by electrophoresis. Blots were individually immunoblotted with anti-Flag monoclonal antibodies (Sigma-Aldrich). HRP conjugates were detected with Denville Hyglo.

Transient Transfections and Luciferase Reporter Assays: COS cells were plated at 5×10^4 cells/well density (70% confluency). Following adhesion, cells were co-transfected as indicated with *HLA-DRA*, Renilla, pcDNA, Myc-CIITA, Flag-ERK1, and Flag-ERK2 using Genejuice according to the manufacturer's instructions. 24h following transfection, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison, WI) supplemented with complete EDTA-free protease inhibitors. Dual Luciferase assays were performed using the Lmax II³⁸⁴ (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Luciferase readings were normalized to Renilla readings for protein normalization.

RNA expression: COS cells were plated at a cell density of 8×10^5 cells/10 cm tissue culture plates and following adhesion, cells were transfected with pcDNA, Myc-CIITA, Myc-S280A CIITA, Flag-ERK1, and Flag-ERK2 as indicated using Genejuice. 18h following transfections, cells were harvested, washed with PBS, centrifuged at 1200 rpm at 4°C for 5 min, and 9/10th of the cell volume was used for RNA extraction. Total RNA was prepared with 1ml of QIAzol (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was reconstituted in 50µL of RNase-free water (Sigma) and was stored at -80°C. An Omniscript reverse transcription kit (Qiagen) was used to reverse transcribe 1µg of RNA into cDNA. MHC II specific anti-sense primers (Sigma) were used for reverse transcription reactions (RT) and PCR was performed using a Mastercycler thermal cycler (Eppendorf, Hauppauge, NY). Real-time PCR reactions were carried out on an ABI prism 7900 (Applied Biosystems, Foster City, CA)

using primers and probes for MHC II (Bhat, Turner et al. 2008, Koues, Dudley et al. 2008) and GAPDH (Medhurst, Harrison et al. 2000). GAPDH RNA was used to normalize mRNA values. Presented values from real-time PCR reactions were calculated from generated standard curves from each gene in triplicate reactions and were analyzed via the SDS 2.0 program. $1/10^{\text{th}}$ of the cell volume was lysed in 100 μL of 1% NP40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and the total protein concentration was determined using the Nano Photometer P-class (Implen). Western blots confirmed equal expression of expressed plasmids.

Flow Cytometry: COS cells were plated at a cell density of 8×10^5 cells/10 cm tissue culture plates. Following cell adhesion, cells were transfected as indicated. 72h following transfection, cells were trypsinized and washed with cold PBS. Following the wash, $9/10^{\text{th}}$ of the cell volume was resuspended in incubation buffer (0.5% bovine serum albumin in PBS w/v) and 10 μg of Phycoerythrin (PE)-labeled anti-human *HLA-DR* (clone L243, Biolegend, San Diego, CA) antibody or PE mouse IgG2a κ isotype control antibody (Biolegend) was added and the cell suspension was rotated at 4°C. Following antibody incubation, cells were washed twice with PBS, fixed with 2% paraformaldehyde, and were stored at 4°C. MHC class II cell surface expression was measured by LSR Fortessa (BD) and analyzed using FlowJo. All samples were analyzed using 10,000 events per sample. $1/10^{\text{th}}$ of the cell volume harvested for flow analysis was lysed in 1% NP40 buffer containing complete EDTA-free protease inhibitors (Roche) on ice. Proteins were denatured and separated by SDS-PAGE gel electrophoresis. Gels were transferred and individually immunoblotted with anti-Myc antibody (Abcam) or anti-Flag antibody (Sigma). HRP conjugates were detected with HyGlo (Denville) to determine

expression of CIITA, S280A CIITA, or ERK1/2 as indicated. Proteins were normalized using the Nano Photometer P-class (Implen) to determine equal loading in lysates.

Half-life Assays: COS cells were plated at a cell density of 8×10^5 in 10 cm tissue culture plates. Following adhesion, cells were transfected as indicated. 24h following transfections, cells were treated with 100 μ M cycloheximide for 0-8h. Following cycloheximide treatment, cells were harvested and lysed as previously described. Lysates were normalized for protein concentration. As controls, COS cells were transfected as indicated and treated with 100 μ M of cycloheximide and MG132 (EMD Biosciences, San Diego, CA) for 8h. Proteins were denatured and separated by SDS-PAGE gel electrophoresis. Blots were individually immunoblotted. Proteins were normalized using the Nano Photometer P-class (Implen) and equal loading was determined.

Ubiquitination Assays: COS cells were plated at a cell density of 8×10^5 in 10cm tissue culture plates. Following adhesion, cells were transfected with Myc-CIITA, Flag-ERK1, Flag-ERK2, Myc Control (empty plasmid), HA-Mono Ub., HA-Poly Ub., HA-K63 Ub., and HA-K48 Ub. as indicated (*Fig 4A*). Following transfection, cells were harvested and were lysed as previously described. Lysates were normalized for protein concentration. Cells were precleared with Protein G (Thermo) and Mouse IgG agarose beads (Sigma Aldrich), and were immunoprecipitated with EZ view Red anti-Myc agarose beads (Sigma Aldrich). Immune complexes were denatured and separated by SDS-PAGE gel electrophoresis. Immunoblot gels were transferred to PVDF membranes and were individually immunoblotted with anti-Ub. antibody (Life Sensors, Malvern, PA). Lysate gels were transferred to nitrocellulose and were

individually immunoblotted with anti-Myc (Abcam) and with anti-Flag (Sigma) antibodies. Proteins were normalized and equal loading was determined in lysates. (*Fig 4C*) 10 μ M U0126 was added as indicated at time of transfections. 24h following transfections, 10% FBS media was removed from indicated samples and was washed with 1X PBS, and 1% FBS was added for 4 hours. 100 μ M of MG132 was added 4h prior to harvest to indicated samples. 200nM of Phorbol 12-Myristate 13-Acetate (PMA) (Sigma) was added for 30min to indicated samples. Following PMA treatment, cells were harvested and lysed as above. Samples were immunoprecipitated with EZ view Red anti-Myc agarose beads (Sigma). Immune complexes were denatured and separated by SDS-PAGE gel electrophoresis. Immunoblots were transferred to PVDF membranes and were individually immunoblotted with anti-Ub. antibody (Life Sensors). Lysate gels were blotted with anti-Myc (Abcam), anti-P42/44 ERK, or (PhosphoSolutions, Aurora, CO), anti-actin (Cell Signaling, Danvers, MA). HRP conjugates were detected as previously described. Proteins were normalized and equal loading was determined in lysates. (*Fig, 4E*) 24h following transfections, cells were harvested and were lysed as previously described. Lysates were normalized for protein concentration, precleared with Protein G (Thermo), and Mouse IgG (Sigma), and were immunoprecipitated with EZ view Red anti-Myc agarose beads (Sigma). Immune complexes were denatured and separated as previously described. Blots were immunoblotted with anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) anti-Myc (Abcam), or anti-Flag (Sigma) antibodies. HRP conjugates were detected as previously described. Proteins were normalized and equal loading was determined in the lysates.

Cell Fractionation assays: COS cells were plated at a cell density of 8×10^5 in 10cm tissue culture plates. Following adhesion, cells were transfected with Myc-CIITA and HA-K63 Ub. as indicated. 24h following transfection, 10% FBS media was removed, cells washed with PBS, and 1% FBS DMEM media was replaced for 6h serum starvation where indicated. 30 min prior to harvest, cells were treated with 200nM of PMA (Sigma). Following treatments, cell fractionation was completed with ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany), following manufacturer's protocol. Following fractionation, proteins were normalized and immunoprecipitated with EZ view Red anti-Myc agarose beads (Sigma Aldrich). Immune complexes were denatured and separated as previously described. Blots were individually immunoblotted with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Lysate and fractionation controls were immunoblotted with anti-Myc (Abcam), anti-HSP 90, anti-H3 (Cell Signaling Technology, Danvers, Massachusetts), anti-p44/42 MAPK (ERK1/2) (Thermo Scientific), and anti-ERK1/2 rabbit polyclonal antibody (Proteintech, Chicago, IL).

Immunofluorescence Confocal Imaging: COS cells were plated at a density of 5×10^4 in 6-well plates with glass coverslips. 18h following adhesion, cells were transfected with Myc-CIITA and HA-K63 Ub. 24h following transfections, 10% FBS media was removed and washed with PBS and 1% FBS media was replaced where indicated for 6h. 30min prior to fixation, 200nM of PMA was added to 1% serum media to activate ERK1/2. All media was then removed and washed with PBS. To fix the cells on coverslips, ice-cold methanol was added and incubated in the -20° for 10min. Methanol was aspirated and 500 μ L of 2% BSA-PBS was added. To stain the cover slips, anti-CIITA (Santa Cruz Biotechnology), anti-K63 Ub. (Abcam),

anti-p44/42 MAPK (Thermo Scientific), or anti-ERK1/2 (Proteintech) was added to indicated samples for 1h. Following staining, antibody was removed and coverslips were washed 5x with PBS. Goat anti-rabbit Alexa 594 and goat anti-mouse Alexa 488 (Invitrogen) were added for 1h in the dark. Following secondary antibody incubation, antibody was removed and samples were washed 5X with PBS. DAPI (1:1000 in PBS) (Life Technologies) was added to coverslips and incubated for 10min in the dark. DAPI was removed and coverslips were washed 5X with PBS. Coverslips were mounted with ProLong Gold Antifade (Life Technologies). Samples were allowed to dry overnight in 4°. Images were obtained using the LSM 700 Confocal Microscope (Zeiss) using 40X magnification.

2.4 RESULTS:

The kinase complex ERK1/2 associates with CIITA IF3 and phosphorylates CIITA within its degron sequence- CIITA is a large multidimensional protein that is tightly regulated through a series of posttranslational modifications (Spilianakis, Papamatheakis et al. 2000, Cressman, O'Connor et al. 2001, Li, Harton et al. 2001, Drozina, Kohoutek et al. 2006, Voong, Slater et al. 2008, Wu, Kong et al. 2009, Bhat, Truax et al. 2010). Previously, we identified a regulatory phosphorylation site, S280 that lies within the degron sequence (Bhat, Truax et al. 2010). Phosphorylation at S280 leads to subsequent mono-ubiquitination on three degron proximal lysine residues, leading to increased CIITA transactivity (Bhat, Truax et al. 2010). CIITA isoform I (IF1) contains a homologous serine residue (S357) that lies within a degron sequence phosphorylated by ERK1/2 (Drozina, Kohoutek et al. 2006). We sought to determine if CIITA IF3 S280, located within the degron sequence, was similarly targeted for phosphorylation by ERK1/2. First, to determine association of CIITA and the kinase complex; a co-immunoprecipitation (co-IP) assay was performed. Expression of WT-CIITA with ERK1/2 in COS-7 cells, followed by co-IP, demonstrates CIITA association with the individual components of the kinase complex, ERK1, ERK2, or with the ERK1/2 complex (**Fig. 2.1A**).

The phosphorylated form of CIITA migrates as a doublet at 145kDa and 149kDa (Greer, Harton et al. 2004, Voong, Slater et al. 2008). The upper band is indicative of the phosphorylated form (**Fig. 2.1B i and ii first lanes**) (Tosi, Jabrane-Ferrat et al. 2002, Sisk, Nickerson et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008). WT-CIITA treated with λ phosphatase shows removal of phosphate groups, leaving the lower unphosphorylated

form (**Fig. 2.1B i, second lane**). WT-CIITA treated with U0126, a selective MEK inhibitor, is also void of the higher molecular weight band, indicating ERK1/2 contributes to CIITA phosphorylation (**Fig. 2.1B ii, second lane**). The serine to alanine mutant of CIITA, S280A, does not show the doublet pattern (**Fig. 2.1B iii and iv, first lanes**), when CIITA S280A is treated with either λ phosphatase or with U0126; CIITA S280A is also less stable as indicated by the decrease in protein expression (**Fig. 2.1B iii and iv, second lanes**). The instability of the S280A CIITA upon treatment with either U0126 or λ phosphatase we believe is due, in part, to depletion of phosphate residues on other ERK1/2 mediated phosphorylation sites (S286, S288, and S293) (Voong, Slater et al. 2008). While ERK1/2 phosphorylation at these sites is known to suppress CIITA, it remains unknown if S280 phosphorylation is required in order for additional sites to be phosphorylated. When all ERK1/2 phosphorylation is blocked, it is likely S280A CIITA is a nonviable and unstable protein.

ERK1/2 phosphorylation leads to increased CIITA IF3 activity and to increased MHC class II expression-The phosphorylation regulation site S280 controls subsequent mono-ubiquitination that is required for increased CIITA transactivity and for increased MHC class II transcription (Bhat, Truax et al. 2010). When CIITA S280 is mutated from serine to alanine, CIITA's activity decreases, leading to an overall decrease in the levels of MHC class II expression (Bhat, Truax et al. 2010). Our observations in Fig. 1 indicate CIITA is phosphorylated at residue S280 by the kinase complex, ERK1/2. To determine if the association between CIITA and ERK1/2 drives CIITA transactivation, and to determine any dependence of this interaction on CIITA S280, we performed luciferase reporter assays. WT-CIITA co-transfected with ERK1/2 leads to a two fold increase in CIITA transactivity and ability to drive

MHC class II transcription; conversely, the CIITA S280A mutant has a decreased ability to drive MHC class II transcription. When the CIITA S280A mutant was co-transfected with ERK1/2, CIITA transactivity levels further decrease (**Fig. 2.2A**). To further identify the impact of ERK1/2, we next addressed the impact of ERK1/2 on CIITA and the S280 mutant by measuring MHC class II mRNA levels in COS cells transfected with WT-CIITA or with S280A CIITA +/- ERK1/2 (**Fig. 2.2B**). In cells transfected with ERK1/2, MHC class II mRNA levels were significantly increased, as compared to cells transfected with WT-CIITA alone. Cells transfected with CIITA S280A indicated decreased levels of MHC class II mRNA as compared to cells transfected with WT-CIITA and the addition of ERK1/2 did not enhance levels of MHC class II mRNA (**Fig. 2.2B**).

Phosphorylation at S280 by ERK1/2 enhances MHC Class II cell surface expression- MHC II molecules are responsible for recognition of extracellular antigens (Matheux and Villard 2004). Prior to MHC class II surface expression CIITA is modified through various posttranslational modifications (Spilianakis, Papamatheakis et al. 2000, Greer, Zika et al. 2003, Sisk, Nickerson et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008, Wu, Kong et al. 2009, Bhat, Truax et al. 2010). To further investigate the role of phosphorylation by ERK1/2 on CIITA activity, we addressed the impact of ERK1/2 on MHC class II cell surface expression. Un-modified CIITA had low transactivity and lead to basal levels of MHC class II surface expression of 16.38% (**Fig. 2.2C i**); WT-CIITA overexpressed with ERK1/2 enhanced MHC class II surface expression to 40.22% (**Fig. 2.2C ii**); transfection of the CIITA-S280A mutant in the presence, and absence, of ERK1/2 decreased the expression of surface expression of MHC class II levels to 6.2% and 6.84%, respectively (**Fig. 2.2C iii, iv**).

Table 2.1.1: CIITA IF3 phosphorylation and ubiquitination modifications

Posttranslational Modification	Modification Site	Enzyme Mediating Modification	Consequence(s)
Phosphorylation	S280	ERK1/2	Regulates subsequent mono-ubiquitination
Phosphorylation	S286, S288, S293	ERK1/2	Inhibits MHC II transcription and regulates nuclear export
Mono-ubiquitination	K315, K330, K333	Undetermined	Increases CIITA activation and overall MHC II expression
K63 linked ubiquitination	Undetermined	Undetermined	Involved in PTM crosstalk with ERK1/2 to mediate movement of CIITA from the cytoplasm to nucleus

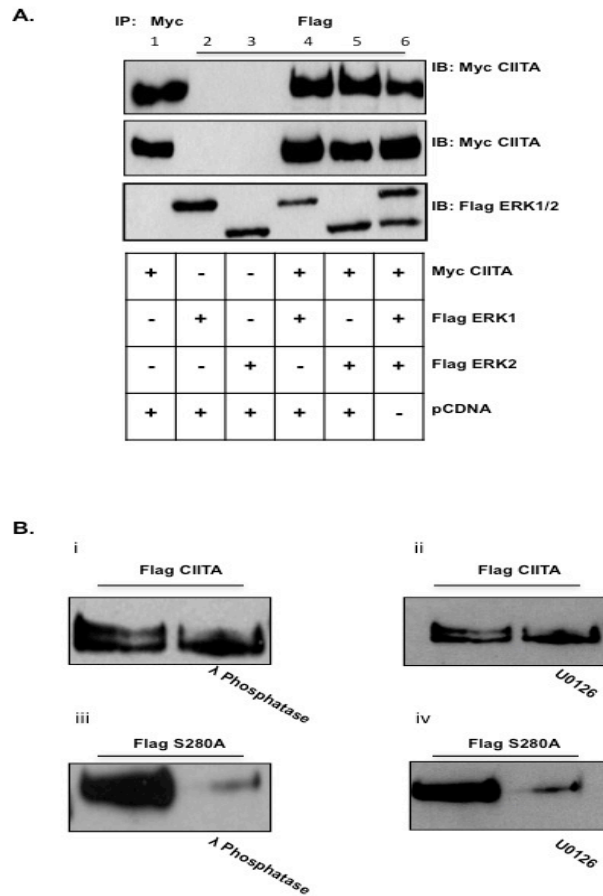


Figure 2.1: Kinase Complex ERK 1/2 associates with CIITA and phosphorylates S280

(A) CIITA IF3 and kinase complex ERK1/2 associate. *Co-immunoprecipitation of CIITA IF3 and ERK1/2*, COS cells were co-transfected with Myc-WT-CIITA and Flag-ERK1 or Flag-ERK2 or a combination of both. Cells were harvested, lysed, precleared and immunoprecipitated (*IP'd*) with anti-Myc or anti-Flag antibody as indicated. Western blots were performed and *IP'd* samples were immunoblotted (*IB*) using anti-Myc antibodies. Lysate controls demonstrate expression of Myc-CIITA and Flag-ERK1/2. Data shown are cropped images from one *IP* gel and one lysate gel and are representative of 3 individual experiments. (B) Phosphorylated CIITA is represented by a doublet at 145 kDa and 149 kDa (*first lane in i and ii*). Wild type CIITA IF3 treated with λ -phosphatase or U0126 (specific MEK inhibitor) loses the upper band (*second lane in i and ii*) indicating loss of phosphorylation. CIITA S280A does not migrate as a doublet (*first lane in iii and iv*). The CIITA S280A mutant treated with λ phosphatase or U0126 indicates loss of protein (*second lane in iii and iv*).

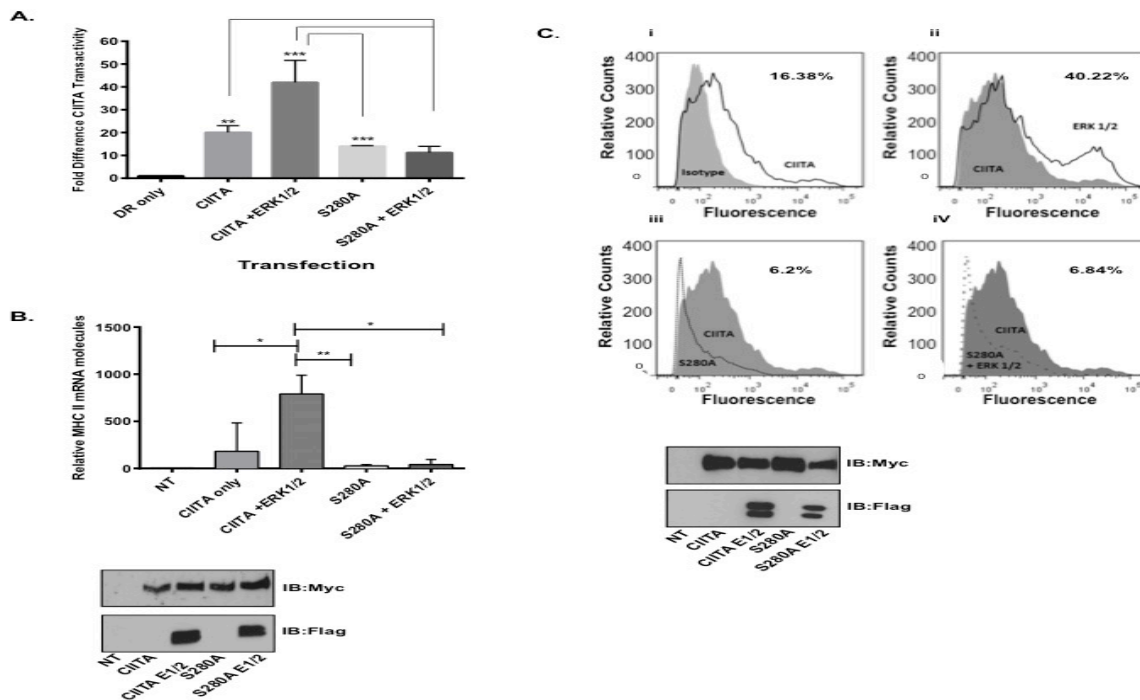


Figure 2.2: ERK 1/2 expression increases CIITA transactivity leading to increased MHC II mRNA and surface expression.

(A) CIITA transactivation increases in the presence of expressed ERK1/2. *Reporter Assay*. COS cells were transfected as indicated with MHC II *HLA-DR-Luc* reporter construct, Renilla, CIITA, CIITA S280A, and ERK 1/2. Luciferase assays were performed in triplicate in three independent experiments, data are presented as fold increase in luciferase activity. Results are standardized to Renilla values and represent the mean \pm S.D. * < 0.05 . (B) Expression of MHC II mRNA is enhanced in the presence of expressed WT CIITA and ERK 1/2. *mRNA Assay*. COS cells were transfected as indicated with Myc-CIITA, Myc-CIITA S280A, Flag-ERK1/2. Cells were harvested, RNA extracted, and cDNA was prepared and quantified by real-time PCR. Levels of MHC II mRNA were normalized to GAPDH mRNA. Data presented are results of three independent experiments and represent \pm SD. * < 0.05 , ** < 0.005 . Western blots indicate equal transfection and expression of Myc-CIITA and Flag-ERK1/2. (C) Expression of CIITA and ERK1/2 leads to an increase in cell surface expression of MHC II. *Flow cytometry*. COS cells were transfected with Myc-CIITA, Myc-CIITA S280A, and Flag-ERK1/2. Seventy-two hours following transfection, cells were trypsinized, washed, and incubated with PE-labeled anti-human HLA-DR antibody. Following incubation, cells were fixed and PE cell surface staining was measured by LSR Fortessa. (i) COS cells were transfected with Myc-CIITA (dark grey line), and COS cells were stained for isotype control (grey shaded). (ii) COS cells were transfected with Myc-CIITA (grey shaded) and was used to compare with Myc-CIITA and Flag-ERK1/2 (grey line). (iii) COS cells were transfected with Myc-CIITA (grey shaded) and were compared to cells transfected with CIITA S280A (grey dotted line). (iv) COS cells were transfected with Myc-CIITA (grey shaded) and compared to cells transfected with Myc-CIITA S280A and Flag-ERK1/2 (grey dotted line). Western blots indicate equal transfection and expression of Myc-CIITA, Myc-CIITA S280A, and Flag-ERK1/2. Results shown are representative of three separate experiments.

WT-CIITA half-life is increased when phosphorylated by ERK1/2- CIITA is constitutively expressed at basal levels in B cells and has a half-life of 2-4 hours (Bhat, Truax et al. 2010). During an active immune response, CIITA production is quickly activated to drive increased MHC class II to allow B cells to present antigen to prospective CD4⁺ T cells (Holling, Schooten et al. 2004). Increased requirement for MHC class II, and therefore for CIITA, requires various posttranslational modifications to CIITA (Wu, Kong et al. 2009). ERK1/2 phosphorylates CIITA at S280; this regulatory site increases the half-life of CIITA to 6-8 hours and allows a stabilized CIITA to bind the enhanceosome complex and drive the production of MHC class II (*Fig. 2.3*).

ERK1/2 phosphorylation of CIITA IF3 leads to an increase in global ubiquitination levels-We previously demonstrated levels of mono-ubiquitination are decreased when S280 is mutated to S280A (Bhat, Truax et al. 2010). S280 lies within the CIITA degron sequence, which is necessary for CIITA's triple mono-ubiquitination, leading to increased CIITA transactivity (Bhat, Truax et al. 2010). CIITA IF1 has a similar regulatory phosphorylation site, S357, which is phosphorylated by ERK1/2 (Drozina, Kohoutek et al. 2006). Our data suggest S280 is also phosphorylated by ERK1/2. To determine if overexpression of ERK1/2 effects levels of global (all forms) ubiquitination, we conducted a co-immunoprecipitation experiment. In the absence of expressed ERK1/2, CIITA exhibits very low levels of global ubiquitination (*Fig. 2.4A, lane 1*) compared to lane 2, where cells are treated with a proteasome inhibitor and CIITA exhibits maximum global ubiquitination. Alternatively, the addition of ERK1/2 results in a significant impact on CIITA global ubiquitination (*Fig. 2.4A, lane 4*).

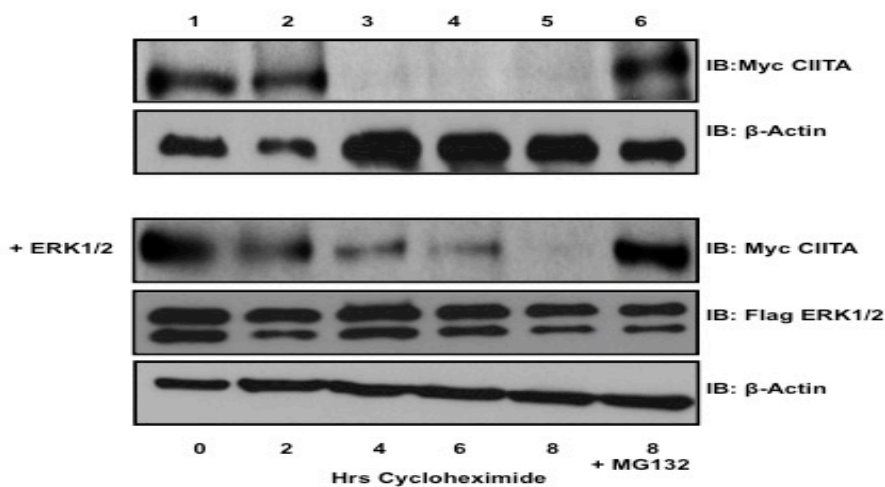


Figure 2.3: ERK1/2 expression stabilizes CIITA half-life

Half-life assays. COS cells were transfected with Myc-CIITA and Flag-ERK1/2 as shown. Following transfections, cells were treated with cycloheximide for 0-8 hrs. Following cycloheximide treatment, cells were harvested, and Western blot analysis was performed to determine the half-life of transfected Myc-CIITA (*top panel*). To determine effect of ERK1/2 expression on CIITA half-life, COS cells were transfected with Myc-CIITA and Flag-ERK1/2 (*lower panel*). As transfection and degradation controls, COS cells were transfected with Myc-CIITA, and/or Flag-ERK1/2 and were treated with cycloheximide and with the proteasome inhibitor MG132 for 8hrs (*last lane*). Experiment shown is representative of three experimental repeats.

Inhibiting endogenous ERK1/2 leads to a decrease in CIITA IF3 global ubiquitination levels-To investigate levels of CIITA ubiquitination when endogenous ERK1/2 is inhibited, COS-7 cells were transfected with Myc-CIITA. In cells incubated with 10% serum media and treated with PMA 18 hours post transfection, CIITA ubiquitination is very low (*Fig. 2.4C, lane 1*) compared to cells serum starved with 1% serum media for 6 hours followed by 30 minutes of PMA stimulation to activate ERK1/2 (*Fig. 2.4C, lane 3*). When treated with MEK inhibitor U0126, ubiquitination levels decrease (*Fig. 2.4C lanes 2 and 4*). Cells serum starved for 6 hours followed by 30 minute PMA incubation, demonstrate significant increases in CIITA ubiquitination levels (*Fig. 2.4C lane 5*).

ERK1/2 leads to increased levels of CIITA IF3 Mono-ubiquitination-The S280 regulatory phosphorylation site on CIITA is necessary for subsequent triple mono-ubiquitination events leading to increased CIITA transactivation (Bhat, Truax et al. 2010). To determine if expression of ERK1/2 alters the levels of CIITA mono- or poly-ubiquitination, *in vivo* ubiquitination assays were performed. Expression of CIITA with HA-mono-ubiquitin and Flag-ERK1/2 significantly increase CIITA mono-ubiquitination levels (*Fig. 2.4E, compare lanes 2 and 4; quantified in Fig. 2.4F*). To determine if ERK1/2 alters CIITA poly-ubiquitination, cells were co-transfected as indicated with Myc-CIITA, HA-poly-ubiquitin and Flag-ERK1/2; only a small increase in CIITA poly-ubiquitination was observed over cells in which Flag-ERK1/2 was not expressed (*Fig. 2.4E, compare lanes 5 and 6; quantified in Fig. 4F*). Western blots demonstrate stable expression of Myc-CIITA even upon poly-ubiquitination, indicating the poly-ubiquitin linkage is not exclusively K48 linked ubiquitin.

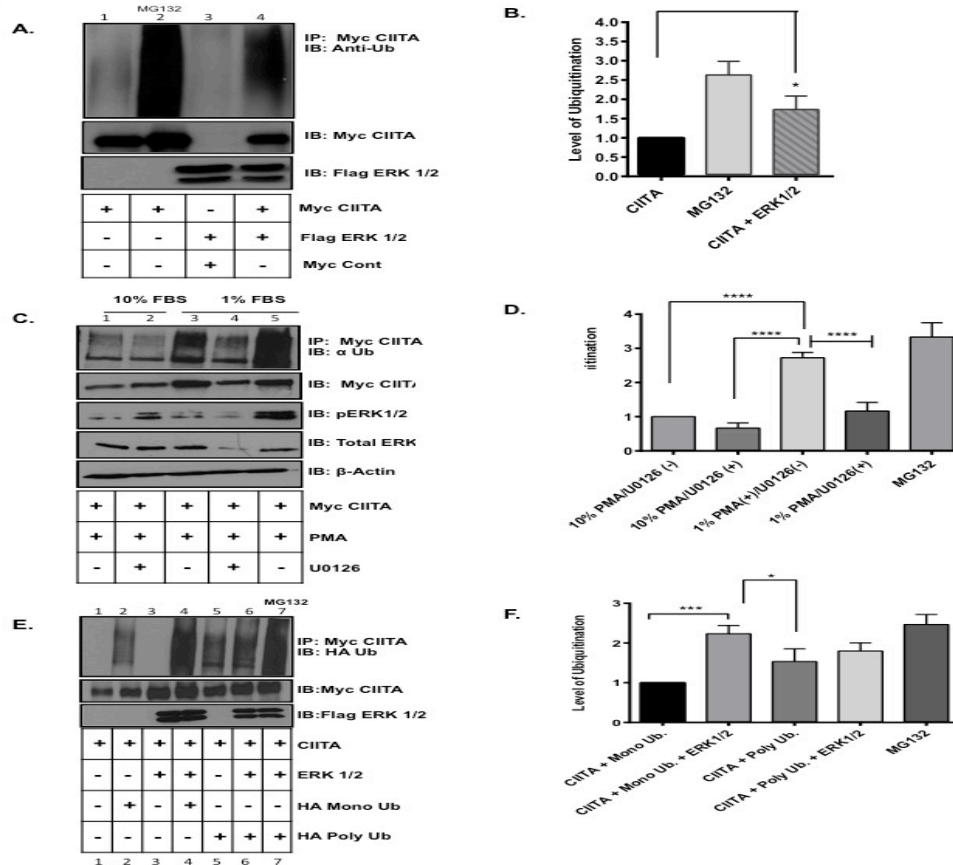


Figure 2.4: CIITA global ubiquitination and mono-ubiquitination is enhanced when ERK1/2 are overexpressed, and inhibiting endogenous ERK1/2 leads to decreases in global CIITA ubiquitination levels.

In vivo ubiquitination assay. (A) COS cells were transfected with Myc-CIITA, and Flag-ERK1/2. Lysate controls (*bottom two panels*) demonstrate expression of Myc-CIITA and Flag-ERK1/2. Data shown are cropped images from one IP gel and one lysate gel and are representative of three experiments. (B) Densitometry and quantification of data in fig. 4A. Densitometry was performed on three independent experiments, \pm SD, $* < 0.05$. (C) COS cells were transfected with Myc-CIITA and indicated samples were treated with U0126 (MEK specific inhibitor) at time of transfections. Eighteen hours following transfections, 10% FBS media was replaced with 1% FBS media for six hours and PMA and MG132 were added as indicated. *Co-immunoprecipitation and ubiquitination analysis.* Following all treatments, cells were harvested, lysed, pre-cleared, and IP'd with anti-Myc antibodies. Lysate controls (*Bottom four panels*) demonstrate expression of Myc-CIITA, total ERK1/2, phosphorylated ERK1/2, and actin as controls. Data shown are cropped images from one IP gel and one lysate gel. (D) Densitometry and quantification of data in fig. 4C. Densitometry was performed on three independent experiments, \pm SD, $**** < 0.0001$. (E) COS cells were transfected with Myc-CIITA, Flag-ERK1/2, HA-Mono Ubiquitin, or HA-Poly Ubiquitin. MG132 was added to control sample for four hours. Lysate controls (*Bottom two panels*) demonstrate expression of Myc-CIITA and Flag-ERK1/2. Data shown are cropped images from one IP gel and one lysate gel. (F) Densitometry and quantification of data in fig. 4E. Densitometry was performed on three independent experiments, \pm SD $*** < 0.001$.

K63 CIITA IF3 ubiquitination is increased when ERK1/2 is overexpressed- Protein degradation is often signaled via the addition of K48 linked ubiquitination onto the targeted protein (Ciechanover 1998). Our data suggests (*Fig. 2.4E*) that CIITA poly-ubiquitination is increased in the presence of expressed ERK1/2, but no change in CIITA expression indicates the ubiquitin linkage is likely not driving CIITA degradation. K63 ubiquitination has recently been implicated in transcription factor regulation (Ishitani, Takaesu et al. 2003, Balkhi, Fitzgerald et al. 2008, Lauwers, Jacob et al. 2009, Emmerich, Ordureau et al. 2013). As we and others have previously shown CIITA to be phosphorylated and ubiquitinated, we next performed ubiquitination assays to determine if the ubiquitination present on CIITA in the presence of expressed ERK1/2 is an alternative linkage to K48. In the presence of expressed ERK1/2, levels of CIITA K63 ubiquitination increased significantly as compared to cells transfected with CIITA and HA-K63 ubiquitin alone (*Fig. 2.5A, compare lanes 1 and 2 and quantification in Fig. 2.5B*). As ERK1/2 also phosphorylates CIITA at additional downstream sites that ultimately lead to the down-regulation in CIITA activity and to CIITA degradation (Greer, Harton et al. 2004, Voong, Slater et al. 2008), we also investigated if ERK1/2 would impact levels of K48 linked ubiquitination. CIITA K48 linked ubiquitination was not significantly increased in the presence of ERK1/2 over CIITA cells expressing CIITA HA-K48 alone (*Fig. 2.5A, compare lanes 3 and 4 and quantified in Fig. 2.5B*).

K63 ubiquitinated CIITA is cytoplasmic; ERK1/2 activation drives CIITA nuclear mobilization- Data from *Fig. 5A*, shows an overall increase in K63 ubiquitination in presence of overexpressed ERK1/2. To better understand the role for K63 CIITA ubiquitination in the presence of activated ERK1/2, we conducted a cellular fractionation assay followed by co-

immunoprecipitation. We co-transfected COS-7 cells with Myc-CIITA and HA-K63, followed by serum starvation and PMA treatment to activate the ERK1/2 kinase complex. Prior to ERK1/2 activation (without serum starvation and PMA), CIITA was highly modified by K63 ubiquitin in the cytoplasmic fraction (*Fig. 2.6A, cytoplasmic fraction, lane 1*) while little K63 modified CIITA is observed in the nuclear fraction (*Fig. 2.6A, nuclear fraction, lane 1*). When cells were serum starved and treated with 200nM of PMA, CIITA K63 ubiquitin levels were significantly decreased in the cytoplasmic fraction (*Fig. 2.6A, cytoplasmic fraction, compare lanes 1 and 2*), but significantly increased in the nuclear fraction (*Fig. 2.6A, nuclear fraction, compare lanes 1 and 2*) as shown in the densitometry analysis (*Fig. 2.6C*). Equal loading, transfection, and cellular fractionation are shown by the lysate controls (*Fig. 2.6B*). To further investigate the changes in K63 ubiquitination observed in the cellular fractionation assay, we performed confocal microscopy. Cells were treated with 10% serum media where ERK1/2 is not activated (*Fig. 2.7G-I*). As with the fractionation assay, when 10% serum media is removed and replaced with 1% serum media and treated with PMA, ERK1/2 is phosphorylated and therefore, activated (*Fig. 2.7J-L*). CIITA was primarily located in the cytoplasm prior to ERK1/2 activation (*Fig. 2.7A-C*). Following serum starvation for 6 hours and treatment with PMA for 30 minutes, ERK1/2 was activated and thus able to phosphorylate CIITA. Following this event, CIITA was primarily located in the nucleus (*Fig. 2.7D-F*). Previous data (*Figs. 2.5A and 2.6A*), demonstrate CIITA is modified via K63 ubiquitination. In the presence of ERK1/2 activation (serum starvation and PMA treatment), CIITA and K63 ubiquitin co-localize to the nucleus (*Fig. 2.7Q-T*) as compared to in-activated ERK1/2 (10% serum and no PMA) and CIITA and K63 ubiquitin both appear in the cytoplasm (*Fig. 2.7M-P*). The merge of nuclear

staining shows CIITA and K63 ubiquitin co-localize, mirroring the results seen in the fractionation assay.

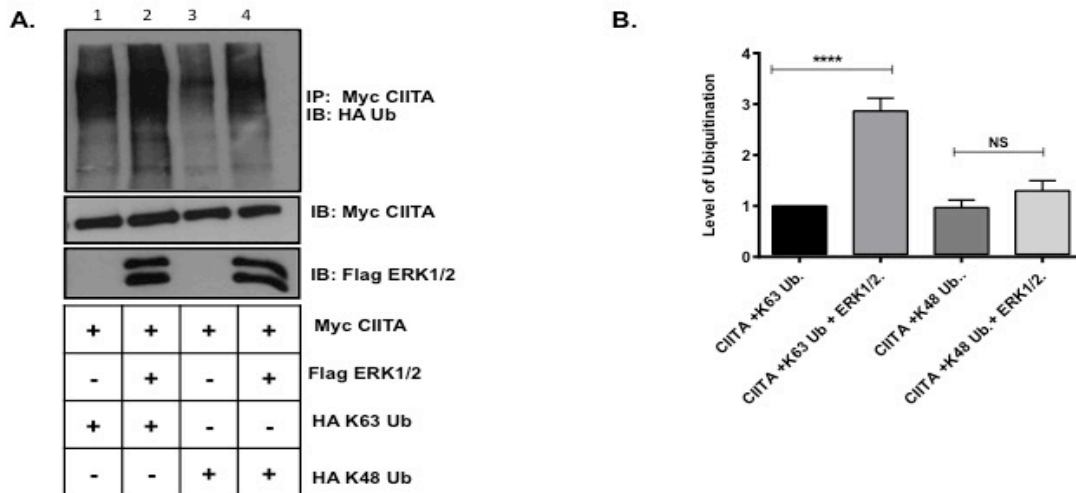


Figure 2.5: K63 linked ubiquitination increases on CIITA in presence of overexpressed ERK1/2.

(A) COS cells were transfected with Myc-CIITA, Flag-ERK1/2, HA-K63 ubiquitin, and HA-K48 ubiquitin. Lysate controls (*Bottom two panels*) demonstrate expression of Myc-CIITA and Flag-ERK1/2. Data shown are cropped images from one IP gel and one lysate gel. (B) Densitometry and quantification of data in fig. 5A, Densitometry was performed on three independent experiments, \pm SD **** < 0.0001 .

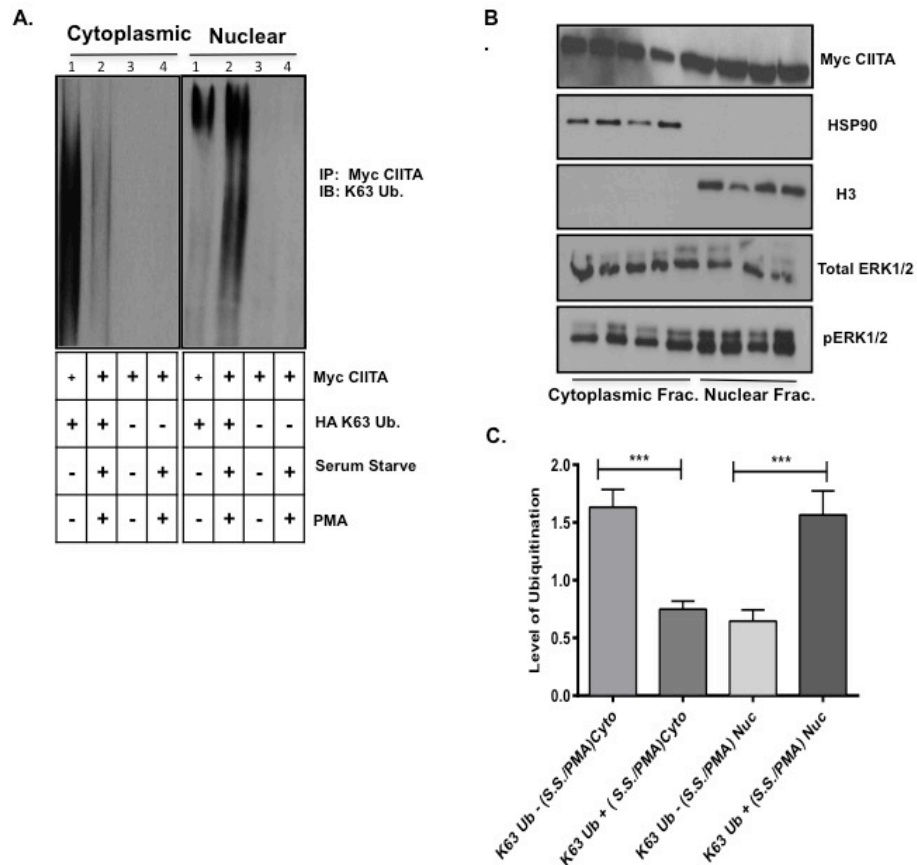


Figure 2.6: Activation of ERK1/2 leads to movement of K63 linked ubiquitinated CIITA from the cytoplasm to the nucleus.

Cell Fractionation and Immunoprecipitation. (A) COS cells were transfected with WT Myc-CIITA and HA-K63 ubiquitin where indicated. Eighteen hours following transfections, media containing 10% serum was removed where indicated for six hours. Thirty minutes prior to harvest, 200nM of PMA was added as indicated. Media was removed from cells, washed, and cells were fractionated and samples were normalized for protein concentration. All samples were then Immunoprecipitated (*IP'd*) with anti-Myc antibody. Data shown are cropped images from one IP gel. (B) Western blots demonstrate equal expression of Myc-CIITA, total ERK1/2, phosphorylated ERK1/2, HSP90 (cytoplasmic fraction) and H3 (nuclear fraction). (C) Densitometry and quantification of data in fig. 6A, densitometry was performed on three independent experiments, \pm SD *** <0.001.

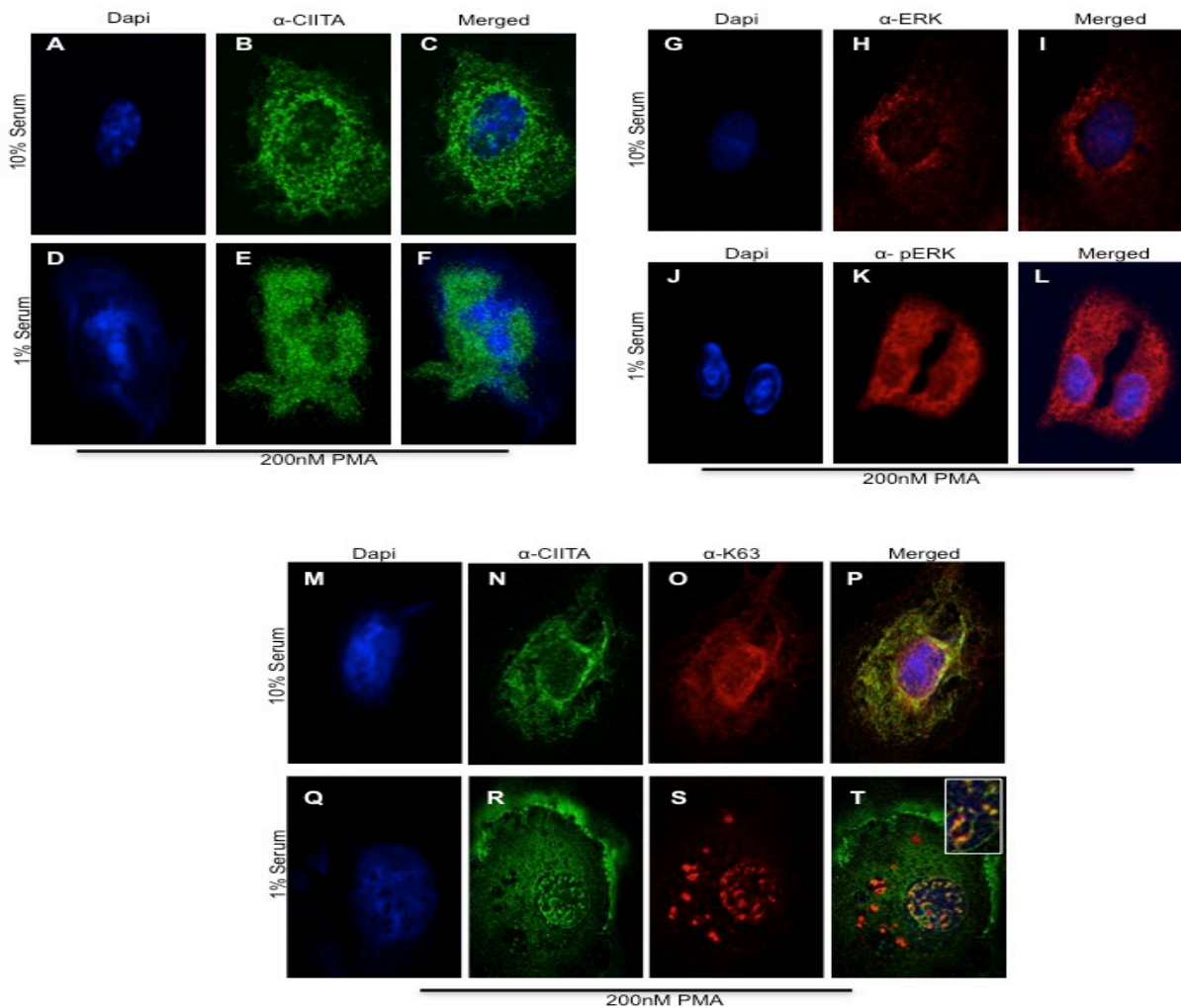


Figure 2.7: K63 ubiquitin co-localizes with CIITA in cytoplasm and upon ERK1/2 activation moves to nucleus.

Immunofluorescence. COS cells were plated in 6-well plates with glass cover slips. Twenty-four hours post seeding, cells were transfected with Myc-CIITA and HA-K63 ubiquitin. Eighteen hours following transfections, 10% serum media was removed where indicated and was replaced with 1% serum media for 6 hours. Thirty minutes prior to harvest, 200nM PMA was added to indicated samples. Cells were fixed in ice-cold methanol for 10 minutes, and were stained with anti-CIITA, anti-K63, anti-ERK1/2, and anti-pERK1/2 as indicated. (A-C): cells in 10% serum media, stained for Dapi, CIITA, and merge (D-F): Cells in 1% serum free media, stained for Dapi, CIITA, and merge (G-I): 10% serum media, stained for Dapi, ERK1/2, and merge (J-L): 1% serum free media, stained for Dapi, pERK1/2, and merge (M-P): 10% serum media, stained for Dapi, CIITA and K63 ubiquitin, and merge (Q-T): 1% serum free media, stained for Dapi, CIITA and K63 ubiquitin, and merge. Images shown are representative images.

2.5 DISCUSSION:

We sought here to identify the kinase responsible for phosphorylating CIITA IF3, the isoform seen constitutively expressed in B cells, at the regulatory site S280 and to further determine the ubiquitination conjugation driven by CIITA phosphorylation. Phosphorylation and ubiquitination are both dynamic and reversible posttranslational modifications that lead to alterations of target proteins. Phosphorylation allows for changes in protein-protein interactions, localization, and activity. Ubiquitination has traditionally been known for its role in tagging proteins for eventual degradation via the 26S proteasome (Ciechanover 1998). More recently, ubiquitination has been shown to have non-proteolytic roles including receptor internalization, multi-protein complex assembly, and intracellular trafficking (Sun and Chen 2004). More recently phosphorylation and ubiquitination have been shown to regulate posttranslational crosstalk; NF- κ B, Cyclin E, and EGFR have all been shown to be regulated through multiplexed crosstalk (Hunter 2007). Here we have identified the kinase responsible for phosphorylating CIITA IF3 S280 (Bhat, Truax et al. 2010). Phosphorylation at S280 leads to non-degradative ubiquitination and increases CIITA's IF3 transactivity (Bhat, Truax et al. 2010).

We and others have shown the interaction between CIITA IF3 and the kinase complex ERK1/2 (Voong, Slater et al. 2008). Phosphorylated CIITA appears as a doublet, with the upper band representing phosphorylated CIITA (Greer, Harton et al. 2004, Voong, Slater et al. 2008). The mutation of serine to alanine on residue 280 (S280A) shows a lack of phosphorylation at

that particular site and further phosphorylation does not occur on CIITA (**Fig 2.2B, iii and iv**). S280 is therefore critical for additional phosphorylation to occur on CIITA. Treatment with U0126 prohibits ERK1/2 phosphorylation, and the S280A CIITA IF3 mutant is unstable (**Fig.2.2 B ii, and iv**). Previous findings identify ERK1/2 as kinases responsible for phosphorylation of CIITA at residues S286, 288, and 293, leading to down regulation of CIITA activity and eventual export from the nucleus (Voong, Slater et al. 2008). Voong *et al.*, show that inhibition of ERK1/2 leads to an increase in CIITA's transactivity; however, we failed to obtain similar results. These differences may be attributed to the activities of additional kinases, such as Cdc2, which was also shown to phosphorylate CIITA. Additionally, ERK5 kinase phosphorylates similar targets as ERK1/2 when ERK1/2 is unavailable (Schweppe, Cheung et al. 2006). Finally, Voong *et al.* did not confirm ERK1/2 expression nor activation, in assays used to determine CIITA transactivity therefore, it is difficult to directly compare our results. We now demonstrate phosphorylation by ERK1/2 on the regulatory site S280 it is not only necessary, but also critical for CIITA transactivity. Phosphorylation of S280 by ERK1/2 leads to significant increase in MHC II mRNA and cell surface expression (**Fig. 2.2B and 2C**) leading to an increased ability to present antigens.

The regulation of CIITA is accomplished through a series of dynamic PTM's (Greer, Zika et al. 2003, Greer, Harton et al. 2004, Wu, Kong et al. 2009, Bhat, Truax et al. 2010). The phosphorylation of CIITA by ERK1/2 leads to a sustained half-life, and thus an active CIITA for a greater period of time. Active CIITA leads to increased MHC II and an immune response. Ours and other reports identify ERK1/2 phosphorylation on various sites throughout CIITA and indicate multiple roles for ERK1/2 phosphorylation in regulating activation and suppression of

CIITA (Voong, Slater et al. 2008). While there is still a great deal to uncover in regards to phosphorylation of CIITA, including the order in which modifications are made, data clearly indicates ERK1/2 targets multiple sites on CIITA and that this kinase complex is necessary in regulating overall activity of CIITA.

Previously we linked CIITA phosphorylation at S280 and CIITA mono-ubiquitination in regulation of CIITA transactivity. To further our insight into the mechanisms regulating phosphorylation/ubiquitination crosstalk on CIITA, we investigated the influence of ERK 1/2 on CIITA global ubiquitination level. Levels of global ubiquitination increase when ERK1/2 is overexpressed (**Fig. 2.4A**). Furthermore, inhibition of endogenous ERK1/2 with U0126, a specific inhibitor for MEK, results in reduction in global ubiquitination. These findings indicate links between ubiquitination and phosphorylation as previously observed by our group (Bhat, Truax et al. 2010). Several reports link phosphorylation and ubiquitination in regulating transcription factors such as FOXO4, NF- κ B, and Cyclin E (Hunter 2007).

Ubiquitination is a diverse modification that yields multiple types of poly-ubiquitin conjugates, and mono-ubiquitination. Poly-ubiquitination is determined by the ubiquitin lysine residue added to a target protein (K6, 11, 27, 29, 33, 48, and 63) in which an additional ubiquitin molecule is attached to the already existing ubiquitin on the target protein (Ciechanover 1998). Our data demonstrates CIITA is modified with a different ubiquitin linkage (**Fig 2.4C, first blot**) than either mono-ubiquitination or K48 linked ubiquitination based on the lack of protein degradation of CIITA (**Fig. 2.4A second blot**). Additionally, we show an increase in CIITA protein concentration when ERK1/2 is activated following serum starvation

and treatment with PMA (**Fig. 2.4C, second blot**). Together, these data suggests CIITA phosphorylation by ERK1/2 participates in PTM crosstalk with a unique ubiquitination modification, leading to increased CIITA transactivity.

The process of ubiquitination has been heavily studied; it is well known that the type of ubiquitin linkage added to a target protein determines a proteins fate. K48 linked ubiquitination tags proteins for degradation via the 26S proteasome (Ciechanover 1998). K63 ubiquitination of TNFR-associated factor 6 (TRAF6) links IL-1R signaling to the activation of NF- κ B (Habelhah 2010). TRAF6 modification by K63 ubiquitination allows additional proteins, including TAB2, to bind directly to the K63 ubiquitin chain resulting in additional downstream effects including phosphorylation and nuclear translocation (Balkhi, Fitzgerald et al. 2008, Hayakawa 2012, Emmerich, Ordureau et al. 2013, Ori, Kato et al. 2013, Zhou, Shen et al. 2013). IRAK1 is also modified with K63 ubiquitination, resulting in multiple protein-protein interactions and downstream phosphorylation events in the NF- κ B pathway (Balkhi, Fitzgerald et al. 2008). In both cases, K63 ubiquitination brings two-protein complexes together to act as a scaffold. K63 ubiquitination has also been implicated in endocytosis, protein trafficking, and DNA damage repair (Wang, Gao et al. 2012). Our observation of K63 ubiquitination is the first of any isoform of CIITA and here we show CIITA K63 ubiquitination followed by phosphorylation by ERK1/2, which leads to cellular localization changes (**Fig. 2.5A**). Additionally, we also note increased levels of CIITA K48 ubiquitination (**Fig. 2.4E**), observations in line with previous reports in which ERK1/2 have been indicated in phosphorylating S286, 288, and 293 leading to the down regulation of CIITA (Greer, Harton et al. 2004).

To gain insight into the role of K63 ubiquitination in regulating CIITA activity, we conducted cellular fractionation assays. Our data shows heavily modified K63 ubiquitinated CIITA in the cytoplasm followed by a decrease once CIITA has been phosphorylated by ERK1/2 (*Fig. 2.6A, cytoplasmic frac.*). The nuclear fraction shows the inverse pattern with an increase in the amount of K63 ubiquitinated CIITA following phosphorylation by ERK1/2 (*Fig. 2.6A, nuclear frac.*). Similar trends were seen in immunofluorescence images (*Fig. 2.7P and 2.7T*). K63 ubiquitinated CIITA was predominately located in the cytoplasm, and following ERK1/2 activation, K63 ubiquitinated CIITA translocate to the nucleus. When the images are merged, yellow punctate loci can be seen. In sum, these data support K63 ubiquitination as an important modification for phosphorylation of CIITA by ERK1/2. Several scenarios exist; K63 ubiquitination could act as a scaffold driving CIITA nuclear localization where ERK1/2 then binds and phosphorylates CIITA, or K63 ubiquitination could be assisting in the activation of ERK1/2, as has been seen with other kinases, as is the case with Akt (Wang, Gao et al. 2012).

Because CIITA is dynamically regulated by multiple PTM's, and the intertwined nature of these modifications has yet to be fully defined, it is important to decipher these modifications to gain a better understanding of the regulation of this critical adaptive immune protein. Phosphorylation of CIITA occurs on various residues (Li, Harton et al. 2001, Tosi, Jabrane-Ferrat et al. 2002, Sisk, Nickerson et al. 2003, Greer, Harton et al. 2004). Numerous kinases are involved in the regulation of these phosphorylation events, however, our data also indicates CIITA is regulated by the same kinase at multiple locations, but with very different impacts on protein activity. While links between phosphorylation and mono-ubiquitination have previously been identified, the kinase responsible for initiating the chain of reactions was unknown (Bhat,

Truax et al. 2010). We identify here CIITA is phosphorylated by ERK1/2 at residue S280, leading to a significant increase in CIITA mono-ubiquitination resulting in increased CIITA activity and MHC II expression and activity. Additionally, we are the first one to show a novel ubiquitination linkage on CIITA, K63. Further, we demonstrate a novel crosstalk between phosphorylation by ERK1/2 and K63 ubiquitination of CIITA IF3 and the combined role of these PTMs have in facilitating movement of CIITA from the cytoplasm to the nucleus and overall increase in activity (**Fig. 2.8**). Our findings contribute to a greater understanding of the regulation of IF3 that is derived from CIITA pIII, however as we utilized an overexpression system, the data contained within will need to be recapitulated in B cells in order to further validate the findings observed. Relevant clinical applications of our findings include CIITA IF3's activation PTMs are largely mediated by ERK1/2 phosphorylation. Based on these observations, dysregulation in the MAPK pathway can contribute to the development of many autoimmune diseases (Tidyman and Rauen 2009). While the immune related effects are not well understood, MEK inhibitors, provide a promising novel therapeutic approach for treatment of many autoimmune diseases (Rogers, Serafin et al. 2012). Future work will focus on identifying the E3 ligases involved in ubiquitination of CIITA. While there have been strides in mapping CIITA's PTMs network, considerable ground remains to be covered in understanding the web of modifications and the crosstalk occurring to regulate this "Master Regulator" of MHC II genes and the adaptive immune response.

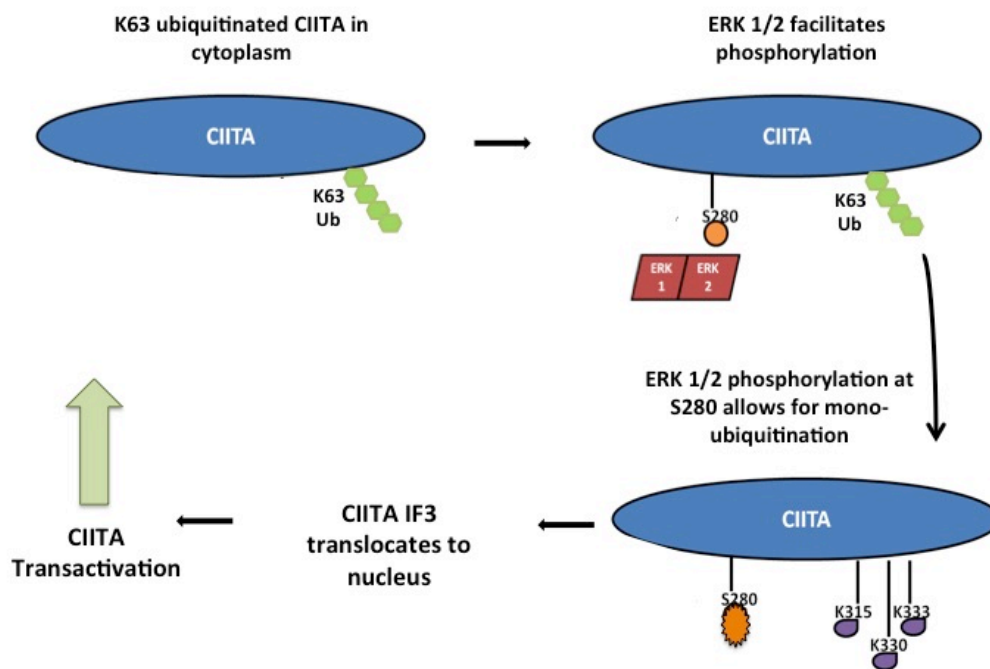


Figure 2.8: Mechanism for CIITA IF3 PTMs

We propose CIITA IF3 is modified by K63 linked ubiquitination while in the cytoplasm by an unknown E3 ubiquitin ligase. K63 linked ubiquitinated CIITA is phosphorylated by the kinase complex, ERK1/2 at S280, which allows for CIITA to translocate to the nucleus where CIITA acts as the master regulator for the MHC II genes. It is clear that mono-ubiquitination is necessary for CIITA transactivity; however, it is unclear if mono-ubiquitination occurs in the cytoplasm or nucleus.

2.6 ACKNOWLEDGEMENT:

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3 CHAPTER 3: Pulling a ligase out of a “HAT”: pCAF mediates ubiquitination of the Class II Transactivator, CIITA

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In Review

3.1 ABSTRACT:

The Class II Transactivator (CIITA) is essential to the regulation of Major Histocompatibility Class II (MHC II) genes transcription. As the “Master Regulator” of MHC II transcription, CIITA regulation is imperative and requires various posttranslational modifications (PTMs) itself in order facilitate its role. Previously we identified various ubiquitination events on CIITA. Mono-ubiquitination is important for CIITA transactivity, while K63 linked ubiquitination is involved in crosstalk with ERK1/2 phosphorylation; where together they mediate cellular movement from the cytoplasm to nuclear region. Further, CIITA is also modified by degradative K48 poly-ubiquitination. However, the E3 ligase responsible for these modifications was unknown. We show CIITA ubiquitination and transactivity is enhanced with the histone acetyltransferase (HAT), p300/CBP Associated Factor (pCAF) and the E3 ligase region within pCAF is necessary for both. Additionally, pCAF mediated ubiquitination is independent of pCAF’s HAT domain, and acetylation deficient CIITA is K48 poly-ubiquitinated and degraded in the presence of pCAF. Lastly, we identify the histone pCAF as the E3 ligase responsible for CIITA’s ubiquitination including mono, K48, and K63 linked poly-ubiquitination.

3.2 INTRODUCTION:

Major histocompatibility class II (MHC II) genes are essential for the initiation of adaptive immune responses to extracellular pathogens, thus their expression and activation are of critical importance and are tightly regulated (Abbas and Janeway 2000, Schnappauf, Hake et al. 2003, Drozina, Kohoutek et al. 2005). Coordinated orchestration of multiple proteins

accomplishes transcription of MHC II, however one protein in particular, known as the “master regulator” of MHC II genes, the Class II Transactivator is particularly important (Benoist and Mathis 1990, Chang, Fontes et al. 1994, Fontes, Kanazawa et al. 1999, Boss and Jensen 2003). In addition to CIITA, various other chromatin-remodeling enzymes are required for “opening” of the MHC II promoter thus allowing the transcriptional machinery to bind. In particular, two histone acetyltransferase, (HATs), the CREB binding protein (CBP/p300), and p300/CBP associated factor, (pCAF), are recruited to the MHC II promoter where they assist in the remodeling of chromatin, which occurs before, and in the presence of, CIITA (Harton, Zika et al. 2001, Zika, Fauquier et al. 2005).

CIITA is an 1130 amino acid protein, and is dynamically regulated through an intricate series of posttranslational modifications (PTMs)(Morgan JE. 2015). PTMs on CIITA include phosphorylation, ubiquitination, and acetylation (Spilianakis, Papamatheakis et al. 2000, Tosi, Jabrane-Ferrat et al. 2002, Greer, Zika et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008, Wu, Kong et al. 2009, Bhat, Truax et al. 2010, Wu, Kong et al. 2011). These modifications precisely regulate CIITA’s location, function, and stability within the cell, and 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, Greer, Zika et al. 2003, Jabrane-Ferrat, Nekrep et al. 2003, Sisk, Nickerson et al. 2003, Wright and Ting 2006, Morgan JE. 2015) HATS including pCAF and CBP are responsible for acetylation of CIITA at lysine(s) (K) 141 and K144 (Spilianakis, Papamatheakis et al. 2000). It has further been shown that acetylation plays important roles in the ubiquitination of CIITA (Spilianakis, Papamatheakis et al. 2000, Greer, Zika et al. 2003). Located at the N-terminal region of pCAF lies a domain containing ubiquitin E3 ligase activity (Linares, Kiernan et al. 2007).

Ubiquitination requires three enzymes: an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase, which is responsible for the ligation of ubiquitin onto a substrate in conjunction with the E2 (Ciechanover 1994). Previously pCAF's intrinsic ubiquitination domain was identified and shown to play a role in the ubiquitination and stability of the critical cell cycle protein, Human double minute 2 (the human ortholog of Mdm2) (Wang, Taplick et al. 2004) (Linares, Kiernan et al. 2007), and Gli1 a transcription factor that mediates hedgehog signaling (Mazza, Infante et al. 2013). Thus, pCAF is not only a HAT, but also an ubiquitin E3 ligase thus far shown to ubiquitinate only a few substrates: Hdm2, Gli1, and pCAF itself (Wang, Taplick et al. 2004, Linares, Kiernan et al. 2007, Mazza, Infante et al. 2013). As pCAF is known to affect the activity of many transcription factors and cofactors through its HAT activities, it is likely that pCAF also has additional targets for its ubiquitin E3 ligase activities. As CIITA has previously been shown to be a substrate for pCAF's HAT activity, and, as observations have been made of CIITA's increased ubiquitination in the presence of pCAF (Greer, Zika et al. 2003), we sought to determine if pCAF was a potential E3 ligase for CIITA.

We hypothesized pCAF is playing a novel role as an ubiquitin E3 ligase for CIITA in addition to its traditional role as a HAT. We show here that both CIITA transactivity levels and global ubiquitination (all ubiquitin types) significantly decline in the absence of the pCAF E3 ligase domain. Further, we demonstrate CIITA ubiquitination does not rely on the HAT domain of pCAF. Acetylation null CIITA mutants lacking the signal to become nucleus bound and therefore, are ubiquitinated in a K48 linked fashion leading to degradation. In vitro ubiquitination assays confirm pCAF's ability to facilitate CIITA ubiquitination. Lastly, we identify that CIITA mono, K63, and K48 linked ubiquitination are mediated by pCAF in vivo. These results demonstrate pCAF's capacity to facilitate various topologies of CIITA

ubiquitination. These results indicate pCAF, via its E3 ligase activity, plays additional important roles in the regulation of CIITA activity, and thus in regulating the expression of MHC II genes. Further, identification of the E3 ligase responsible for ubiquitination of CIITA is critical for gaining added understanding of CIITA regulation by PTMs. Identifying enzymes responsible for these PTMs allows for valuable insight into the regulation of the adaptive immune response and for identifies potential therapeutic targets.

3.3 MATERIALS AND METHODS:

Cell Culture- COS cells (Monkey fibroblast) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco's modified Eagle (DMEM) medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 µg/ml of streptomycin and 2mM of L-glutamine. Cells were maintained at 37° with 5% CO₂.

Plasmids and purified proteins- Flag-K141R, K144R, and K141/144R, and Myc-CIITA were kindly provided by Dr. Jenny Ting. Flag-pCAF was a generous gift from Drs. O. Coux and M. Benkirane (Linares, Kiernan et al. 2007). Myc-pCAF was sub cloned into Myc tagged pCMV-3 using the EcoR1 restriction site. HA-K48 Ub and K63 Ub was a gift from Dr. Ted Dawson. Both HA-K48 and K63 ubiquitin have all internal lysine residues of ubiquitin mutated to arginine except K48 or K63, allowing poly-ubiquitination to only occur on those lysine residues. The HLA-DRA luciferase reporter construct was described previously (Bhat, Turner et al. 2008). The E1 activating enzyme UBE1 Boston Biochem, Boston, MA), E2 conjugating

enzyme, UbcH5b (Boston Biochem), and Flag ubiquitin (Boston Biochem), Hdm2 (Boston Biochem), His-pCAF (Proteinone, Rockville, MD) were all obtained commercially.

GST-protein production and purification- BL21 star (DE3) competent cells (Invitrogen, Carlsbad, CA) were transformed with pGEX constructs. Transformed colonies were selected and inoculated in 5mL LB supplemented with AMP overnight at 37°. One ml preps were added to 100ml fresh LB supplemented AMP and bacterial were allowed to grow for three and a half hours at 37° to OD₆₀₀ of 0.8. IPTG was added to induce expression. Cells were centrifuged and the pellet was washed with chilled PBS and was centrifuged again. The cell pellet was frozen for one hour at -80°, was thawed on ice and was resuspended in buffer A (PBS + 1% Triton-X100 + 0.1M NaCl + Protease Inhibitor (Roche)). Cells were sonicated on ice and were centrifuged to obtain the soluble fraction. The insoluble fraction was then resuspended in buffer B (buffer A + 25% (w/v) sucrose and the mixture was centrifuged at 20,000rpm for 20 minutes. The supernatant was then collected as the insoluble fraction. Solubilization and refolding of inclusion bodies was performed in 8M urea + 5mM DTT to dissolve the pellet. The protein-urea mixture was then dialyzed in PBS at 4° for two days. GST-CIITA protein was added to a Glutathione Resin column and the protein was eluted in 10mM glutathione elution buffer (0.154g reduced glutathione dissolved in 50ml of 50mM Tris-HCL, pH 8.0). GST-CIITA, flow through, wash and elutes were analyzed by SDS-PAGE and were stained with Coomassie and elutes were dialyzed to remove free glutathione.

Co-immunoprecipitations- COS cells were plated at a cell density of 8×10^5 /10cm on tissue culture plates. Cells were transfected using GeneJuice (Merck Millipore, Darmstadt, Germany) as indicated with 5 μ g of Myc-CIITA, Flag-pCAF, Flag-K141R, K144R, K141/144R CIITA, HA-K48 Ub, K63 Ub, HA-mono Ub, or pCDNA control. Twenty-four hours after transfections, cells were lysed in 1% NP40 buffer supplemented with EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged, normalized for protein concentration, and pre-cleared with mouse IgG (Sigma-Aldrich) and Protein G (Thermo Fisher) followed by immunoprecipitation with either EZ view anti-c Myc affinity gel beads (Sigma-Aldrich) or with anti-Flag M2 affinity gel (Sigma-Aldrich). Immune complexes were denatured with Laemmli buffer, boiled, and were separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and were individually immunoblotted with anti-Myc (Abcam, Cambridge, MA), anti-Flag (Sigma-Aldrich), anti-ubiquitin (Life Sensors, Malvern, PA), anti-K48 ubiquitin (Cell Signaling, Danvers, MA), anti-K63 ubiquitin (Millipore), or with anti-GST (Abcam, Cambridge, MA). HRP conjugates were detected using HyGlo Chemiluminescent substrate (Denville). Protein normalization and equal loading was determined in lysates.

Luciferase Reporter Assays- COS cells were plated at 5×10^4 cells/well density (70% confluency). Following adhesion, cells were co-transfected as indicated with *HLA-DRA*, Renilla, pCDNA, Myc-CIITA, and Flag-pCAF using GeneJuice (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Twenty four hours following transfections, cells were lysed with 1X Passive lysis buffer (Promega, Madison, WI) supplemented with EDTA-free protease inhibitor (Roche). Dual luciferase assays were performed using the Lmax II³⁸⁴

(Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Luciferase readings were normalized to Renilla readings for protein normalization.

***In Vitro* ubiquitination assay-** The CIITA *in vitro* ubiquitination assay was carried out in 150 μ L of reaction mixture containing 40mM Tris-HCl (pH 7.5), 5mM MgCl₂, 2mM dithiothreitol, 1mM Creatine Phosphate, 2mM ATP, 400ng of Recombinant GST-CIITA (Substrate), 400ng of GST-Hdm2 (Boston Biochem), 400ng Recombinant His-pCAF (E3 ligase candidate) (Proteinone Rockville, MD), 500ng Flag-ubiquitin (Boston Biochem, Boston, MA), 200ng E1 activating enzyme, UBE1 (Boston Biochem), 200ng E2 conjugating enzyme, UbcH5b (Boston Biochem). All components were added and incubated at 37° C for 60 minutes and were analyzed via SDS-PAGE. Ubiquitinated CIITA was detected by immunoblot using Flag antibody (Sigma), and CIITA ubiquitination was verified with GST (Abcam), and pCAF with monoclonal α pCAF antibody (Santa Cruz). Verification of Hdm2 ubiquitination was detected using GST (Abcam).

3.4 RESULTS:

CIITA and pCAF co-immunoprecipitate- CIITA and pCAF previously have been shown to associate (Spilianakis, Papamatheakis et al. 2000), and pCAF is known to acetylate CIITA on lysines (K) 141 and 144. These residues lie within a nuclear localization signal (NLS) region and acetylation is necessary to shuttle CIITA to the nucleus. Once pCAF acetylates CIITA, CIITA accumulates in the nucleus; where it binds to the enhanceosome complex at the MHC II promoter (Spilianakis, Papamatheakis et al. 2000) and drives MHC II transcription. To confirm previous findings, we conducted co-immunoprecipitation assays to verify interactions between

WT CIITA and WT pCAF. Lane one indicates association of WT Myc-CIITA and WT Flag-pCAF through co-immunoprecipitation analysis (**Fig 3.1, Top panel, lane one**).

pCAF's E3 ligase domain is necessary for CIITA transactivity- While pCAF is known primarily for its HAT role, pCAF is also considered to be an ubiquitination factor with intrinsic E3 ligase capabilities (Linares, Kiernan et al. 2007, Mazza, Infante et al. 2013). Interestingly, pCAF does not have any homology to other known E3 ligases. Linares et. al., performed a series of deletion mutations and identified a region (amino acids 121-242) that possesses E3 ligase capability (Linares, Kiernan et al. 2007). Previous reports suggest pCAF is able to mediate both acetylation and ubiquitination of the same target (Greer, Zika et al. 2003, Linares, Kiernan et al. 2007, Kass, Poyurovsky et al. 2009, Mazza, Infante et al. 2013). Thus, we next wanted to determine if pCAF's E3 ligase domain is necessary for CIITA's increased transactivity. Levels of transactivity were determined using a dual luciferase reporter assay. Transactivity levels of CIITA alone are low and insufficient to drive MHC II transcription. When CIITA and WT Flag-pCAF are co-expressed, transactivity increases 2-fold; however, deletion of the E3 ligase domain of pCAF leads to a significant decrease in CIITA transactivity levels (**Fig 3.2**).

E3 ligase domain deficient pCAF is unable to ubiquitinate CIITA- We next investigated if CIITA ubiquitination would be impaired or altered in the absence of the E3 ligase domain of pCAF. pCAF contains an auto-ubiquitination domain that is able to mediate self-ubiquitination, but has not been shown to be involved in ubiquitination of other substrates (Linares, Kiernan et al. 2007). To determine if the E3 ligase domain is necessary to facilitating CIITA ubiquitination, we performed an in vivo ubiquitination assay. WT-Myc CIITA, WT-Flag

pCAF, or the $\Delta E3$ pCAF mutant were co-transfected into COS cells. Ubiquitination levels of WT CIITA co-transfected with WT pCAF show a significant increase over those of WT CIITA transfected alone (**Fig 3.3, compare lanes 1 and 2**). However, the ubiquitination levels of CIITA co-transfected with the $\Delta E3$ pCAF mutant show levels of ubiquitination that are significantly decreased when compared to those of CIITA co-transfected with WT pCAF (**Fig. 3.3 compare lanes 2 and 3**). These data support that the E3 ligase domain of pCAF is important for CIITA ubiquitination and is involved in mediating CIITA ubiquitination.

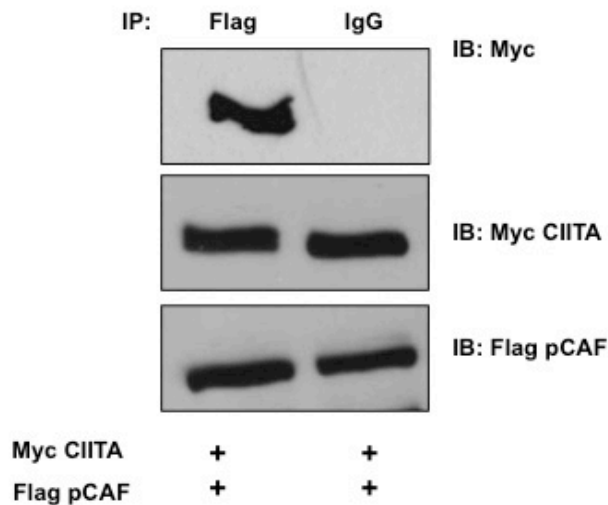


Figure 3.1 CIITA associates with the E3 ligase pCAF.

Co-immunoprecipitation of CIITA and pCAF: COS cells were co-transfected with Myc-CIITA and Flag-pCAF. Cells were harvested, lysed, pre-cleared and immunoprecipitated (IP'd) with anti-Flag and anti-Mouse IgG. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-Myc antibodies. Lysate controls demonstrate expression of Myc-CIITA and Flag-pCAF. Data shown are cropped images from one IP gel and one lysate gel and are representative of three individual experiments.

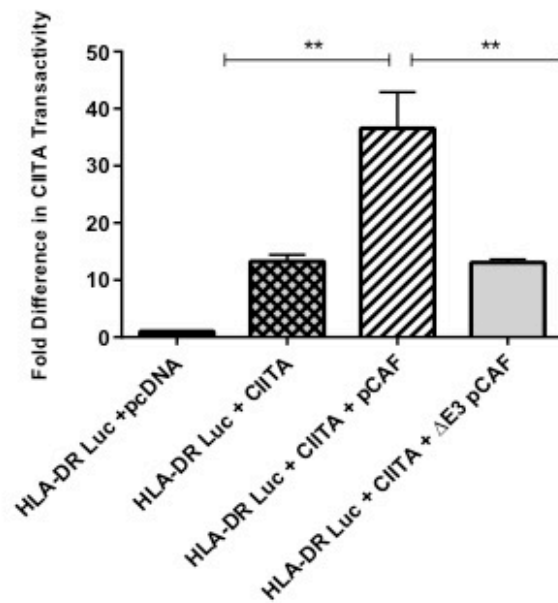


Figure 3.2 The pCAF E3 ligase domain is necessary for enhanced CIITA transactivity.

CIITA transactivation increases in the presence of expressed WT pCAF. *Reporter Assay:* COS cells were transfected as indicated with the MHC II HLA-DR-Luc reporter construct, Renilla, CIITA, pCAF, and Δ E3 pCAF. Luciferase assays were performed in triplicate in three independent experiments, data are presented as fold increase in luciferase activity. Results are standardized to Renilla values and represent the mean \pm SD, **< 0.01.

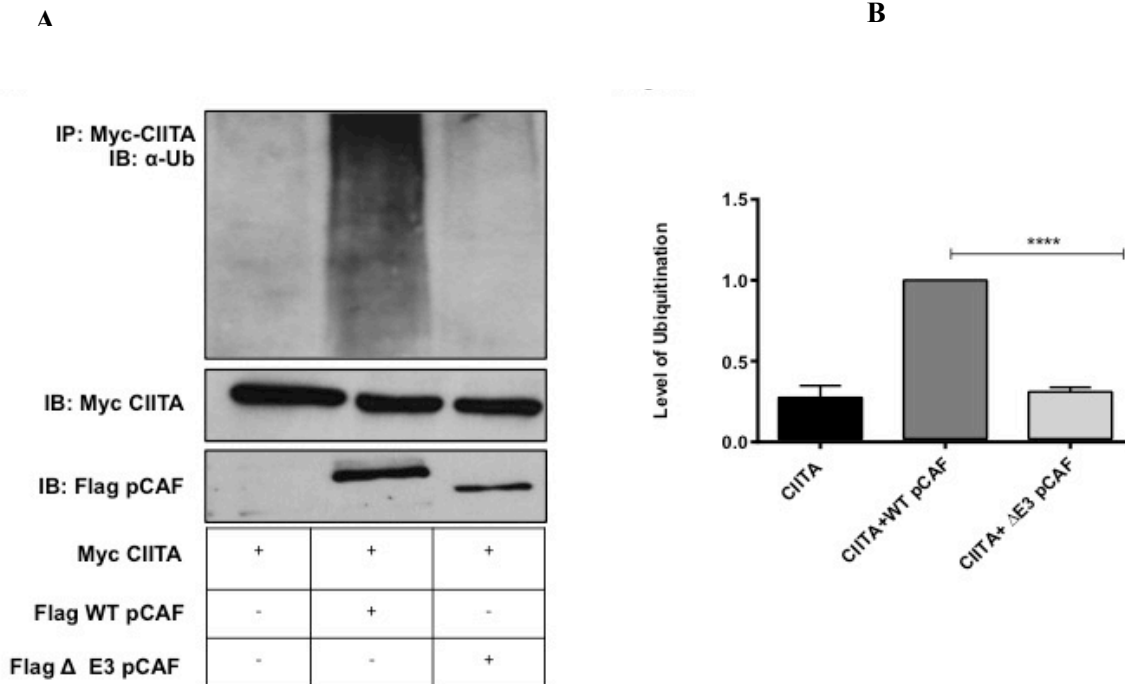


Figure 3.3 pCAF facilitates CIITA ubiquitination independent of its HAT domain.

(A) CIITA is ubiquitinated by pCAF. *In vivo ubiquitination assay*: COS cells were co-transfected as indicated with Myc-CIITA, Flag-pCAF, and Flag-pCAF Δ HAT mutant. Eighteen hours following transfections, cells were harvested, lysed, pre-cleared and were immunoprecipitated (IP'd) with the anti-Myc antibody. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-ubiquitin antibodies. Lysate controls (bottom two panels) demonstrate expression of Myc-CIITA, Flag-pCAF, and Flag-pCAF Δ HAT. Data shown are cropped images from one IP gel and one lysate gel and are representative of three independent experiments. (B) Densitometry and quantification of data in fig 2A. Densitometry was performed on three independent experiments, mean \pm SD, *** $<$ 0.001.

pCAF facilitates CIITA ubiquitination independent of its HAT domain- pCAF is a well known HAT and is involved in many aspects of CIITA and MHC II regulation (Spilianakis, Papamatheakis et al. 2000, Li, Harton et al. 2001, Chou, Khan et al. 2005). pCAF assists in remodeling the chromatin structure of the MHC II promoter where it acetylates histones H3 and H4 (Li, Harton et al. 2001) and also regulates CIITA's nuclear relocation by acetylating CIITA K141 and K144, leading to increased activation of CIITA and increased expression of MHC II (Spilianakis, Papamatheakis et al. 2000). To determine if pCAF's role in ubiquitination of CIITA was independent of pCAF's HAT activities, we performed in vivo ubiquitination assays using WT CIITA and a HAT deletion mutant of pCAF. In this assay, expression of WT CIITA alone indicates low level ubiquitination (***Fig. 3.4A, lane 1***), and ubiquitination levels significantly increase when WT pCAF is overexpressed (***Fig. 3.4A lane 3***). However, in the absence of the pCAF HAT domain, there is no measureable difference in CIITA ubiquitination levels when CIITA ubiquitination is compared in to that generated in the presence of WT pCAF (***Fig. 3.4A compare lanes 3 and 4***). Thus, we conclude that pCAF's ability to ubiquitinate CIITA is independent of the pCAF HAT domain.

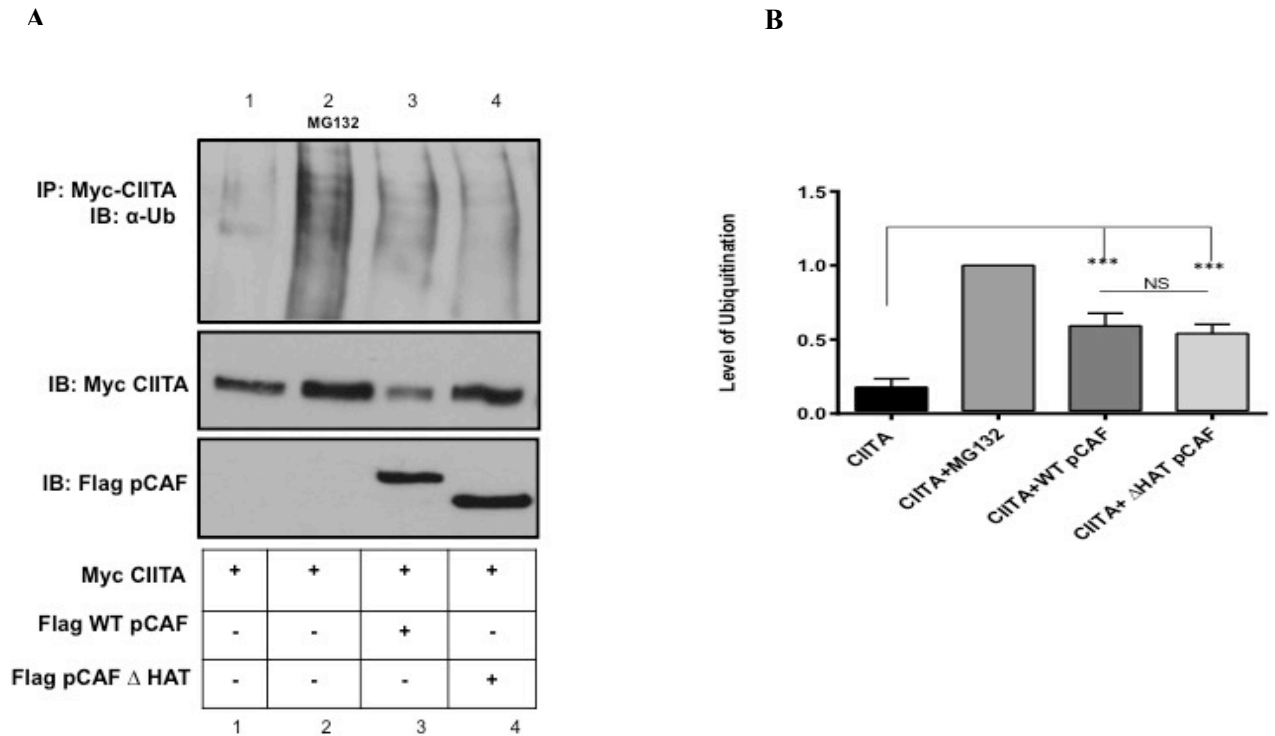


Figure 3.4 CIITA ubiquitination depends on the E3 ligase domain of pCAF.

(A) The E3 ligase null mutant pCAF negates CIITA ubiquitination. *In vivo ubiquitination assay*: COS cells were co-transfected with Myc-CIITA, Flag-pCAF, and Flag-pCAF Δ E3 ligase mutants as indicated. Eighteen hours following transfections, cells were harvested, lysed, pre-cleared and were immunoprecipitated (IP'd) with the anti-Myc antibody. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-ubiquitin antibodies. Lysate controls (bottom two panels) demonstrate expression of Myc-CIITA, Flag-pCAF, and Flag-pCAF Δ E3. Data shown are cropped images from one IP gel and one lysate gel and are representative of three independent experiments. (B) Densitometry and quantification of data in fig 2A. Densitometry was performed on three independent experiments, mean \pm SD, **** $<$ 0.0001.

pCAF associates with CIITA acetylation null mutants- As pCAF acts in a dual role as both HAT and ubiquitin ligase, and as acetylation drives CIITA nuclear import, we next determined if acetylation of CIITA is necessary for CIITA ubiquitination mediated by pCAF. To begin to investigate the relationship between acetylation and ubiquitination on CIITA, we first wanted to identify if acetylation null CIITA mutants and pCAF are able to associate. Co-immunoprecipitation analysis indicates CIITA acetylation mutants deficient at K141R, K144R individually, or K141/144R double mutant sites all remain capable of interaction with pCAF (**Fig 5**).

pCAF mediates K48 linked ubiquitination of acetylation null CIITA mutants- Acetylation at K141 and K144 of CIITA are critical in signaling the movement of CIITA from the cytoplasm to the nucleus (Spilianakis, Papamatheakis et al. 2000). To investigate pCAF's role in mediating acetylation independent ubiquitination, we utilized acetylation null mutants of CIITA. These CIITA mutants are incapable of being acetylated at K141 and K144, and thus likely targets for K48 linked poly-ubiquitination and degradation. Our results indicate K141R and K144R and K141/144R CIITA mutants display low levels of ubiquitination and the addition of pCAF yields significantly lower levels of detectable ubiquitination with lysate blots indicating CIITA transfections and a decrease in protein expression (**Fig 3.6A**). These data suggests CIITA is being degraded at greater rate with the overexpression of pCAF (**Fig 3.6A compare lanes 1 and 2**). Proteasome inhibition by MG132 indicates an accumulation of ubiquitinated CIITA when pCAF is present (**Fig 3.6A lane 3**), indicating the lack of ubiquitination smear seen in lane 2 is likely due to CIITA degradation. Further, the K144R mutant and the double K141/K144R mutants indicate similar trends, respectively (**Fig 3.6A lanes 4-6 and 7-9**). To further determine

if pCAF mediates CIITA K48 linked ubiquitination, we took 15uL of the same IP sample and IB was assayed for K48 ubiquitination using specific antibodies recognizing K48 ubiquitination (**Fig 3.6B**). Similar trends were observed as seen in Fig 6A, where CIITA acetylation mutants were ubiquitinated, however, when pCAF is introduced we again observe that ubiquitination diminish and samples treated with proteasome inhibitor show accumulation of ubiquitinated CIITA. Densitometry assays indicate significant differences in ubiquitination levels in both global ubiquitination and K48 specific ubiquitination (**Fig 3.6 C and D**).

In vitro ubiquitination assays indicates pCAF's ability to mediate ubiquitination of CIITA- To elucidate if CIITA was a substrate for pCAF as an E3 ligase, we conducted an *in vitro* ubiquitination assay using purified human recombinant proteins. As a positive control, we used GST-Hdm2, which has previously been seen to be ubiquitinated by pCAF (Linares, Kiernan et al. 2007) (**Fig. 3.7 C**). Purified human recombinant proteins E1 (UbA-1), E2 (UbC-H5B), and Flag-Ubiquitin, GST-WT CIITA, and His-WT pCAF (E3) were all used in the presence with a reaction mixture (see materials and methods). As shown in figure 3.7A and 3.7B, when all ubiquitination components (E1, E2, pCAF (E3), and ubiquitin) were present along with the substrate in question, CIITA ubiquitination occurs (**3.7A and 3.7B, Lane 5**), however in the absence of any of these components, ubiquitination did not occur. We IB'd for both, anti-flag ubiquitin and anti GST-CIITA. These data reveal that CIITA is a substrate for the ubiquitin E3 ligase pCAF.

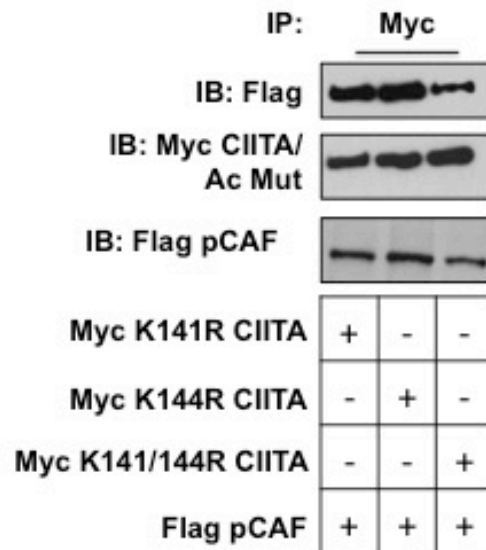


Figure 3.5 Acetylation null CIITA mutants co-immunoprecipitate with pCAF.

CIITA acetylation null mutants associate with WT pCAF. *Co-immunoprecipitation of acetylation null CIITA mutants with pCAF*: COS cells were co-transfected with Myc-CIITA, K141R CIITA, K144R CIITA, or K141/144R CIITA, and Flag-pCAF. Cells were harvested, lysed, pre-cleared and were immunoprecipitated (IP'd) with anti-Myc or Mouse IgG antibodies as indicated. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-Myc antibodies. Lysate controls demonstrate expression of Myc-CIITA and acetylation null mutants and Flag-pCAF (bottom two panels). Data shown are cropped images from one IP gel and one lysate gel and are representative of 3 individual experiments.

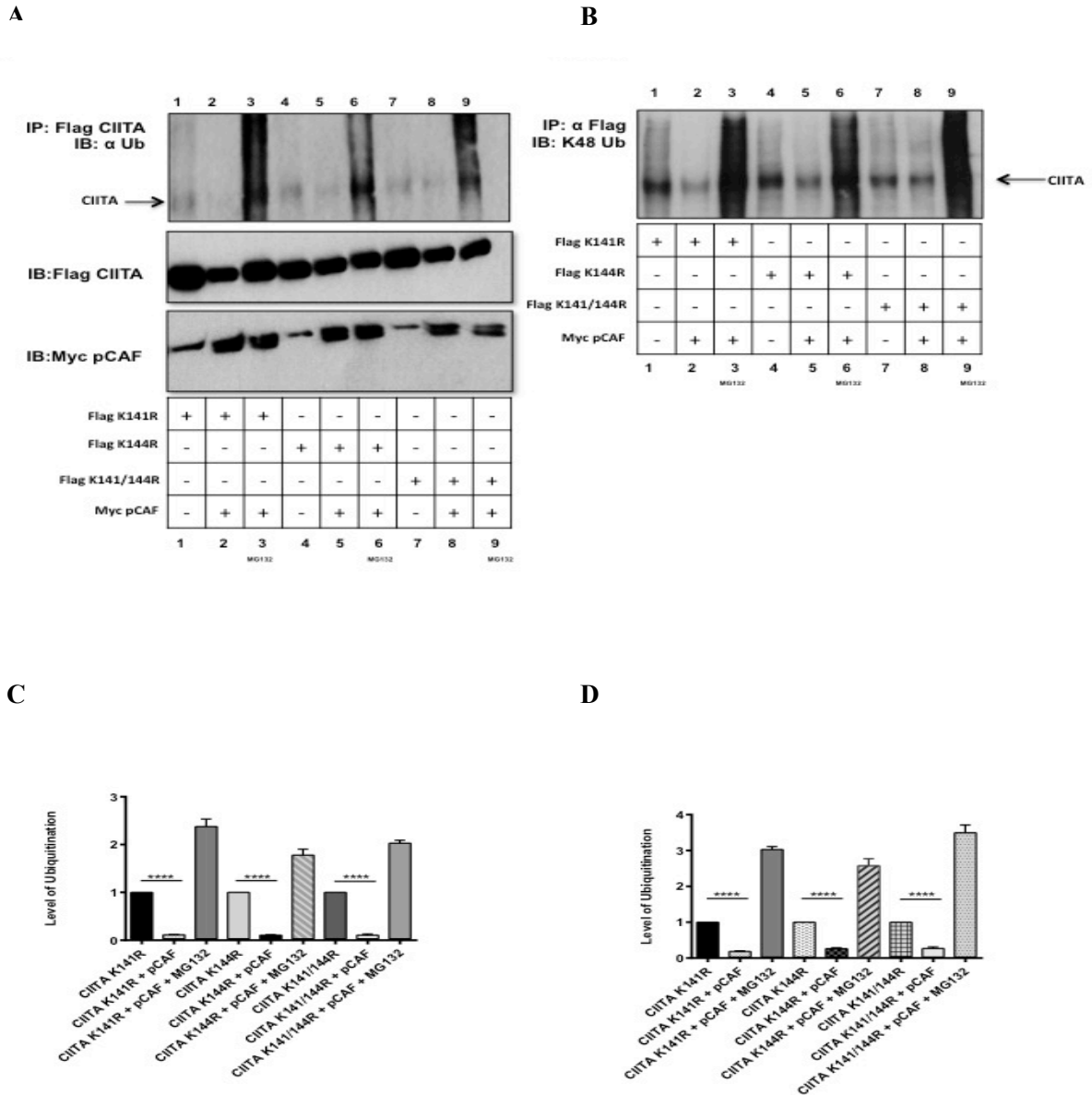


Figure 3.6 pCAF enhances ubiquitination of CIITA acetylation null mutants.

(A) Acetylation null CIITA mutants have increased levels of ubiquitination in the presence of pCAF. *In vivo ubiquitination assay*: COS cells were co-transfected with Flag-K141R, K144R, K141/144R CIITA, and with Myc-pCAF. Eighteen hours following transfections, MG132 was added to indicated samples in order to inhibit the 26S proteasome. Cells were harvested, lysed, pre-cleared, and immunoprecipitated (IP'd) with anti-Flag antibody. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-ubiquitin antibodies. Lysate controls (bottom two panels) demonstrate expression of Flag-K141R, K144R, K141/144R and Myc-pCAF. Data shown are cropped images from one IP gel and one lysate gel and are representative of three individual experiments. (B) CIITA acetylation null mutants have increased levels of K48 specific linkage ubiquitination in the presence of pCAF. (C) Densitometry and quantification of data in Fig 6A. Densitometry was performed on three independent experiments, mean \pm SD, **** $<$ 0.0001. (D) Densitometry and quantification of data in Fig 6B. Densitometry was performed on three independent experiments, mean \pm SD, **** $<$ 0.0001.

CIITA mono, K63 and K48 linked ubiquitination is increases in vivo with pCAF expression- We previously determined CIITA's ubiquitination status to include Mono, K63, and K48 ubiquitination (Bhat, Truax et al. 2010, Morgan JE. 2015). Bhat et al., showed the three sites of mono ubiquitination on CIITA are necessary for CIITA stability and for increased CIITA transactivity. Additionally, we previously demonstrated CIITA modified by K63 linked ubiquitination plays a role in CIITA movement from the cytoplasm to the nucleus. Knowing that several types of ubiquitination linkages modify CIITA, we wanted to determine if pCAF was able to facilitate all three types of CIITA ubiquitination. Previously, we demonstrated, pCAF is able to mediate K48 linked CIITA ubiquitination in the absence of CIITA acetylation. To investigate if pCAF is able to mediate various ubiquitin linkages on CIITA, we conducted in vivo ubiquitination assays. We demonstrate CIITA mono ubiquitination, K63 and K48 linked ubiquitination all increase in the presence of WT pCAF as compared to assays performed when WT CIITA is expressed alone (***Fig 3.8 A, B, and C, compare lanes 1 and 2***). When the proteasome inhibitor MG132 inhibits the 26S proteasome, all three forms of ubiquitinated CIITA accumulated indicating maximum ubiquitination levels (***Fig 3.8 A, B, and C, lane 3***). These data indicate pCAF's ability to mediate multiple forms of ubiquitination of CIITA.

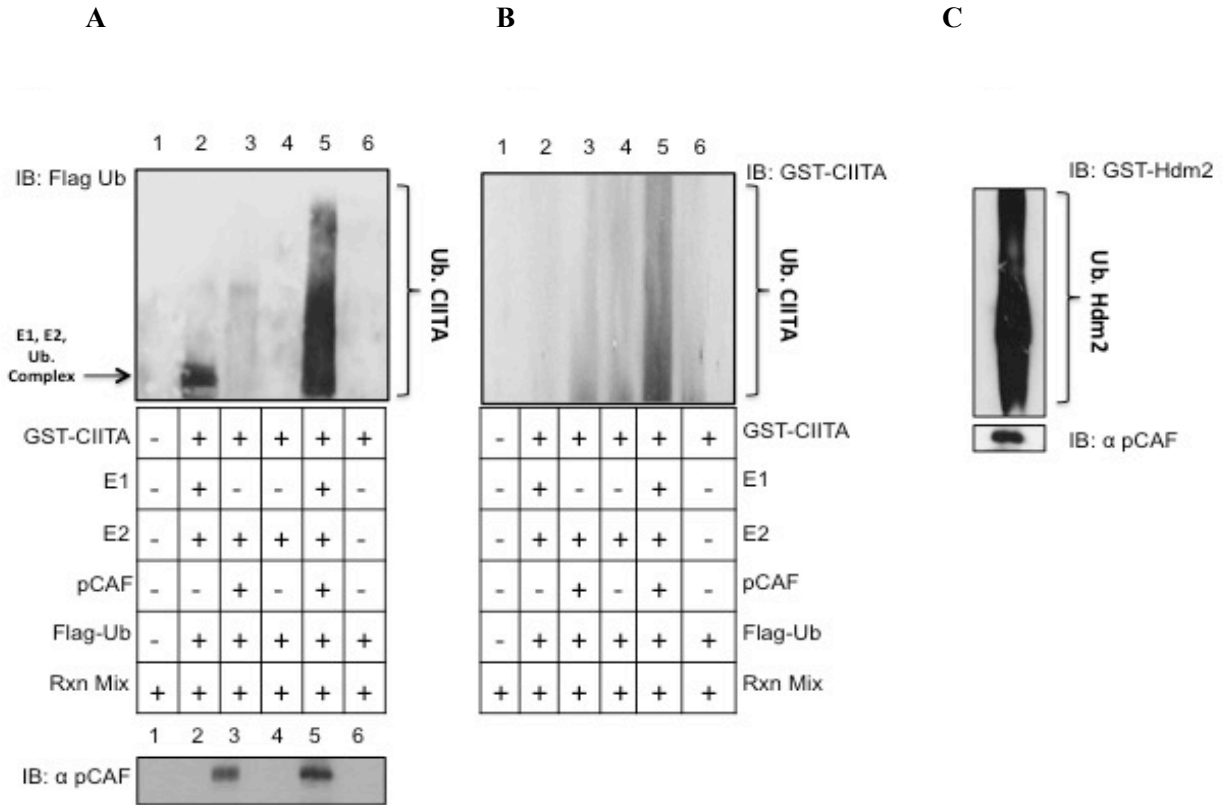


Figure 3.7 pCAF ubiquitinates CIITA *in vitro*.

(A) Ubiquitination of CIITA by pCAF *in vitro*. *In vitro ubiquitination assay*: Reactions components are as indicated and were performed as described in Material and Methods. Ubiquitination was detected with anti-Flag antibody (top panel), pCAF was detected using anti-pCAF antibody (bottom panel) (B) Ubiquitinated CIITA was further confirmed with anti-GST antibody. (C) Positive control demonstrating pCAF's ability to facilitate ubiquitination *in vitro* on Hdm2.

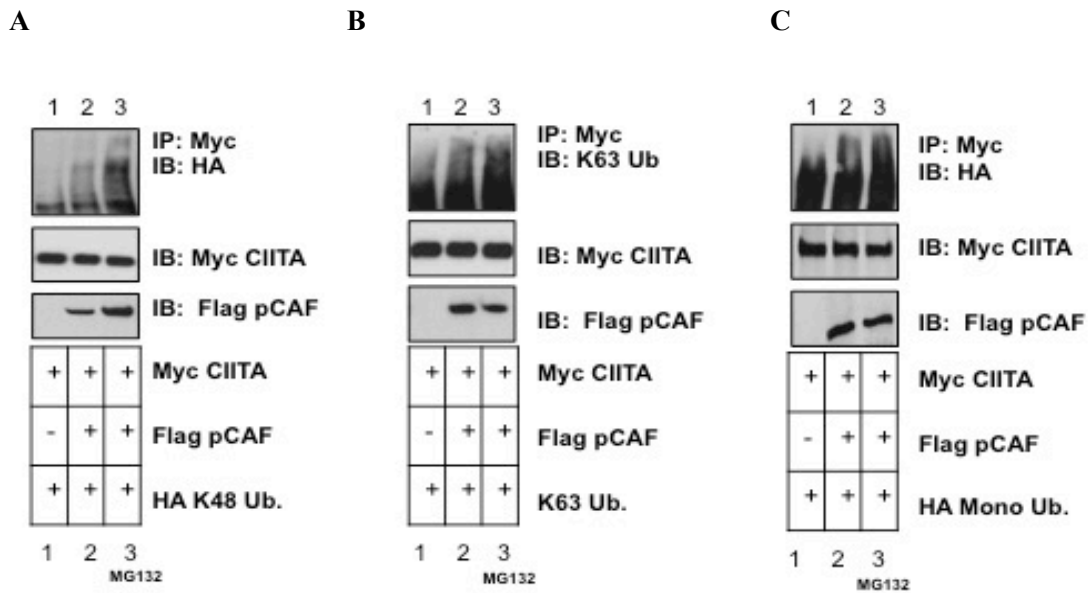


Figure 3.8 pCAF enhances K48, K63, mono ubiquitination of CIITA.

(A) pCAF facilitates multiple ubiquitination types. *In vivo ubiquitination assay*: COS cells were co-transfected with Myc-CIITA, Flag- pCAF, HA-K48 ubiquitin, HA-K63 ubiquitin or HA-Mono Ub. Eighteen hours following transfections, MG132 was added to indicated samples to inhibit the 26S proteasome in treated cells. Cells were harvested, lysed, pre-cleared, and immunoprecipitated (IP'd) with anti-Myc antibody. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-HA antibody. Lysate controls (bottom two panels of A, B, and C) demonstrate expression of Myc-CIITA and Flag-pCAF. Data shown are cropped images from one IP gel and one lysate gel and are representative of three individual experiments.

3.5 DISCUSSION:

We sought here to identify the ubiquitin E3 ligase mediating CIITA ubiquitination. CIITA is known as the “master regulator” of MHC II genes. MHC II is critically important for proper presentation of extracellular pathogen to CD4⁺ T cells in adaptive immune responses. While MHC II is regulated at the level of transcription, CIITA is tightly regulated at the level of posttranslational modification. CIITA is heavily modified through a complex series of PTMs that dynamically regulate its location, function, stability, and activity.

Previous reports identify CIITA as being modified by mono, K63, and K48 linked ubiquitination (Bhat, Truax et al. 2010, Morgan JE. 2015). Ubiquitination of CIITA has been demonstrated as an essential posttranslational modification. Mono ubiquitinated CIITA is more stable and active at the MHC II promoter (Greer, Zika et al. 2003, Drozina, Kohoutek et al. 2006, Bhat, Truax et al. 2010). K63 linked ubiquitination is also important as this particular ubiquitin linkage demonstrates enhanced crosstalk with phosphorylation and together these modifications are important for movement of CIITA from the cytoplasm to the nucleus (Morgan JE. 2015). Additionally, CIITA is modified by K48 linked ubiquitination, leading to recognition and degradation by the proteasome (Bhat, Truax et al. 2010).

pCAF is a well known histone acetyltransferase or HAT (Zika and Ting 2005) and has previously been demonstrated to be recruited to and to activate transcription of the MHC II promoter via pCAF's HAT activities (Drozina, Kohoutek et al. 2005, Wright and Ting 2006). pCAF must be localized to the MHC II promoter where pCAF cooperates with CIITA to drive

MHC II transcription. The interaction of pCAF and CIITA is independent of pCAF's HAT domain (Harton, Zika et al. 2001, Drozina, Kohoutek et al. 2005, Zika and Ting 2005). We confirm here the addition of pCAF drives increased CIITA transactivation levels at the MHC II promoter (**Fig.3.2**).

Reports by several groups of pCAF's ability to act as an E3 ubiquitin ligase, facilitating ubiquitination on targets such as Hdm2/Mdm2 and Gli1 (Linares, Kiernan et al. 2007, Mazza, Infante et al. 2013) where pCAF has also been shown to act as a HAT, raised the possibility to us that pCAF could have dual enzyme activity on CIITA. We next sought to determine if pCAF was participating as an E3 ligase and able to mediate CIITA ubiquitination. Levels of CIITA ubiquitination significantly increase in the presence of WT pCAF, while in the absence of the pCAF E3 ligase region; ubiquitination is abolished (**Fig. 3.3**). The region of pCAF containing the E3 ligase domain, does not conform to any known E3 ligase structures, many questions remain as to how pCAF may function as an E3 ligase. Possible mechanisms are many E3 ligases are regulated through auto-ubiquitination (de Bie and Ciechanover 2011). pCAF's auto-ubiquitination domain has previously been shown to not be involved in substrate ubiquitination, but does promote self-ubiquitination (Linares, Kiernan et al. 2007). E3 ligases also can be regulated by phosphorylation, leading to either activation or deactivation (Hunter 2007, Woelk 2007). pCAF is phosphorylated at tyrosine (Y) 729 and threonine (T) 731 and the role for phosphorylation has yet to be determined (Hornbeck, Zhang et al. 2015), however, phosphorylation at these residues could be involved in pCAF's E3 ligase function.

We further show CIITA ubiquitination by pCAF is independent of pCAF's HAT domain (**Fig 3.4A**). When CIITA acetylation is blocked, CIITA is ubiquitinated by K48 linked poly-ubiquitination and subsequently degraded. This data is in line with previous reports indicating treatment with Trichostatin A (TSA) and HDAC1 which show reduced levels of CIITA ubiquitination (Greer, Zika et al. 2003). By blocking the proteasome, we were able to visualize accumulated levels of ubiquitination (**Fig 3.6A & 3.6B**). These data indicate pCAF's ability to ubiquitinate independently of its HAT function.

In vitro analysis confirmed pCAF's role in ubiquitination of CIITA when all necessary ubiquitin components were available, and pCAF as the E3, ubiquitination of CIITA occurred (**Fig. 3.7A & B**). Further, In vivo ubiquitination assays revealed pCAF's ability to mediate multiple types of CIITA ubiquitination, including mono, K63, and K48 linked poly-ubiquitination (**Fig. 3.8 A, B, & C**). pCAF utilizes the E2 conjugating enzyme UbcH5b, which is capable of synthesizing ubiquitin chains containing all of the possible seven linkages (K6, 11, 27, 29, 33, 48, and 63) of ubiquitin (Li and Ye 2008).

Our study increases understanding of the regulation of the Class II Transactivator, thus leading directly to the molecular events, which contribute to the regulation of MHC II genes. Ubiquitination has been shown to be one of the many important and necessary PTMs, which regulate CIITA. Our previous identification of both Mono and K63 linked ubiquitination provided valuable insight that ubiquitination regulates the stability, location, and activity of CIITA (Bhat, Truax et al. 2010, Morgan JE. 2015). Here we identify a novel substrate for the E3 ligase function of pCAF and identify pCAF's ability to mediate various ubiquitination moieties

on CIITA. Future work will focus on understanding the regulation of pCAF as an E3 ligase. pCAF does not contain homologous domains of other known E3 ligases and it remains unknown how enzymes with “dual” HAT and E3 ligase activity are regulated. Understanding the mechanism controlling each of these functions and how they are simultaneously regulated will be important to further understanding the regulation of CIITA and the adaptive immune response.

3.6 ACKNOWLEDGEMENT:

We would like to thank Dr. Ya-Shu Huang for producing the GST-CIITA recombinant protein. Funding was provided by Georgia State University Molecular Basis of Disease area of focus fellowship to Julie E. Morgan.

4 CHAPTER 4: CONCLUSIONS:

PTMs are critically important to protein diversity; they alter the function, location, and stability of proteins (Hunter 2007, Benayoun and Veitia 2009, Filtz, Vogel et al. 2014). The reversible nature of PTMs allows dynamic protein regulation to occur as many PTMs are governed by enzymatic reactions (Filtz, Vogel et al. 2014). The addition or removal of a PTM allows for increased diversity in a protein's activity. Within the last decade, PTMs have grown to be an intense area of focus and have been cited as having roles in many diseases including heart disease (Smith and White 2014), cancer (Jin and Zangar 2009), neurodegenerative diseases (Karve and Cheema 2011), and various auto-immune diseases (Cohen 2000, Sollid and Jabri 2011, Dunne, Overbergh et al. 2012, Burska, Hunt et al. 2014, Ryan, Nissim et al. 2014, Mastrangelo, Colasanti et al. 2015). Early research of PTMs focused on modifications occurring on histone tails and the roles these modifications have in regulating changes in chromatin states (Nightingale, O'Neill et al. 2006, Lee, Smith et al. 2010). More recently focus has turned to understanding roles PTMs have in the regulation of non-histone proteins, especially in the regulation of transcription factors (O'Malley, Qin et al. 2008, Benayoun and Veitia 2009, Filtz, Vogel et al. 2014).

Transcription factors are an assorted class of proteins that, while diverse in their activities, make up only a small percentage (7%) of the human proteome (Filtz, Vogel et al. 2014). Transcription factors are key to cellular activity as they oversee cellular functions while integrating external signals into gene expression. Many of these external signals lead to the addition or removal of PTMs on transcription factors themselves, which in turn govern the activity of the modified transcription factor (Kim, Bae et al. 2012). PTMs, which are added to

transcription factors occur individually or can be sequentially, linked and can either inhibit or promote the establishment of additional PTM sites on the modified transcription factor (Benayoun and Veitia 2009, Kim, Bae et al. 2012, Zhang, Vogel et al. 2012, Filtz, Vogel et al. 2014). These types of multiple modifications are known as crosstalk among PTMs (Hunter 2007). These “interconnections” between PTMs are excellent therapeutic targets for disease treatments as dysregulated PTMs are often associated with disease development (Karve and Cheema 2011).

Tight regulation of transcription factors is necessary as many of these proteins are involved in numerous unrelated cellular processes. PTMs act as “molecular switches,” communicating with each other and generating “on/off” signals, which regulate in turn other transcription factors. For example, the transcription factor Forkhead-box protein 4 (FOXO4) is modified by ubiquitination. Ubiquitination of FOXO4 is necessary for its translocation and transcriptional activation (van der Horst, de Vries-Smits et al. 2006, Filtz, Vogel et al. 2014). The transcription factor FOXO1 is regulated by PTM crosstalk between acetylation, phosphorylation and ubiquitination. FOXO1 is a negative regulator in insulin sensitivity (Nakae, Biggs et al. 2002) and acetylation of FOXO1 promotes its phosphorylation by various kinases and leads to its reduced nuclear accumulation (Nakae, Biggs et al. 2002). Phosphorylation results in retention of FOXO1 in the cytoplasm where it interacts with its ubiquitin E3 ligase and is degraded (Matsuzaki, Daitoku et al. 2005). The PTM modifications that “regulate” these critical regulators are important contributors to transcription regulation. PTMs can be used as biomarkers of disease states; however, current therapeutics that target PTMs are few, and even fewer are those that specifically target transcription factors, mostly due to a lack of specificity.

Identifying PTMs and PTM crosstalk gives new hope in identifying novel drug targets (Filtz, Vogel et al. 2014).

CIITA is a transcription factor which is heavily modified by multiple PTMs (Spilianakis, Papamatheakis et al. 2000, Li, Harton et al. 2001, Greer, Zika et al. 2003, Greer, Harton et al. 2004, Drozina, Kohoutek et al. 2006, Linares, Kiernan et al. 2007, Voong, Slater et al. 2008, Wu, Kong et al. 2009, Bhat, Truax et al. 2010), which ultimately regulate CIITA activity. CIITA, as the “master regulator” of MHC II genes, is required to be present at the MHC II promoter to drive MHC II transcription (Mach, Steimle et al. 1996, Masternak, Muhlethaler-Mottet et al. 2000, Boss and Jensen 2003). CIITA is transcribed from three distinct promoters, yielding three MHC II cell specific isoforms (Muhlethaler-Mottet, Otten et al. 1997). This dissertation focuses on CIITA pIII, which is constitutively expressed in B cells (Piskurich, Linhoff et al. 1999).

Proper transcription of MHC II leads to antigenic peptides being presented to CD4⁺ T cells and thus, activation other adaptive immune responses (Cresswell and Howard 1997, Janeway, Travers et al. 2001, Morris, Beresford et al. 2002, Owen, Punt et al. 2013). Early work focused on understanding the transcriptional components involved in MHC II transcription, including uncovering the necessary enhanceosome components that CIITA; a non-DNA binding transcription factor binds at the MHC II promoter (Ting and Trowsdale 2002, Boss and Jensen 2003, Drozina, Kohoutek et al. 2006). Focus then shifted to dissecting and understanding how CIITA itself is regulated; as many diseases have been linked to dysregulation of MHC II, it has become apparent that greater understanding of the regulation of CIITA will lead to greater ability to manipulate MHC II expression.

Initial observations revealed CIITA is modified by PTMs including phosphorylation, acetylation, and ubiquitination (Spilianakis, Papamatheakis et al. 2000, Greer, Zika et al. 2003, Greer, Harton et al. 2004, Bhat, Truax et al. 2010). Work by Spilianakis et. al., determined CIITA is acetylated by HATs CBP and pCAF. Acetylation mediated by these HATS on CIITA K141 and K144 contributes to nuclear trafficking of CIITA (Spilianakis, Papamatheakis et al. 2000), where CIITA binds the MHC II enhanceosome complex driving MHC II transcription (Spilianakis, Papamatheakis et al. 2000). Several additional groups identified phosphorylation sites on CIITA mediated by multiple kinases including PKA, Cdc2, and ERK1/2 (Li, Harton et al. 2001, Sisk, Nickerson et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008, Bhat, Truax et al. 2010). The results of phosphorylation on these sites include inhibition of MHC II transcription, increased MHC II transcription, and the promotion of CIITA nuclear export (Li, Harton et al. 2001, Sisk, Nickerson et al. 2003, Greer, Harton et al. 2004, Voong, Slater et al. 2008, Bhat, Truax et al. 2010).

One particular site of importance is the regulatory phosphorylation site, CIITA S280 (Bhat, Truax et al. 2010). This site was shown to be phosphorylated, allowing for three mono-ubiquitination events, which result in activation of CIITA and ultimately result in initiation of MHC II expression (Bhat, Truax et al. 2010). However, the kinase responsible for this important phosphorylation modification remains to be identified.

Ubiquitination has also been explored as an important modification occurring on CIITA (Greer, Zika et al. 2003, Bhat, Truax et al. 2010). Recent evidence shows CIITA is modified by ubiquitination, which is central for enhanced CIITA transactivation and for MHC II transcription

(Greer, Zika et al. 2003, Bhat, Truax et al. 2010). Greer et. al., indicated CIITA is modified by ubiquitination, that mono-ubiquitinated CIITA is important in CIITA transactivity and MHC II transcription, and that mono-ubiquitination is necessary for CIITA to associate with MHC class II enhanceosome components NF-Y and RFX5 (Greer, Zika et al. 2003). Greer et. al. further established links between the HAT pCAF and ubiquitination. CIITA's ubiquitination is enhanced in the presence of pCAF; however the relationship between the two proteins remained to be fully elucidated (Greer, Zika et al. 2003).

To fully understand the scope and impact of CIITA's PTM landscape, we investigated the enzymes responsible for regulating CIITA phosphorylation and ubiquitination. Our initial goal was to identify the kinase responsible for phosphorylating CIITA at the regulatory site, S280. Previous work in our lab identified this serine residue as a "gate keeper" for CIITA transactivation (Bhat, Truax et al. 2010). Work by Drozina et. al. established that CIITA isoform I, which is expressed in dendritic cells and macrophages, also contains a regulatory site at S357 (Drozina, Kohoutek et al. 2006) which is similar to the regulatory site in CIITA IF3 and is phosphorylated by the kinase complex ERK1/2 (Drozina, Kohoutek et al. 2006).

To begin our investigation we focused on the kinase complex ERK1/2, as the two isoforms are similar in function, domain structure, and activity (Muhlethaler-Mottet, Otten et al. 1997). Our initial observations showed CIITA IF3 co-precipitates with either ERK1, ERK2, or as a complex of ERK1/2. We established ERK1/2 as responsible for mediating phosphorylation of CIITA S280; as revealed in *in vivo* phosphorylation assays. Phosphorylated CIITA appears as a doublet at 145kDa and 149kDa, with the upper band being indicative of the phosphorylated

form. Treatment with λ phosphatase or the specific MEK inhibitor U0126 resulted in the loss of the upper band, indicating loss of phosphorylation. Inhibition of phosphorylation by U0126 demonstrates ERK1/2 involvement in phosphorylation. The S280A CIITA mutant failed to be phosphorylated and further, the S280A CIITA mutant was unstable when treated with either inhibitor. Together these data indicate the CIITA regulatory site S280 is phosphorylated by the kinase complex ERK1/2.

We next investigated the role of phosphorylation in regulating CIITA's transient activation. Previous findings identified CIITA S280 phosphorylation is required for mono-ubiquitination, and increased CIITA activity. Overexpression of ERK1/2 increased CIITA activity by 2 fold as compared to when the CIITA S280A mutant was overexpressed with ERK1/2. Further, MHC II mRNA levels of WT CIITA overexpressed in the presence of ERK1/2 are significantly increased over levels of MHC II mRNA generated when CIITA S280A is overexpressed with ERK1/2. The role of MHC II in presenting extracellular antigen to CD4⁺ T cells requires that MHC II molecules are properly transcribed, translated, and shuttled to the cell surface. Flow cytometry analysis further confirmed phosphorylation of CIITA at S280 by ERK1/2 is a crucial PTM as surface MHC II is also increased when ERK1/2 is overexpressed. ERK1/2 phosphorylation also contributed to increased CIITA half-life. Together these observations support that ERK1/2 phosphorylation of CIITA at S280 is vital to CIITA regulation, and thus to MHC II transcription.

We next investigated the relationship between CIITA phosphorylation and ubiquitination. Triple mono-ubiquitination of CIITA at K315, 330, and 333 leads to increased CIITA activity following phosphorylation of CIITA S280. ERK1/2 phosphorylated CIITA increases global

CIITA ubiquitination; however, inhibition of endogenous ERK1/2 resulted in decreased global CIITA ubiquitination, indicating ERK1/2 phosphorylation is required for CIITA ubiquitination. We determined CIITA's ubiquitination landscape is composed of both mono and poly-ubiquitination and identified K48 and K63 linked poly-ubiquitination marks on CIITA. These are novel observations, as K63 poly-ubiquitination had previously not been identified on this immune regulator. We further noted that CIITA K63 ubiquitination levels were amplified when ERK1/2 was overexpressed. Further investigation identified that CIITA is heavily K63 poly-ubiquitinated in the cytoplasm and, upon ERK1/2 activation, that CIITA is phosphorylated. K63 poly-ubiquitinated CIITA then translocates to the nucleus. Our data uncovers a new modification and another layer to PTM regulation of CIITA and further establishes K63 poly-ubiquitination as an important modification for phosphorylation of CIITA by ERK1/2.

While ubiquitination is often associated with degradation (Ciechanover 1994, Ciechanover 1994, Ciechanover 1998, Ciechanover 2010), roles for ubiquitin go far beyond signaling protein degradation (Ciechanover 1994, Ciechanover 1998). Our previous findings identified a novel CIITA ubiquitination modification and linked it to ERK1/2 phosphorylation. We next sought to determine the E3 ligase involved in mediating ubiquitination of CIITA. Previous reports from Greer et. al., showed an increase in CIITA ubiquitination when the HAT pCAF is overexpressed (Greer, Zika et al. 2003). Recent investigations identified pCAF as a dual functioning enzyme, able to act as both HAT and ubiquitin E3 ligase (Linares, Kiernan et al. 2007, Mazza, Infante et al. 2013). Hdm2/Mdm2 and Gli1 have thus far been the only identified substrates of pCAF's E3 ligase function (Linares, Kiernan et al. 2007, Mazza, Infante et al. 2013). To determine if pCAF could also be the ligase for CIITA, we first showed CIITA

ubiquitination and transactivity levels significantly decreased in the presence of the Δ E3 ligase pCAF mutant. CIITA ubiquitination levels were unchanged in the presence of the Δ HAT pCAF mutant, indicating pCAF's ability to mediate CIITA ubiquitination are independent of its HAT domain and functions. Additionally, CIITA acetylation null mutants, displayed K48 ubiquitination in the presence of pCAF, indicating pCAF has the ability to mediate the degradative form of ubiquitination on a non-viable protein. *In vitro* ubiquitination analysis with purified human recombinant proteins demonstrates pCAF's ability to facilitate ubiquitination of CIITA and further identifies CIITA as an additional target for pCAF's E3 ligase function. CIITA ubiquitin modifications include mono, K48, and K63 poly-ubiquitination (Bhat, Truax et al. 2010) and *in vivo* analysis indicate pCAF's ability to mediate all of the above ubiquitination reactions.

Based on our observations, we propose un-activated CIITA is located in the cytoplasm and, once activated, is modified with K63 linked ubiquitination by pCAF. The role of K63 linked ubiquitination for regulating CIITA phosphorylation is complex, however several potential scenarios exist: K63 ubiquitination could be acting as a scaffold where ERK1/2 then binds and phosphorylates CIITA driving CIITA nuclear localization. Alternatively, K63 poly-ubiquitination may assist in the activation of ERK1/2, as has been seen in other kinases. We favor the former of these scenarios. Once CIITA is K63 poly-ubiquitinated by pCAF and phosphorylated by ERK1/2, CIITA is shuttled to the nucleus. Acetylation by pCAF is also an important driver of this process (Spilianakis, Papamatheakis et al. 2000). Mono-ubiquitination of CIITA at K315, 330, and 333 are also required for CIITA transactivity (Bhat, Truax et al. 2010) and we now know pCAF is responsible for these modifications, however, it remains unclear if

these modifications occur prior to CIITA nuclear localization. We believe once CIITA is modified by K63 poly-ubiquitination, phosphorylation at S280, mono-ubiquitination at K315, 330, and 333, and acetylation at K141 and K144, CIITA is then signaled to the nucleus where CIITA then is able to drive MHC II transcription.

Our findings contribute to the greater understanding of how CIITA is regulated and identifies interconnected PTMs important for CIITA's regulation. As the "master regulator" of MHC II genes, CIITA is a crucial transcription factor. CIITA's ultimate responsibility is to ensure transcription of MHC II at levels that will allow the adaptive immune response to be great enough to combat any assault. Involvement of CIITA in transcriptional regulation in a wide array of immune response genes like IL-4 (Sisk, Gourley et al. 2000), IL-10 (Yee, Yao et al. 2005), Fas ligand (Gourley and Chang 2001), MMP-9 (Nozell, Ma et al. 2004), E-cathepsin (Yee, Yao et al. 2004), and plexin (Wong, Brickey et al. 2003) indicates that CIITA may be involved in disease development. Thus, dysregulation of CIITA's PTMs can contribute to either too much/little, or no CIITA activity, resulting in an ineffective MHC II transcription. Therefore, identifying CIITA's PTMs and enzymes involved, like E3 ligase pCAF; moves us towards uncharted territory of deciphering specific PTMs involved in various diseases in which CIITA regulation is involved. CIITA has been shown to be involved in a host of diseases including multiple tumor types and autoimmune conditions (Yazawa, Kamma et al. 1999, Drozina, Kohoutek et al. 2005, Truax, Thakkar et al. 2012, Osborn and Greer 2015). Our findings combined with previous studies of the PTMs of CIITA contribute to a greater understanding of the landscape of modifications and their roles in regulating CIITA. Additionally, identification of the enzymes facilitating these crucial and necessary modifications provides excellent novel

targets for therapeutic strategies to manipulate the expression of CIITA and MHC II. Our findings further bring to light many unanswered questions regarding the regulation of CIITA. While, many modifications have been determined, limitations of methods have hampered the ability to determine the sequence of modifications. While we identified a novel ubiquitination modification to CIITA, the sites for the K63 poly-ubiquitination have yet to be determined. However, mass spectrometry analysis is an excellent and reliable method for identifying specific amino acid residues modified by ubiquitination and will assist in identifying K63 poly-ubiquitination site(s) on CIITA (Denis, Vasilescu et al. 2007). While the PTM profile of CIITA has expanded considerably, understanding the immune modulated signals that trigger and regulate CIITA's PTMs is an area that needs to be further explored. Identification of pCAF as an E3 ligase involved in CIITA ubiquitination is an important step in understanding CIITA's regulation; however, the enzyme involved in deubiquitinating (DUB) CIITA remains elusive. Identifying the DUB is important in understanding CIITA's ubiquitin portfolio, and could be used as therapeutic drug targets. Future work will focus on identifying the DUB enzyme involved in removal of CIITA's ubiquitination, additionally future work will focus on deciphering the sequential order of CIITA's PTMs.

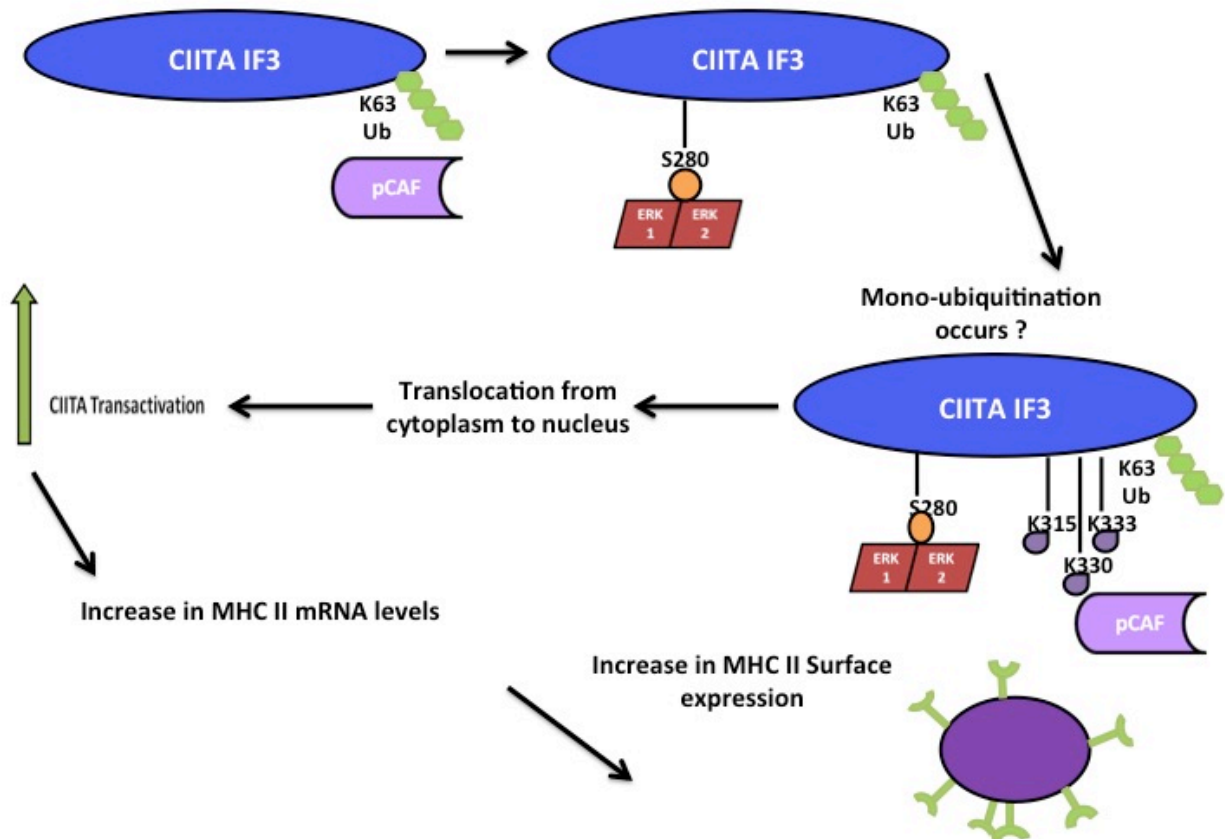


Figure 4.1: CIITA Posttranslational Modifications and mediating enzymes.

CIITA is regulated through a series of posttranslational modifications. Here we show that CIITA is modified by Lysine (K) 63 poly-ubiquitination by the E3 ligase pCAF, which allows for the regulatory phosphorylation site Serine (S) 280 to be phosphorylated by the kinase complex, ERK1/2. Additionally, CIITA is also mono-ubiquitinated by pCAF at K315, 330, and 333. Together these modifications allow for CIITA to translocate to the nucleus and increase CIITA's transactivity levels. Increased transactivity allows for more MHC II mRNA and ultimately increase in MHC II cell surface expression, which allows for antigen to be presented to CD⁺ T cells and ultimately the initiation of the adaptive immune response.

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