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# ROLES OF THE UBIQUITIN-PROTEASOME SYSTEM AND MONO-UBIQUITINATION IN REGULATING MHC CLASS II TRANSCRIPTION

by

KAVITA PURNANDA BHAT

Under the Direction of Susanna F. Greer, PhD

## ABSTRACT

Major Histocompatibility Complex (MHC) class II molecules are indispensable arms of the immune system that present extracellular antigens to CD4<sup>+</sup>T cells and initiate the adaptive immune response. MHC class II expression requires recruitment of a master regulator, the class II trans-activator (CIITA). How this master transcriptional regulator is recruited, stabilized and degraded is unknown. The 26S proteasome, a master regulator of protein degradation, is a multi-subunit complex composed of a 20S core particle capped on one or both ends by 19S regulatory par-

ticles. Previous findings have linked CIITA and MHC class II transcription to the ubiquitin proteasome system (UPS) as mono-ubiquitination of CIITA increases its transactivity whereas poly-ubiquitination targets CIITA for degradation. Increasing evidence indicates individual ATPase subunits of the 19S regulator play non-proteolytic roles in transcriptional regulation and histone modification. Our initial observations indicate proteasome inhibition decreases CIITA transactivity and MHC class II expression without affecting CIITA expression levels. Following cytokine stimulation, the 19S ATPase Sug1 associates with CIITA and with the MHC class II enhanceosome complex. Absence of Sug1 reduces promoter recruitment of CIITA and proteasome inhibition fails to restore CIITA binding, indicating Sug1 is required for CIITA mediated MHC class II expression. Furthermore, we identify a novel N-terminal 19S ATPase binding domain (ABD) within CIITA. The ABD of CIITA lies within the Proline/Serine/Threonine (P/S/T) region of CIITA and encompasses a majority of the CIITA degron sequence. Absence of the ABD increases CIITA half-life, but blocks MHC class II surface expression, indicating that CIITA requires interaction with the 19S ATPases for both its deployment and destruction. Finally, we identify three degron proximal lysine residues, lysines (K): K315, K330 and K333, and a phosphorylation site, serine (S), S280, located within the CIITA degron, that regulate CIITA ubiquitination, stability and MHC class II expression. These are the first lysine residues identified as sites of CIITA ubiquitination that are essential for MHC class II expression. These observations increase our understanding of the role of the UPS in modulating CIITA mediated MHC class II transcription and will facilitate the development of novel therapies involving manipulation of MHC class II gene expression.

## INDEX WORDS:

Major Histocompatibility Complex class II, Class II transactivator, Transcription regulation, 26S proteasome, 19S proteasome, Ubiquitination, Degradation, 19S ATPases, Sug1

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REGULATING MHC CLASS II TRANSCRIPTION

by

KAVITA PURNANDA BHAT

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

2010

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Kavita Purnanda Bhat  
2010

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REGULATING MHC CLASS II TRANSCRIPTION

by

KAVITA PURNANDA BHAT

Committee Chair: Susanna Greer, PhD

Committee: Margo Brinton, PhD

Zhi-Ren Liu, PhD

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2010

## **DEDICATION**

I would like to dedicate this document to my husband Purna, my loving daughter Diya and my parents Jaya and Laxman. They have supported me during rough times in life and I would not be able to do this without their love, support and guidance.



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I am grateful to have the opportunity to work in a stimulating and friendly environment like the Greer lab. We spend most of our time in the lab and having a great work environment is absolutely essential for success. We have all gone through bad data, stressful exams and manuscript rejections, but we have always stood beside each other like a family and supported each other. For that I would like to thank all my colleagues, especially Aga Truax.

Last but never the least; I would like to thank my husband Purna Bhat and my daughter Diya Bhat for their unconditional love and support through all these years. Without their continuing support, it would have been impossible for me to achieve this. My parents, Jaya and Laxman Nayak have provided great support and love through my life. I would like to take this opportunity to thank my family for always being there for me.

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## LIST OF ABBREVIATIONS

Acetylation (Ac)  
Acidic Activation Domain (AAD)  
Adenosine triphosphate (ATP)  
ADP-ribosylation factor-binding protein1 (ARF-BP1)  
Anaphase-promoting complex (APC/C)  
Antigen Presenting Cells (APCs)  
Apoptosis-antagonizing transcription factor (AATF)  
ATP dependent proteolysis factor 1 (APF-1)  
Bare Lymphocyte Syndrome (BLS)  
Base-excision repair (BER)  
BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE)  
Camp Responsive Element Binding Protein (CREB)  
CDK-inhibitor (CKI)  
Cell division control protein (Cdc),  
Chromatin Immunoprecipitation (ChIP)  
Class II Transactivator (CIITA)  
Co-Immunoprecipitation (Co-IP)  
Constitutive photomorphogenesis protein 1(COP1)  
C-Terminal Domain (CTD)  
Cyclin Dependent Kinases (Cdks)  
Deubiquitinating enzymes (DUBs)  
Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1)  
Double stranded break (DSB)  
DRB-Sensitive Inducing Factor (DSIF)  
E6 associated protein (E6-AP)  
Endosomal sorting complex for transportation machinery (ESCRT)  
Epidermal growth factor receptor (EGFR)  
Fanconi anemia (FA)  
Fanconi anemia complementation group I protein (FANCI)



Fanconi anemia group D2 protein (FANCD2)  
Flag (Fg)  
Foxhead box O4 (FOXO4)  
General control of amino-acid synthesis 5 (GCN5)  
Glutathione-S-Transferase (GST)  
GTP-Binding Domain (GBD)  
Hexamethylene Bisacetamide Inducible Protein (Hexim)  
Histone Acetyltransferase (HAT)  
Histone Methyltransferase (HMT)  
Huntington's disease (HD)  
Immunoblot (IB)  
Immunoprecipitate (IP)  
Interferon- $\gamma$  (IFN-  $\gamma$ )  
Isoform I (IFI)  
Isoform III (IFIII)  
Leucine Rich Region (LRR)  
Major Histocompatibility Complex (MHC)  
Membrane-associated RING-CH (MARCH1)  
Metastasis-associated protein (MTA)  
Methyltransferase (Mgt1)  
Modulator of immune recognition (cMIR)  
Mouse double minute 2 (Mdm 2)  
Multivesicular endosomal (MVE)  
Multivesicular body (MVB)  
Negative Transcription Elongation Factor (N-TEF)  
Neural precursor cell expressed developmentally down regulated (NEDD8)  
Neurofibrillary tangles (NFT)  
Nuclear Factor-Y Complex (NFY)  
Nuclear Localization Sequences (NLS)  
Nuclear receptor subfamily 4 group A member2 (NR4A2)  
Nucleosome remodeling histone deacetylation complex (NuRD),  
Nucleotide excision repair (NER)  
O<sup>6</sup>-Alkylguanine-DNA alkyltransferase (AGT)

O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT)  
Paired helical filaments (PHF)  
Parkinson's disease (PD)  
Phosphorylation (P)  
Poly-ADP ribose polymerase-1 protein (PARP1)  
Polyglutamine (polyQ)  
Positive Transcription Elongation Factor (P-TEFb)  
Proline/Serine/Threonine Rich (P/S/T) Domain  
Proteasome-Associated Protein (PAAF-1)  
Protein Kinase (PKA)  
Radiation sensitivity protein 6 (Rad6)  
Regulatory Factor X Complex (RFX)  
Retinoic acid (RA)  
Ring finger protein (RFP)  
RNA Polymerase II (RNA Pol II)  
Serine 2 (Ser2)  
Serine 5 (Ser5)  
Severe combined immunodeficiency (SCID)  
Short Hairpin RNA (shRNA)  
Short Interfering RNA (siRNA)  
Skp1-culin-F-box complex (SCF)  
Small ubiquitin like modifier (SUMO)  
Topoisomerase I-binding RING protein (Topors)  
Transcription Factor II H (TFIIH)  
Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ )  
Ub-Activating Enzyme (E1)  
Ub-Conjugating Enzyme (E2)  
Ubiquitin (Ub)  
Ubiquitin carboxyl-terminal esterase (UCHL-1)  
Ubiquitin proteasome system (UPS)  
Ubiquitination (Ubn)  
Ub-Ligase (E3)  
Untreated (NT)

## CHAPTER 1

### INTRODUCTION

THE 26S PROTEASOME, IT'S NOT JUST DEGRADING ANYMORE!

PUBLICATION: KAVITA P. BHAT AND SUSANNA F. GREER AS SUBMITTED TO BBA

– GENE REGULATORY MECHANISMS, JANUARY 22, 2010.

## THE UBIQUITIN PROTEASOME SYSTEM

Work in the late 1930's by Schoenheimer first indicated the cellular pool of proteins is in a "dynamic state" involving constant synthesis and degradation (Olson 1997). We now know the process of protein degradation carried out by the ubiquitin proteasome system (UPS) plays a critical role in the maintenance of this cellular homeostasis. Ubiquitination is the term applied to the enzymatic cascade that covalently attaches ubiquitin, a 76 amino acid protein, to a substrate protein. The 26S proteasome is itself a multi-subunit complex known as the master regulator of protein degradation. The role of ubiquitin and the 26S proteasome in protein turnover was comprehensively described in 1970s and early 1980s (Hochstrasser 1996; Ciechanover 2009) and in the last two decades the ubiquitin proteasome pathway has been demonstrated to play crucial roles in cellular activities as diverse as cell cycle regulation, DNA damage repair, signal transduction, membrane trafficking, neural development and transcription. While it is well accepted that changes in proteolytic functions of the UPS lead to deregulation of cellular function and disease development, recent studies have introduced differential ubiquitination pathways and non-proteolytic functions of the UPS and have demonstrated their equally significant impact on cellular function and disease.

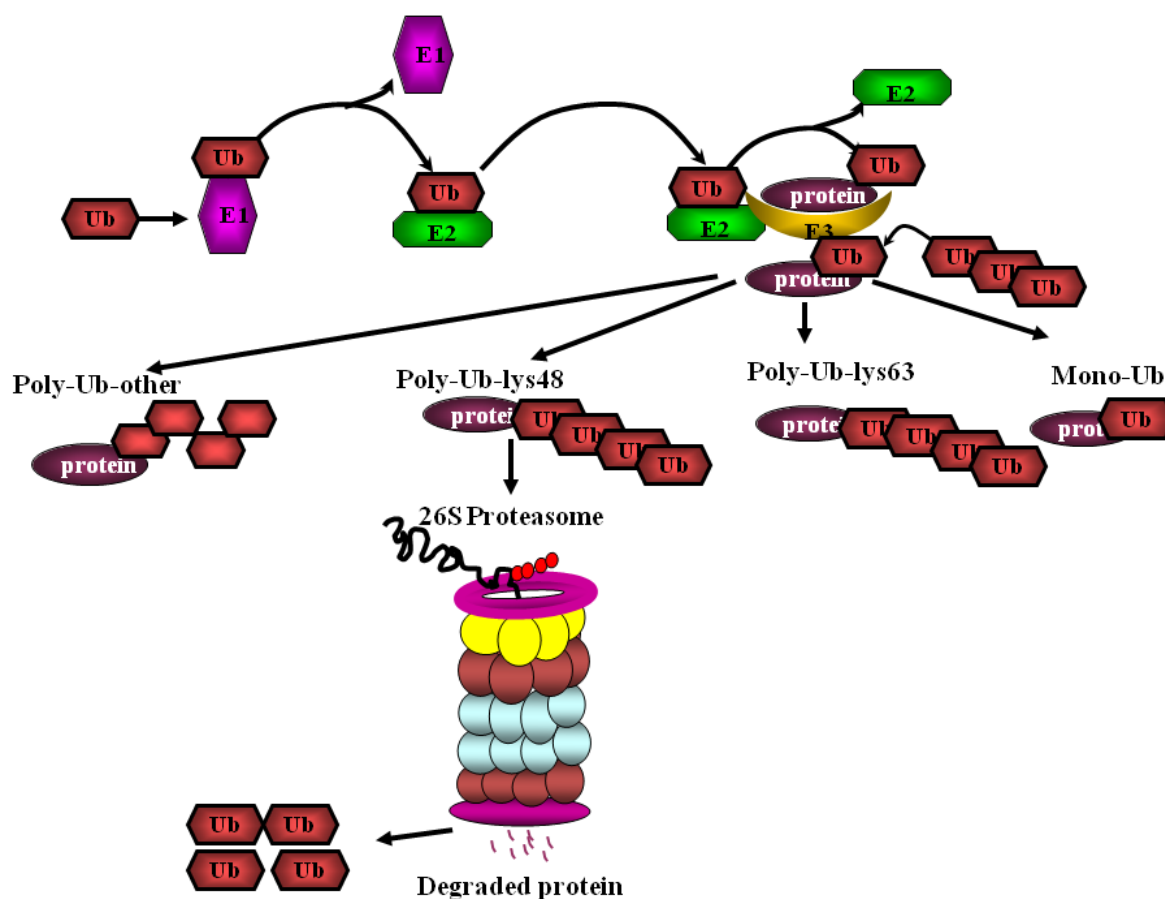
Here, the ubiquitin pathway and the 26S proteasome are discussed with emphasis on the process and forms of ubiquitination, the structural components of the 26S proteasome and the impact of proteasome diversity. Furthermore, we summarize recent findings indicating non-proteolytic roles for components of the UPS in regulating a multitude of cellular processes and also indicate their significant impact on disease development. The complex roles of the UPS in these diverse cellular functions impact epigenetics, transcription and post-translational modifications. The proteolytic and non-proteolytic roles of UPS discussed herein are intended to provide

increased understanding of the significant contributions of the UPS in regulating cellular functions.

### THE UBIQUITIN PATHWAY

The process of protein ubiquitination and degradation was first identified by Aron Ciechanover, Avram Hershko and Irwin Rose for which they received the Nobel prize in Chemistry (Hershko and Ciechanover 1998). Ubiquitin, initially termed adenosine triphosphate (ATP) dependent proteolysis factor 1 (APF-1) (Ciechanover, Hod et al. 1978), is a highly conserved, 8.5kD, 76 amino acid protein which is ubiquitously expressed in eukaryotic cells (Ciechanover 1998; Hershko and Ciechanover 1998; Yang and Yu 2003; Zhang, Wang et al. 2004; Shmueli and Oren 2005; Taylor and Jobin 2005; Xu and Peng 2006). Although cells have multiple copies of ubiquitin genes tandemly arranged without intronic sequences, ubiquitin proteins are expressed as monomers (Hegde 2004). However, ubiquitin contains a unique propensity to form multimeric chains once attached to target substrates (Ciechanover 1998; Hershko and Ciechanover 1998; Yang and Yu 2003; Zhang, Wang et al. 2004; Shmueli and Oren 2005; Taylor and Jobin 2005; Xu and Peng 2006).

Ubiquitination is a multi-step process involving the covalent addition of ubiquitin molecules to lysine residues within target proteins. Substrate ubiquitination is mediated by an ATP-dependent enzymatic cascade involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-ligase (E3) (**Figure 1.1**)



**Figure 1.1: Ubiquitination.**

Ubiquitination is an enzymatic cascade involving a ubiquitin (Ub)-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-ligase (E3). During ubiquitination, Ub is activated by the E1 Ub-activating enzyme and then transferred to an E2 Ub-conjugating enzyme. The E3 Ub-ligase enzyme recognizes and binds both the target substrate and the Ub-E2 enzyme and transfers Ub to the target substrate. Ubiquitination of target substrates is associated with new cellular functions (via monoubiquitination and lys 63 linked poly-ubiquitination) or degradation (via lys 48 linked poly-ubiquitination). Figure adapted from Hegde et.al., 2004, Taylor et.al., 2005, Wickliffe et.al., 2009, Haglund et.al., 2005.


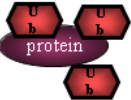




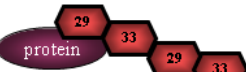

(Deshaies 1995; Hochstrasser 1995; Hochstrasser 1996; Hershko and Ciechanover 1998). E1 is encoded by a single highly conserved gene with two isoforms, E1a and E1b (Cook and Chock 1992). Both E1 isoforms lack specificity for target proteins, but are essential for cellular function as inactivation of E1 is lethal to cells (Ciechanover 1994). By comparison, there are more than 50 genes encoding for E2 and more than 500 genes encoding for E3 (Chen, Seth et al. 2006; Lehman 2009), and, as each E2 can interact with multiple E3s, numerous E2/E3 combinations make possible increased substrate specificity (Lehman 2009).

To initiate substrate ubiquitination, the C-terminal glycine residue of free ubiquitin is activated by the E1 ubiquitin-activating enzyme. Ubiquitin activation involves the formation of an ubiquitin-adenylate intermediate, the release of  $PP_i$ , transfer of ubiquitin-adenylate to a cysteine residue in E1 via a thiolester bond, and subsequent release of AMP (Deshaies 1995; Hochstrasser 1995; Hochstrasser 1996). Activated ubiquitin is transferred to a cysteine residue on the E2 ubiquitin-conjugating enzyme via a thiolester bond (Deshaies 1995; Hochstrasser 1995; Hochstrasser 1996; Hershko and Ciechanover 1998). Transfer of activated ubiquitin from E2 to target protein is orchestrated by an E3 ubiquitin ligase which recognizes the target substrate and catalyzes transfer via an isopeptide bond between the C-terminal glycine residue of ubiquitin and the  $\epsilon$ -amino group of an internal lysine residue of the substrate (Deshaies 1995; Hochstrasser 1995; Hochstrasser 1996; Hershko and Ciechanover 1998).

The product of this enzymatic cascade is a mono-ubiquitinated protein. Mono-ubiquitination has been linked to roles in transcription via regulation of histones and transcription factors (Greer, Zika et al. 2003; Muratani and Tansey 2003; Rasti, Grand et al. 2006; Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007) and to various cellular functions including receptor transport, viral replication, cell cycle regulation, localization of proteins and DNA repair (Hicke

2001; Di Fiore, Polo et al. 2003; Greer, Zika et al. 2003; Gregory, Taniguchi et al. 2003; Haglund, Sigismund et al. 2003; Brenkman, de Keizer et al. 2008). Consecutive addition of ubiquitin molecules to internal lysine residues of substrate conjugated mono-ubiquitin leads to the formation of poly-ubiquitin chains (Pickart and Fushman 2004). The formation of poly-ubiquitin chains is frequently catalyzed by substrate specific E3 ligases; however, recent studies have demonstrated the involvement of an additional conjugating enzyme, E4, in the formation of lysine 48 linked polyubiquitin chains (Koegl, Hoppe et al. 1999). The first E4 to be identified was the yeast protein, ubiquitin fusion degradation protein 2 (UFD2) which catalyzes ubiquitin chain extensions (Koegl, Hoppe et al. 1999) and harbors the U-box motif that renders ubiquitin ligase activity (Koegl, Hoppe et al. 1999). Although, poly-ubiquitin chains can be formed at any of the seven internal lysine residues (K 6, 11, 27, 29, 33, 48 and 63) in ubiquitin (Johnson, Ma et al. 1995; Peng, Schwartz et al. 2003; Kim, Kim et al. 2007), in eukaryotes poly-ubiquitination can occur via lysine 48 or lysine 63 or can be atypical. Proteins poly-ubiquitinated via lysine 48 are targeted for protein degradation by the 26S proteasome, whereas proteins poly-ubiquitinated via lysine 63 are directed towards various cellular processes and are eventually degraded in lysosomes (Tan, Wong et al. 2007). Atypical ubiquitination includes mixed chain ubiquitination, heterologous ubiquitination, and multiple mono-ubiquitination (Ikeda and Dikic 2008). Mixed chain ubiquitination occurs when poly-ubiquitin chains are formed via different lysine residues within ubiquitin (lysine 29/48 ubiquitin chains for example) (Kim, Kim et al. 2007). Specific pairs of E2 Ub-conjugating enzymes and E3 Ub-ligating enzymes, including the E2 Ubch5 and the E3 mouse double minute 2 (Mdm 2), are capable of forming mixed-linkage poly-ubiquitin chains (Kim, Kim et al. 2007). Heterologous ubiquitin chains involve ubiquitin like modifiers such as the small ubiquitin like modifier (SUMO) (Tatham, Jaffray et al. 2001) or the neural



<b>Homotypic ubiquitination</b>		<b>Mono-ubiquitination</b>
		<b>Multiple Mono-ubiquitination</b>
		<b>K48-linked poly-ubiquitination</b>
		<b>K11-linked poly-ubiquitination</b>
		<b>K63-linked poly-ubiquitination</b>
		<b>Others</b>
<b>Mixed chain ubiquitination</b>		<b>K29/48, 27/29, 29/33 mixed chain poly-ubiquitination</b>
<b>Heterologous ubiquitination</b>		<b>SUMOylation or NEDDylation</b>

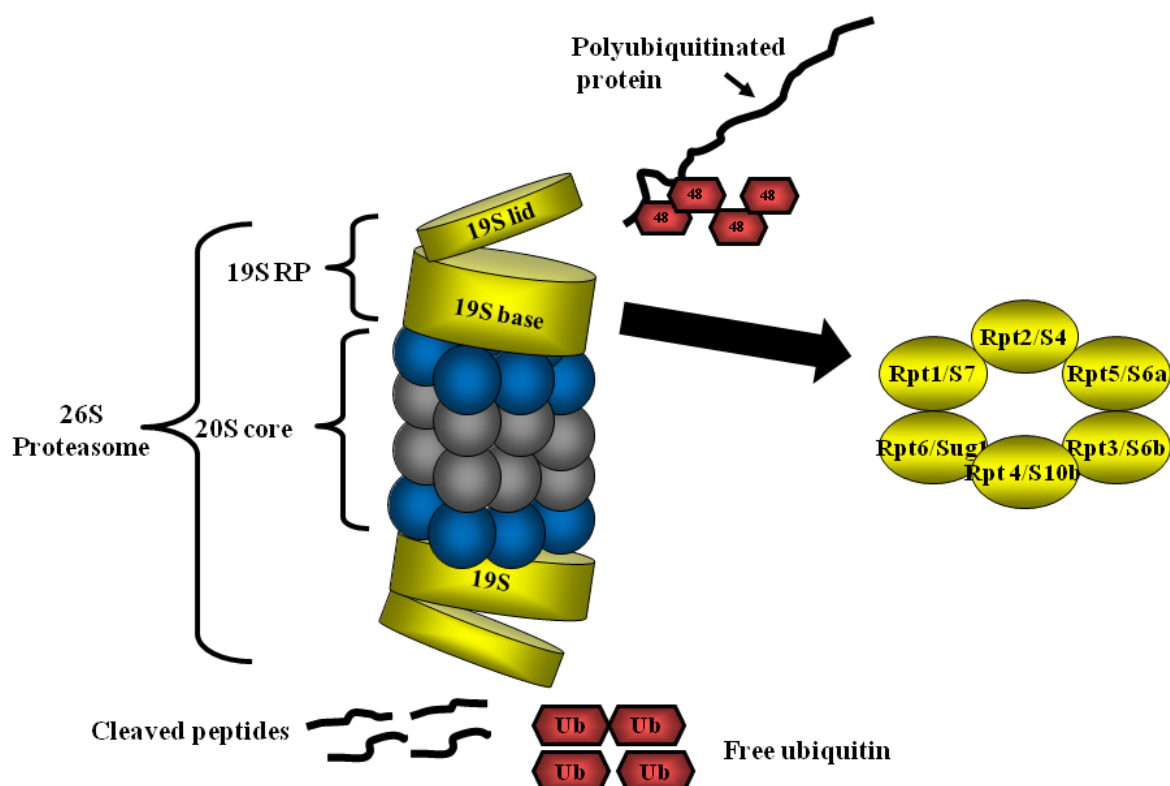
**Figure 1.2: Ubiquitination status determines protein fate.**

Addition of one ubiquitin to target protein leads to mono-ubiquitination. Multiple additions of single ubiquitin moieties to different lysine residues on target proteins is termed multiple mono-ubiquitination. Subsequent addition of ubiquitin to internal lysine residues of the previously added ubiquitin moiety is termed poly-ubiquitination. Poly-ubiquitination occurs at one of the 7 internal lysine residues in ubiquitin (lys 6, 11, 27, 29, 33, 48 and 63). Poly-ubiquitination via lysine 48 is considered to be the typical form of poly-ubiquitination. Atypical forms of ubiquitination include homotypic ubiquitination, mixed chain ubiquitination, heterologous ubiquitination and multiple mono-ubiquitination. Homotypic ubiquitination occurs when poly-ubiquitin chains are formed via the same lysine residues within ubiquitin (for example: a lysine 63 poly-ubiquitin chain). Alternatively, mixed chain ubiquitination occurs when poly-ubiquitin chains are formed via different lysine residues within ubiquitin (for example: a lysine 29/48 poly-ubiquitin chain). Formation of heterologous ubiquitin chains involves additional ubiquitin like modifiers such as SUMO and NEDD8 in addition to ubiquitin.

precursor cell expressed developmentally down regulated (NEDD8) (Xirodimas, Sundqvist et al. 2008) in addition to ubiquitin. Multiple mono-ubiquitination involves the addition of mono-ubiquitin to multiple lysine residues in target proteins (Liu, Aneja et al. 2008). A comprehensive chart of the various ubiquitination patterns and their known roles in cellular function is shown in **Figure 1.2**.

### THE PROTEASOME

The 26S proteasome is an approximately 2.5 MDa complex composed of more than 31 subunits which was partially purified from rabbit reticulocytes by Hough *et al* (1986) and was characterized by Waxman and colleagues as a “high molecular weight alkaline protease” which was renamed as the 26S proteasome (Hough, Pratt et al. 1986; Waxman, Fagan et al. 1987). The 26S is composed of a 20S core particle capped on one or both ends by 19S regulatory particles (RP) or PA700 (**Figure 1.3**) (Hough, Pratt et al. 1987; Hoffman, Pratt et al. 1992; Tanaka 2009). The 19S and 20S sub-complexes can exist together or independently of each other in both nuclear and cytoplasmic compartments (Peters, Franke et al. 1994). Discrepancies exist regarding the nomenclature assigned to the 26S, which was originally named based on sedimentation coefficients as measured by density-gradient centrifugation (Tanaka 2009). Yoshimura and colleagues have shown that the sedimentation coefficient of the 26S proteasome in solution is “30S” according to physicochemical calculations (Yoshimura, Kameyama et al. 1993). The difference may be due to the “26S” proteasome containing one 19S regulatory particle on either end of the 20S core while the “30S” proteasome contains two 19S regulatory particles on either end of the 20S core



**Figure 1.3: The 26S Proteasome.**

The 26S proteasome is composed of a 20S proteolytic core and a 19S regulatory particle (RP). The 20S core particle is the proteolytic center of the proteasome and is a 670kDa complex composed of 28 subunits. The core is a hollow cylindrical structure composed of two heptameric rings of  $\alpha$  subunits and two heptameric rings of  $\beta$  subunits ( $\alpha\beta\alpha$ ). Capping one or both of the 20S core are 19 S regulators. The 19S regulatory particle, also known as “PA 700” or “Proteasome activator 700,” is a large complex of approximately 700 KDa. The 19S is composed of a “base” component and a “lid” component with nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15) in the lid and four non-ATPase (Rpn1, Rpn2, Rpn10 and Rpn13) and six ATPase subunits (Rpt1-6) in the base. Polyubiquitinated proteins are tagged for degradation, recognized and unfolded by the 19S ATPases, and are translocated to the 20S core. The 20S cleaves the protein into peptides and free ubiquitin is released.

(Tanaka 2009). For the remainder of this review, ‘26S’ will be used to describe the functional proteasome with a 19S regulatory particle on either end of the 20S core.

### The 20S Core Particle

The 20S core particle is a 670kDa complex composed of 28 subunits and is the proteolytic center of the proteasome (Navon and Ciechanover 2009). This hollow cylindrical structure is composed of two heptameric rings of  $\alpha$  subunits and two heptameric rings of  $\beta$  subunits with an  $\alpha_{1-7} \beta_{1-7} \beta_{1-7} \alpha_{1-7}$  structure (Burkhardt, Lothar et al. 1992; Groll, Ditzel et al. 1997). The  $\alpha$ -subunits in the outer rings of the 20S core serve to recognize and direct poly-ubiquitinated substrates into the proteolytic center. The N-terminus of the  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_4$  subunits forms a gate that protects the opening to the proteolytic center. Binding of the regulatory 19S particle to the 20S core triggers a conformational change in the N-terminus of the  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_4$  subunits which causes the gate to open and allows the passage of proteins into the proteolytic center (Jung, Catalgol et al. 2009). Each of the  $\alpha$ -subunits has a highly conserved YRD-motif (Tyr8-Asp9-Arg10) which functions as a hinge and is essential for the opening and closing of the gate.

Poly-ubiquitinated proteins targeted for degradation are processed by deubiquitinating enzymes (DUBs) before they are channeled into the proteolytic core (Hegde 2004). The DUBs remove the poly-ubiquitin tag from the substrate, following which the protein is channeled through the proteolytic core for degradation (Hegde 2004). This step is crucial to prevent “clogging” of the small proteolytic chamber (13Å) by the poly-ubiquitin chain (Hegde 2004). Although the 20S proteolytic core has two  $\beta$  rings with seven  $\beta$  subunits in each ring ( $\beta_{1-7} \beta_{1-7}$ ), only three  $\beta$ -subunits:  $\beta_1$ ,  $\beta_2$  and  $\beta_5$ , which reside in each of the two  $\beta$  rings, are proteolytically active. These  $\beta$ -subunits have distinct proteolytic activities.  $\beta_1$  has a peptidyl-glutamyl-peptide-hydrolyzing, or caspase-like activity (Kisselev, Akopian et al. 1999), which cleaves after acidic

amino acids;  $\beta_2$  has a trypsin-like activity which cleaves after basic amino acids, and  $\beta_5$  has a chymotrypsin-like activity which cleaves after neutral amino acids (Loidl, Groll et al. 1999; Groll and Huber 2004). The proteolytic activities of the three  $\beta$ -subunits are due to the presence of a threonine residue in their N-terminus which acts as a nucleophile in the hydrolysis of substrate proteins (Jung, Catalgol et al. 2009; Navon and Ciechanover 2009). In addition to the N-terminal threonine residue, aspartate 17 and lysine 33 in the three  $\beta$ -subunits are also essential for the proteolytic activity of 20S core particle (Jung, Catalgol et al. 2009). The result of protein degradation via the 26S proteasome are peptides with average lengths of 8-12 amino acids and free ubiquitin that is recycled in the ubiquitination process by E1 activating enzyme (Jung, Catalgol et al. 2009).

#### The 19S Regulatory Particle (PA700)

The 20S core particle is capped on one or both sides by a 19S regulatory particle to form the active 26S proteasome. The 19S regulatory particle is a large 700 KDa complex, also known as the “proteasome activator 700” or “PA 700” (Jung, Catalgol et al. 2009; Navon and Ciechanover 2009), composed of rings of subunits that make up the “19S base” and the “19S lid” of the regulatory particle. Nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15) are found in the lid and four non-ATPase subunits (Rpn1, Rpn2, Rpn10 and Rpn13) along with six ATPase subunits (Rpt1-6) are found in the base (Jung, Catalgol et al. 2009; Tanaka 2009). The six ATPases in the 19S base interact with the  $\alpha$ -subunits of 20S core particles and, along with the non-ATPases of the 19S base and lid, have various functions in ubiquitin dependent degradation. Recent studies have also highlighted non-proteolytic role of the 19S ATPases in tran-

scription regulation which will be discussed in detail in later portions of this review. Rpt2, Rpt3 and Rpt5 contain a HbYX motif which enables them to function as “gate opening” subunits (Tanaka 2009). Rpn10 and Rpn13 function as “ubiquitin receptors” (Tanaka 2009), while Rpn11 functions as a “deubiquitinating enzyme” in the removal of poly-ubiquitin chains from substrate proteins (Tanaka 2009). Rpn1 and Rpn2, along with the six ATPases, function to translocate substrate proteins into the gate of the 20S proteolytic core (Tanaka 2009).

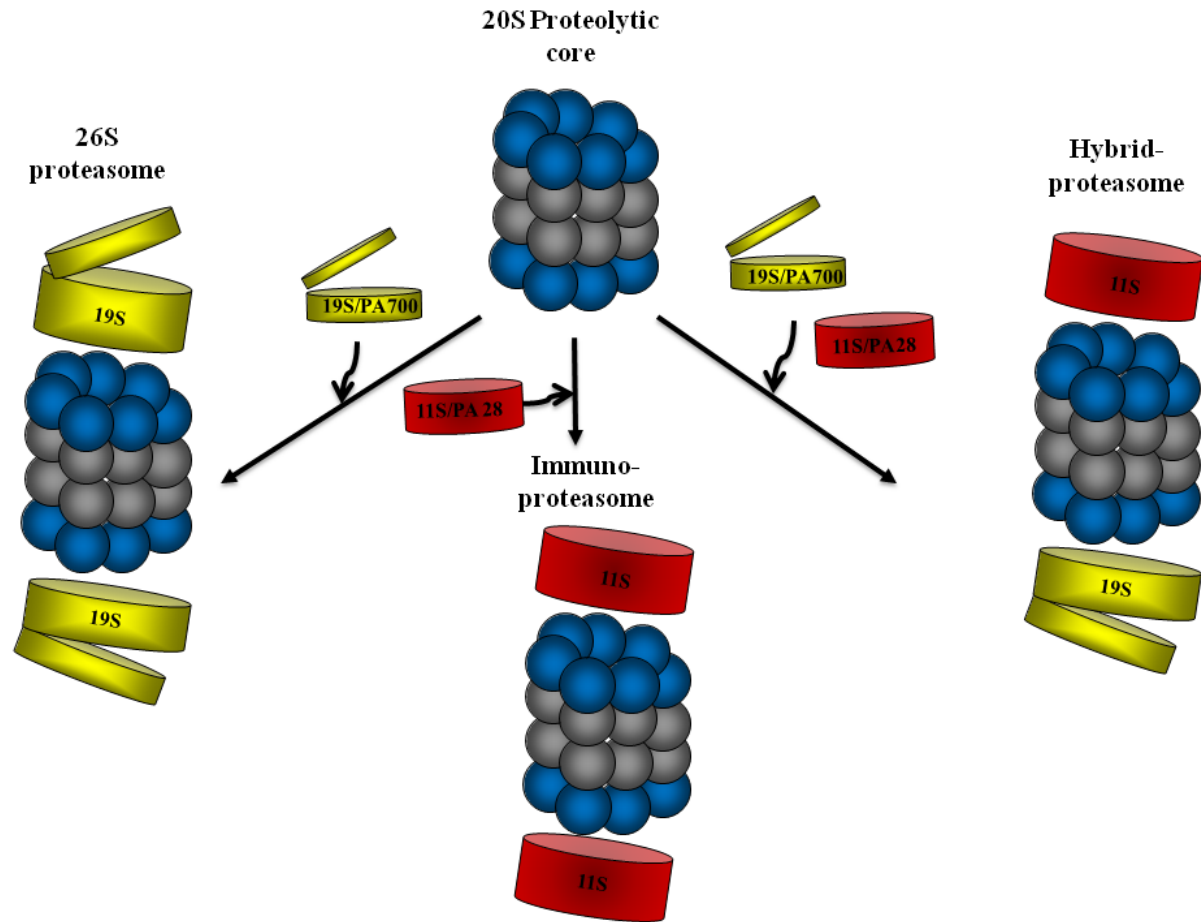
### The 11S Regulatory particle (PA28)

The 11S regulator, also known as “PA28” or “REG,” also forms a conical cap by binding to  $\alpha$ -rings on both sides of the 20S core (Jung, Catalgol et al. 2009). Once PA28 binds the 20S core, the complex demonstrates increased degradation of small or mid-sized poly-peptides and unfolded proteins in an ATP-independent manner (Jung, Catalgol et al. 2009; Tanaka 2009). PA28 is composed of  $\alpha$ ,  $\beta$  or  $\gamma$  subunits which share 50% structural homology with each other but differ in subcellular localization (Tanaka 2009). PA28 $\alpha$  and PA28 $\beta$  are primarily cytoplasmic (Jung, Catalgol et al. 2009; Tanaka 2009) and when bound to the 20S core participate in processing polypeptide antigens for Major Histocompatibility Complex (MHC) class I mediated immunity; thus their expression is induced by the inflammatory cytokine interferon- $\gamma$  (IFN- $\gamma$ ) (Tanahashi, Yokota et al. 1997). PA28 $\gamma$  is predominantly localized in the nucleus, is thought to play roles in regulating nuclear proteolysis (Jung, Catalgol et al. 2009; Tanaka 2009), and has been shown to be important in ubiquitin-independent degradation of proteins involved in cell cycle regulation, apoptosis, and mitotic progression (Tanaka 2009). For example, the cell cycle regulator p21<sup>Cip1</sup> is degraded independently of poly-ubiquitination in a PA28 $\gamma$ -dependent manner

(Jung, Catalgol et al. 2009). As PA28 $\gamma$  is also involved in degradation of lysine-free or lysine-low native proteins (which cannot be poly-ubiquitinated), PA28 $\gamma$  may also be a key player in degradation of viral proteins low in lysine residues (Jung, Catalgol et al. 2009). These diverse functions of PA28 $\gamma$  are plausibly due to its nuclear localization.

### Proteasome Diversity

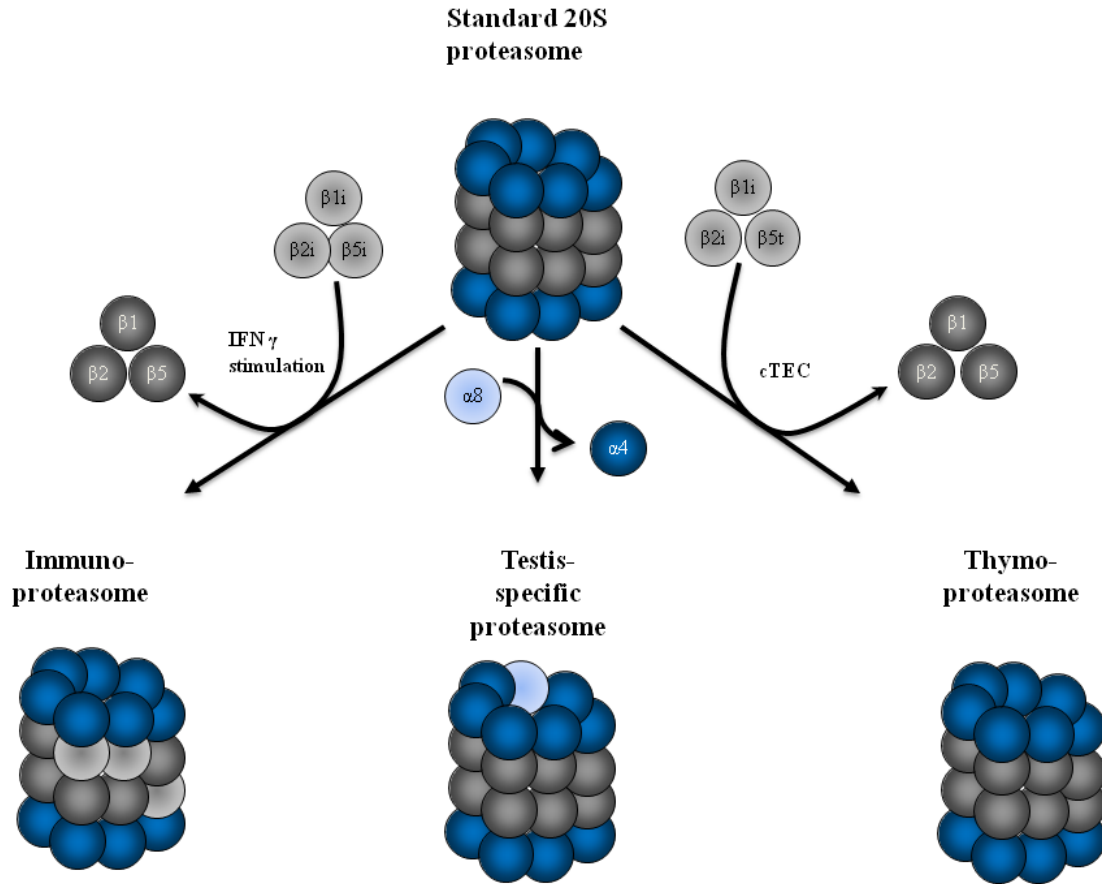
In addition to the traditional 26S proteasome (PA700-20S-PA700), Tanahashi and colleagues have shown that 11S/PA28 and 19S regulators can bind to either sides of the 20S core to form a “Hybrid Proteasome” (PA700-20S-PA28) (**Figure 1.4A**) (Tanahashi, Murakami et al. 2000). IFN- $\gamma$  increases the expression of PA28 $\alpha/\beta$  (11S), which in turn increases the formation of hybrid proteasomes (Tanahashi, Yokota et al. 1997; Tanahashi, Murakami et al. 2000). The hybrid proteasome functions in antigen processing through efficient hydrolysis to generate peptides with unique hydrolysis patterns from those produced by the traditional 26S (Cascio, Call et al. 2002; Tanaka 2009). The “Immunoproteasome” refers to the proteasome that is dedicated to processing peptide antigens for MHC class I mediated immunity (Tanaka 1994; Tanahashi, Yokota et al. 1997; Tanaka and Kasahara 1998). IFN- $\gamma$  upregulates the expression of  $\beta$ 1,  $\beta$ 2i and  $\beta$ 5i subunits which replace three homologous catalytic subunits:  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 of the 20S proteasome, to form the immunoproteasome (Tanaka 1994; Tanahashi, Yokota et al. 1997; Tanaka and Kasahara 1998; Yewdell 2005). The “Thymoproteasome,” was recently identified by Murata and colleagues in cortical thymic epithelial cells (**Figure 1.4B**) (Murata, Takahama et al. 2008). The thymoproteasome contains a  $\beta$ 5t subunit homologous to, and structurally related to,  $\beta$ 5 and  $\beta$ 5i (Murata, Takahama et al. 2008). Unique features of  $\beta$ 5t include that it is encoded by an intronless gene and that it is transcribed and translated only in cortical thymic epithelial cells



**Figure 1.4 A: Diversity in the proteasome lid.**

The 20S core particle is capped on one or both sides by 19 S regulatory particles to form the 26S proteasome. The 11S regulator, or PA28, forms a conical cap on either end of the 20S core to form the immunoproteasome. PA28 and PA700 can actively bind to either sides of the 20S core to form the hybrid proteasome. Figure adapted from Jung et.al., 2009, Tanaka et.al., 2009.





**Figure 1.4B: Diversity in the proteasome base.**

The standard 20S proteasome, or proteolytic core, is composed of four heptameric rings of  $\alpha_{1-7} \beta_{1-7} \beta_{1-7} \alpha_{1-7}$  arrangement. Upon IFN- $\gamma$  stimulation, the  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  subunits of the standard proteasome are replaced by  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5i$  subunits to form the immunoproteasome. In specialized thymic epithelial cells, the  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  subunits of the standard proteasome are replaced by  $\beta 1i$ ,  $\beta 2i$  and a unique  $\beta 5t$  to form a thymoproteasome. In the testis-specific proteasome, the  $\alpha 4$  subunit is replaced by a novel subunit,  $\alpha 8$ . Figure adapted from Tanaka et.al., 2009.

(cTECs) (Murata, Takahama et al. 2008). The  $\beta 5t$  subunit lowers chymotrypsin like activity of the proteasome as compared to  $\beta 5$  or  $\beta 5i$ . The thymoproteasome in cTECs produces MHC class I ligands of moderate avidity, thus supporting positive selection of  $CD8^+$  T cells (Murata, Takahama et al. 2008; Tanaka 2009). Lastly, Tanaka and colleagues have identified an  $\alpha 8$  subunit homologous to the  $\alpha 4$  subunit of the 20S core particle. Expression of the  $\alpha 8$  subunit is restricted to cells and tissues of testis and thus the term “Mammalian Testis-Specific Proteasome” has been coined for the proteasome containing an  $\alpha 8$  subunit in place of an  $\alpha 4$  subunit (**Figure 1.4B**) (Tanaka 2009).

## ROLES OF THE UPS

Thus far we have discussed contributions of ubiquitination, the structures elements of proteasomal subunits and proteasome diversity to the regulation of multiple cellular pathways and to the maintenance of cellular homeostasis. Proteolytic roles of the UPS in regulating cellular processes including DNA repair, cell cycle control, membrane trafficking, transcription and signal transduction are well known and are highlighted below. More recently identification of non-proteolytic functions of the UPS has identified new pathways of cellular regulation. The complex contributions of the UPS in these diverse cellular functions include roles in epigenetics, and transcriptional and post-translational modifications. The proteolytic and non-proteolytic roles of the UPS in regulatory mechanisms and in disease development are also discussed in the following sections and are intended to provide a better understanding of the magnitude of contributions of the UPS.

## Roles in DNA repair

Internal and external genotoxic agents pose a constant threat to the genetic information stored in DNA. In order to combat these threats, cells have DNA damage checkpoints and DNA repair mechanisms (Panagiotis, Anna et al. 2009) activated to prevent cells from dividing and expressing damaged genes; failure to correct damaged DNA leads to induction of senescence or cell death (Sheikh and Fornace 2000; Zamzami and Kroemer 2005). Thus, DNA repair plays crucial roles in maintaining the integrity of DNA encoded genetic material. Roles of ubiquitin in modulating DNA repair pathways was first demonstrated when the DNA repair gene RAD6 was shown to encode for a ubiquitin-conjugating enzyme, E2 (Jentsch, McGrath et al. 1987). Following this, multiple signal transducers involved in DNA damage response and DNA repair have been shown to be enzymes either capable of catalyzing ubiquitination or to harbor a ubiquitin binding domain. Herein we will review the roles of UPS in various DNA repair pathways including double stranded break repair, nucleotide excision repair, Fanconi Anemia DNA repair, and MGMT/ AGT repair pathways.

Roles for the UPS in **double stranded break (DSB) repair** have been well documented in previous reviews (Motegi, Murakawa et al. 2009; Panagiotis, Anna et al. 2009) that illustrate how various DNA repair pathways interact and are regulated by the UPS, with particular emphasis on pathways involved in tumors and chemotherapeutic regimes. Recent studies have identified a component of the nucleosome remodeling histone deacetylation complex (NuRD), metastasis-associated protein 1 (MTA1), to play novel roles in DSB repair caused by ionizing radiation (Li, Ohshiro et al. 2009). MTA2, an additional member of the MTA family, is a known co-regulator of cellular growth and apoptosis via deacetylation and inactivation of the tumor sup-

pressor protein p53 (Li, Divijendra Natha Reddy et al. 2009; Toh and Nicolson 2009). MTA1 has recently been shown to be a DNA damage responsive protein involved in DSB repair (Li, Divijendra Natha Reddy et al. 2009). MTA1 competes with Mdm2 (Li, Ohshiro et al. 2009) and with the RING domain E3 ligase COP1 (constitutive photomorphogenesis protein 1) to bind to p53 to inhibit p53 ubiquitination and/or to repress Mdm2 and COP1 activity, resulting in stabilization of p53 (Li, Divijendra Natha Reddy et al. 2009). MTA1 also promotes COP1 destabilization by promoting its auto-ubiquitination (Li, Ohshiro et al. 2009). Deficiency of MTA1 reduces p53 stability and p53-dependent expression of downstream genes, including the ribonucleotide reductase, p53R2, which is involved in nucleotide supply for DNA repair (Li, Divijendra Natha Reddy et al. 2009). Thus, MTA1 regulates p53 dependent DNA repair. MTA1 is targeted for ubiquitination by COP1 and is subsequently degraded via the UPS, and over expression of MTA1 has been correlated with tumor formation and increased metastasis in breast cancer (Li, Ohshiro et al. 2009; Toh and Nicolson 2009). In addition to regulating p53 dependent DNA repair process, MTA1 regulates DNA repair independent of p53 by directly binding to the p53-downstream gene, p21<sup>WAF1</sup> (Li, Pakala et al. 2010). A complex of MTA1-HDAC2 co-represses p21<sup>WAF1</sup> expression, resulting in increased levels of proliferating cell nuclear antigen (PCNA) during DNA damage response, which negatively impact DNA damage repair (Li, Pakala et al. 2010). Additionally, ubiquitination (and deubiquitination) of histones H2A and H2AX, well known for roles in chromatin remodeling and transcription, have increasingly been found to play important roles in DNA damage responses. Pinato and colleagues have recently identified a novel chromatin associated E3 ligase, RNF168, involved in histone ubiquitination (Pinato, Scandiuzzi et al. 2009). RNF168 is recruited to DSB sites where it preferentially poly-ubiquitinates H2A and H2AX resulting in the formation of lysine linked 63 poly-ubiquitin chains

which stabilize binding of the DSB repair machinery to the damaged site (Doil, Mailand et al. 2009; Pinato, Scandiuizzi et al. 2009).

The **nucleotide excision repair (NER) pathway** is involved in the removal of large adducts caused by UV damage or by exposure to genotoxic agents (Panagiotis, Anna et al. 2009). While roles for the 26S proteasome in NER pathway regulation have also been well documented (Motegi, Murakawa et al. 2009; Panagiotis, Anna et al. 2009), recent work has identified novel NER-dependent chromatin ubiquitination of H2A induced by UV damage. (Bergink, Salomons et al. 2006; Zhu, Wani et al. 2009). Although similar chromatin modifications of H2A and H2AX have been observed in DSB repair, the mechanisms and signaling involved in NER-dependent H2A ubiquitination are less understood. Marteijn and colleagues have shown that ubiquitin ligases Ubc13 and RNF8 ubiquitinate H2A (Marteijn, Bekker-Jensen et al. 2009). These ligases work downstream of the NER pathway as a depletion of these ligases increases UV sensitivity without affecting NER (Marteijn, Bekker-Jensen et al. 2009). It is interesting to note that although double stranded breaks and UV lesions recruit independent repair mechanisms, they generate the same epigenetic mark of H2A ubiquitination (Marteijn, Bekker-Jensen et al. 2009), thus amplifying the DNA damage response.

Fanconi anemia (FA) is a multi-organ human disorder of the **Fanconi Anemia DNA repair pathway** in which patients are highly sensitive to DNA inter cross-linking agents such as mitomycin C (Smogorzewska, Matsuoka et al. 2007). Eight proteins form the Fanconi anemia complex, a nuclear E3 ubiquitin ligase which mono-ubiquitinates FA protein Fanconi anemia group D2 protein (FANCD2) and its paralog, Fanconi anemia complementation group I protein (FANCI) both of which are both involved in the FA DNA repair pathway (Smogorzewska, Matsuoka et al. 2007). FANCD2 and FANCI form an “ID complex” that co-localizes to chroma-

tin upon DNA damage. Following DNA damage, FANCD2 is phosphorylated at S331 by the S-phase checkpoint kinase, CHK1 which triggers DNA damage inducible mono-ubiquitination of FANCD2 (Zhi, Wilson et al. 2009). FANCI is subsequently mono-ubiquitinated, DNA damage induced foci are formed and additional proteins involved in DNA damage response are recruited. Interestingly, FANCD2 and FANCI demonstrate a “dual ubiquitin-locking mechanism” where maintenance of mono-ubiquitination of either protein is dependent on the mono-ubiquitination status of the other (Smogorzewska, Matsuoka et al. 2007). Thus phosphorylation and mono-ubiquitination of the ID complex is required for recruitment to the damage site on chromatin where it directs the removal of damaged DNA and DNA repair (Smogorzewska, Matsuoka et al. 2007).

Recent studies indicate roles for the UPS in regulating the **MGMT or AGT repair pathway**. Upon DNA damage by carcinogenic alkylating agents, multiple types of adducts are formed including O<sup>6</sup>-methylguanine which causes G:C to A:T mutations in DNA and induces cell death. In such situations, the DNA repair protein human O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), or the more commonly used O<sup>6</sup>-Alkylguanine-DNA alkyltransferase (AGT), participates in a one-time transfer of an alkyl group from DNA to an internal cysteine residue (Xu-Welliver and Pegg 2002). Following alkyl group transfer, modified AGT is recognized and ubiquitinated by an unidentified ubiquitin ligase and targeted for degradation (Xu-Welliver and Pegg 2002). The yeast homolog of AGT, DNA repair methyltransferase Mgt1, is targeted for proteolysis via two ubiquitin dependent pathways, the N-end pathway and the ubiquitin fusion dependent pathway (Hwang, Shemorry et al. 2009). The two pathways work synergistically to eliminate persistent Mgt1 following DNA repair and to simultaneously protect

DNA from further damage due to accumulated alkyl groups (Hwang, Shemorry et al. 2009; Li, Ohshiro et al. 2009).

Poly-ADP ribose polymerase-1 protein (PARP1), a key regulator of the base-excision repair (BER) pathway, has also been shown to be poly-ubiquitinated via lysine 48 and degraded by the 26S proteasome (Tao, Cynthia et al. 2008). PARP is recruited to single stranded breaks where it coordinates with various members of BER pathway including the scaffolding protein XRCC1, DNA ligase III, and DNA polymerase  $\beta$ , to remove damaged bases (Panagiotis, Anna et al. 2009). Cleavage of PARP1 by caspases 3 and 7 is a prerequisite to apoptosis and PARP1 is poly-ubiquitinated and degraded by the 26S proteasome (Tao, Cynthia et al. 2008). Upon DNA damage, PARP1 poly-ubiquitination is down regulated and thus stabilized PARP1 participates in nuclear functions, including DNA repair (Tao, Cynthia et al. 2008). Martin and colleagues have recently identified RNF4 as an ubiquitin ligase for PARP1 that regulates its stability and transcriptional activity in response to heat shock (Martin, Schwamborn et al. 2009). Thus stabilization of PARP1 increases its transactivation potential and functions in the DNA damage response.

### Roles in cell cycle control

Normal cells divide in response to proliferative stimuli including developmental and mitotic signals. The cell cycle involves G1, S, G2 and M phases which are coordinately regulated to reduce errors during cell division. The cell cycle is tightly regulated by checkpoint mechanisms which ensure accurate completion of each phase prior to beginning the next. Activation of these checkpoints stalls cell cycle progression and provides time for activation of damage repair

mechanisms. Checkpoint activation frequently involves post-translational modifications including phosphorylation and ubiquitination of various proteins, including histone H2AX, scaffold protein Mdc1, cell division control protein 20 (Cdc20), and cell division control protein 25 (Cdc25), at different stages of the cell cycle which in turn activate downstream checkpoint mechanisms to ensure cell cycle fidelity (Wickliffe, Williamson et al. 2009). Alternatively, unique cyclin dependent kinases (CDKs) play phase specific regulatory roles in promoting cell cycle progression. Each CDK has a cyclin partner to promote its activity and a CDK-inhibitor (CKI) to inhibit its activity. Periodic synthesis of cyclins determines the capability of CDKs to phosphorylate their substrate proteins and subsequent cyclin degradation shuts down CDK activity and leads to an exit from mitosis. Levels of both cyclin-CDK complexes and their CKI inhibitors are maintained by periodic synthesis and UPS mediated degradation. The role of the UPS in the cell cycle extends far beyond protein degradation as the UPS controls progression through cell cycle programs as well as the function of cell cycle checkpoints, all to ensure coordination of cell proliferation and developmental programs.

Multiple E2 ubiquitin-conjugating enzymes regulate cell cycle control (Wickliffe, Williamson et al. 2009) including roles for ubiquitin conjugating enzyme UbcH10 in the progression of mitosis and roles for ubiquitin conjugating enzyme 3 (Ubc3)/cell division control protein (Cdc34) in controlling progression of the G1-S phase (Whitcomb and Taylor 2009). The E2 UbcH10 co-operates with the E3 ubiquitin ligase anaphase-promoting complex (APC/C) to form lysine 11 linked poly-ubiquitin chains on target proteins containing a TEK (threonine-glutamate-lysine residue) motif (Haas and Wilkinson 2008; Wickliffe, Williamson et al. 2009). Lysine 11 linked poly-ubiquitin chains are recognized by proteasomal substrate receptors, following which these APC/C substrates are degraded (Wickliffe, Williamson et al. 2009). The E2



Cdc34 co-operates with the E3 ubiquitin ligase Skp1-culin-F-box complex, SCF, to form lysine 48 linked poly-ubiquitin chains during the transition from quiescent to proliferative phase and the transition from G1 to S phase (Wickliffe, Williamson et al. 2009). These lysine 48 poly-ubiquitinated proteins are subsequently degraded by the UPS.

The BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) is an E2 ligase involved in cell cycle progression, UPS mediated degradation of which leads to initiation of apoptosis (Liu, Goldberg et al. 2007). BRUCE has been shown to be a “chimeric E2/E3 ubiquitin ligase” due to its ability to function as a ubiquitin conjugating (E2) and a ubiquitin ligase (E3) in ubiquitinating the pro-apoptotic protein, Smac (Bartke, Pohl et al. 2004). Reduction in levels of BRUCE triggers activation of caspase 3, release of pro-caspase 9 and Smac, and promotion of cellular apoptosis, indicating the importance of BRUCE in maintaining cell viability (Liu, Goldberg et al. 2007). Nrdp1 (or RFP41-Ring finger protein 41) is a known E3 ligase that ubiquitinates and targets BRUCE for proteasome-mediated degradation (Qiu, Markant et al. 2004; Liu, Goldberg et al. 2007). As BRUCE is essential in regulating cell viability, maintenance of appropriate levels of this protein complex is crucial for cell cycle progression (Liu, Goldberg et al. 2007).

Numerous E3 ubiquitin ligases, including the anaphase-promoting complex (APC/C) and the Skp1-culin-F-box (SCF), also play vital roles in cell cycle control (Wickliffe, Williamson et al. 2009). The APC/C complex ubiquitinates and targets cyclins A and B for degradation in the mitotic stage, thus inhibiting major mitotic kinase, Cdk1 activity (King, Peters et al. 1995) and initiating exit from the mitotic phase. SCF is crucial in the G1 to M transition as SCF ubiquitinates and degrades proteins including the CDK inhibitor p27, cyclin E, cyclin D1 and Sic1 (Nakayama and Nakayama 2006) (Feldman, Correll et al. 1997; Carrano, Eytan et al. 1999;

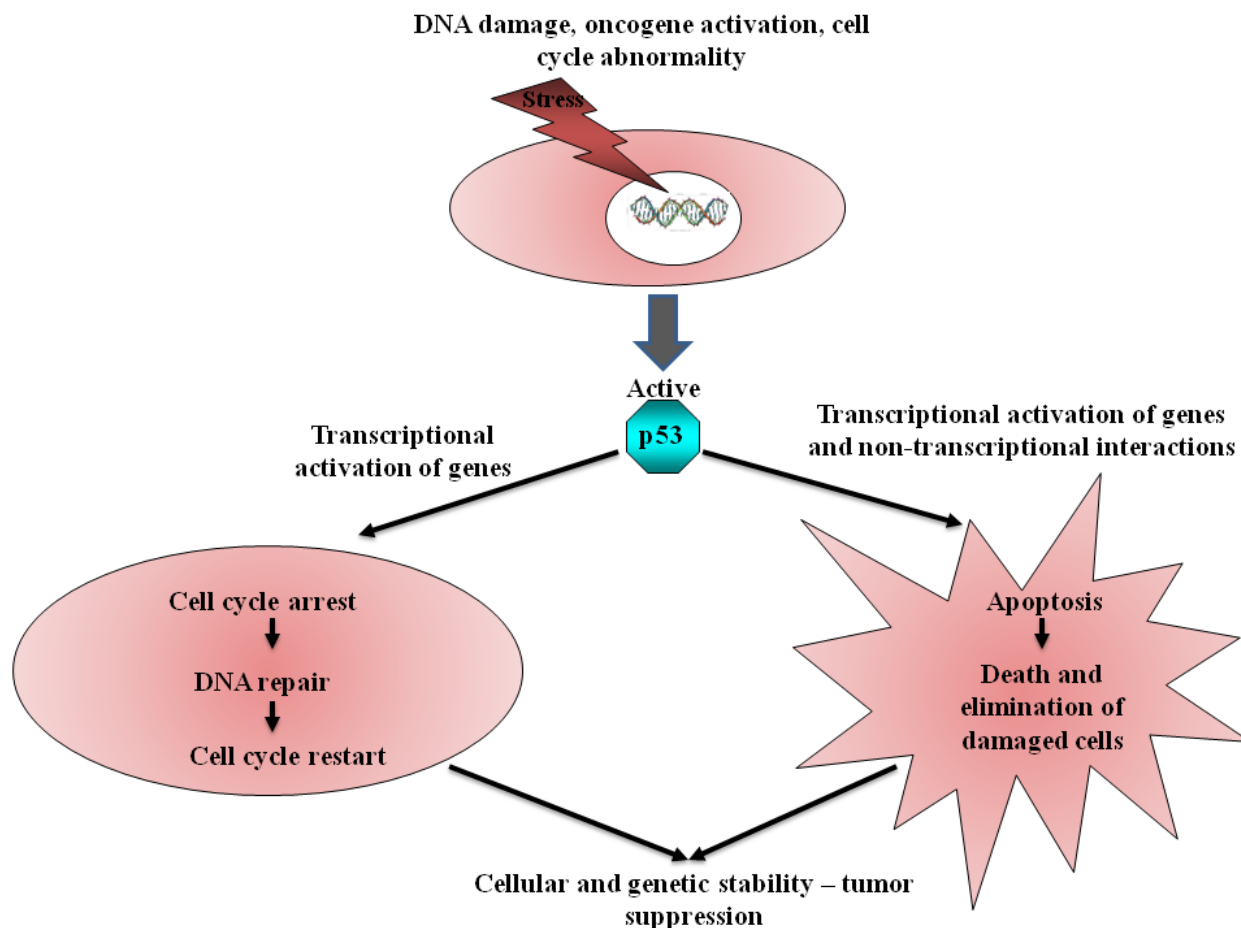
Koepp, Schaefer et al. 2001; Barbash, Egan et al. 2009). Of these, p27 is a critical SCF target as p27 is the Cdk inhibitor whose degradation is essential for the transition from G1 to S phase (Wickliffe, Williamson et al. 2009).

### Roles in tumor development

Regulation of the cell cycle plays fundamental roles in the prevention of tumor development. During G1, when cells commit to the next round of the cell cycle, Src and Abl kinases are activated and phosphorylate p27 (Wickliffe, Williamson et al. 2009). Phosphorylated p27 is targeted for further phosphorylation by cyclin E/Cdk2, recognized by an F-box protein of the SCF complex, ubiquitinated, and degraded by the 26S proteasome (Wickliffe, Williamson et al. 2009). Following p27 degradation, Cdk2 and the transcription factor E2F are activated leading to an increase in CDK inhibitors cyclin A and cyclin E as the cell irreversibly enters S phase (Wickliffe, Williamson et al. 2009). Degradation of p27 is a vital checkpoint; it offers directionality to cell cycle progression and also “releases a brake” in the cell cycle (Wickliffe, Williamson et al. 2009). As such, aberrant expression or degradation of both p27 and cyclin E is frequently seen in tumors including pituitary tumors, breast carcinoma and oral squamous cell carcinoma (Kudo, Kitajima et al. 2005; Nakayama and Nakayama 2006; Porter, Barlow et al. 2006; Wickliffe, Williamson et al. 2009). In the case of cyclin E, mutations in the ligase human F-box protein (hCdc4), which poly-ubiquitinates cyclin E and targets it for degradation, lead to the development of breast cancer (Yu, Ding et al. 2009). In addition to p27 and cyclin E, the UPS mediates degradation of many additional oncogenic proteins involved in cell cycle control, including Myc and Myb,  $\beta$ -catenin, S-phase kinase-associated protein 2 SKP2, hypoxia-

inducible factors, epidermal growth factor receptor (EGFR), and multiple tyrosine kinases (Nakayama and Nakayama 2006; Kyoko, Yojiro et al. 2009). Thus the UPS plays crucial roles in ensuring proper cell cycle progression and in inhibiting tumor development.

Failures in cell cycle regulation are deleterious, as is demonstrated by the development of tumors due to deregulation of critical cell cycle control pathways involving tumor suppressor protein p53 (Mahalingam, Mita et al. 2009; Polager and Ginsberg 2009). Dysregulation of p53 is a major cause of tumor development, with more than 50% of tumors containing a defective or mutated p53 (Irwin and Kaelin 2001). The p53 family of proteins include tumor repressor proteins p53, p63 and p73 (Irwin and Kaelin 2001) with p53 being the most studied. Upon stress induction, p53 is stabilized, following which it activates cellular responses through transcriptional activation of genes responsible for angiogenesis, cell-cycle arrest, DNA- repair, or apoptosis (**Figure 1.5**) (Midgley, Owens et al. 1995; Agarwal, Taylor et al. 1998; Carol Prives 1999). Thus, p53 activation and levels of expression are tightly regulated and increase in response to cellular stresses including DNA damage and oncogene activation (Lane 1992). Levels of p53 are regulated by ubiquitin-mediated degradation by the ubiquitin ligase Mdm2 (Fang, Jensen et al. 2000) which can both mono- and poly-ubiquitinate p53 (Li, Brooks et al. 2003). At low levels, Mdm2 catalyzes mono-ubiquitination of p53 whereas at elevated levels, Mdm2 catalyzes poly-ubiquitination and 26S proteasome-mediated p53 degradation (Li, Brooks et al. 2003). Mono-ubiquitinated p53 is transported out of the nucleus and serves as a reservoir of p53 which can be used under stress conditions. Alternatively, poly-ubiquitinated p53 is rapidly degraded in the nucleus by the 26S proteasome (Li, Brooks et al. 2003; Brooks and Gu 2004). Thus, a fine-regulation of MDM2 mediated p53 ubiquitination is crucial in maintaining p53 function. Mutations in MDM2 leading to deregulation of p53 protein stability and transactivity have been

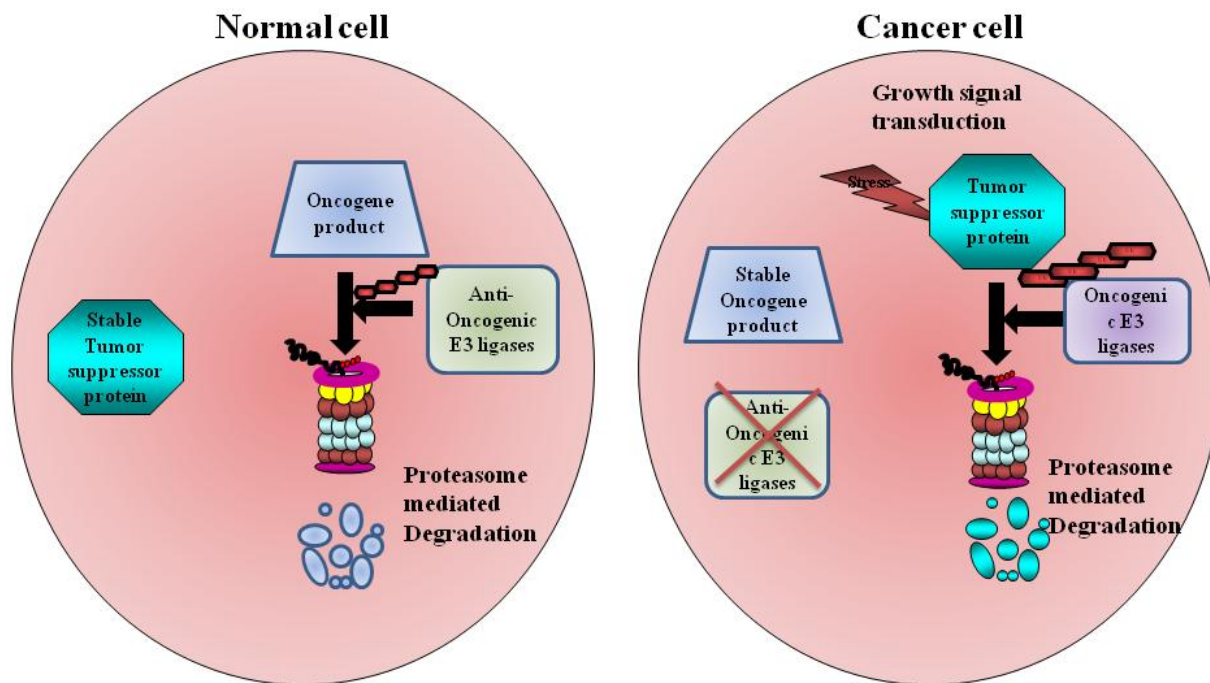


**Figure 1.5: p53 mediated cell cycle control.**

p53 is activated by UV radiation, oncogenes, and DNA-damaging agents. Upon activation, p53 itself activates DNA repair proteins including p21, cyclin B1, and GADD45. P53 activation drives cell cycle arrest at G1/S and simultaneously activates DNA repair mechanisms. Upon successful repair, cell cycling begins. Alternatively, if the DNA damage is irreparable, p53 initiates apoptosis by inducing gene transcription and through direct interactions with mitochondrial proteins. Thus p53 maintains cellular and genetic stability to prevent tumor growth.

demonstrated in 40-80% of late-stage metastatic cancers, in breast cancer, and in endometrial cancer (Petroski 2008; Araki, Eitel et al. 2009; Ogino, Nosho et al. 2009; Osamu, Masatsugu et al. 2009). Recent studies have highlighted the importance of p53 family members, p63 and p73 in tumor development. While p63 and p73 share little homology with p53 in their transcription activation and oligomerization domains, they share high homology in their DNA binding domains with p53, and thus are functionally similar. p63 and p73 are thus able to transcriptionally regulate p53 downstream genes and can induce cell cycle arrest or apoptosis (Pietsch, Sykes et al. 2008). Altered protein expression of p63 and p73 are commonly observed in cancerous growths. For example, 80% of head and neck squamous cell carcinomas demonstrate elevated levels of p63 and neuroblastoma, breast cancer, and lung cancer have each displayed mutations in p73 or p63 that impaired their transactivation potential (Irwin and Kaelin 2001; Pietsch, Sykes et al. 2008).

In addition to a dysregulated cell cycle, tumor development is directly linked to UPS mediated accumulation of oncogenic proteins and degradation of tumor suppressors (Kyoko, Yojiro et al. 2009). In normal cells, oncogenic proteins are poly-ubiquitinated by E3 ligases and targeted for degradation via the 26S proteasome. Thus these E3 ligases function as anti-oncogenic ligases and include the ubiquitin ligase complex APC/C and the ligase SCF. Likewise, tumor suppressor proteins including p53 inhibit cancerous growths and thus prevent tumor onset. Thus, activation of oncogenic E3-ligases that degrade tumor suppressor proteins also leads to cancerous growth and tumors. Examples of the intricate relationships between E3 ubiquitin ligase and target protein turnover in cell cycle regulation and tumor development are the E3 ubiquitin ligase Mdm2 and p53; the E3 ubiquitin ligase complex APC/C and cyclin A and B; and the E3 ubiquitin ligase complex SCF and Cyclin E (**Figure 1.6**).



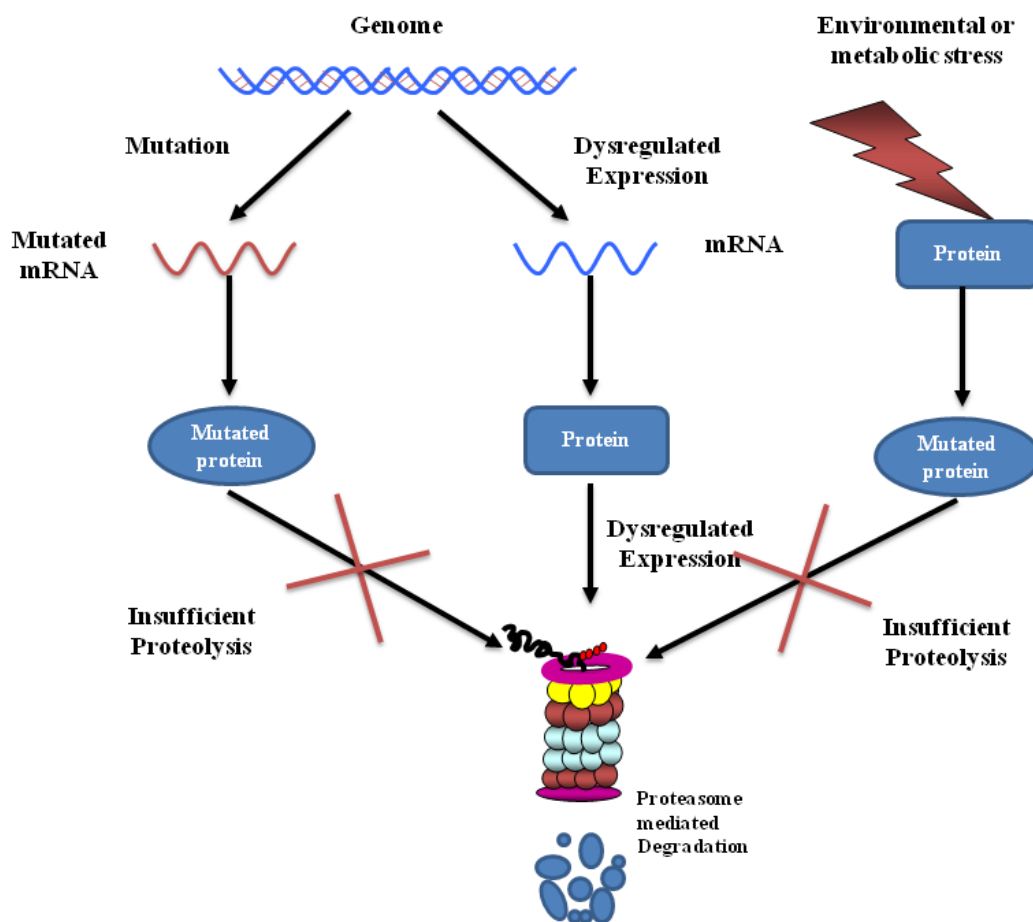
**Figure 1.6: Dysfunction of the UPS leads to tumor growth.**

Tumor development is linked to accumulation of oncogenic proteins and degradation of tumor suppressors. In normal cells, oncogenic proteins are poly-ubiquitinated by anti-oncogenic E3 ligases and are degraded via the 26S proteasome. Malfunctions in E3 ligases which result in accumulation of oncogenic proteins or that target tumor suppressors for degradation, lead to tumor growth. Figure adapted from Kitagawa et.al., 2009

### Roles in neurodegenerative disease

The importance of the UPS is further highlighted by the direct relationship between onset of neurodegenerative diseases and perturbations in the UPS (Ciechanover, Orian et al. 2000; Hegde 2004; Kyoko, Yojiro et al. 2009). A majority of neurodegenerative diseases show increased protein accumulation in extracellular and/or intracellular compartments of neuronal cells (Jung, Catalgol et al. 2009). This accumulation of proteins is caused by genetic or environmental factors in combination with imbalances in anabolic and/or catabolic pathways (**Figure 1.7**). Genetic mutations can lead to the production of mutated proteins that have different rates of degradation or a tendency to form persistent cellular aggregates (Jung, Catalgol et al. 2009). Furthermore, deregulation of gene expression also leads to accumulation of protein aggregates due to increased protein synthesis and reduced protein degradation by the 26S proteasome (Jung, Catalgol et al. 2009). Intraneuronal aggregates contain ubiquitinated proteins and accumulation of these ubiquitinated aggregates is due to their impaired clearance by the 26S proteasome. The link between the UPS and neurodegenerative diseases is further strengthened by observations that several mutations linked to neurodegenerative disease onset are found in genes that code for proteins involved in the UPS. Thus the UPS plays an important role in the development of neurodegenerative diseases.

**Alzheimer's Disease** is a neurodegenerative disorder involving the development of twisted neurofibrils termed “neurofibrillary tangles” (NFT), which involve extracellular accumulation of toxic  $\beta$ -amyloid aggregates, and the appearance of cellular plaques (Hegde 2004; Jung, Catalgol et al. 2009).



**Figure 1.7: Intracellular accumulation of proteins during neurodegenerative disease.**

Post-translational modifications of proteins due to genetic or environmental factors, or to imbalances in anabolic and/or catabolic pathways, leads to an intracellular accumulation of proteins. Genetic mutations can produce proteins with different rates of degradation or tendencies to form aggregates which persist longer in cells. Dysregulation of gene expression may also lead to increases in protein aggregates due to increased protein synthesis or reduced protein degradation. Post-translational modifications caused by environmental factors including stress enhance protein stability or ability to aggregate. Figure adapted from Jung et.al., 2009.



NFTs result from the accumulation of hyperphosphorylated tau proteins, which form large insoluble aggregates called paired helical filaments (PHF) that impair proteasomal function (Hegde 2004; Jung, Catalgol et al. 2009). Tau proteins and  $\beta$ -amyloid aggregates are highly poly-ubiquitinated but are resistant to degradation by the UPS. Resistance stems from mutation of the ubiquitin moiety involved such that there are 19 extra amino acid in C-terminal end of ubiquitin caused by “molecular misreading” (Lindsten, de Vrij et al. 2002). This “molecular misreading” involves deletion of 2 nucleotides in the mRNA for the ubiquitin B pre-cursor. The deletion results in a +1 frame shift near the C-terminal end of the first ubiquitin and results in a 19 amino acid extension. The 19 amino acid extension prevents the activation and conjugation of the mutated ubiquitin, but the presence of internal lysine residues in mutated ubiquitin still serve as sites for addition of ubiquitin molecules (Lindsten, de Vrij et al. 2002). These additions result in a general accumulation of ubiquitinated proteins and cause cell cycle arrest (Lindsten, de Vrij et al. 2002). The decreased proteasomal regulation of tau and  $\beta$ -amyloid proteins leads to accumulation of NTF's in neurons and eventual development of Alzheimer's disease.

**Parkinson's disease** (PD) is another prevalent neurodegenerative disease and has been linked to 13 genes: polyubiquitinated  $\alpha$ -synuclein, ubiquitin carboxyl-terminal esterase L1 (UCH-L1), Parkinson disease 7 (DJ-1), the E3 ligases parkin -2, -3, -4, -5, -6, -7, -8, -9, -10 and Nuclear receptor subfamily 4 group A member2 (NR4A2) (Hegde 2004; Jung, Catalgol et al. 2009). Histology reports from PD patients demonstrate a loss of dopaminergic neurons and the presence of inclusion bodies, termed Lewy bodies, within brain cells. Lewy bodies are formed due to accumulation of multiple proteins, one of them being poly-ubiquitinated form of mutated  $\alpha$ -synuclein (Hegde 2004; Jung, Catalgol et al. 2009). Although the function of  $\alpha$ -synuclein proteins is unclear, they are thought to be involved in neuronal plasticity and in the release of neuro-

transmitters, and mutations in  $\alpha$ -synuclein increase neuronal susceptibility to oxidative stress (Jung, Catalgol et al. 2009). In addition to  $\alpha$ -synuclein, the E3 ligase Parkin is also found in Lewy bodies, and interacts with the Rpn10 subunit of the 19S regulatory particle (Jung, Catalgol et al. 2009). Parkin ubiquitinates the  $\alpha$ -synuclein interacting protein synphilin-1 leading to the formation of Lewy bodies. Thus, in some PD cases, mutations in the coding region of Parkin lead to development of Parkinsonian syndrome in the absence of Lewy bodies (Davie 2008). UCHL-1, a neuronal ubiquitin carboxyl-terminal esterase protein involved in hydrolysis of poly-ubiquitin chains to release ubiquitin monomers, is also found associated with Lewy bodies.

Although the presence of Lewy bodies is pathological, the primary reason for development of PD is thought to be oxidative stress (Jung, Catalgol et al. 2009). Accumulation of reactive oxygen species and depletion in ATP levels, affect ubiquitination and protein degradation via UPS and thus the functioning of neurons (Jung, Catalgol et al. 2009). Dopaminergic neurons demonstrate reduced expression of 19S/11S regulatory particles and of 20S proteasomal  $\alpha$ -subunits, thus negatively affecting the formation of functional proteasomes (Jung, Catalgol et al. 2009). Although the exact mechanism for reduced expression of proteasomal subunits is unclear, proteasomal function is clearly affected and likely contributes to the accumulation of Lewy bodies within neurons.

**Huntington's disease** (HD) is caused by mutations in the gene *Huntingtin* and is a progressive neurodegenerative disease (Hegde 2004; Jung, Catalgol et al. 2009). Mutations in *Huntingtin* involve insertion of CAG triplets and leads to the addition of polyglutamine (polyQ) sequences at the N-terminus of Huntingtin proteins. Addition of polyQ chains to Huntingtin has also been linked to other neurodegenerative diseases including spinocerebellar ataxias 1, 2, 3, 6, 7 and 17, spinobular muscle atrophy, and dentatorubro-pallidoluysian atrophy (Jung, Catalgol et

al. 2009). Histologies from HD patients demonstrate abnormal accumulation of polyQ and the formation of inclusion bodies, with HD severity depending on the length of polyQ chains (Hegde 2004; Jung, Catalgol et al. 2009). In HD and other polyQ diseases, the length of polyQ chains ranges from 40-300 residues and this overwhelms the function of the 26S proteasome (Venkatraman, Wetzel et al. 2004). PolyQ chain extensions of Huntingtin protein interact with the  $\alpha$ -subunits of 20S proteasome thus preventing the binding of 19S regulatory particle, assembly of 26S proteasomes, and protein degradation (Jung, Catalgol et al. 2009). Failure of proteasome assembly leads to accumulation of proteins aggregates within neurons and the onset of neurodegeneration. Additionally, the degradation of the long polyQ chains may produce small peptides that are toxic to the cell (Bennett, Bence et al. 2005).

### Roles in protein localization and membrane trafficking

The UPS is intricately involved in protein localization and membrane trafficking (Hicke 1999; Hicke 2001; Hicke 2001). The canonical poly-ubiquitin lysine 48 chain targets proteins for 26S proteasome-mediated degradation, where as poly-ubiquitin chains formed via lysine 63 target proteins for diverse cellular activities (Hicke 1999; Lauwers, Jacob et al. 2009). Levels of membrane proteins are maintained by lysine 63 linked poly-ubiquitination which functions as a signal for selective trafficking to the lysosomal lumen via multivesicular endosomal (MVE) or multivesicular body (MVB) degradation pathways (Raiborg and Stenmark 2009). This process of endosomal sorting is dependent on ubiquitination of membrane proteins, or “cargo” (Raiborg and Stenmark 2009). Ubiquitinated cargo is recognized by the endosomal sorting complex for transportation machinery (ESCRT) which blocks recycling of ubiquitinated cargos (Raiborg and

Stenmark 2009). The ESCRT facilitates sorting of ubiquitinated cargo into endosomal invaginations to form intraluminal vesicles (ILV) which contain the ubiquitinated cargos (Raiborg and Stenmark 2009). Mutations in subunits of ESCRT III lead to intraneuronal accumulation of ubiquitinated protein aggregates and development of neurodegenerative diseases including amyotrophic lateral sclerosis and frontotemporal dementia (Raiborg and Stenmark 2009). Additionally, subunits of ESCRTs function as tumor suppressors in human embryonic kidney and ductal pancreatic tumors, indicating roles in tumorigenesis (Li, Belogortseva et al. 2008). Thus, the ESCRTs prevent the onset of various neurodegenerative diseases and cancers (Raiborg and Stenmark 2009). Lauwers and colleagues recently demonstrated roles for lysine 63 linked ubiquitination in multivesicular body (MVB) sorting in yeast (Lauwers, Jacob et al. 2009). Although mono-ubiquitination of the general amino acid permease (Gap1) permease triggers its internalization into MVB, lysine 63 linked poly-ubiquitination of Gap1 is crucial for its maximal internalization and sorting in the MVB pathway as mono-ubiquitinated Gap1 is efficiently internalized, but not sorted, in the MVB pathway unless poly-ubiquitinated via lysine 63 (Lauwers, Jacob et al. 2009).

An additional example of role of the UPS in protein trafficking involves EGFR. Following growth factor stimulation, ligand-bound EGFR is rapidly internalized through clathrin-dependent and independent pathways. Ligand-dependent endocytosis and sorting of EGFR is dependent on its ubiquitination status, and Huang and colleagues have provided new evidence for the role of lysine 63 linked poly-ubiquitin chains in this process (Huang, Kirkpatrick et al. 2006). EGFR mutants lacking ubiquitination sites undergo internalization; however, their turnover is dramatically affected as a lysine 63 linked poly-ubiquitination event is essential for ubiquitin mediated EGFR lysosomal degradation (Huang, Kirkpatrick et al. 2006).

Major histocompatibility (MHC) class I and MHC class II molecules are additional examples of ubiquitinated membrane proteins (Geetha, Jiang et al. 2005; Duncan, Piper et al. 2006; Shin, Ebersold et al. 2006). MHC class I and II are cell surface glycoproteins that express antigenic peptides and are involved in the inflammatory response. MHC class I proteins bind and present cytosolic peptides, including viral peptides; thus viruses frequently downregulate MHC class I expression in order to evade immune surveillance (Duncan, Piper et al. 2006). For example, cells infected with Kaposi's sarcoma-associated herpesvirus express viral protein K3 which is an ubiquitin ligase. K3 poly-ubiquitinates MHC class I via lysine 63 additions, resulting in endocytosis and degradation of MHC class I and a reduction in cell surface presentation of virally derived peptides (Duncan, Piper et al. 2006). Ubiquitination of MHC class II plays a crucial role in the development of dendritic cells. In immature DC cells, the cytoplasmic tail of the MHC class II  $\beta$ -chain is ubiquitinated, which facilitates the endocytosis and sequestration of MHC class II in the MVB (Shin, Ebersold et al. 2006). E3 ligases; modulator of immune recognition (cMIR) and membrane-associated RING-CH (MARCH1) poly-ubiquitinate lysine 225 on the cytoplasmic tail of the MHC class II  $\beta$ -chain and therefore decrease MHC class II cell surface expression by regulating trafficking through the endosomal compartment (Ohmura-Hoshino, Matsuki et al. 2006; Ohmura-Hoshino, Matsuki et al. 2009). When an immature dendritic cell encounters foreign stimuli, the dendritic cell matures and dramatically increases cell surface expression of MHC class II (van Niel, Wubbolts et al. 2008). Upon maturation, ubiquitination of MHC class II is reduced through deubiquitination which stabilizes the surface expression of MHC class II on mature dendritic cells (Shin, Ebersold et al. 2006). Thus, the rapid increase of MHC class II on the mature dendritic cell surface is due to rapid changes in the localization of MHC class II from endosomes to the cell surface.

## Roles in Transcription

Transcriptional regulation involves a synchronized interplay of multiple protein complexes which must be recruited to, and ultimately removed from, DNA. Though intensive studies have been undertaken to elucidate the mechanisms involved, how cells perform the tasks required to initiate, maintain and terminate transcription remains largely enigmatic. Swaffield and colleagues first linked the proteasome to transcription regulation in 1992 by identifying mutations in Rpt6 (Sug1) and Rpt4 (S10b) capable of rescuing a mutant Gal4 transactivator phenotype in yeast (Swaffield, Bromberg et al. 1992). Rpt6 (Sug1) was later demonstrated to be a subunit of the 26S proteasome (Rubin, Coux et al. 1996), a seminal finding that encouraged others to investigate roles for the proteasome in transcription regulation. Peters and colleagues showed localization of the intact 26S, the 19S regulatory particle, and the 20S proteolytic core in both cytoplasmic and nuclear components in multiple cell types, including *Xenopus laevis* oocytes (Peters, Franke et al. 1994), indicating the 26S, 19S and 20S could each exist separately and might play independent roles in the regulation of transcription. To date, Sikder and colleagues have demonstrated association of subunits of the 19S and/or 20S proteasome with in excess of 6400 genes (Sikder, Johnston et al. 2006). Components of the UPS have now been linked, via proteolytic and non-proteolytic roles, to transcription factor and co-factor processing, chromatin modifications, initiation, elongation and termination. In this section we will highlight the many functions of UPS in transcriptional regulation.

### Regulation of transcription factors

Recent findings have shed light on the roles played by the 19S ATPases in regulating mammalian gene transcription. In 2006, Rasti and colleagues established a non-proteolytic role for 19S ATPases in adenovirus E1A-dependent transcription. S8 (Sug1) is recruited to the adenoviral promoter and enhances transcription by interacting with the transactivation domain of viral transcription factor E1A. The 20S proteolytic core also interacts with E1A, but has distinct roles in mediating efficient E1A transactivation (Rasti, Grand et al. 2006). Zhu and colleagues next identified non-proteolytic roles for the 26S in p53 mediated stress responses as p53 recruitment to p21waf1 responsive promoters is enhanced upon proteasome inhibition (Zhu, Wani et al. 2007). p53 physically interacts with the 19S proteasomal ATPase Sug1 and is recruited to p21waf1 responsive promoters along with Sug1 and the S1 (a non-ATPase component of 19S) component of the 19S regulatory particle (Zhu, Wani et al. 2007).

Lassot and colleagues have also demonstrated proteolytic and non-proteolytic roles of the proteasome in regulating HIV-1 transcription (Lassot, Latreille et al. 2007). In the absence of the HIV-1 transactivator Tat, proteolytic activity of the 26S proteasome limits basal levels of transcription from HIV-1 promoters (Lassot, Latreille et al. 2007). Upon expression, Tat recruits the proteasome-associated protein PAAF-1 which facilitates separation of the 19S lid and the 20S core and thus switches the proteasome to a non-proteolytic mode (Lassot, Latreille et al. 2007). Following dissociation of the 19S lid, Tat recruits the 19S lid, but not the 20S proteolytic core, to the promoter in a PAAF dependent process (Lassot, Latreille et al. 2007). Once recruited to the HIV-1 promoter, the 19S regulatory particle plays a non-proteolytic role in facilitating transcrip-

tional elongation and HIV-1 transcription (Nelbock, Dillon et al. 1990; Shibuya, Irie et al. 1992; Bres, Kiernan et al. 2003; Lassot, Latreille et al. 2007).

The UPS has also been implicated in regulating retinoic acid (RA)-mediated transcription (Ferry, Gianni et al. 2009). Sug1 physically interacts with the retinoic acid receptor, a transcriptional coactivator, and is recruited to RA-target promoters in order to facilitate the recruitment of additional complexes (vom Baur, Zechel et al. 1996; Gianni, Bauer et al. 2002; Ferry, Gianni et al. 2009). We have recently established the importance of Sug1 in regulating the transcription of MHC class II molecules (Bhat, Turner et al. 2008). Sug1 binds to the MHC class II proximal promoter and to the master regulator of MHC class II transcription, CIITA, via interactions independent of the 20S proteolytic core (Bhat, Turner et al. 2008). In the absence of Sug1, MHC class II transcription is dramatically decreased and CIITA recruitment at the MHC class II promoter is abolished (Bhat, Turner et al. 2008). The 20S proteasome is also recruited to the MHC class II promoter following prolonged cytokine stimulation, suggesting the 26S proteasome may be reassembled at active promoters.

### *Mono-ubiquitination of transcription factors*

The role of ubiquitin in transcription activation was first demonstrated by Salghetti and colleagues who showed ubiquitination of the transactivation domain of viral protein-16 (VP-16) by the Met-30 ubiquitin ligase is essential for transactivity (Salghetti, Caudy et al. 2001). Mono-ubiquitination of LexA-VP16 (the fusion product of the LexA DNA binding domain and the VP16 transactivation domain) was later demonstrated to enhance recruitment of positive tran-

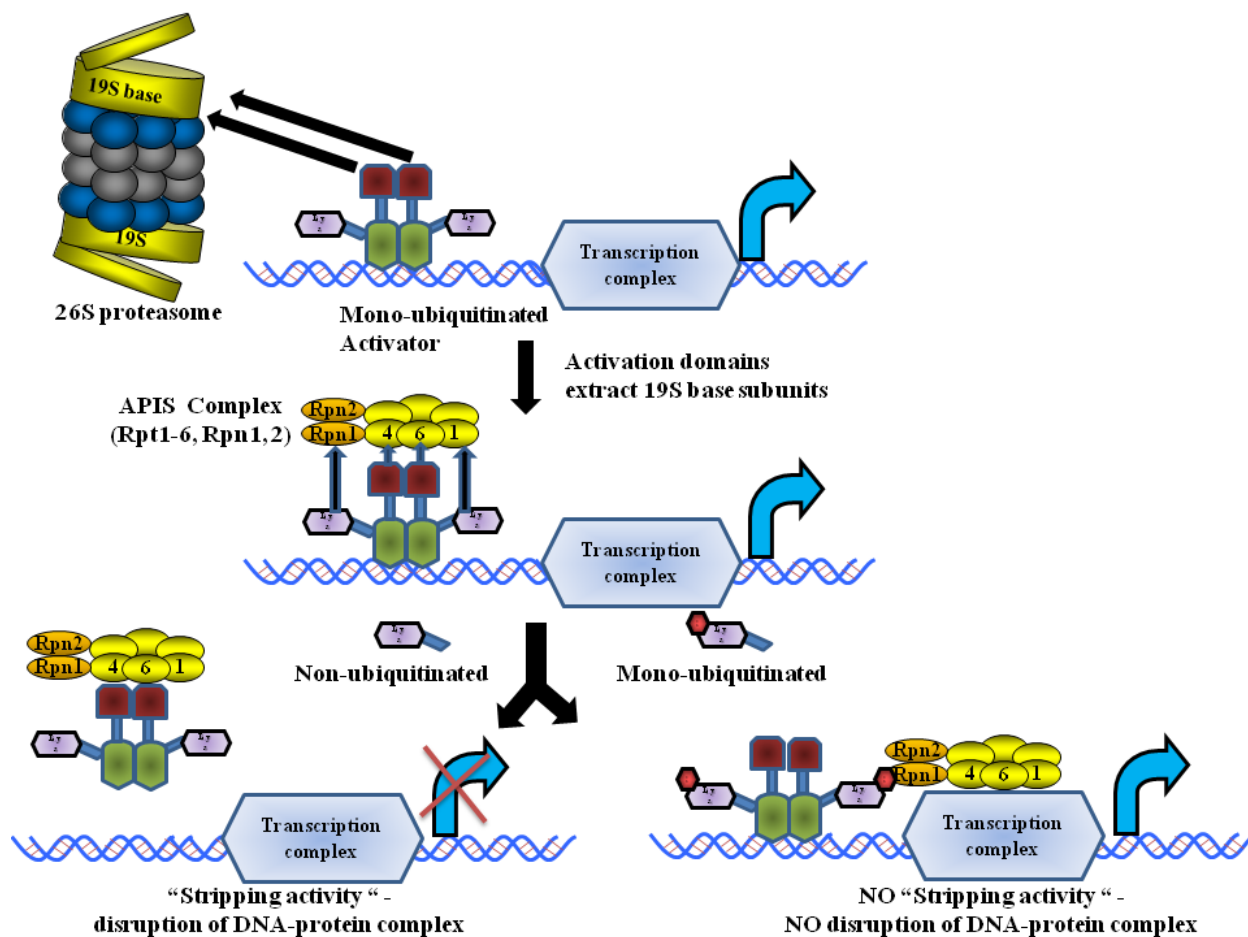


scription elongation factor (PTEFb) to target promoters (Kurosu and Peterlin 2004). We have demonstrated that CIITA, the master regulator of MHC Class II transcription, has increased transactivity and promoter binding when mono-ubiquitinated (Greer, Zika et al. 2003) and others have demonstrated enhanced degradation of CIITA upon poly-ubiquitination (Schnappauf, Hake et al. 2003). Levels of the potent stress-responsive tumor repressor and transactivator p53 are tightly regulated through the actions of multiple E3 ligases including Mdm2, COP1, ADP-ribosylation factor-binding protein1 (ARF-BP1), E6 associated protein (E6-AP), p300 and topoisomerase I-binding RING protein (Topors) (Kim, Wu et al. 2009). Under steady state conditions, E3 ligases maintain low levels of p53 through poly-ubiquitination and degradation (Scheffner, Huibregtse et al. 1993; Haupt, Maya et al. 1997; Honda and Yasuda 1999; Weger, Hammer et al. 2002; Grossman, Deato et al. 2003). During cell stress, E3 ligases including Mdm2 and COP1, mono-ubiquitinate p53 thus increasing p53 transactivity and stability (Kubbutat, Jones et al. 1997; Lai, Ferry et al. 2001; Lin, Ozaki et al. 2005).

The HIV-1 transactivator Tat is also regulated by ubiquitination (Bres, Kiernan et al. 2003). The E3 ligase Hdm2 positively regulates Tat-mediated transcription through the addition of lysine 63 linked poly-ubiquitin chains which increase Tat transactivity without affecting turnover (Bres, Kiernan et al. 2003). Mono-ubiquitination of the atypical nuclear receptor DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) is implicated in transcriptional repression during steroidogenesis (Ehrlund, Anthonisen et al. 2009). Ring finger protein 31 (RNF31) is a E3 ubiquitin ligase that targets DAX-1 and is crucial for regulating DAX-1 activity and stability (Ehrlund, Anthonisen et al. 2009). Foxhead box O4 (FOXO4) is a transcription factor involved in cellular responses to oxidative stress (van der Horst, de Vries-Smits et al. 2006) that is also modified by multiple post-translational modifica-

tions, including mono-ubiquitination, in response to stress. Mono-ubiquitinated FOXO4 translocates to the nucleus with increased transactivity (van der Horst, de Vries-Smits et al. 2006; Brenkman, de Keizer et al. 2008) and Mdm2, a well known regulator of cell cycle control, has been identified as an E3 ligase responsible for mono-ubiquitination of FOXO4 (Brenkman, de Keizer et al. 2008). Finally, apoptosis-antagonizing transcription factor (AATF) is also involved in cell cycle regulation and has been shown to interact with tumor susceptibility gene protein (TSG101), a subunit of the ESCRT subunit, upon activation by hormones. TSG101 functions as a ubiquitin conjugating enzyme (E2) and increases mono-ubiquitination of AATF (Burgdorf, Leister et al. 2004). Mono-ubiquitination stabilizes AATF, increases its transactivity, and positively regulates cell cycle progression (Burgdorf, Leister et al. 2004).

Regulation of the ubiquitination status of activators is critical in the control of transcription factor promoter function. How mono-ubiquitination of transactivators “licenses” their activation, and how this status is balanced with poly-ubiquitination and degradation, has been the focus of intense investigation. Studies in yeast on Gal4-dependent genes first indicated that 19S proteasomal ATPases bind to the Gal4 activation domain independently of both the non-ATPase 19S subunits and the 20S proteolytic core (Gonzalez, Delahodde et al. 2002). Binding of the 19S ATPases to active promoters leads to increases in efficient transcription initiation and elongation (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Ferdous, Sikder et al. 2007). In 2007, Ferdous and colleagues demonstrated a non-proteolytic “destabilizing” function of the APIS (19S ATPase proteins independent of 20S) complex involving an ATP-dependent, rapid, and reversible disassociation of the transactivator-promoter complex which was inhibited in presence of mono-ubiquitin (Ferdous, Sikder et al. 2007; Archer, Delahodde et al. 2008).



**Figure 1.8: Role of mono-ubiquitin in protecting activator from “Stripping Activity” of Proteasomal ATPase**

Protein-DNA destabilization process is initiated by the extraction of APIS complex (ATPases and Rpn1 and Rpn2) from the 26S proteasome leaving 20S and 19S lid subunits. APIS complex bound to activation domain of activator via Rpt4/Rpt6 would start to unfold the protein and thus perform its “stripping activity” to inhibit transcription. Mono-ubiquitination of DNA binding domain of activator would block the “stripping activity” of APIS as this ubiquitin moiety would interact with Rpn1 and Rpt1 following which the interaction between activation domain and Rpt4/Rpt6 is disrupted by allosteric mechanism. The APIS complex then proceeds to execute its function in promoter escape and transcription elongation. Figure adapted from Archer et.al., 2008.

The destabilizing activity of the APIS complex was dependent on direct interaction the 19S ATPase Sug1/Rpt6 and Sug2/Rpt4 with activation domains of Gal4 protein and the presence of ATP (Ferdous, Sikder et al. 2007; Archer, Delahodde et al. 2008). Destabilization is initiated by the extraction of the APIS complex (the 19S ATPases and non-ATPases Rpn1 and Rpn2) from the 26S proteasome leaving 20S and 19S lid subunits behind. When the APIS complex binds to the activation domain of an activator via Rpt4/Rpt6, the APIS begins to unfold the protein, this acts as a “stripping activity” that inhibits transcription (Archer, Burdine et al. 2008) (**Figure 1.8**). Interestingly, the destabilizing activity of APIS is inhibited when Gal4 is mono-ubiquitinated or is genetically fused with mono-ubiquitin (Ferdous, Sikder et al. 2007; Archer, Delahodde et al. 2008). Mono-ubiquitination of the DNA binding domain of Gal4 thus blocks the “stripping activity” of APIS. The mono-ubiquitin moiety interacts with Rpn1 and Rpt1, and via allosteric hindrance, disrupts the interaction between the activation domain and Rpt4/Rpt6 (Archer, Burdine et al. 2008) (**Figure 1.8**). Finally, Kim and colleagues have established the presence of similar mechanisms in mammalian transcription, where p53 mediated regulation of p21waf1 expression is shown to be negatively modulated by the “stripping activity” of 19S proteasomal ATPases (Kim, Wu et al. 2009).

To summarize the complex contributions of subunits of the 19S proteasome to transcription; initial findings have demonstrated that subunits of the APIS complex bind and recruit transcription factors to promoters where they enhance transcription initiation and elongation (Ferdous, Kodadek et al. 2002; Rasti, Grand et al. 2006; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Truax, Koues et al. 2009). Further studies have shown mono-ubiquitination of transcription factors increases transactivation; however, the mechanisms involved in this process were unclear (Salghetti, Caudy et al. 2001; Greer, Zika et al. 2003; van der Horst, de Vries-Smits

et al. 2006). Recent studies have elucidated additional non-proteolytic roles of the 19S ATPase Sug1 and the APIS complex involving stripping of non-mono-ubiquitinated transcription factors. Sug1/Rpt6 binds to unmodified transcription factors and performs a “stripping activity” to halt transcription (Gonzalez, Delahodde et al. 2002; Ferdous, Sikder et al. 2007; Archer, Burdine et al. 2008; Archer, Delahodde et al. 2008; Ferdous, O'Neal et al. 2008; Kim, Wu et al. 2009). In the presence of mono-ubiquitin, Sug1/Rpt6 binding to transcription factor is inhibited, allowing and transcription initiation (Archer, Burdine et al. 2008). Together, these studies shed light on non-proteolytic and proteolytic roles of Sug1, the APIS complex, and mono-ubiquitin in modulating transcription.

### Regulation of elongation

Both degradative and non-degradative roles of the 26S proteasome are important in regulating elongation (Ferdous, Gonzalez et al. 2001). Gcn4, Gal4 and Ino2/4 yeast transcription factors require UPS-mediated proteolytic activity in order to mediate transactivation, as proteasome inhibition decreased transcription of downstream genes (Lipford, Smith et al. 2005). Temperature sensitive mutations in the E3 ligase SCF, or lysine 48 to arginine mutations of ubiquitin, both block Gcn4 mediated transcription of target genes although Gcn4 was abundant at the target promoters. Finally, proteasome inhibition causes a reduction in RNA pol II recruitment to multiple promoters regulated by Gcn4, Gal4 and Ino2/4, further indicating the importance of the proteolytic function of UPS in regulation of efficient transcription elongation (Lipford, Smith et al. 2005).

The 19S proteasomal ATPase subunits have been shown to play roles in pol II dependent yeast elongation that are independent of the 20S proteasome. Initial observations indicated that absence of the 19S decreases transcription elongation whereas absence of the proteolytic 20S increases transcription elongation (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004; Lipford, Smith et al. 2005). These data signified that while the two subunits of proteasome can function independently of each other, each is essential for proper transcription elongation and termination (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004; Lipford, Smith et al. 2005). The APIS complex has also been shown to be essential for efficient transcription elongation by RNA pol II, although the exact mechanism by which the APIS complex aids transcription elongation is unknown (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002). Ferdous and colleagues have demonstrated roles for the 19S ATPase Sug1 in RNA pol II mediated elongation as Sug1 physically interacts with the yeast elongation factor Cdc8 and mutations in Sug1 lead to defective elongation (Ferdous, Gonzalez et al. 2001). Finally, studies in yeast indicate the existence of a holo-RNA pol II composed of a core RNA pol II and a mediator complex which enables enhanced transcription activity of the core RNA pol II (Kim, Bjorklund et al. 1994). In this study, the 19S ATPase Sug1 was identified as a subunit of the mediator complex, providing additional support for a role for Sug1 in transcription initiation and elongation (Kim, Bjorklund et al. 1994). Thus, proteolytic and nonproteolytic activities of proteasome are necessary for efficient RNA pol II mediated elongation.

### Regulation of epigenetics

Ezhkova and colleagues first demonstrated roles for 19S ATPase heterodimer subunits Rpt6 (Sug1) and Rpt4 (S10b) in yeast chromatin remodeling (Muratani and Tansey 2003). Binding of functional Rpt6 and Rpt4 to chromatin links histone H2B ubiquitination to the activating modifications of histone H3 lysine 4 (H3K4) and H3K79 methylation as mutations in Rpt6 and Rpt4 prevent methylation of H3K4 and H3K79 but do not affect H2B ubiquitination (Ezhkova and Tansey 2004). Mono-ubiquitination of histone H2B may direct the stable recruitment of 19S ATPases to chromatin, where the ATPases facilitate recruitment of methyl transferases to histones leading to histone H3K4 and H3K79 methylation and open chromatin (Ezhkova and Tansey 2004; Collins and Tansey 2006).

Lee and colleagues have demonstrated links between 19S ATPases and the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, a co-activator involved in yeast transcription initiation (Lee, Ezhkova et al. 2005). SAGA physically interacts with Rpt6 (Sug1) and this interaction further enhances recruitment of the SAGA complex to transcription factors and target promoters (Lee, Ezhkova et al. 2005). The SAGA complex catalyzes histone H3 acetylation and thus plays crucial roles in opening chromatin structure for transcription initiation (Lee, Ezhkova et al. 2005). Mutations in Rpt6 (Sug1) lead to reduced H3 acetylation due to decreases in promoter recruitment of general control of amino-acid synthesis 5 (GCN5), a histone acetylase component of SAGA (Lee, Ezhkova et al. 2005). The deubiquitinase Ubp8 of the SAGA complex is a structurally non-essential component of SAGA which has been implicated in deubiquitination of histone H2B, as disruption of the *ubp8* gene leads to a significant increase in histone H2B ubiquitination (Daniel, Torok et al. 2004). Ubp8 physically associates with upstream activation se-

quence of GAL10 gene when radiation sensitivity protein 6 (Rad6) dissociates, and this process has been shown to be essential for trimethylation of histone H3K4 (Daniel, Torok et al. 2004). In sum, these data indicate the 19S ATPases mediate initial chromatin reorganization events by modulating various post-translational modifications to regulate transcription at yeast promoters.

We have investigated roles played by the 19S ATPases in epigenetic modifications occurring at MHC class II and CIITA mammalian promoters. Sug1 physically interacts with histone acetyltransferase CREB-binding protein (CBP) and recruits CBP to the MHC class II promoter (Koues O.I. 2008). Recruitment of Sug1, and subsequent recruitment of CBP, to the promoter is essential for histone H3 acetylation, as knocking down Sug1 decreases histone H3 acetylation and reduces MHC class II expression (Koues O.I. 2008). We have also demonstrated roles for Sug1 and additional 19S ATPases in activating methylation events at the MHC class II *HLA-DRA* gene (Koues, Dudley et al. 2009). Sug1 modulates chromatin activation events including histone H3 trimethylation at lysine4 and histone H3 dimethylation at arginine 17 at both MHC class II and CIITA promoters (Koues, Dudley et al. 2009).

The 19S ATPases exist as heterodimeric complexes of Sug1-S10b, S7-S4, and S6a-S6b. Evidence that these heterodimers exist in the nucleus independent of the non-ATPase 19S lid subunits (Richmond, Gorbea et al. 1997; Russell, Steger et al. 1999; Adams 2003) suggests these heterodimers may also have roles independent of the 26S proteasome. We have shown that the 19S ATPase S6a regulates cytokine inducible expression of MHC class II by regulating expression of its master regulator CIITA (Agnieszka D.Truxax 2009). This regulation is likely at the level of transcription as knocking down S6a reduces acetylation at histone H3 and H4 preferentially at lysine 18 on histone H3 and lysine 8 on histone H4 (Agnieszka D.Truxax 2009). S6b the 19S ATPase that heterodimerizes with S6a, was determined to have parallel functions to S6a in



regulating histone acetylation events and to be dependent on S6a for these functions (Agnieszka D. Truax 2009), indicating S6a and S6b may function as heterodimers in regulating epigenetic events at mammalian promoters. Thus, the UPS plays important roles in regulating the dynamics of transcription by controlling the assembly of regulatory complexes and the turnover of transcription factors and cofactors involved in multiple stages of transcription. This regulation is further complicated by the multiple regulatory mechanisms employed by UPS and observations that components of the 26S proteasome have differential association and involvement in different stages of transcription.

## MHC CLASS II AND IMMUNE SYSTEM

### Immune system

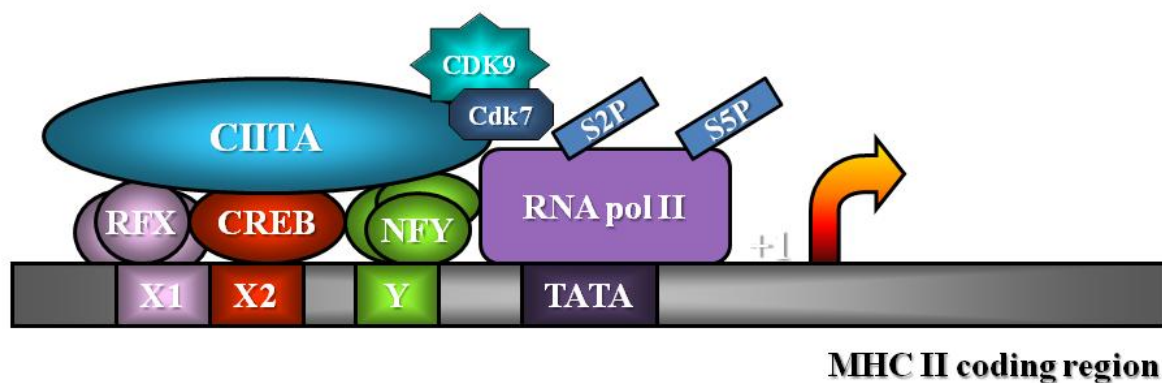
Proper functioning of the adaptive immune system requires fine regulation of various immune proteins. One of the major determinants of the immune response is the major histocompatibility complex (MHC). MHC are cell surface glycoproteins involved in antigen presentation (Kvist and Levy 1993). MHC class I is constitutively expressed by all nucleated cell types (York and Rock 1996), while constitutive expression of MHC class II is restricted to antigen presenting cells (APCs) (Matheux and Villard 2004). Cytokines including interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) upregulate MHC class II expression on almost all other cell types (Weber and Rosenberg 1988; Matheux and Villard 2004). The antigens presented by the two classes of MHC differ in that MHC class I presents cytosolic antigen on the cell surface to activate CD8<sup>+</sup> T cells (cytotoxic T cells) (Rock 1996) while MHC class II presents processed exogenous antigens on the cell surface to activate CD4<sup>+</sup> T cells (Matheux and Villard 2004).

Activation of CD4<sup>+</sup> T cells is required for cell mediated and antibody mediated immune responses (Glimcher and Kara 1992). Due to the crucial roles in adaptive immunity played by MHC class II molecules, deficiencies in MHC class II expression lead to the development of bare lymphocyte syndrome (BLS) (Reith and Mach 2001), an autosomal recessive disease fatal in early childhood. Severe combined immunodeficiency (SCID) is also an outcome of MHC class II deficiency (Reith and Mach 2001). SCID patients lack CD4<sup>+</sup> T cells and, in the absence of these T cells, many B cells also fail to function, thus SCID patients are severely immunocompromised. Overexpression of MHC class II is associated with increased vulnerability to the development of autoimmune diseases including multiple sclerosis, rheumatoid arthritis and myocardial infarction (Swanberg, Lidman et al. 2005). In addition, MHC class II molecules play critical roles in anti-tumor immunity as tumor specific CD4<sup>+</sup> T cells help recruit and activate CD8<sup>+</sup> T cells at the tumor sites (Wang 2003) and tumor cells frequently decrease MHC class II expression to evade immune recognition (Guy, Krajewski et al. 1986). Because MHC class II is a critical regulator of adaptive immune response, its expression is tightly regulated (Benoist and Mathis 1990).

### Regulation of MHC class II

There are three types of MHC class II proteins expressed in humans HLA -DR, -DP and -DQ (Trowsdale 2001) expression of which is regulated primarily at the level of transcription (Benoist and Mathis 1990; Glimcher and Kara 1992). The MHC class II proximal promoter contains a conserved cis-acting region consisting of W (or S), X1, X2 and Y boxes (Benoist and Mathis 1990; Reith and Mach 2001). Ubiquitous transcription factors including regulatory factor

X (RFX5, RFX-AP, RFX-ANK) (Steimle, Durand et al. 1995; Ting and Trowsdale 2002) (Nagarajan, Louis-Pence et al. 1999) (Masternak, Barras et al. 1998), cAMP responsive element binding protein (CREB) (Moreno, Beresford et al. 1999) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Mantovani 1999) bind respectively to the X1, X2 and Y elements to form the “enhanceosome complex” (Ting and Trowsdale 2002) (**Figure 1.9**). Although recruitment of these transcription factors is essential for MHC class II transcription, it is not sufficient (Masternak, Muhlethaler-Mottet et al. 2000). MHC class II transcription initiation requires recruitment of the master regulator CIITA to the enhanceosome complex (Steimle, Otten et al. 1993; Mach, Steimle et al. 1996; Muhlethaler-Mottet, Otten et al. 1997; Masternak, Muhlethaler-Mottet et al. 2000). CIITA does not directly bind to DNA but acts as a co-factor that binds the pre-assembled enhanceosome complex (Fontes, Jiang et al. 1997; Mahanta, Scholl et al. 1997; Kretsovali, Agaloti et al. 1998; Moreno, Beresford et al. 1999; Zhu, Linhoff et al. 2000; Jabrane-Ferrat, Nekrep et al. 2003) (Zhu, Linhoff et al. 2000). Upon cytokine stimulation, CIITA is recruited to the MHC class II proximal promoter and co-ordinates multiple steps to initiate MHC class II transcription (Zhu, Linhoff et al. 2000). Chromatin remodeling enzymes including BRG1/ATPase (Mudhasani and Fontes 2005) and histone acetyltransferases (CBP/p300) are recruited by CIITA to open the chromatin structure (Drozina, Kohoutek et al. 2005) (Wright and Ting 2006). Various basal transcription factors and cyclin dependent kinases (CDKs), which phosphorylate the C-terminal domain of RNA polymerase II (RNA pol II), are also recruited in a CIITA dependent fashion; following which MHC class II transcription initiates (Spilianakis, Kretsovali et al. 2003).



**Figure 1.9: MHC class II HLA-DRA promoter.**

MHC class II proximal promoter region has a conserved cis-acting region consisting of W (or S), X1, X2 and Y boxes. Ubiquitous transcription factors including regulatory factor X (RFX5, RFX-AP, RFX-ANK), cAMP responsive element binding protein (CREB) and nuclear factor-Y (NF-Y) bind to the X1, X2 and Y elements respectively to form the “enhanceosome complex”. MHC class II transcription requires recruitment of master regulator, class II transactivator (CIITA) to the enhanceosome complex. CIITA does not directly bind to DNA but acts as a co-factor that binds the pre-assembled enhanceosome complex and recruits kinases that phosphorylate

CIITA interaction with the enhanceosome complex and transcription factors is further enhanced by phosphorylation by c-AMP-dependent protein kinase (PKA) (Chang CH 2002; Sisk, Nickerson et al. 2003). Transcription initiation then occurs, however elongation is soon paused due to the presence of negative transcription elongation factor (N-TEF) and DRB-sensitive inducing factor (DSIF) (Kanazawa and Peterlin 2001; Peterlin and Price 2006). To enable proper transcription elongation, CIITA binds to the CycT1 subunit of positive transcription elongation factor (P-TEFb) complex, following which the P-TEFb complex is recruited to the MHC class II promoter (Kanazawa, Okamoto et al. 2000). The cdk9 subunit of P-TEFb complex phosphorylates N-TEF and releases the paused complex leading to elongation (Kohoutek, Blazek et al. 2006; Oven, Brdickova et al. 2007). These multiple interactions are crucial steps in the initiation of MHC class II transcription and their regulation plays a critical role in initiation of the immune response. The steps involved in activation of MHC class II transcription are well understood, but how CIITA itself is recruited, stabilized and degraded at the MHC class II proximal promoter region remains unknown.

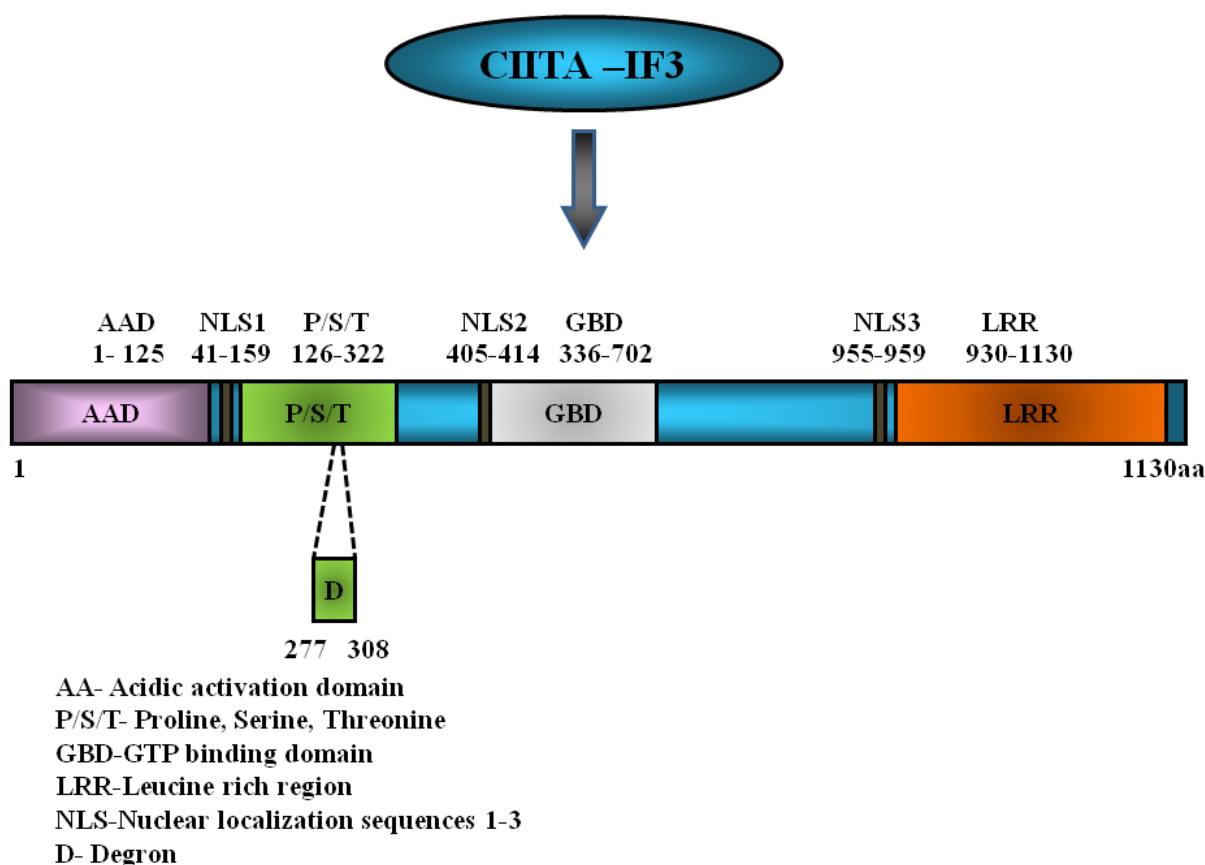
### CIITA, The Master Regulator of MHC class II

CIITA is a non-DNA binding co-factor that is absolutely required for initiation of MHC class II genes (Masternak, Muhlethaler-Mottet et al. 2000). CIITA is not only essential for MHC Class II transcription, but also plays crucial roles in transcriptional regulation of additional diverse immune response genes including IL-4 (Sisk, Gourley et al. 2000), IL-10 (Yee, Yao et al. 2005), E-cathepsin (Yee, Yao et al. 2004), MMP-9 (Nozell, Ma et al. 2004), plexin (Wong, Brickey et al. 2003) and Fas ligand (Gourley and Chang 2001). As CIITA plays critical roles in

regulating expression of multiple immune response genes, it is inevitable that CIITA might play a role in disease development. A selection from the many diseases in which CIITA has been implicated include head and neck cancer (Meissner, Whiteside et al. 2009), small cell lung cancer (Yazawa, Kamma et al. 1999), Erwins sarcoma (Dagmar, Alfons et al. 2009), autoimmune Addison's disease (Skinningrud, Husebye et al. 2008), and arteriosclerosis (Buttice, Miller et al. 2006). Due to the role of CIITA in regulating expression of multiple genes and in development of multiple diseases, intense studies have focused on understanding CIITA regulation. Despite this, due to the complex structure of CIITA and the roles CIITA plays in transcriptional regulation of multiple genes in various cell types, much regarding CIITA regulation remains unknown.

CIITA is an 1130 amino acid protein with four functional domains: an N-terminal transcriptional acidic activation domain (AAD), a proline/serine/threonine rich (P/S/T) domain which is required for CIITA transactivity, a GTP-binding domain (GBD), and a C-terminal leucine rich region (LRR) that is required for localization (Tosi, Jabrane-Ferrat et al. 2002) (**Figure 1.10**).

CIITA has three nuclear localization sequences (NLS) that are required for nuclear translocation and activity (Cressman, O'Connor et al. 2001). The P/S/T rich region of CIITA contains a proteolytic signal site called a degron, specific sequences that frequently target proteins for degradation (Drozina, Kohoutek et al. 2006). CIITA is expressed from three distinct promoters (pI, pIII and pIV) to ensure the presence of CIITA in different cell types under different conditions (Muhlethaler-Mottet, Otten et al. 1997). CIITA isoform I (IF1) is expressed from pI in dendritic cells and macrophages, isoform III (IF III) is expressed from pIII in B cells, and isoform IV (IF IV) is expressed from pIV in IFN- $\gamma$  induced cells (Muhlethaler-Mottet, Otten et al. 1997). Although transcriptional control of CIITA is well understood, the post-translational code (different combinations of post-translational modifications) of CIITA remains to be explored.



**Figure 1.10: Schematic representation of domain structure of CIITA protein.**

CIITA is a 1130 amino acid long protein with four functional domains, the N-terminal transcriptional acidic activation domain (AAD) required for CIITA transactivity, proline/serine/threonine rich (P/S/T) domain required for binding to various factors and cofactors, GTP-binding domain (GBD) and C-terminal leucine rich region (LRR) required for its localization and oligomerization. CIITA has three nuclear localization sequences (NLS) that are required for its nuclear translocation and its activity. The P/S/T rich region of CIITA contains proteolytic signal sites called degrons (D) that are specific sequences that target proteins for ubiquitination at proximal lysine residues and degradation. This figure is adapted from Ting J.T. and Trowsdale J., 2002.

## Post-translational modification of CIITA

Transcription factors bind to specific sites on promoters to initiate transcription of target genes. Therefore, transcription factors and co-activators are highly regulated in order to allow fine-tuning of gene expression (Kodadek, Sikder et al. 2006). Regulation of CIITA is achieved through multiple levels of control including genetic, transcriptional and post-translational pathways. Amongst these regulations, post-translational regulation is the most crucial as in order to perform; CIITA is highly 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).

Post-translational modifications including phosphorylation (P), ubiquitination (Ub) and acetylation (Ac) have been shown to regulate CIITA activity (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). A comprehensive table of the various post-translational modifications of CIITA and their known roles in cellular function is shown in **Table 1.1**. Phosphorylation at serine 384 (S834) and serine 1050 (S1050) in the LRR domain of CIITA leads to a reduction in MHC Class II expression (Li, Harton et al. 2001). Alternatively, phosphorylation at serine 374 (S374) and serine 375 (S375), that lie between the P/S/T and GBD domains of CIITA, increases MHC class II expression (Sisk, Nickerson et al. 2003). These different phosphorylation events are catalyzed by PKA, thus it is thought that PKA activates CIITA at the MHC class II proximal promoter by phosphorylating S374 and S375 and inactivates CIITA by phosphorylating S834 and S1050 (Dummer, Bastian et al. 1996; Li, Harton et al. 2001; Sisk, Nickerson et al. 2003; Wu, Kong et al. 2009). Xu and colleagues have demonstrated that kinases GSK1 and CKI both phosphorylate serine 373 (S373) and serine 377 (S377), and that these phosphorylation



events increase CIITA mediated collagen transcription without affecting MHC class II expression, indicating that these post-translational modifications play alternative roles in modulating CIITA transactivity (Xu, Harton et al. 2008). The P/S/T domain of CIITA also has multiple phosphorylation sites, and Greer and colleagues have shown that CIITA is phosphorylated at sites serines 286, 288 and 293 (S286, S288 and S293), and that these phosphorylation events regulate CIITA nuclear localization but not oligomerization (Greer, Harton et al. 2004). Following this observation, Voong and colleagues subsequently identified ERK1/2 as the kinase involved in phosphorylating these sites (Voong, Slater et al. 2008).

Acetylation, a reversible post-translational modification, has also been shown to regulate CIITA activity. Histone acetyl transferases p300 and PCAF acetylate lysine 141 (K141) and lysine 144 (K144) in the N-terminus of CIITA and increase MHC Class II expression (Spilianakis, Papamatheakis et al. 2000). Histone deacetylases (HDACs) catalyze the removal of acetyl groups and have also been shown to modulate CIITA transactivity. HDAC2 mediated deacetylation of CIITA targets it for degradation and thus reduces CIITA mediated gene expression whereas SIRT1 mediated deacetylation enhances CIITA transactivity (Kong, Fang et al. 2009; Wu, Kong et al. 2009). Finally, CIITA has been shown to have an intrinsic histone acetyl transferase activity that resides in its N-terminus, although target proteins for this activity remain to be identified (Raval, Howcroft et al. 2001).

The posttranslational modification of mono-ubiquitination has been shown to increase CIITA transactivity (Greer, Zika et al. 2003). Upon mono-ubiquitination, CIITA demonstrates increased association with the enhanceosome complex at the MHC class II promoter where it drives increased MHC class II transcription (Greer, Zika et al. 2003). CIITA has also been shown to be degraded by polyubiquitination and the ubiquitin-proteasome pathway (Schnappauf,

Hake et al. 2003). Although these studies have demonstrated ubiquitination to be a regulatory mechanism of CIITA transactivity, sites of ubiquitination in CIITA remain unknown. Identifying ubiquitination sites is a significant task as CIITA has multiple ubiquitination sites (42 lysine residues). Although much is known about the post-translational modifications of CIITA, how CIITA is recruited to, stabilized on and eventually removed from the MHC class II promoter remains enigmatic.

**Table 1.1 Post-translational code of CIITA**

<b>Post-translational modifications</b>	<b>Sites of modifications</b>	<b>Effect of modifications</b>
<b>Phosphorylation</b>	S834, S1050	Inhibition of MHC class II transcription
<b>Phosphorylation</b>	S286, S673, S942, S943, S944, S1128	Not determined
<b>Phosphorylation</b>	S374, S375	Increase MHC class I transcription, interaction with co-activator p300 and oligomerization
<b>Phosphorylation</b>	S373, S377	Inhibition of collagen transcription by interacting with co-repressor Sin3B
<b>Phosphorylation</b>	S286, S288, S293	Inhibition of MHC class II transcription and Nuclear export
<b>Phosphorylation</b>	S293	Repression of CD36
<b>Ubiquitination</b>	Not determined	Increase in MHC class II transcription
<b>Acetylation</b>	K141, K144	Enhance nuclear localization of CIITA and increase MHC Class II expression
<b>Deacetylation</b>	Not determined	HDAC2 mediated deacetylation decreases CIITA stability and its interaction with RFX5
<b>Deacetylation</b>	Not determined	SIRT1 mediated deacetylation increases MHC class II transcription

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

## SUMMARY

The master regulator of degradation, the 26S proteasome, regulates transcription through proteolytic and non-proteolytic pathways. The 19S regulatory particle and the 20S proteolytic core of the 26S proteasome can function independently of each other and each component has distinct roles in transcription regulation (Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Rasti, Grand et al. 2006; Zhu, Wani et al. 2007). The 19S proteasomal ATPases associate with actively transcribing genes in yeast (Gonzalez, Delahodde et al. 2002; Auld, Brown et al. 2006) and have been shown to regulate the elongation process carried out by RNA-polymerase II (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002). The role of the 19S proteasomal subunit in transcription is widely studied in yeast, it remains unclear if the 19S proteasomal subunits play similar roles in mammalian gene transcription.

Because MHC class II plays a crucial role in initiating the adaptive immune response to infection and in anti-tumor responses, it is of utmost importance to understand how MHC class II transcription and CIITA stability and activity are regulated. It is known that mono-ubiquitination of CIITA increases its transactivity and that CIITA is eventually degraded via the ubiquitin-proteasome pathway (Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003). These findings link CIITA and regulation of MHC class II transcription to the ubiquitin proteasome system (UPS). In the light of the novel non-proteolytic roles of various 26S proteasomal subunits in transcription regulation in yeast, it is probable that these subunits have roles to play in MHC class II gene transcription. Thus, studying the roles played by the UPS in regulating MHC class II transcription will not only aid in the development of novel therapies to manipulate MHC class II gene expression but will also further our knowledge of the roles of ubiquitin and 19S proteasomal subunits in mammalian gene transcription.

## CHAPTER 2

### THE 19S PROTEASOME ATPASE SUG1 PLAYS A CRITICAL ROLE IN REGULATING MHC CLASS II TRANSCRIPTION

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## SUMMARY

Emerging evidence in yeast suggests roles for ATPases of the 19S proteasome as mediators of transcriptional systems through their association with actively transcribed promoters, facilitation of clearance of paused elongation complexes and recruitment of coactivators. Although 19S subunits also regulate mammalian transcription, their role in recruiting transcription factors remains unclear. Here, we demonstrate for the first time a role for the 19S proteasome ATPase Sug1 in regulating transcription of the critical adaptive immune gene, MHC class II. Sug1 associates with the class II transactivator, CIITA, and with the MHC class II proximal promoter. In the absence of Sug1, *HLA-DR* promoter activity and MHC class II transcription are decreased. Critically, CIITA association with the MHC II promoter is dramatically decreased when Sug1 expression is reduced, even under conditions of proteasome inhibition. In contrast to the rapid promoter association of the 19S subunit, a 20S proteasome subunit associates with the MHC class II proximal promoter following prolonged cytokine stimulation and its association corresponds with pronounced promoter disassociation of CIITA. Taken together, these data demonstrate that both 19S and 20S subunits of the 26S proteasome play specific and critical roles in regulating CIITA activity and MHC class II transcription.

## BACKGROUND

MHC class II gene products encode cell-surface glycoproteins that bind and present antigenic peptides derived from extracellular proteins. Molecules of the MHC class II complex, HLA-DR, HLA-DP and HLA-DQ in humans, present these antigenic peptides specifically to CD4<sup>+</sup> T helper lymphocytes, resulting in initiation of T helper cell activation and proliferation. This basic premise forms an essential arm of the adaptive immune response and is critical to the activation of both cellular and antibody mediated immune mechanisms (Glimcher and Kara, 1992). Tight regulation of MHC class II protein expression is necessary for control of the adaptive immune response and for the prevention of autoimmune diseases. MHC class II expression is regulated primarily at the level of transcription of MHC class II genes (Benoist and Mathis, 1990) (Reith and Mach, 2001) by a group of *cis* acting elements, W(or S), X1, X2 and Y boxes commonly referred to as the W-X-Y module, that are highly conserved among promoters of MHC class II genes (Glimcher and Kara, 1992). The W-X-Y module is recognized and bound by the trimeric RFX and NFY complexes (Steimle et al., 1995; Ting and Trowsdale, 2002) (Nagarajan et al., 1999) (Mantovani, 1999) (Masternak et al., 1998) and by CREB (Moreno et al., 1999) (Muhlethaler-Mottet et al., 2004). These transcription factors are ubiquitously expressed and, while their binding is insufficient for MHC class II expression (Masternak et al., 2000), they form a platform that recruits the Class II Transactivator (CIITA) to the proximal promoter, a step required for MHC class II expression (Steimle et al., 1993).

Unlike the requisite MHC class II transcription factors mentioned above, CIITA gene expression proceeds and parallels that of MHC class II (Ting and Trowsdale, 2002). CIITA does not bind directly to the MHC class II promoter, but it is known as the master regulator of MHC class II transcription as its association with the pre-assembled enhanceosome complex coordi-

nates several additional steps leading to transcriptional initiation (Moreno et al., 1999) (Zhu et al., 2000) (Masternak et al., 2000) (Bewry et al., 2007). CIITA directs MHC class II transcription initiation through interactions with the BRG1 ATPase of the hSWI/SNF remodeling complex (Mudhasani and Fontes, 2002) and through promoter recruitment of histone acetyltransferases (p300, CBP, pCAF and SRC-1) (Drozina et al., 2005; Wright and Ting, 2006), components of the basal transcriptional machinery (Fontes et al., 1997) (Mahanta et al., 1997) and kinases that phosphorylate the carboxyl terminus of RNA polymerase II (Spilianakis et al., 2003). Phosphorylation of CIITA further increases its interactions with the co-activator CBP/p300, with RFX and with CIITA itself, thus increasing MHC class II promoter activity (Sisk et al., 2003). Therefore, interactions of CIITA with the MHC class II enhanceosome complex, with the basal transcriptional machinery, and with chromatin remodeling enzymes play a critical role in regulating MHC class II transcription.

Localization of the enhanceosome proteins, chromatin remodeling enzymes, basal transcriptional machinery and CIITA at the transcribing MHC class II proximal promoter makes this, as are most promoters, a complex regulatory region. How cells manage to regulate and recruit multiple protein:protein interactions like these has become an increasingly important question. Recent evidence has supported a role for components of the 26S proteasome as mediators of the assembly of transcriptional systems in yeast. The 26S proteasome is a multi-protein complex that regulates the degradation of polyubiquitinated proteins and consists of two basic parts: a 20S proteolytic core complex and a 19S regulatory particle (Baumeister et al., 1998). The 19S can be further divided into base and lid subunits. The base is composed of six homologous AAA ATPases (S4, S6a, S6b, S7, Sug1(Sug1) and S10b) and three non-ATPases (S1, S2 and S5a) while the lid is composed of eight non-ATPase subunits (Ciechanover, 1998). Studies in yeast



have demonstrated that ATPase components of the 19S proteasome play a role in activating gene transcription. The ATPases associate with actively transcribed genes where they may link promoter recruitment of transcription factors to their eventual degradation by polyubiquitination and the proteasome (Makino et al., 1999) (Gonzalez et al., 2002). In yeast, the most novel implications for a role for the proteasome in regulating transcription come from observations that 19S ATPases directly associate with genes that are actively being transcribed. These ATPases, but not other proteins from the 19S lid or the 20S proteolytic core, have been shown to bind to the yeast Gal4 activation domain *in vitro*, suggesting that components of the 19S complex, and not the 26S holoenzyme, are intimately involved with transcription (Gonzalez et al., 2002). In addition, the 26S proteasome associates with RNA polymerase II (RNA Pol II) (Gillette et al., 2004) and is critical for efficient RNA Pol II mediated elongation (Ferdous et al., 2001). In fact, specific inhibition of activity of the 19S subunit decreases elongation while inhibition of the 20S subunit increases elongation, indicating that a balance between these two subunits and their activities may be important for transcriptional regulation in yeast (Gillette et al., 2004) (Ferdous et al., 2001) (Ferdous et al., 2002). While most research to date has been performed in yeast systems, several very recent publications have highlighted the significance of the 19S in regulating transcription in mammalian cells. The 19S ATPase Sug1 is recruited to the viral transcription factor E1A and enhances transcription from viral promoters during Adenoviral infection of human cells (Rasti et al., 2006). Sug1 also associates with the p53 transcription factor and is recruited to p53 responsive  $p21^{waf1}$  promoters in a manner that correlates with p53 recruitment (Zhu et al., 2007). Finally, the HIV-1 trans-activator protein, Tat, regulates the conversion of the 26S proteasome into separate 19S and 20S components by recruiting a proteasome-associated

protein, PAAF-1, which results in decreased proteasome activity and increased association of multiple 19S, but not 20S, subunits with the HIV-1 promoter (Lassot et al., 2007)

While the evidence for 19S involvement in mammalian transcription is tantalizing, many unanswered questions remain. It is primarily unclear if 19S subunits play a role in recruiting transcription factors to promoters and, if so, how these factors are targeted for recruitment. To understand the role of the proteasome system in regulating transcription of MHC class II genes, we investigated the role of the 19S ATPase subunit Sug1 in regulating CIITA activity, promoter recruitment and MHC class II transcription. Our data show that Sug1 associates with CIITA and with the MHC class II proximal promoter. Sug1 plays important roles in regulating CIITA activity and MHC class II expression, in part by regulating promoter recruitment of CIITA. The 20S proteasome is also strongly recruited to the proximal promoter, but only following prolonged cytokine stimulation, suggesting that the entire 26S proteasome is recruited to the MHC II proximal promoter at various times during active transcription. Our data show for the first time that CIITA, the master regulator of MHC class II transcription, is in turn regulated by components of the master regulator of protein destruction, the 26S proteasome. These data define novel roles for proteasomal subunits in transcriptional programs regulated by CIITA and offer further insights into the function of the proteasome in mammalian transcription.

## MATERIALS AND METHODS

**Cell culture:** HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% FCS, 50U/ml of penicillin, 50µg/ml of streptomycin and 2mM of L-

glutamine. The cells were maintained at 37°C with 5% CO<sub>2</sub>. Raji cells (Burkitts lymphoma-derived cell line) from ATCC were maintained in Roswell Park Memorial Institute (RPMI) Media (Mediatech) supplemented with 10% FCS, 50U/ml of penicillin, 50µg/ml of streptomycin and 5mM of L-glutamine at 37°C with 5% CO<sub>2</sub>.

**Plasmids:** The following plasmids used were described previously: Flag-CIITA, pCDNA3 and *HLA-DRA*-Luc (Cressman et al., 1999; Cressman et al., 2001; Greer et al., 2003). The C-Myc Sug1 construct was a generous gift from Dr. A.Wani (30).

**Transient transfection and luciferase reporter assays:** HeLa cells were plated at  $5 \times 10^4$  cells/well density (60% confluency) in 6-well plates and were incubated for 18 hours at 37°C. Transfection of indicated plasmids was carried out using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. 18 hours following transfection, cells were lysed with 100µl of 1x cell culture lysis reagent (Promega, Madison, WI). Following addition of cell lysis reagent, cells were scraped and the lysed cell suspension was centrifuged for 2 minutes at 12,000 rpm (Thermo electron 851, Thermo INC, Needham Heights, MA) at 4°C and luciferase assays were performed according to the manufacturer's instructions. *Luciferase reporter assays on Sug1 knockdowns.* HeLa cells were plated as above, transfected with 1.0µg of siRNA (diluted in buffer EC-R, Qiagen) using the RNAi transfection reagent (Qiagen) according to manufacturer's instruction. Following 30 hours of siRNA transfection, Flag-CIITA, *HLA-DRA*-Luc and pCDNA3 plasmids were transfected, the cells were lysed as above and luciferase assays and western blots were performed. 10% of total lysates were normalized for protein concentration, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with mAb to Sug1 (Novus Biologicals, Littleton, CO) and secondary goat anti-mouse horseradish peroxidase (HRP) conjugated antibody (Promega).

**Generation of siRNA duplexes:** Short interfering RNA duplexes were used for transient knockdown of Sug1 expression. siRNA sequences were designed with a GC content of 35-55% containing dTdT overhangs and were blasted using NCBI nucleotide BLAST. The target sequence of siRNA used was 5'-AAGGTACATCCTGAAGGTAAA-3' (Qiagen, Valencia, CA). siRNA for Lamin protein was used as a positive control siRNA (Qiagen) and scrambled sequence siRNA was used as a negative control (Qiagen; Santa Cruz Biotechnology, Santa Cruz, CA).

**RNA expression:** HeLa cells were plated at a cell density of  $4 \times 10^5$  cells/plate. Cells were harvested, washed with cold PBS, centrifuged at 3,000rpm at 4°C for 5 minutes, and total RNA was prepared with 1ml of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reconstituted in 60µl DEPC water (MP Biomedical, LLC, Aurora, OH) and stored at -80°C. The Omniscript reverse transcription kit (Qiagen) was used to make 20µl of cDNA from 1µg of RNA. Gene specific antisense primers (Sigma, Saint Louis, MO) were used for reverse transcription (RT). Q-PCR was done in an eppendorf microcycler. PCR reactions for all reactions included an initial 10 minute incubation step at 65°C followed by a 60 minute incubation step at 37°C according to the manufacturer's instructions (Qiagen). Real-time PCR reactions were carried out on an ABI prism 7900 (Applied Biosystems, Foster City, CA). MHC class II and CIITA promoter IV probes were labeled 5' with 6-carboxyfluorescein (FAM) reporter dye and 3' with N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) quencher dye. The primer and probe sequences are as follows: MHC class II sense sequence, 5'-AA GCCAACCTGGAAAT-CA-3'; antisense sequence, 5'-GGCTGTTCGTGAGCAC AGTT-3'; probe sequence, 5'-6 FAM-CTCCGATCACCAATGTACCTCCAGA-TAMRA-3'; human CIITA pIV sense sequence, 5'-GGGAGAGGCCACCAGCAG-3' ; antisense sequence, 5'-GCTCCAGGTAGCCACCTTCT-3' ;

probe sequence, 5'-6 FAM-CTGTGAGCTGCCGCTGTTCCC-TAMRA-3'. The housekeeping gene 18S ribosomal RNA was used to normalize mRNA values. 18S rRNA probes were labeled with tetrachloro-6-carboxyfluorescein (TET) reporter dye at the 5' end and with TAMRA quencher dye at the 3' end. Primer and probe sequence for 18S rRNA are as follows: sense sequence, 5'-GCTGCTGGCACCAGACTT-3'; antisense sequence, 5'- CGGCTACCACATCCA AGG-3'; probe sequence, 5'-6 TET-CAAATTACCCAC TCCCGACCCG-TAMRA-3'. Presented values from real-time PCR reactions were calculated on the basis of standard curves generated for each gene, were run in triplicate reactions and were analyzed using the SDS 2.0 program. *RNA Expression in Sug1 Knockdowns.* HeLa cells were plated at a cell density of  $4 \times 10^5$  cells and 18 hours later were transfected with siRNA. 24 hours following siRNA transfection, cells were stimulated with IFN- $\gamma$  (25 $\mu$ g/ml) for 20 hours. Cells were harvested and 15% of the cells were lysed with 1% Nonidet P-40 buffer ( NP-40:1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH<sub>2</sub>O) with protease inhibitor and were analyzed by western blot for Sug1 knockdown as above. The remaining fraction of cells was subjected to RNA extraction as described above.

**Co-immunoprecipitation:** *In transiently transfected cells.* HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10cm tissue culture plates. Following cell adhesion, cells were transfected using Fugene 6 (Roche) as indicated with 5 $\mu$ g of Flag-CIITA and/or 5 $\mu$ g c-Myc-Sug1 or transfection reagent alone. 20 hours following transfection, cells were untreated or were stimulated with IFN- $\gamma$  (25 $\mu$ g/ml) for four hours. Four hours following stimulation, or 24 hours following transfection, cells were lysed in RIPA lysis buffer (RIPA: 1M tris pH8.0, 5M NaCl, 10% NP-40, 5% DOC, 10% SDS, 1M DTT, dH<sub>2</sub>O) with protease inhibitors for 30 minutes on ice. Lysates were centrifuged, normalized for protein concentration, pre-cleared with 50  $\mu$ l mouse IgG (Sigma-

Aldrich, Saint Louis, MO) and immunoprecipitated with 50ul of anti-c-Myc agarose beads (Sigma-Aldrich) or with 5µg anti-CIITA (Santa Cruz Biotechnology). Immune complexes were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-Flag or anti-Myc monoclonal antibodies (Sigma-Aldrich). HRP conjugates were detected with Supersignal West Pico Chemiluminescent substrate to determine associations between Sug1 and CIITA. Equal loading was determined in non-immunoprecipitated lysates by immunoblot of total protein. *Endogenous co-immunoprecipitations.*  $15 \times 10^6$  Raji B cells were lysed in RIPA lysis buffer (RIPA: 1M Tris pH 8.0, 5M NaCl, 10% NP-40, 5% DOC, 10% SDS, 1M DTT, dH<sub>2</sub>O) with protease inhibitors for 30 minutes on ice. Lysates were centrifuged, normalized for protein concentration, pre-cleared with 50µl mouse IgG (Sigma-Aldrich, Saint Louis, MO) and immunoprecipitated with 10µg of mAb against Sug1. Positive control samples were immunoprecipitated with 10µg of mAb against CIITA and negative control samples were immunoprecipitated with 10µg of mouse IgG (Upstate Biotechnology). Isolated immune complexes were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted for endogenous CIITA. Equal loading was determined in non-immunoprecipitated lysates by immunoblot of total protein for CIITA.

**Chromatin immunoprecipitation (ChIP):** HeLa cells were plated at a cell density of  $2 \times 10^6$  and, at the end of IFN- $\gamma$  stimulation, cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature and crosslinking was stopped by the addition of 0.125 M glycine for 5 minutes at room temperature. Cells were lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, dH<sub>2</sub>O) and protease inhibitor for 30 minutes on ice and sonicated at constant pulse to generate an average of 500-750 bp sheared DNA. ChIP assays were performed as pre-

viously described (Greer et al., 2003). Briefly, the sonicated lysates were precleared with salmon-sperm coated agarose beads (Upstate Biotechnology) and half of the lysate was immunoprecipitated with 10µg of antibody to CIITA or individual proteasome components overnight at 4°C. The remaining half of the lysate was used as a control and was immunoprecipitated with control antibody. Following an additional 2 hour immunoprecipitation with 50µl of salmon-sperm coated agarose beads, samples were washed for 5 minutes at 4°C with the following buffers: Low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 150mM NaCl, dH<sub>2</sub>O), High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH<sub>2</sub>O), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH<sub>2</sub>O) and 1xTE buffer and were eluted with SDS elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>, dH<sub>2</sub>O). Following elution, crosslinks were reversed overnight with 5M NaCl at 65°C and immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using primers spanning the W-X-Y box of the MHC class II *HLA-DRA* promoter. MHC-II *HLA-DRA* promoter primers and probe sequences are as follows: MHC-II promoter probe, 5'-6 FAM-CTGGACCCTTTGCAAGAACCCTTCCC-TAMRA-3'; sense primer, 5'-TCCAATGAACGGAGTATCTTGTG T-3'; and antisense primer, 5'-TGAGATGACGCATCTGTTGCT-3'. Values were calculated based on standard curves generated.

*Chromatin immunoprecipitation in Sug1 knockdowns.* HeLa cells were plated at a cell density of  $8 \times 10^5$  in 15cm tissue culture plates and were transfected with Sug1 siRNA. Cells were treated with proteasome inhibitor MG132 for 4 hours and then harvested. Nine% of the total cell volume was lysed with 1% Nonidet P-40 buffer with protease inhibitor and analyzed by western

blot for Sug1 knockdown as described. The remaining fraction of cells was subjected to ChIP as described above.

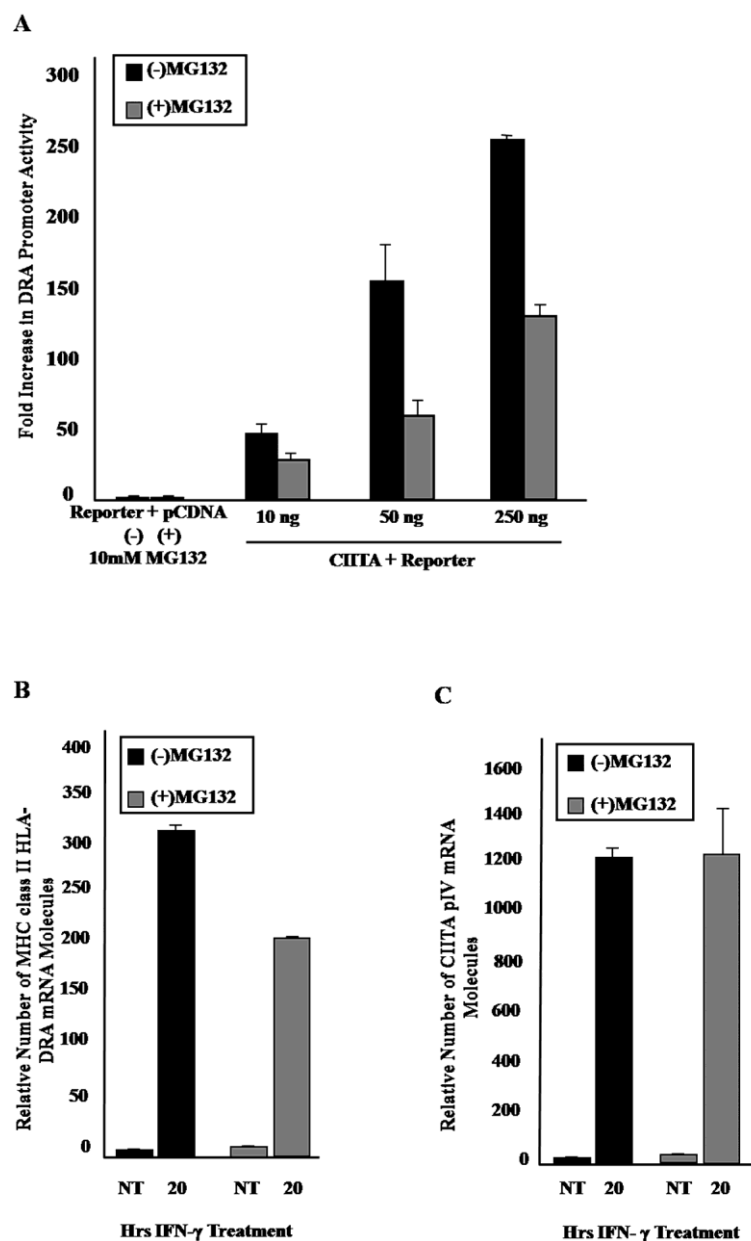
## RESULTS

### **Proteasome inhibition reduces CIITA-mediated MHC class II transcription.**

If components of the 26S proteasome positively regulate MHC class II gene transcription in a degradation-independent manner as has been proposed in yeast, then inhibition of the 26S proteasome should decrease, rather than increase, transcription. We used transient transfection to test the effect of the potent 26S proteasome inhibitor MG132 on the activation of the human MHC class II promoter, *HLA-DRA*, associated with a luciferase (Luc) reporter gene by CIITA. As shown in **Figure 2.1A**, transfection of HeLa cells with MHC class II *HLA-DRA*-Luc reporter and increasing amounts of CIITA resulted in dose dependent increases in *HLA-DRA* promoter activity, while treatment with the proteasome inhibitor MG132 for 16 hours decreased *HLA-DRA* promoter activity. Controls were transfected with the *HLA-DRA* reporter construct and empty pCDNA3 vector in the absence or presence of MG132. Inhibition of proteasome activity resulted in impaired CIITA mediated transcription of its downstream gene. Thus, we hypothesized that a component of the 26S proteasome is required for full CIITA transactivation.

We next assessed the effects of proteasome inhibition on the activation of endogenous MHC class II *HLA-DRA* genes. We stimulated HeLa cells with IFN- $\gamma$  to induce expression of CIITA (Pattenden et al., 2002) and determined the effects of proteasome inhibition on endogenous MHC class II transcription by simultaneously treating with MG132 for 4 additional hours. Real-time RT-PCR conducted on mRNA isolated from these cells showed that proteasome inhibition decreased IFN- $\gamma$  induction of MHC class II mRNA expression (**Figure 2.1B**).





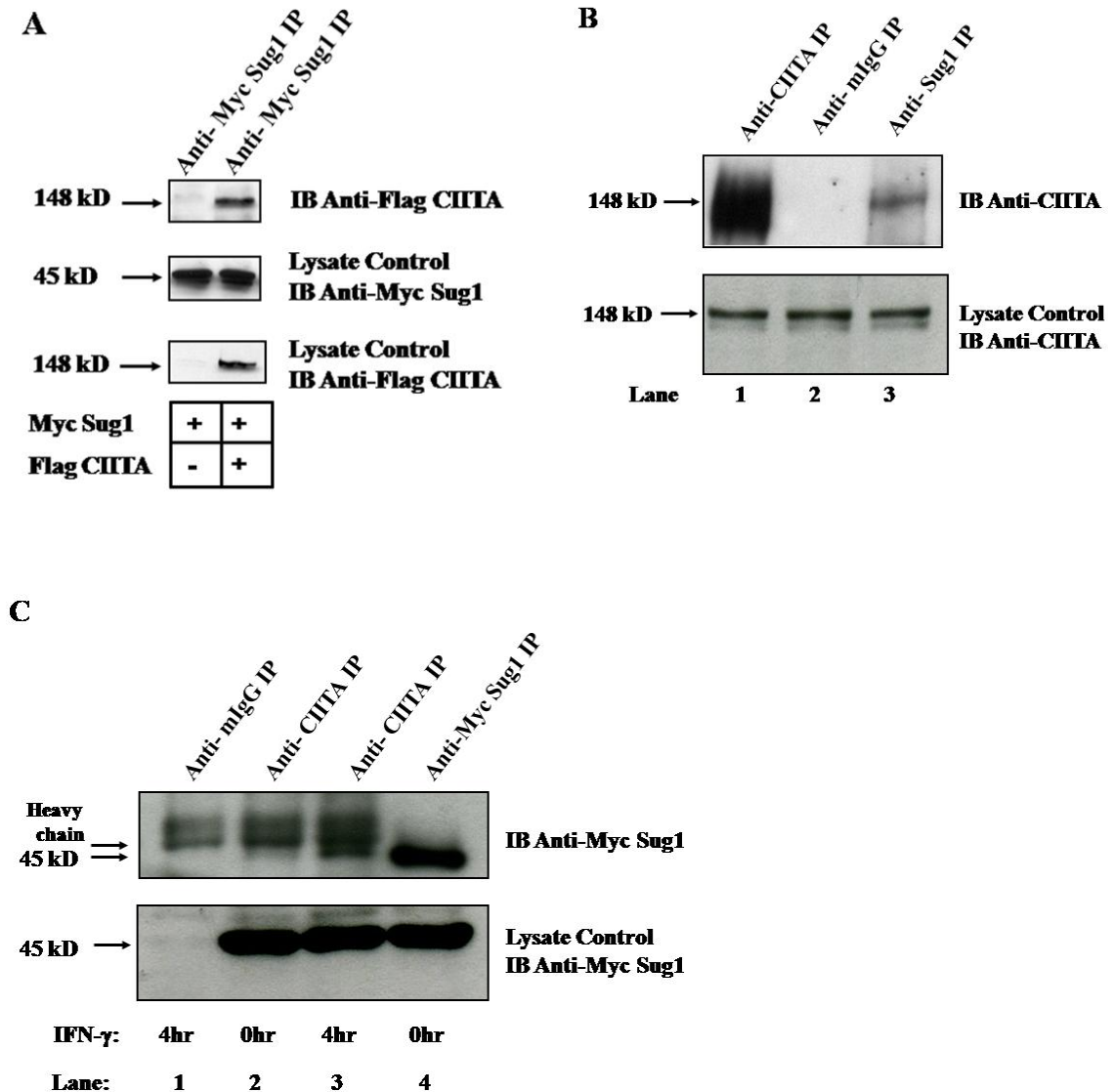
**Figure 2.1: Proteasome inhibition represses CIITA mediated MHC class II transcription.**

**(A) Proteasome inhibition decreases CIITA transcriptional activity.** HeLa cells were transfected with MHC II *HLA-DRA*-Luc reporter construct and increasing amounts of CIITA. Controls were transfected with reporter, empty pCDNA3 vector and were both untreated (black bars) and treated (grey bars) with the proteasome inhibitor MG132. Luciferase assays were performed in triplicate and data are presented as fold increase in the luciferase activity. Results presented represent the mean  $\pm$  S.D. and are representative of three independent experiments. **(B) and (C) Proteasome inhibition results in decreased levels of MHC class II mRNA but does not affect levels of CIITA mRNA.** HeLa cells were either unstimulated (NT) or were stimulated with IFN- $\gamma$  for 20 h and were simultaneously treated (grey bars) with MG132 for 4 h. Control cells were stimulated with IFN- $\gamma$ , but did not receive MG132 treatment (black bars). Levels of MHC class II mRNA were measured by real-time PCR and were normalized to 18S rRNA. Real-time PCR was performed in triplicate and results, which are representative of three experiments, represent the mean  $\pm$  S.D.

This reduction in MHC class II mRNA expression does not reflect stabilization of a general repressor or RNAase as CIITA mRNA expression levels remained stable in presence or absence of MG132 (**Figure 2.1C**). These results indicate that proteasome activity is required for optimal MHC class II expression in the presence of intact chromatin and under conditions of physiological CIITA induction and further suggest an important proteolysis-independent role of the proteasome in regulating MHC class II transcription.

### **The 19S ATPase subunit Sug1 physically interacts with CIITA *in vivo*.**

19S subunits play a critical role in binding ubiquitinated proteins and directing these proteins to the hydrolase activity of the 20S catalytic core (Rubin et al., 1998). The base component of the 19S has been shown to exist as a separate functional species, the APIS complex, apart from the 19S lid. The yeast 19S ATPase subunit and APIS complex member Rpt6 associates with both active and inactive yeast genes and undergoes substantial enrichment at multiple promoters under gene activation (Ezhkova and Tansey, 2004; Ferdous et al., 2001). Rpt6 stimulates promoter escape and elongation and is required for transcription of several stress induced proteins in yeast (Sulahian et al., 2006) and has been found to interact with transcription factors including the TFIID subunits TAF<sub>II</sub>90 and TBP (Sun and Allis, 2002; Yanagi et al., 2000), the XPB (Weeda et al., 1997) and Tfb2 (Sun et al., 2002) subunits of TFIIH, the Cdc68 subunit of the transcription elongation factor FACT (Sun et al., 2002) and the Rpb1 subunit of pol II (Sun et al., 2002). Given the interactions of Rpt6 with various transcription factors, we sought to determine if its mammalian homolog, Sug1, is involved in regulating MHC class II transcription. In order to address specific mechanisms by which Sug1 might interact with the MHC class II



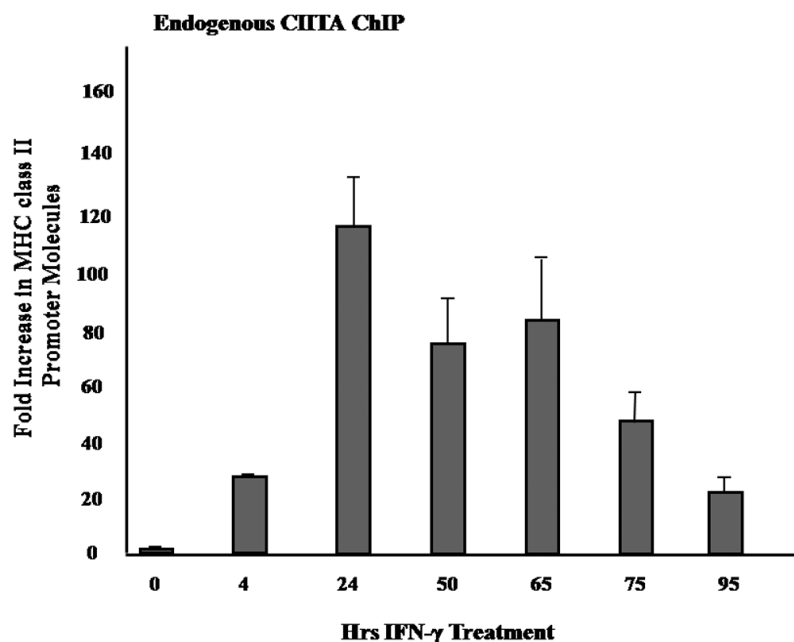
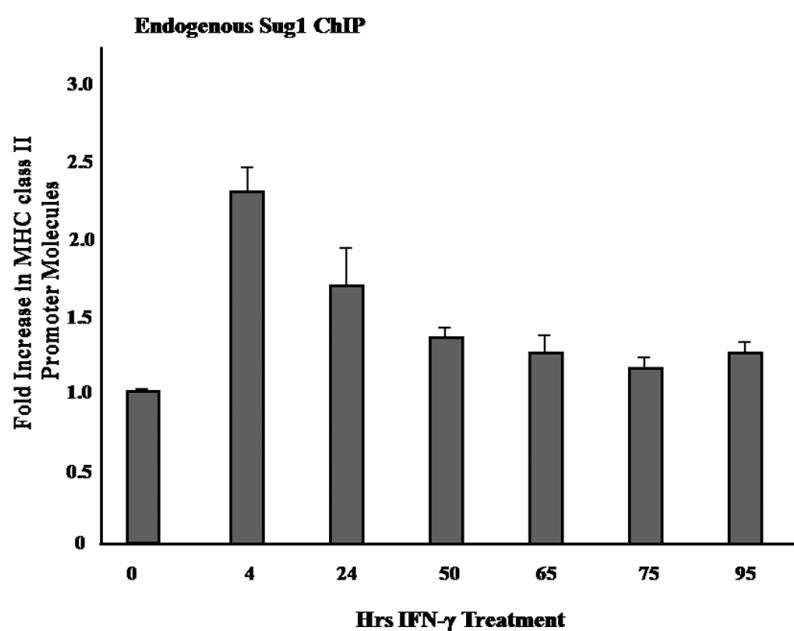
**Figure 2.2: The 19S ATPase Sug1 associates with CIITA.**

(A) HeLa cells were transfected with Flag-CIITA and c-Myc-Sug1 as indicated. Cells were lysed and immunoprecipitated (IP) with anti-c-Myc agarose beads (lanes 1 and 2). IP samples were immunoblotted (IB) for Flag-CIITA. Lysates were IB for Flag-CIITA and c-Myc. (B) Raji cells were lysed and immunoprecipitated with mAb against CIITA as a positive control (lane 1), with rabbit IgG as a negative control (lane 2) and with mAb against Sug1 (lane 3). IP (top panel) and lysate (bottom panel) samples were immunoblotted with antibody against endogenous CIITA. (C) HeLa cells were transfected with c-Myc-Sug1 (lanes 2–4). Twenty hours following transfection, cells were stimulated with IFN- $\gamma$  as indicated. Four hours following stimulation, cells were lysed and immunoprecipitated with anti-CIITA mAb (lane 1, negative control; lanes 2 and 3, experimental) or anti-Myc-Sug1 as a positive control (lane 4). IP (top panel) and lysate (bottom panel) samples were immunoblotted for Myc-Sug1. All results reported in this figure are representative data of five experiments.

promoter, we examined interactions of Sug1 with CIITA. Sug1 and CIITA immunoprecipitated both from HeLa cells overexpressing tagged and versions of the proteins (**Figure 2.2A**) and endogenously from Raji B cells (**Figure 2.2B**). Association of endogenous CIITA and overexpressed Sug1 was enhanced upon stimulation with IFN- $\gamma$  (**Figure 2.2C**), and thus parallels increased transcription of MHC class II genes (Piskurich et al., 2006). Equal transfection and loading of cell lysates was confirmed by immunoblot (IB) analysis of total protein (**Figure 2.2A-C, lower panels**).

### **The 19S proteasome Sug1 subunit is recruited to the endogenous MHC class II promoter in parallel to CIITA.**

Yeast 19S ATPases, including Rpt6, associate with the *GAL1/10* promoter and the *GAL1* gene upon stimulation of transcription (Gonzalez et al., 2002). In mammalian cells, the Rpt6 homolog Sug1 is recruited to *p21<sup>waf1</sup>* promoters in response to ultraviolet-induced DNA damage (Zhu et al., 2007). To determine if Sug1 directly associates with the endogenous MHC class II promoter, and to compare this association with that of CIITA, we performed ChIP assays on an endogenous MHC class II (*HLA-DRA*) promoter (**Figure 2.3**). First, to evaluate CIITA association with the MHC class II proximal promoter, HeLa cells were stimulated with IFN- $\gamma$ , immunoprecipitated with antibody to endogenous CIITA, and analyzed by real-time PCR with primers spanning the W-X-Y box of the MHC class II *HLA-DRA* promoter. Endogenous CIITA associated with the *HLA-DRA* promoter by 4 hours of stimulation with IFN- $\gamma$ ; association dramatically increased by 24 hours, decreased and remained through 65 hours of IFN- $\gamma$  stimulation and was rapidly declining at 95 hours (**Figure 2.3A**).

**A****B**

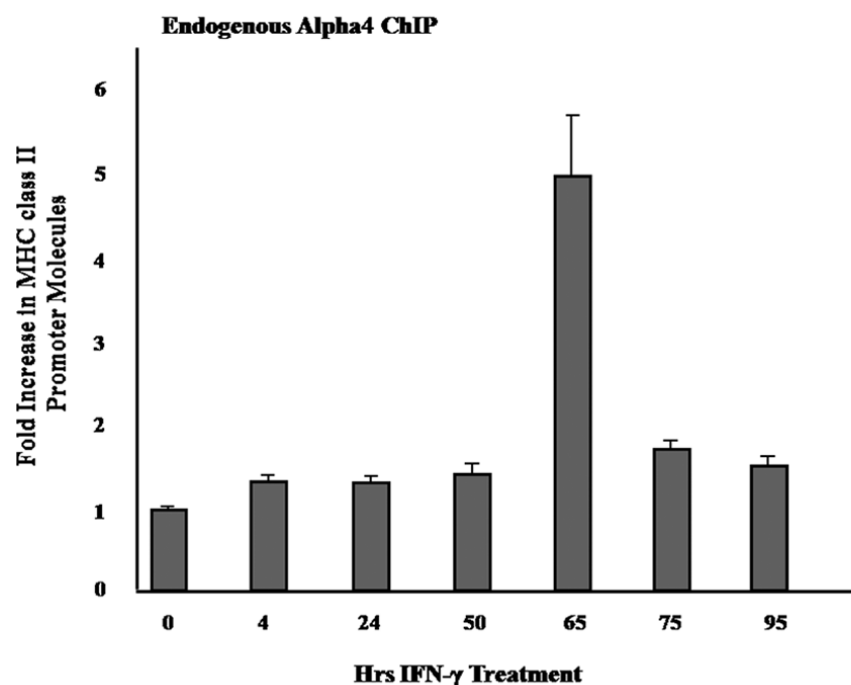
**Figure 2.3: The 19S ATPase Sug1 associates with the MHC class II proximal promoter.**

A and B, ChIP assays were performed in HeLa cells stimulated with IFN- $\gamma$  for 0 to 95 h. Lysates were immunoprecipitated (IP) with control antibody, antibody to endogenous CIITA (A) or to endogenous Sug1 (B), and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC class II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of *HLA-DRA* promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as fold increase in the MHC class II promoter DNA relative to unstimulated IP samples. Control IP values for (A) and (B) were  $0.5 \pm 0.05$ . Values for control IP's, CIITA and Sug1 IP's represent mean  $\pm$  S.E.M. of ( $n = 3$ ) independent experiments.

These *in vivo* kinetic data are consistent with published results demonstrating that elevated levels of histone acetylation remain at the MHC class II promoter for a minimum of 48 hours following IFN- $\gamma$  stimulation (Beresford and Boss, 2001). Low levels of Sug1 associated with the *HLA-DRA* promoter by 30 minutes of stimulation with IFN- $\gamma$ ; association peaked at 4 hours and low but reproducible Sug1 association was sustained throughout 95 hours of IFN- $\gamma$  stimulation (**Figure 2.3B**). These findings indicate that Sug1 is inducibly recruited to MHC class II proximal promoters.

**The 20S proteasome is recruited to the MHC class II proximal promoter following prolonged IFN- $\gamma$  stimulation.**

The 20S contains fourteen subunits that assemble as a stack of four rings, two alpha and two beta rings, each having seven distinct subunits arranged in a barrel structure: alpha 1-7, beta 1-7, beta 1-7 and alpha1-7. The alpha subunits form the outer rings and, although they have no catalytic activity, they are critical for interactions of the 19S regulator with the 20S core and thus for assembly of the intact 26S proteasome (Baumeister et al., 1998). In yeast, components of the 20S catalytic core of the 26S proteasome are recruited to actively transcribing genes, potentially to remodel transcription complexes or to degrade transcription factors once transcription of the gene has been initiated (Gillette et al., 2004) (Morris et al., 2003). In mammalian cells, the 20S subunit alpha 4 is present at the HIV-1 promoter and coding regions under low transactivation conditions (Lassot et al., 2007). To determine if the 20S directly associates with the endogenous MHC class II promoter, we performed ChIP assays on an endogenous MHC class II (*HLA-DRA*) promoter using antibodies against endogenous alpha 4 (**Figure 2.4**).



**Figure 2.4: The 20S proteasome associates with the MHC class II proximal promoter following extensive IFN- $\gamma$  stimulation.**

ChIP assays were carried out in HeLa cells stimulated with IFN- $\gamma$  for 0–95 h. Lysates were immunoprecipitated (IP) with control antibody or antibody to the endogenous alpha four component of the 20S and associated DNA was isolated and analyzed via real-time PCR using primers spanning the W–X–Y box of the MHC class II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of *HLA-DRA* promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as fold increase in the MHC class II promoter DNA relative to unstimulated CIITA IP samples. Control IP values were  $0.5 \pm 0.5$ . Values for control and alpha four I IP's represent mean  $\pm$  S.E.M. of ( $n = 3$ ) independent experiments.

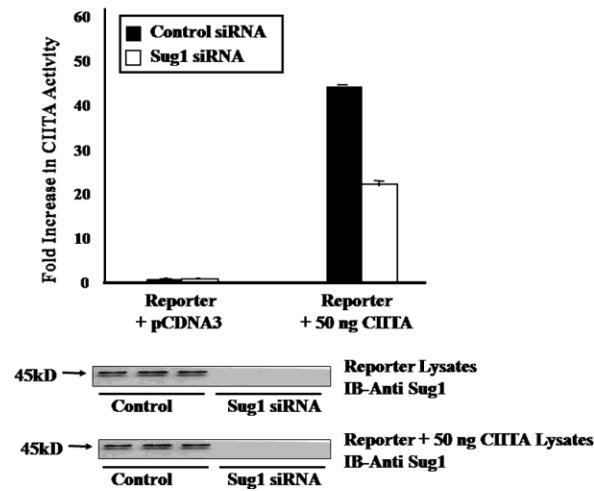
In contrast to CIITA and Sug1, alpha 4 required 65 hours of IFN- $\gamma$  stimulation to associate with the MHC class II proximal promoter, indicating that the 20S core does not associate with the MHC class II promoter during initiation of transcription (**Figure 2.4**). These results support our observations that CIITA and alpha 4 do not interact following short periods of IFN- $\gamma$  stimulation (data not shown). Interestingly, low Sug1 association with the MHC class II proximal promoter throughout the time course demonstrates the potential for reconstitution of the intact 26S proteasome. Indeed, early association of 19S ATPase Sug1 but delayed association of 20S with the MHC class II promoter suggests that these two proteasome components might be mediating distinct events.

#### **The 19S ATPase Sug1 is required for CIITA activation.**

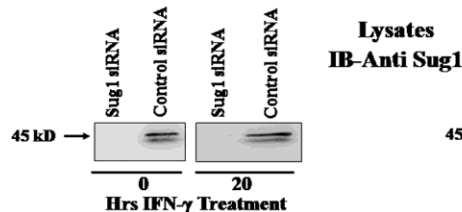
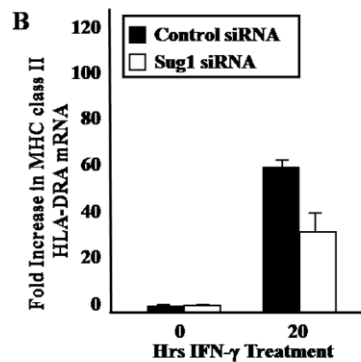
Mutation of the yeast 19S ATPase Rpt6 results in a block in transcription of long transcripts and prevents transcription of multiple insult induced genes (Sulahian et al., 2006). In mammalian cells, decreased expression of Sug1 blocks transactivation of the HIV-1 transactivator protein Tat (Lassot et al., 2007). Our observations of cytokine inducible association of Sug1 with CIITA and of Sug1 with the MHC class II proximal promoter indicate a potential role for Sug1 in the regulation of CIITA. To investigate potential roles for Sug1 in MHC class II transcription, we designed a siRNA duplex to specifically knockdown endogenous Sug1 expression (**Figure 2.5A, lower panels**). siRNA-mediated reduction of endogenous Sug1 decreased Sug1 expression by approximately 95-99% relative to cells transfected with scrambled control siRNA sequence (**Figure 2.5A, middle panel**). The reduction of Sug1 expression by siRNA is not affected by the presence of transfected CIITA (**Figure 2.5A, bottom panel**).



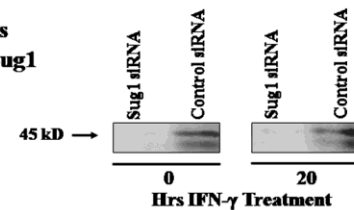
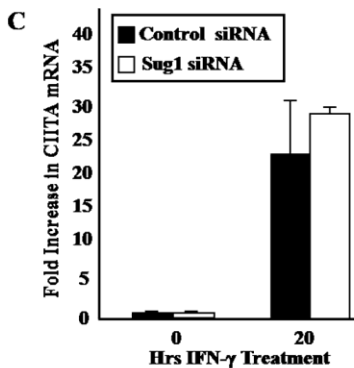
A



B



C



**Figure 2.5: The 19S ATPase Sug1 is required for optimal CIITA activity and MHC class II gene expression.**

(A) Reduced expression of Sug1 in siRNA transfected cells decreases the transcriptional activity of CIITA. HeLa cells were transfected with scrambled siRNA control or Sug1 specific siRNA duplexes and were co-transfected 48 h later with *HLA-DRA-Luc* and CIITA. Luciferase activity is reported as fold activation relative to that of the luciferase reporter alone (top). Luciferase readings were normalized to lysate protein concentrations as determined by Bradford assay. Luciferase assays were performed in triplicate and results represent the mean  $\pm$  S.D. ( $n = 3$ ). Western blots showed 95 % knockdown efficiency of Sug1 (bottom). (B) and (C) **Reduced expression of Sug1 in siRNA transfected cells decreases endogenous MHC class II mRNA expression but not CIITA mRNA expression.** Twenty-four hours following Sug1 siRNA expression, HeLa cells were unstimulated or were stimulated with IFN- $\gamma$  for 20 h. Control cells were transfected with scrambled control siRNA sequence. Fractions of cell lysates were analyzed for Sug1 expression by Western blot (bottom) or for levels of MHC class II mRNA or CIITA mRNA by real-time PCR (top). Western blots show 90–95% knockdown of endogenous Sug1. Levels of MHC class II mRNA and CIITA mRNA were measured by real-time PCR and were normalized to 18S rRNA. Real-time PCR was performed in triplicate and results represent the mean  $\pm$  S.D. Results are representative data of four experiments.

In cells transfected with the negative control siRNA, CIITA efficiently activated an MHC class II driven luciferase reporter, whereas in cells transfected with Sug1-specific siRNA, CIITA activation of the MHC class II promoter was reduced (**Figure 2.5A, top**).

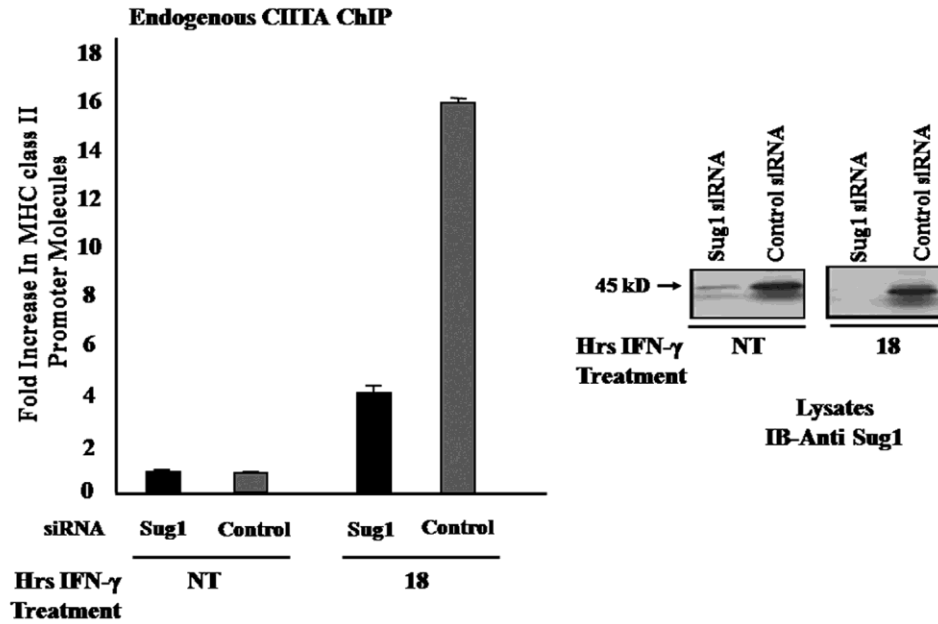
### **The 19S ATPase Sug1 is required for MHC class II mRNA expression.**

We addressed the role of Sug1 in regulating endogenous MHC class II expression by using real-time PCR to measure endogenous MHC class II mRNA in HeLa cells. Twenty hours following Sug1 or control siRNA expression, HeLa cells were stimulated with IFN- $\gamma$ . Fractions of cell lysates were analyzed for Sug1 expression by western blot (**Figure 2.5B, bottom**) or for levels of MHC class II mRNA by real-time PCR (**Figure 2.5B, top**). Loss of expression of Sug1 decreased the expression of endogenous MHC class II, further indicating a functional relationship between Sug1 and CIITA. As a control for the specificity of the effects on MHC class II mRNA levels, we addressed the role of Sug1 in regulating endogenous CIITA expression. Twenty hours following Sug1 or scrambled control siRNA expression, HeLa cells were stimulated with IFN- $\gamma$ , lysed and analyzed for Sug1 expression (**Figure 2.5C, bottom**) or for levels of CIITA mRNA (**Figure 2.5C, top**). Real-time PCR analysis indicated that the levels of CIITA induced by IFN- $\gamma$  were not affected by Sug1 siRNA. Therefore, the effects of decreased expression of Sug1 on physiologically induced MHC class II expression were not due to decreased expression of CIITA.

### **CIITA recruitment to the MHC class II proximal promoter is dramatically decreased in the absence of Sug1.**

The above data strongly suggest that Sug1 enhances the transcription of MHC class II genes in a degradation independent manner. Though obviously required for efficient and effective transcription, we do not know the function of Sug1 during transcription of MHC class II genes. One possibility is that the Sug1 ATPase generates necessary energy to drive assembly of the large, multiprotein enhanceosome complex at the proximal promoter. There is precedence for this scenario as related ATPases in the Clp/HSP 100 chaperone family found in bacteria, mitochondria and chloroplasts, are involved in both degradation and remodeling processes (Ottosen et al., 2002). Therefore, a scenario by which Sug1 may control MHC class II transcription is by recruiting or stabilizing CIITA at the proximal promoter. To determine whether Sug1 regulates association of endogenous CIITA with the enhanceosome complex at the MHC class II promoter, we used siRNA duplexes to specifically knock down endogenous Sug1 expression in HeLa cells and then performed ChIP experiments to detect endogenous CIITA associated with the promoter. siRNA-mediated knock down of Sug1 resulted in an approximate 90% decrease in endogenous Sug1 expression (**Figure 2.6, right panels**). Association of endogenous CIITA with the MHC class II proximal promoter was decreased in the absence of Sug1, indicating that Sug1 plays a critical role in regulating MHC class II transcription by recruiting and/or stabilizing CIITA (**Figure 2.6, left panel**).

Proteasome inhibition results in decreased levels of *HLA-DRA* promoter activity (**Figure 2.1A**), but does not reduce the level of CIITA transcripts in IFN- $\gamma$  induced cells (**Figure 2.1C**). To determine the effects of proteasome inhibition on CIITA binding to the MHC class II proximal promoter, CIITA ChIPs were performed. In the presence of wild-type levels of Sug1,

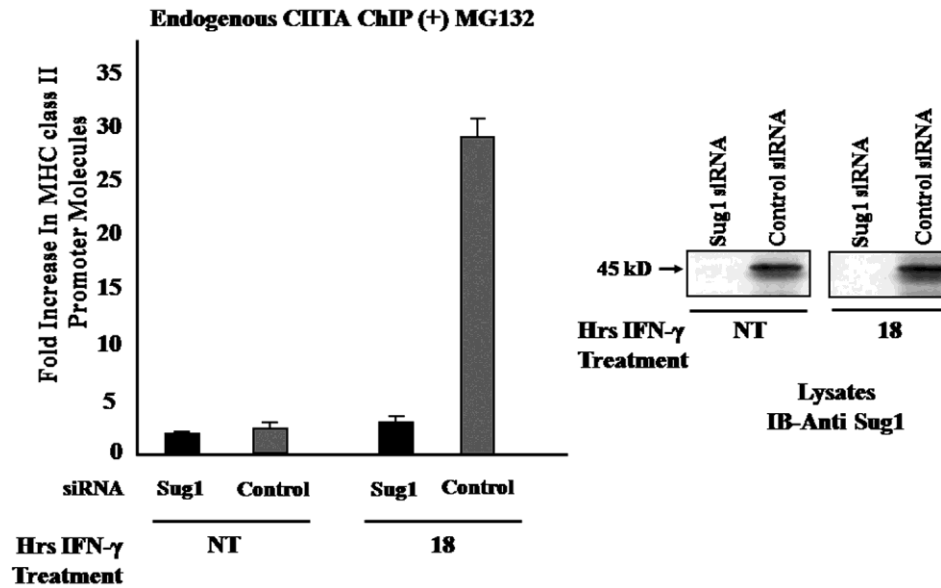


**Figure 2.6: CIITA recruitment to the MHC class II proximal promoter is dramatically decreased in the absence of Sug1.**

HeLa cells were transfected with scrambled siRNA control or with Sug1 specific siRNA duplexes and 24 h later were stimulated with IFN- $\gamma$  for 18 h or left untreated (NT). Cells were harvested and 9% of the lysate was removed for Western blot analysis of endogenous Sug1 expression. Western blots shows 90% knockdown of Sug1 (right panel). The remaining lysate was immunoprecipitated with control antibody or antibody to endogenous CIITA (left panel) and associated DNA was isolated and analyzed via real-time PCR as above. Real-time PCR values were normalized to the total amount of *HLA-DRA* promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. Data are presented as fold increase in the MHC class II promoter DNA relative to unstimulated CIITA IP samples. Control IP values were  $1.0 \pm 0.025$ . Values for control and CIITA IP's represent mean  $\pm$  S.E.M. of ( $n = 3$ ) independent experiments. Statistical significance was determined by Student  $t$ -test and the  $p$ -value was calculated to be  $<0.001$ .

treatment of IFN- $\gamma$  stimulated cells with the proteasome inhibitor MG132 results in increased binding of CIITA to the promoter when compared to non-MG132 treated cells (**Figure 2.7** grey bars versus **Figure 2.6** grey bars). Despite the increased levels of CIITA binding, MHC mRNA expression is ultimately reduced (**Figure 2.1B**), indicating that MG132 also negatively impacts non-proteolytic components of the proteasome that are involved in transcriptional regulation.

The addition of a siRNA-mediated knock down of Sug1 resulted in an approximate 95% decrease in endogenous Sug1 expression (**Figure 2.7, right panels**). To determine whether or not blocking 26S proteasome activity would also enhance CIITA promoter recruitment in the absence of Sug1, we treated Sug1 knockdown cells with MG132 with or without the addition of IFN- $\gamma$  prior to performing CIITA ChIPs. In the absence of Sug1, CIITA association with the MHC class II proximal promoter in MG132 treated cells remained low upon IFN- $\gamma$  stimulation (**Figure 2.7, left**). These data indicate that Sug1 functions specifically to stabilize CIITA at the MHC class II proximal promoter as proteasome inhibition in the absence of Sug1 could not restore CIITA promoter association.



**Figure 2.7: Proteasome inhibition does not reconstitute CIITA promoter association in the absence of Sug1.**

HeLa cells were transfected with scrambled siRNA control or with Sug1 specific siRNA duplexes and 24 h later were stimulated with IFN- $\gamma$  for 18 h or left untreated (NT). All cells were treated with the proteasome inhibitor MG132 4 h prior to cell harvest. ChIP assays and Western blots were performed as above to determine CIITA promoter association (left panel) and extent of Sug1 knockdown (right panels). Real-time PCR values obtained from ChIP assays were normalized to the total amount of *HLA-DRA* promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. Data are presented as fold increase in the MHC class II promoter DNA relative to unstimulated CIITA IP samples. Control IP values were  $2.0 \pm 0.5$ . Values for control and CIITA IP's represent mean  $\pm$  S.E.M. of ( $n = 2$ ) independent experiments. Statistical significance was determined by Student *t*-test and the *p*-value was calculated to be  $<0.01$ .

## DISCUSSION

Our data has provided evidence suggesting a model in which 19S and 20S components of the 26S proteasome associate with the MHC class II proximal promoter at different stages of transcription where they both positively and negatively regulate CIITA mediated transcription. In support of this model, ChIP analysis demonstrates that low levels of the 19S ATPase Sug1 rapidly associate with the MHC class II proximal promoter within 4 hours of cytokine stimulation. Following 4 hours of cytokine stimulation, CIITA is also recruited to the MHC class II promoter. CIITA and Sug1 also co-precipitate, thus suggesting that a regulatory interaction exists between these two proteins. The 20S alpha 4 subunit also associates with the MHC class II promoter, but requires 65 hours of cytokine stimulation to do so. Association of the 19S Sug1 subunit peaks at the MHC class II promoter between 4 and 24 hours, then declines and shows low but reproducibly sustained for 95 hours; indicating potential reassembly of the 26S proteasome at the less transcriptionally active promoter. CIITA association with the MHC class II promoter remains relatively stable until 65 hours of IFN- $\gamma$  stimulation, after which time CIITA promoter association begins to decline; also suggesting local assembly of a functional 26S.

Our observations correlate 20S recruitment to and CIITA loss from the promoter, and are consistent with a role for the reconstituted 26S proteasome in mediating transcriptional termination by degrading promoter bound CIITA. Previous studies have demonstrated that CIITA remains bound to the MHC class II proximal promoter for extended periods of time following stimulation with inflammatory cytokines. Our results suggest that assembly of the 26S proteasome at the MHC class II promoter serves to degrade promoter bound CIITA and to suppress MHC class II transcription. It has recently been reported that the 20S proteasome associates with the promoter region of adenovirus (Ad) early region genes and that the Ad transactivator 13S E1A

utilizes the 19S and 20S proteasomes independently and at several points during transcription to regulate Ad transcription (Rasti et al., 2006). Several studies in yeast have suggested that the 20S is associated with actively transcribing genes indicating that the 20S proteasome is required for transcriptional elongation (Ferdous et al., 2001; Gillette et al., 2004; Gonzalez et al., 2002). Recruitment of the 20S to 3' coding regions of MHC class II genes in a transcription dependent manner remains to be determined. However, our reporter assays show that proteolytic function is required for efficient transcriptional activation by CIITA and therefore for cytokine inducible MHC class II expression. It is therefore possible that the proteasome functions both proteolytically and non-proteolytically at promoter and coding regions of MHC class II during different stages of transcription.

Data presented within this report provide further evidence that the 19S proteasome ATPase Sug1 regulates MHC class II transcription by controlling CIITA promoter association. We determined by ChIP that Sug1 associates with the MHC class II promoter and showed by immunoprecipitation that Sug1 binds CIITA. Using RNAi, we established a role for endogenous Sug1 in CIITA transactivation in reporter assays. The ability of CIITA to regulate transcription from the MHC class II proximal promoter is reduced in cells where Sug1 expression has been reduced by RNAi. Consequently, expression of MHC class II mRNA is also reduced in the absence of Sug1, however CIITA expression remains stable, indicating selectivity in transcriptional systems regulated by Sug1. We also demonstrate by ChIP that in the absence of Sug1, severely reduced levels of CIITA bind the MHC class II promoter. Although diminished, MHC class II transcription still occurs despite very low levels of CIITA binding the MHC class II promoter in the absence of Sug1. These data further implicate the importance of degradation (Drozina et al., 2006) in regulating MHC class II transcription as even small amounts of CIITA function to in-



itiate transcripts from this promoter. In data not shown, we have demonstrated that each of the additional 19S lid ATPases binds the MHC class II promoter and plays a positive role in regulating MHC class II transcription. The combined actions of these ATPases in the 19S lid likely play overlapping roles in regulating CIITA recruitment and MHC class II transcription. A full understanding of the mechanistic contributions of individual components of the proteasome in regulating MHC class II will require further in depth studies of the events and molecular interactions occurring at this promoter.

We propose that the following series of events occurs to regulate MHC class II transcription: following cytokine stimulation, Sug1 is recruited to the proximal promoter where it then recruits and/or stabilizes CIITA. Following extensive gene activation, the 20S catalytic core is recruited to the proximal promoter where the 26S proteasome may be reassembled resulting in degradation of local cofactors, including CIITA. As proteasome inhibition restored CIITA association but not CIITA function at the MHC class II proximal promoter, additional roles for the 26S proteasome in regulating CIITA activity must be fully explored.

Although roles for 19S ATPases in mammalian transcription have been suggested previously (Lassot et al., 2007; Rasti et al., 2006; Zhu et al., 2007), mechanisms of action and independent function of the 19S lid, the APIS complex and/or 19S ATPases on promoters are unclear and remain to be fully addressed. Observations in yeast that 19S ATPases link histone ubiquitination and histone methylation (Ezhkova and Tansey, 2004) and that the 19S recruits histone modifying enzymes to promoters (Lee et al., 2005) suggest that the 19S may independently regulate transcription by controlling chromatin modifications. A model of proteasome subcomplexes performing different functions at different locations on a gene is supported by the observation in yeast that although 26S proteins are associated with a majority of genes, proteins from the 19S

lid or 20S core, but not both, crosslink individually to several hundred genes (Auld et al., 2006; Sikder et al., 2006). It is also important to note that the 26S disassociates into 19S and 20S components in yeast cells (Babbitt et al., 2005) and in mammalian nuclear extracts (Lassot et al., 2007), indicating that the 19S or 19S subcomplexes could independently regulate discrete states of the transcription cycle. The contextual contributions of individual 19S subunits, the 20S and 26S proteasome to mammalian transcriptional processes remain to be determined and will likely reflect both the differences in function and regulation of target genes.

#### ACKNOWLEDGEMENTS

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### CHAPTER 3

ASSOCIATION OF THE 19S PROTEASOMAL ATPASES WITH THE ATPASE BINDING  
DOMAIN OF CIITA IS ESSENTIAL FOR CIITA STABILITY AND MHC CLASS II  
EXPRESSION

PUBLICATION: KAVITA PURNANDA BHAT<sup>†</sup>, AGNIESZKA DOROTA TRUAX<sup>†</sup>,  
JEANNE KAYE BROOKS<sup>\*</sup> AND SUSANNA FLETCHER GREER<sup>\*†</sup> IN PRESS IN IMMUNOLOGY AND CELL BIOLOGY, FEBRUARY 26, 2010.

## SUMMARY

Major histocompatibility class II (MHC class II) molecules are glycoproteins that present extracellular antigens to CD4<sup>+</sup> T cells and are essential for initiation of adaptive immune responses. MHC class II expression requires recruitment of a master regulator, the class II transactivator (CIITA), to the MHC class II promoter. We and others have previously linked CIITA to the ubiquitin proteasome system (UPS) by demonstrating that mono-ubiquitination of CIITA increases its transactivity whereas polyubiquitination of CIITA leads to its degradation. We have further shown that the 26S proteasome also has non-proteolytic roles in MHC class II transcription as 19S ATPase subunits of the 26S proteasome positively regulate MHC class II transcription and are necessary for stable promoter binding of CIITA. Although these basic requirements of the proteasome to initiate MHC class II transcription are known, how CIITA is recruited, stabilized and degraded remains unclear. Here, we identify a novel N-terminal 19S ATPase binding domain of CIITA. The ATPase binding domain lies within the P/S/T region of CIITA and encompasses a majority of the CIITA degron sequence. Absence of the ATPase binding domain increases the half-life of CIITA, but blocks MHC class II surface expression, indicating that CIITA requires interaction with the 19S ATPases for both appropriate deployment and destruction.

## BACKGROUND

MHC class II molecules are cell surface glycoproteins which present extracellular antigens to CD4<sup>+</sup>T helper cells and elicit specific adaptive immune responses (Matheux and Villard 2004). MHC class II expression is essential for protection against invading pathogens, and also serves in anti-tumor responses as tumor specific CD4<sup>+</sup> T cells are often required for CD8<sup>+</sup> T cell

activation (Marzo, Kinnear et al. 2000). In addition to these important functions, MHC class II molecules play critical roles in tolerance induction and transplant rejection (Harton and Ting 2000). Over-expression of MHC class II is thus correlated with increased vulnerability to the development of autoimmune diseases including multiple sclerosis, rheumatoid arthritis and myocardial infarction (Swanberg, Lidman et al. 2005) and a lack of MHC class II expression results in the fatal disease, Bare Lymphocyte Syndrome (Harton and Ting 2000). Due to these and other critical roles played by MHC class II molecules in regulating adaptive immune responses and disease susceptibility, MHC class II gene expression is tightly regulated (Benoist and Mathis 1990).

MHC class II transcription requires recruitment of ubiquitously expressed transcription factors including the regulatory factor X complex (RFX), (Steimle, Durand et al. 1995; Ting and Trowsdale 2002), (Nagarajan, Louis-Pence et al. 1999), (Masternak, Barras et al. 1998), the cAMP responsive element binding protein (CREB) (Moreno, Beresford et al. 1999) and the nuclear factor-Y complex (NFY) (Mantovani 1999) which bind respectively to the X1, X2 and Y gene elements to form the “enhanceosome complex” (Steimle, Otten et al. 1993; Mach, Steimle et al. 1996; Ting and Trowsdale 2002). The enhanceosome complex is required, but is insufficient to initiate MHC class II transcription (Steimle, Otten et al. 1993; Mach, Steimle et al. 1996; Muhlethaler-Mottet, Otten et al. 1997). Transcription is however initiated when the master regulator, the class II transactivator (CIITA) is recruited to the preformed enhanceosome complex (Steimle, Otten et al. 1993; Mach, Steimle et al. 1996; Masternak, Muhlethaler-Mottet et al. 2000). CIITA binding to the enhanceosome complex orchestrates a sequence of events to recruit transcription factors and co-factors to initiate transcription of MHC class II genes (Mahanta, Scholl et al. 1997; Kretsovali, Agalioti et al. 1998; Masternak, Muhlethaler-Mottet et al. 2000;

Zhu, Linhoff et al. 2000). Although these basic requirements for initiating MHC class II transcription are fairly well understood, how CIITA is recruited, stabilized and degraded remains unclear.

The 26S proteasome is a complex long known to be responsible for the destruction of a majority of cellular proteins, including transcription factors. The 26S proteasome is composed of a 20S proteolytic core which contains the protease active sites and a 19S regulatory particle (RP) that caps either end of the core (Ciechanover 1994; Hendil, Kristensen et al. 1995; Coux, Tanaka et al. 1996). The 20S proteolytic core is composed of 4 stacked rings of 7- $\alpha$ , 7- $\alpha'$ , 7- $\beta$  and 7- $\beta'$  subunits (Hendil, Kristensen et al. 1995; Coux, Tanaka et al. 1996; Hochstrasser 1996) which receive and degrade poly-ubiquitinated proteins recognized by the 19S (Lam, Lawson et al. 2002; Husnjak, Elsasser et al. 2008; Schreiner, Chen et al. 2008). The 19S consists of a base subunit composed of six ATPase subunits (S4, S6a, S6b, S7, Sug1 and S10b) and three non-ATPase subunits (S1, S2 and S5a), and a lid subunit which consists of eight non-ATPase subunits (Ciechanover 1998).

The 26S proteasome has recently been found to be intimately associated with the initiation of transcription of many genes (Makino, Yoshida et al. 1999; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Muratani and Tansey 2003; Hegde 2004; Rasti, Grand et al. 2006; Sulahian, Sikder et al. 2006; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008) and it is now clear that multiple proteins of the 19S proteasome play a variety of roles in transcription. In yeast, ATPase components of the 19S proteasome associate with actively transcribing genes and regulate the elongation process carried out by RNA-polymerase II (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002). Subunits of the 19S but not the 20S have been shown to associate with Gal4 activators and Gal responsive promoters (Gonzalez, Delahodde et al. 2002).

Specific inhibition of the 19S leads to a decrease in RNA pol II mediated elongation, but inhibition of the 20S increases RNA pol II mediated elongation, indicating that these sub complexes play a role in transcription independent of both each other and of proteolytic activity (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004). Moreover, in mammals, the 19S ATPase Sug1 (S8/Trip1 in humans and Rpt6 in yeast), has been shown to interact with transcription factors including E1A, p53 and HIV-tat and to be recruited to responsive gene promoters (Rasti, Grand et al. 2006; Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007). We have previously demonstrated the importance of Sug1 in MHC Class II expression (Bhat, Turner et al. 2008). That CIITA failed to bind to the MHC class II promoter in the absence of Sug1 indicates a role for Sug1 in CIITA recruitment and stabilization (Bhat, Turner et al. 2008). Sug1 recruits histone modifying enzymes at the MHC class II promoter and positively regulates histone acetylation and methylation events at MHC class II genes (Koues O.I. 2008; Koues, Dudley et al. 2009). In addition to its requirement for MHC class II expression, Sug1 interacted with CIITA *in vivo* (Bhat, Turner et al. 2008). Finally, we recently demonstrated the 19S ATPase S6a has regulates CIITA transcription by controlling histone modifying events at the CIITA interferon gamma (IFN- $\gamma$ ) responsive promoter, CIITA pIV (Agnieszka D. Truax 2009). In sum, these studies indicate that the 19S ATPases play critical roles in the regulation of multiple genes, including CIITA mediated MHC class II expression.

Prior observations from our lab and others have linked MHC class II transcription and the ubiquitin-proteasome pathway. In this study we sought to map the binding of the 19S ATPase Sug1 to CIITA to further determine the role of the ATPases in MHC class II gene expression. We have identified a novel N-terminal ATPase binding domain in CIITA required for CIITA binding to the 19S ATPases. This ATPase binding domain lies within the P/S/T region of CIITA

and encompasses a majority of the degron site. Absence of this domain increases the half-life of CIITA, but dramatically decreases MHC class II surface expression. These findings pose an interesting conundrum: CIITA is dependent on interactions with the 19S for activation and promoter binding, but interaction with 19S ATPases also leads to proteasome mediated degradation of CIITA.

## MATERIALS AND METHODS:

**Cell culture:** HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% FCS, 50U/ml of penicillin, 50µg/ml of streptomycin and 2mM of L-glutamine. The cells were maintained at 37°C with 5% CO<sub>2</sub>.

**Plasmids:** Full length Flag CIITA, truncation mutants flag CIITA 1-224aa, 1-335aa, 1-444aa, 1-949aa, deletion mutant flag CIITA (Δ132-301aa) and pCDNA3 plasmids were kindly provided by Dr. J. Ting. The pGEX-GST Sug1 and Myc Sug1 constructs were a generous gift from Dr. A. Wani. Site specific mutants flag CIITA 1-335aa K1R (K315R), flag CIITA 1-335aa K2R (K330, 333R) and flag CIITA 1-335aa K3R (K315, 330, 333R); deletion mutant flag CIITA (Δ132-301) 1-335aa; and truncation mutant flag CIITA 1-301aa were generated using Quick-Change Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specific primers with the desired mutations were designed using the Stratagene primer design tool. Mutagenesis reactions were performed as per the manufacturer's protocol. Mutagenesis was confirmed by sequence analysis and expression of constructs was analyzed by western blot.



**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 3ml LB<sup>+</sup> Amp<sup>+</sup> 1% glucose. Three ml preps were transferred into 100ml LB<sup>+</sup> Amp<sup>+</sup> 1% glucose and incubated at 37°C for 4 hrs. Following incubation, cells were pelleted and fresh LB<sup>+</sup> Amp media was added. IPTG was used to induce protein expression. Cells were pelleted at the end of 4hrs and resuspended in STE buffer (10mM Tris-Cl pH7.5, 10mM NaCl, 1mM EDTA) with 1mM PMSF, 5mM DTT and 1.5% Sarkosyl. Following incubation, cells were sonicated and centrifuged. The supernatant containing soluble proteins was aliquoted into low retention eppendorph tubes and stored at -80°C.

**GST pull-down assay:** HeLa cells were transfected with 5µg of flag CIITA using Fugene6 (Roche, Indianapolis, IN) as per the manufacturer's protocol. HeLa cell extracts were prepared by lysing cells in 1% Nonidet P-40 buffer ( NP-40:1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH<sub>2</sub>O) supplemented with complete EDTA-free protease inhibitors (Roche) on ice. One ml of GST-tagged protein preps were incubated with pre-washed 50% GST slurry beads for 30 min at room temperature and then for 90 min at 4° C to allow for maximal binding. Following incubation, beads were washed twice with cold phosphate buffered saline (PBS) containing 1mM PMSF. Pooled HeLa cell lysates were added to GST-tagged protein-bound beads and incubated at room temperature for 30 min and then at 4° C for 90 min to allow for maximal exposure of cellular proteins to GST-tagged proteins bound GST beads. Following incubation, beads were washed twice with cold PBS containing 1mM PMSF. Bead complexes were denatured with Laemmli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-flag (Sigma-Aldrich, Saint Louis, MO). HRP conjugates were detected with Immobilon Western Chemilu-

minescent substrate (Milipore, Billerica, MA) to determine associations between Sug1 and CII-TA. Equal loading was determined in lysates immunoblotted with anti-flag (Sigma-Aldrich) or anti-GST monoclonal antibodies (Abcam, Cambridge, MA).

**Co-immunoprecipitation assay:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10 cm tissue culture plates. Following cell adhesion, cells were transfected as indicated with 5 $\mu$ g of flag CIITA full length, truncation or deletion constructs or pCDNA control and/or 2 $\mu$ g myc Sug1 or myc control plasmids. Twenty hours following transfection, cells were lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged, normalized for protein concentration, pre-cleared with 50  $\mu$ l mouse IgG (Sigma-Aldrich) and immunoprecipitated with 30 $\mu$ l of anti Myc agarose beads (Sigma-Aldrich). Immune complexes were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-flag or anti-myc monoclonal antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Milipore) to determine associations between Sug1 and CIITA. Bradford assays were used to normalize for protein and equal loading was determined in non-immunoprecipitated lysates.

**Half-life assay:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10cm tissue culture plates. Following cell adhesion, cells were transfected with 5 $\mu$ g of flag CIITA ATPase binding domain ( $\Delta$ 132-301) deletion construct or flag CIITA. Twenty hrs following transfection, cells were treated with 100 $\mu$ M cycloheximide for 0-7 hrs. At the end of cycloheximide treatment, cells were lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and normalized for protein concentration. As a control, HeLa cells were transfected with flag CIITA ( $\Delta$ 132-301) deletion construct or flag CII-

TA construct and treated with 100 $\mu$ M cycloheximide and MG132 (EMD Biosciences, San Diego, CA) for 7 hrs to stop new protein synthesis and protein degradation. Proteins were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Milipore) to determine half-life of CIITA constructs. Bradford assay was used to normalize for protein and equal loading was determined in lysates. Western blots from half-life assays were quantified using Multi-Gauge V3.1 and plotted as percent (%) protein remaining (after 2, 4, 5, 6 or 7 hours of cycloheximide treatment as compared to 0hr cycloheximide treatment) v/s hours of cycloheximide treatment.

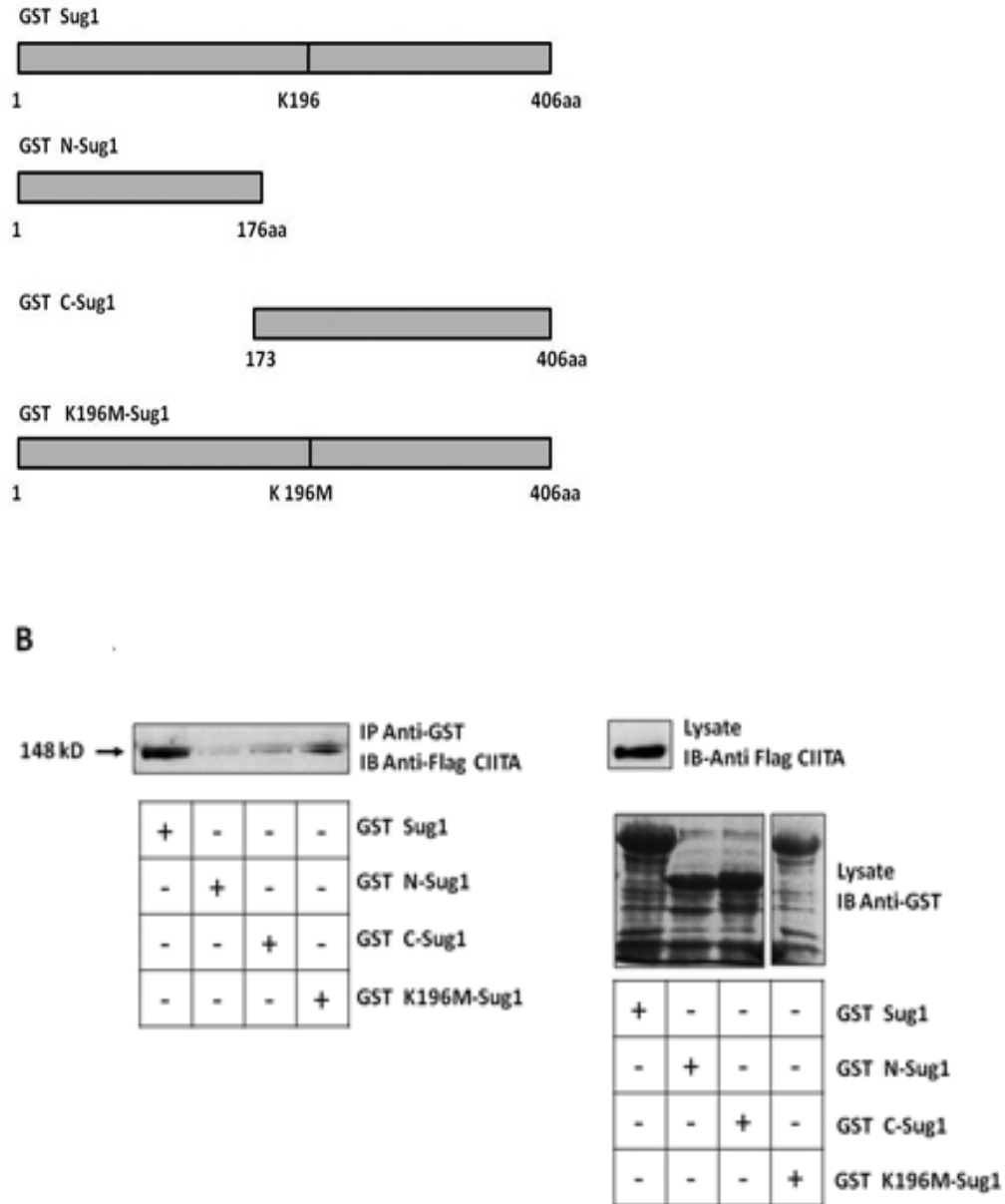
**Flow cytometry:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10cm tissue culture plates. Following cell adhesion, cells were transfected with flag CIITA ATPase binding domain ( $\Delta$ 132-301) deletion construct or with flag CIITA. Forty eight or seventy two hrs following transfection, cells were trypsinized and washed with PBS. Following the wash, the cells were resuspended in incubation buffer (0.5% bovine serum albumin in PBS w/v) and 10 $\mu$ g Phycoerythrin (PE)-labeled anti-human HLA-DR (clone L243, Biolegend, San Diego, CA) antibody or PE mouse IgG2a  $\kappa$  isotype control antibody (Biolegend) was added to the cell suspension and rotated at 4° C. Following antibody incubation, cells were washed twice with PBS, fixed with 2% paraformaldehyde and stored at 4°C. MHC class II cell surface expression was measured by FACS-Canto (Becton Dickinson, San Jose, CA) and analyzed using FlowJo. All samples were analyzed using 10,000 events per sample.  $1/10^{\text{th}}$  of the HeLa cells harvested for flow analysis were lysed in 1% Nonidet P-40 buffer supplemented with Complete EDTA-free protease inhibitors (Roche) on ice. Proteins were denatured with Leammli buffer, boiled and separated by

SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Millipore) to determine half-life of CIITA constructs. Bradford assay was used to normalize for protein and equal loading was determined in lysates.

## RESULTS

### **CIITA interacts with the C-terminal end of Sug1 independent of Sug1 ATPase activity**

Sug1 is an ATPase subunit of the 19S regulatory particle of the 26S proteasome (Rubin, Coux et al. 1996; Fraser, Rossignol et al. 1997; Makino, Yamano et al. 1997). Our previous studies have demonstrated the importance of Sug1 in MHC class II expression (Bhat, Turner et al. 2008). We initially observed that CIITA, the master regulator of MHC class II gene expression, interacts with Sug1 *in vivo* and fails to be recruited to the MHC class II promoter in the absence of Sug1 (Bhat, Turner et al. 2008), suggesting a role for Sug1 in CIITA recruitment and/or stabilization. To further understand the importance of the role of Sug1 and the 19S regulatory particle in CIITA regulation, interactions between CIITA and *in vitro* translated Sug1 were mapped using glutathione S-transferase (GST) tagged wild type Sug1 and Sug1 truncation mutants. Sug1 is a 406 amino acid protein with coiled coil structure in its N-terminal end (Fraser, Rossignol et al. 1997) and with 3'-5' DNA helicase activity and ATPase activity in its C-terminal domain (Fraser, Rossignol et al. 1997; Makino, Yamano et al. 1997).



**Figure 3.1: CIITA interacts with the C-terminal end of Sug1 independent of Sug1 ATPase activity.**

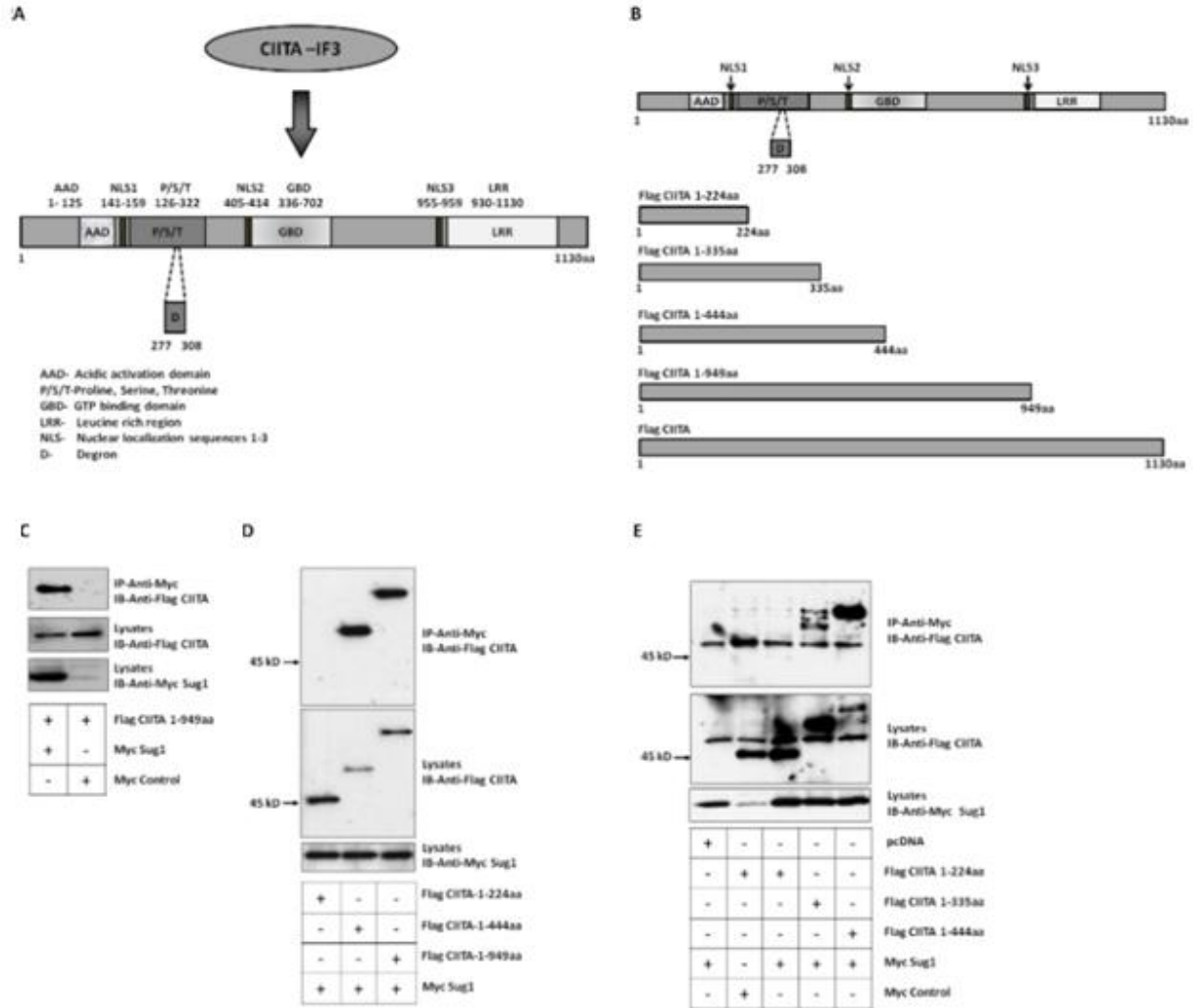
**(A) Schematic representation of Sug1 truncation mutants.** Sug1 is a 406aa protein with ATPase activity located near the C-terminus. Shown are GST tagged full length Sug1, Sug1 N- and C-terminal truncation mutants, and the Sug1 ATPase mutant. **(B) Glutathione S-Transferase (GST) pull-down assay.** HeLa cells were transfected with flag CIITA and HeLa cell lysates were prepared using NP40 lysis buffer. GST bead slurries were incubated with GST tagged protein preps, washed and incubated with HeLa cell lysates. Western blot analysis was performed and the bead-bound complexes were immunoblotted (IB) using anti-flag antibody (left panel). Lysate blots (right panels) demonstrate expression of flag CIITA in pooled HeLa cell lysates (top, right) and GST tagged (bottom, right) proteins. Results are representative of 3 experiments.

Lysine 196 in the C-terminal end of Sug1 lies in the Walker A box and mutation of lysine 196 leads to loss of ATPase and helicase activity of Sug1 (Fraser, Rossignol et al. 1997) without affecting its assembly into the 19S base or the overall structure of the proteasome (Rubin, Glickman et al. 1998) (**figure 3.1A**). GST pull down assays indicate that CIITA interacts primarily with the C-terminal domain of Sug1 (**figure 3.1B**). Interaction with CIITA is also largely independent of the ATPase activity of Sug1, activity which is non-essential for the role of Sug1 in chromatin remodeling at the MHC class II promoter (**figure 3.1B**) (Koues, Dudley et al. 2008).

### **Amino acids within the PST domain of CIITA are critical for Sug1 binding**

While transcriptional regulation of CIITA is well understood, less is known about the post-transcriptional modifications and interactions that control CIITA activity and half life. CIITA is an 1130 amino acid long non-DNA binding co-factor with four functional domains: an N-terminal transcriptional acidic activation domain (AAD), a proline/serine/threonine rich (P/S/T) domain, a GTP-binding domain (GBD), and a C-terminal leucine rich region (LRR) (Masternak, Muhlethaler-Mottet et al. 2000) (Tosi, Jabrane-Ferrat et al. 2002). CIITA also has three nuclear localization sequences (NLS) that are required for its nuclear translocation and activity (Cressman, O'Connor et al. 2001). The P/S/T domain of CIITA contains a proteolytic signal site termed a degron, which typically targets proteins for degradation (Drozina, Kohoutek et al. 2006) (**figure 3.2A**). Interactions between Sug1 and CIITA were initially mapped using flag tagged CIITA C-terminal truncations (**figure 3.2B**). CIITA truncation 1-949aa, which lacks the leucine rich region, associated with Sug1 (**figure 3.2C**) with the same affinity as full length CIITA (data

not shown), indicating that the LRR domain of CIITA is not required for interactions with Sug1. Further analysis indicated that CIITA truncation 1-444aa associated with Sug1 while CIITA truncation 1-224aa failed to associate (**figure 3.2D**) and that the interaction of Sug1 with CIITA 1-444aa was independent of Sug1 ATPase activity (**Supplementary figure 3.7**). These data narrowed ATPase independent Sug1 binding to the amino terminus of CIITA. In order to further narrow the region of association, CIITA truncation 1-335aa was used. CIITA truncations 1-444aa and 1-335aa associated with Sug1, while truncation 1-224aa again failed to associate (**figure 3.2 E**). These data indicate that the C-terminus, including the GBD, NLS2 and 3, and the LRR domains of CIITA are not involved in Sug1 interactions. CIITA truncation 1-224aa failed to bind Sug1 and lacks most of the Proline-Serine-threonine rich (P/S/T) region, a region fully present in the 1-335aa truncation which bound CIITA. Together, these data indicate the P/S/T region of CIITA is important for CIITA- Sug1 interactions.

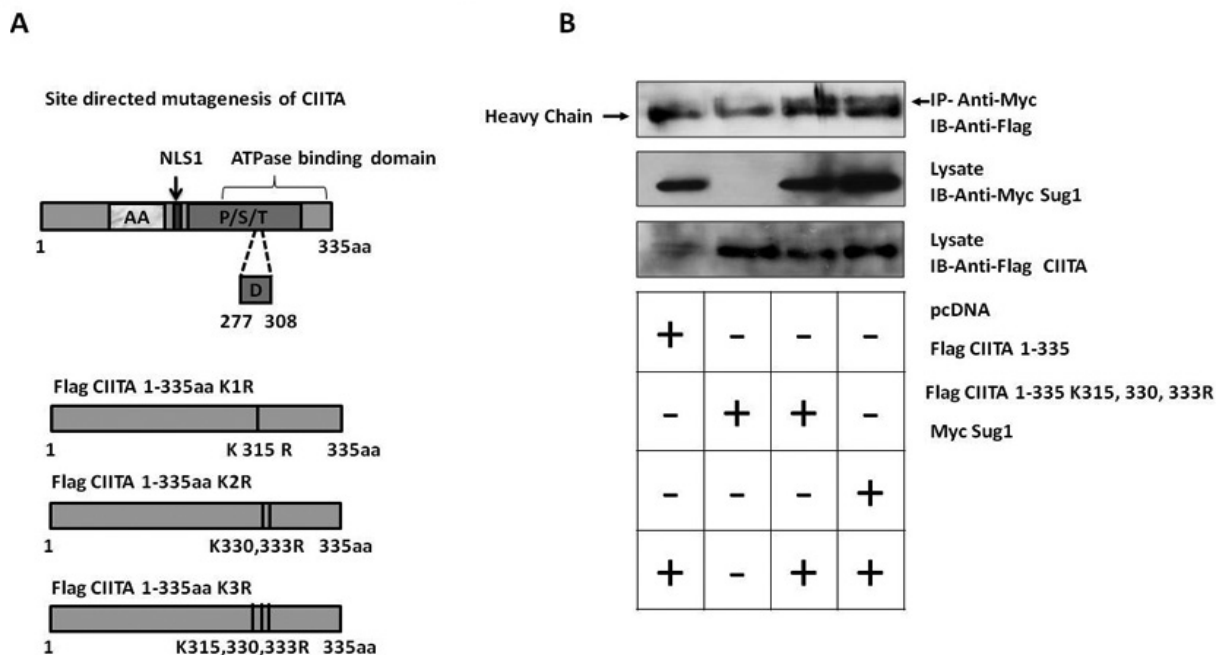


**Figure 3.2: Amino acids within the PST domain of CIITA are critical for Sug1 binding**

(A) **Schematic representation of functional domains of CIITA.** CIITA is a 1130aa protein. CIITA has four major functional domains, an N-terminal transcriptional acidic activation domain (AA) required for CIITA transactivity, a proline/serine/threonine rich (P/S/T) domain whose function is undetermined, a GTP-binding domain (GBD) and a C-terminal leucine rich region (LRR) required for localization. CIITA also has three nuclear localization sequences (NLS 1-3) that are required for nuclear translocation and activity and the P/S/T rich region of CIITA contains a proteolytic signal site termed a degron which typically targets proteins for degradation via unknown mechanisms. This figure is adapted from Ting J.T. and Trowsdale J., 2002. (B) **Schematic representation of N-terminal truncation constructs of CIITA.** Flag CIITA truncation constructs 1-224, 1-335, 1-444 and 1-949aa were used to map association of Sug1 with CIITA. (C, D, E) **Sug1 interacts within 224-335aa of CIITA.** Co-Immunoprecipitation (Co-IP) assays. HeLa cells were co-transfected as indicated with flag tagged truncations of CIITA 1-949aa, 1-444aa, 1-335aa or 1-224aa, and either myc Sug1 or myc control. Cells were harvested, lysed in NP40 buffer, precleared and IP'd with anti-myc antibody. Western blot analysis was performed and the immunoprecipitated (IP'd) samples were IB using anti-flag antibody. Lysate blots indicate equal expression of myc Sug1 and flag CIITA. Results shown are representative of 3 experiments.



The P/S/T region of CIITA contains a highly conserved proteolytic site termed a degron which typically targets host proteins for degradation (**figure 3.3A**) (Drozina, Kohoutek et al. 2006). These N-terminal degron sites can serve as recognition sites for E3 ubiquitin-ligases (Tasaki, Mulder et al. 2005) which ubiquitinate target proteins on nearby lysine residues. The region between 224 and 335aa of CIITA contains three lysine residues which we have shown to be potential sites of CIITA ubiquitination (data not shown). Mono-ubiquitination of CIITA increases CIITA transactivity, increases CIITA binding to P-TEFb and upregulates MHC class II expression (Drozina, Kohoutek et al. 2005) (Greer, Zika et al. 2003). Mono-ubiquitination has recently been shown to protect target proteins from the “stripping” activity of bound 19S proteasomal ATPases and thus to provide a stimulatory effect on protein activity (Archer, Burdine et al. 2008; Archer, Delahodde et al. 2008). If the three lysine residues located immediately downstream from the degron site serve as sites for mono-ubiquitination, they may protect CIITA from “stripping” activity of 19S proteasomal ATPases and/or affect ATPase binding. If so, then mutating these sites to arginine would block ubiquitination and thus potentially the binding of 19S ATPases. To determine the effects of loss of these ubiquitination sites on CIITA binding to Sug1, lysines 315, 330 and 333 in CIITA truncation 1-335aa were mutated to arginine (**figure 3.3A**, bottom 3 schematics). Co-immunoprecipitation assays indicate that the mutant 1-335 K315, 330, 333R associated with Sug1 with the same intensity as the 1-335aa truncation (**figure 3.3B**). These data indicate that these three lysine residues are not involved in Sug1 interactions, although modifications at these sites may be important for CIITA activity.

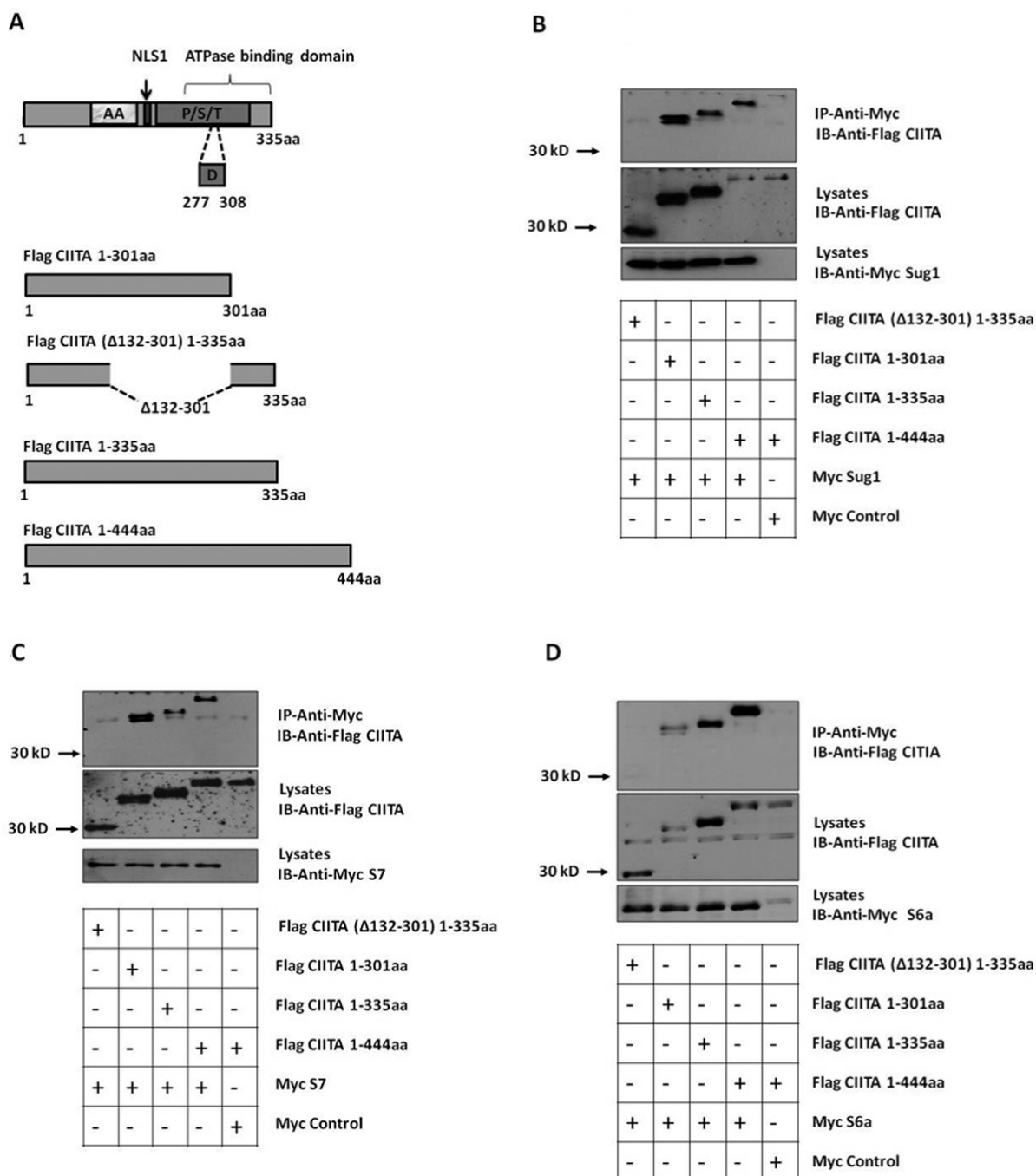


**Figure 3.3: The interaction of Sug1 within the PST domain of CIITA is unaffected by mutating multiple lysine residues.**

**(A) Schematic representation of 1-335 CIITA lysine mutants.** Site-directed mutagenesis was performed to mutate lysine residues 315, 330 and 333 to arginine. The mutants generated were flag CIITA 1-335aa and flag CIITA 1-335aa K3R (K315, 330, 333R). **(B) Sug1 binding to CIITA is not impaired by mutating lysines 315, 330 and 333.** Co-IP. HeLa cells were co-transfected as indicated with flag tagged CIITA 1-335aa or the flag CIITA 1-335aa lysine mutant and myc Sug1 or myc control. Cells were harvested, lysed in NP40 buffer, precleared and IP'd with anti-myc antibody. Western blot analysis was performed and the IP'd samples were IB'd using anti-flag antibody. Lysate blots indicate equal expression of myc Sug1 and flag CIITA. Results shown are representative of 3 experiments.

### **19S ATPases associate with amino acids 132-301 of the PST domain of CIITA**

Proline rich domains in transcription factors are linked to their DNA binding ability and activation potential (Mermod, O'Neill et al. 1989; Harton and Ting 2000). CIITA has a proline rich region (within the P/S/T region), but it is a non-DNA binding transcription factor with an N-terminal transcription activation domain (Steimle, Otten et al. 1993; Zhou and Glimcher 1995; Kretsovali, Agaloti et al. 1998). Thus, functional importance of the P/S/T domain of CIITA remains unclear (Harton and Ting 2000). Here we have shown that amino acids within the P/S/T domain are crucial for interactions between Sug1 and CIITA. We observed that mutating multiple degon proximal lysine residues within amino acids 224-335 (K315, K330, and K333) did not affect Sug1 binding. The region between amino acids 224 and 335 of CIITA contains just the serine and threonine rich domains. Thus, we deleted amino acids 132-301aa in the 1-335 truncation construct of CIITA (**figure 3.4A**) to determine if the deletion of proline rich amino acids 132-301 in the P/S/T domain would affect interaction of CIITA with Sug1. Co-immunoprecipitation assays indicate that deletion of amino acids 132-301 in the CIITA P/S/T domain leads to a loss of Sug1 association (**figure 3.4B**). Sug1 is one of six ATPases of the 19S regulatory particle that exist as heterodimers; Sug1/S10b, S7/S4 and S6a/S6b (Richmond, Gorbea et al. 1997; Adams 2003). We next assayed to determine if additional ATPases of the 19S proteasome associate within aa132-301 of CIITA. Co-immunoprecipitation assays indicate that S7 and S6a also fail to associate with the  $\Delta$ 132-301aa construct of CIITA (**figures 3.4C, D**). These data suggest the 19S ATPases associate as a hexameric complex to this newly identified ATPase binding domain of CIITA.



**Figure 3.4: 19S ATPases associate with amino acids 132-301 of the PST domain of CIITA.**

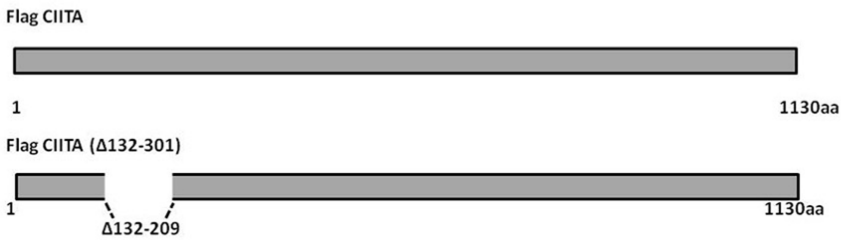
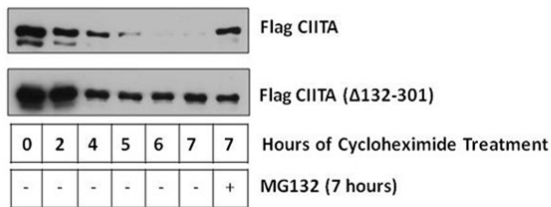
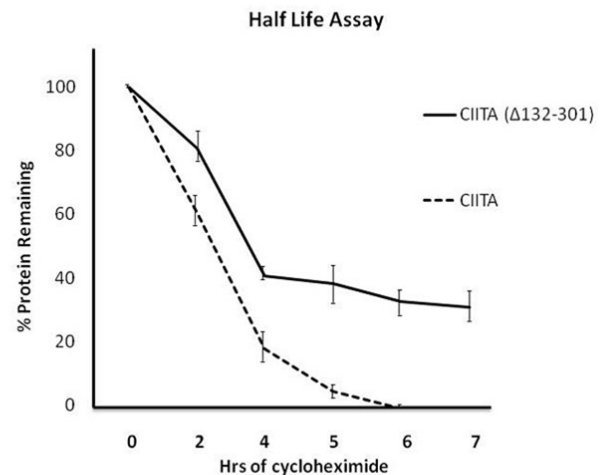
(A) Schematic representation of N-terminal truncation and deletion constructs of CIITA. Flag CIITA (Δ132-301) 1-335aa deletion construct and flag CIITA 1-301, 1-335 and 1-444 aa truncation constructs were used to further narrow the association of Sug1 with CIITA. (B) The 19S ATPases Sug1, S7 and S6a fail to bind (Δ132-301) 1-335aa CIITA. Co-IP. HeLa cells were co-transfected as indicated with flag tagged truncation or deletion constructs of CIITA and myc tagged 19S ATPase (myc Sug1, myc S7, myc S6a) or myc control. Cells were harvested, lysed in NP40, precleared and IP'd with anti-myc antibody. Western blot analysis was performed and the IP'd samples were IB using anti-flag antibody. Lysate blots demonstrate expression of myc 19S ATPase and flag CIITA. All results shown are representative of 3 experiments.

### **Deletion of amino acids 132- 301 stabilizes CIITA**

Deletion of the ATPase binding domain of CIITA (132-301aa) leads to a loss of 19S ATPase association. The region of CIITA between 132-301aa also encompasses a degron site which typically targets proteins for degradation. To further understand the functional importance of the ATPase binding domain on CIITA stability, half-life assays were performed using cycloheximide chase. Half-life studies indicate that full length CIITA with an ATPase binding domain deletion ( $\Delta$ 132-301) was significantly more stable (>40%) than wild-type full length CIITA (**figure 3.5 B-C**). Cells treated for 7 hours with cycloheximide and the proteasome inhibitor MG132 show reconstitution of CIITA expression (**figure 3.5 B, lane 7**). Together, these studies demonstrate binding of 19S ATPases to the CIITA ATPase binding domain is required for normal CIITA turnover.

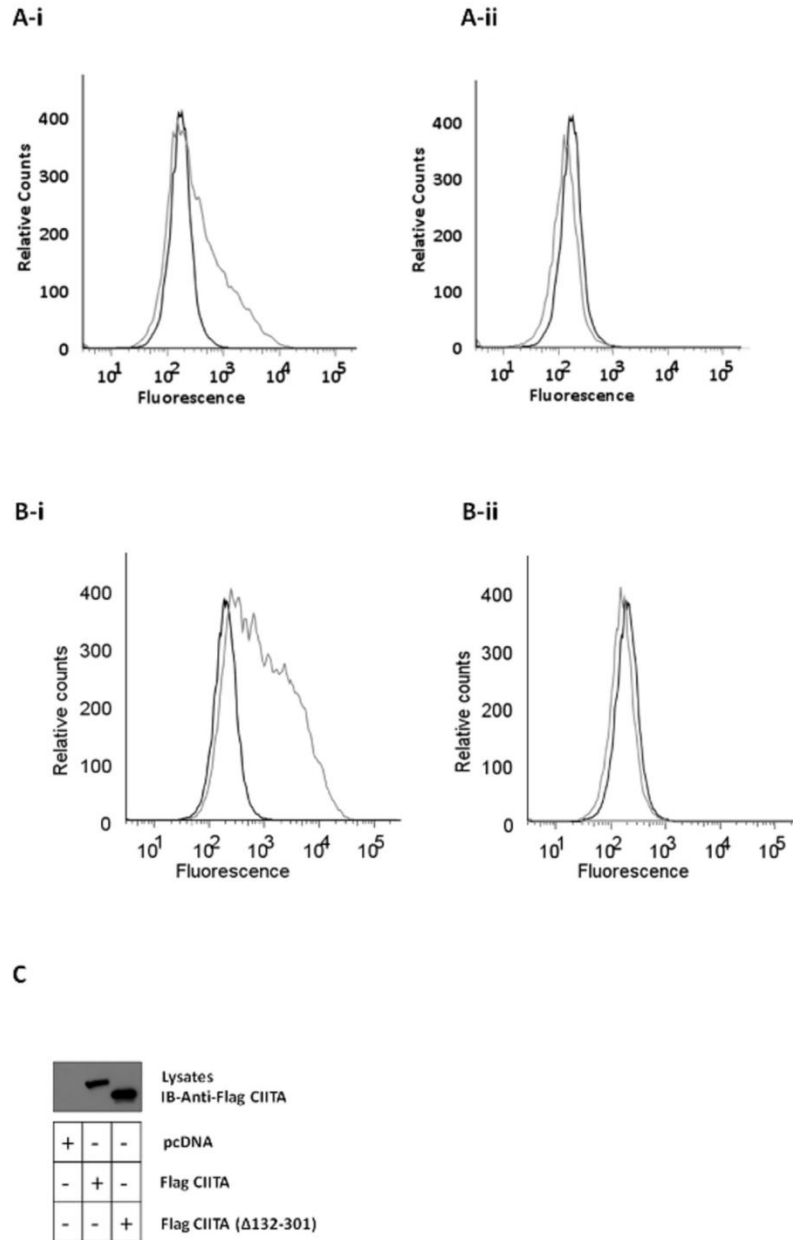
### **Cell surface expression of MHC class II is abolished by deletion of amino acids 132-301**

As deletion of the ATPase binding domain (amino acids 132-301) of CIITA increases CIITA stability, we next assayed for the effect of this deletion on MHC class II cell surface expression. Flow cytometry results indicate that following 48hrs or 72hrs of WT CIITA transfection, MHC class II cell surface expression was dramatically increased (**figure 3.6A-i-B-i**). The robust increase in MHC class II surface expression was abolished when the ATPase binding domain deletion construct of CIITA was transfected (**figure 3.6A-ii-B-ii**), indicating the removal of amino acids 132-301 is deleterious to CIITA mediated MHC class II expression. No MHC class II expression was observed when mouse IgG2a  $\kappa$  isotype control antibody was used (**supplementary figure 3.8**).

**A****B****C**

**Figure 3.5: Deletion of the ATPase binding domain (amino acids 132- 301) stabilizes CIITA.**

(A) **Schematic representation of ATPase binding domain deletion constructs.** Half-life assays were performed using the full length flag CIITA (Δ132-301) deletion construct and flag full length CIITA. (B-C) **The ATPase binding domain deletion construct of CIITA is more stable than full length CIITA.** HeLa cells were transfected with flag CIITA (Δ132-301) deletion construct or flag CIITA and were then treated with cycloheximide for 0-7 hrs. Following cycloheximide treatment, cells were harvested and western blot analysis was performed to determine the half-life of transfected CIITA. As a positive control, HeLa cells were transfected with flag CIITA (Δ132-301) deletion construct or flag CIITA and were treated with both cycloheximide and proteasome inhibitor MG132 for 7 hrs (last lane). Results shown are representative of 3 experiments. (C) **Densitometry and quantitation of data in A.** Western blots from half-life assays were quantified using Multi-Gauge V3.1 and plotted as percent (%) protein remaining (after 2, 4, 5, 6 or 7 hours of cycloheximide treatment as compared to 0hr cycloheximide treatment) v/s hours of cycloheximide treatment. Flag CIITA (Δ132-301) is represented by a solid black line and flag CIITA is represented by a dotted black line. Results shown are average of 3 experiments.



**Figure 3.6: Cell surface expression of MHC class II is abolished by deletion of the ATPase binding domain.**

**(A, B) HeLa cell MHC class II surface expression is dramatically induced upon transfection of CIITA but not upon expression of the CIITA (Δ132-301) deletion construct.** Flow cytometry. HeLa cells were transfected with flag CIITA (Δ132-301) deletion construct or flag CIITA. 48hrs (A) or 72 hrs (B) following transfection, cells were trypsinized, washed and incubated with PE-labeled MHC II antibody. Following antibody incubation, cells were fixed and MHC class II cell surface expression was measured by FACS-Canto. (A, B-i) MHC II expression in cells transfected with CIITA (light gray line) and MHC II expression in cells transfected with pcDNAcontrol (black line). (A, B-ii) MHC II expression in cells transfected with CIITA (Δ132-301) (light gray line) and MHC II expression in cells transfected with pcDNA (black line). Results shown are representative of 3 experiments. **(C) Transfection of deletion and full length constructs of CIITA.** Western blot. 1/10<sup>th</sup> of HeLa cells harvested for flow analysis were lysed in NP40. Western blot analysis was performed to demonstrate expression of transfected constructs. Results shown are representative of 3 experiments.

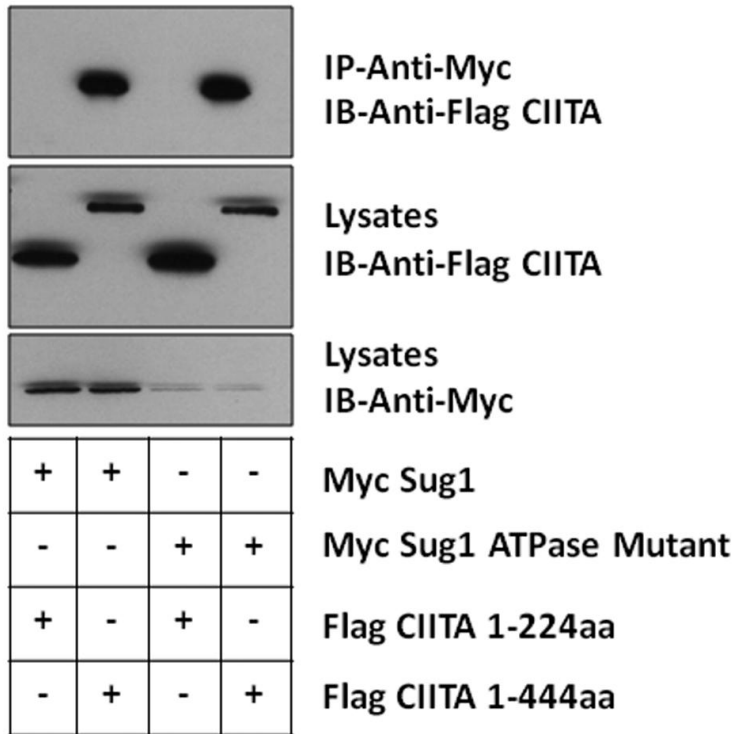
Thus binding of 19S ATPases to the ATPase binding domain, which lies within the CIITA P/S/T domain, is essential for both CIITA turnover and transactivation. These data suggest proteolytic and non-proteolytic roles of 19S ATPases in CIITA mediated MHC class II transcription.

## DISCUSSION

Recruitment of CIITA to the MHC class II promoter is required for both constitutive and inducible expression of MHC class II genes. Further evidence of the complex contributions of CIITA to the inflammatory response is that CIITA also plays crucial roles in transcriptional regulation of additional diverse immune response genes including IL-4 (Sisk, Gourley et al. 2000), IL-10 (Yee, Yao et al. 2005), E-cathepsin (Yee, Yao et al. 2004), MMP-9 (Nozell, Ma et al. 2004), plexin (Wong, Brickey et al. 2003) and Fas ligand (Gourley and Chang 2001). Since the identification of CIITA as a multi-potent master regulator, many studies have focused on understanding the complex domain structure and functions of CIITA. Despite this, due to the intricacy of CIITA structure and the roles CIITA plays in transcriptional regulation of multiple genes in various cell types, CIITA regulation remains enigmatic. We and others have previously linked CIITA to the UPS through demonstration of CIITA mono-ubiquitination (Greer, Zika et al. 2003) and poly-ubiquitination (Schnappauf, Hake et al. 2003), and through observation of functional dependence of CIITA on 19S ATPases (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Agnieszka D. Truax 2009; Koues, Dudley et al. 2009). Here we have identified a novel N-terminal ATPase binding domain in CIITA and show that this domain is crucial for CIITA stability and MHC class II expression.

In mapping studies we observed the 19S ATPase Sug1 associated with CIITA truncations 1-949aa, 1-444aa and 1-335aa but failed to bind truncation 1-224aa.



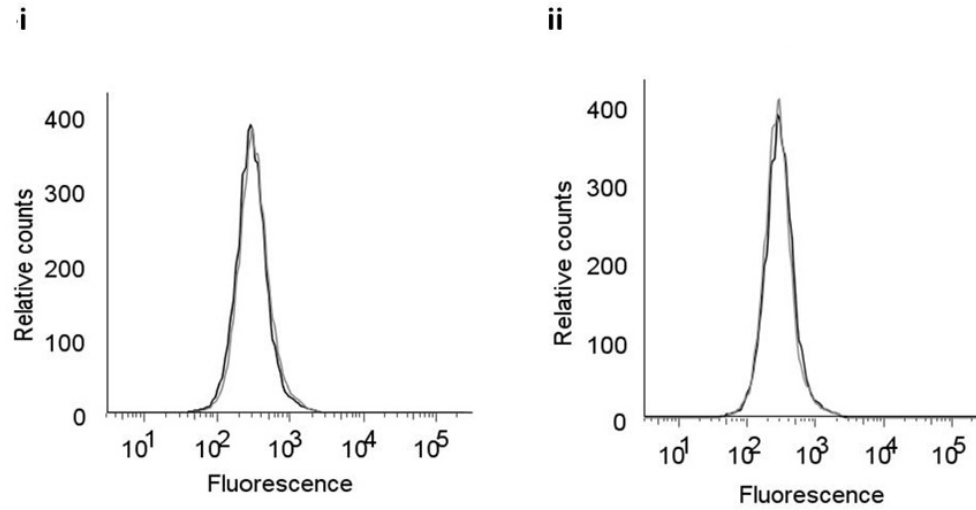


**Figure 3.7: Supplementary data: CIITA truncation of 1-444aa associates with Sug1 independent of Sug1 ATPase activity.**

Co-Immunoprecipitation (Co-IP) assays. HeLa cells were co-transfected as indicated with flag tagged truncations of CIITA 1-224aa or 1-444aa and either myc Sug1 or a myc Sug1 ATPase mutant. Cells were harvested, lysed in NP40 buffer, precleared, and IP'd with anti-myc antibody. Western blot analysis was performed and the immunoprecipitated (IP'd) samples were immunoblotted (IB) using anti-flag antibody. Lysate blots indicate expression of myc Sug1 and flag CIITA constructs. Results shown are representative of 3 experiments.

CIITA truncation 1-224aa lacks a majority of the proline-serine-threonine rich (P/S/T) region which is fully present in the truncations which bind Sug1. These data indicated that while C-terminal domains of CIITA, including the GBD, NLS2 and 3 and LRR domains, are not important in interactions with Sug1; the P/S/T region is important for CIITA association with Sug1.

CIITA has a proline rich region that lies within its P/S/T region, and transcription factors rich in proline residues often demonstrate increased DNA binding ability and activation potential (Mermod, O'Neill et al. 1989; Harton and Ting 2000). However, CIITA is a non-DNA binding transcription factor with an N-terminal transcription activation domain (Steimle, Otten et al. 1993; Zhou and Glimcher 1995; Kretsovali, Agalioti et al. 1998). Although deletion of a part of the domain does not affect CIITA transactivity (Chin, Li et al. 1997), the P/S/T domain has been shown to be essential for interaction with other transcription factors and cofactors (Fontes, Jiang et al. 1997; Fontes, Kanazawa et al. 1999) and deletion of the entire domain reduces CIITA transactivity as measured by reporter assays (Chin, Li et al. 1997). Therefore, the functional importance of the proline rich region and the P/S/T domain of CIITA remains under investigation (Harton and Ting 2000). We show here that the 19S ATPase Sug1, and the S7 and S6a representative members of the additional 19S heterodimers, interacts within this proline rich region. Based on these observations, we have termed the region required for CIITA association with 19S ATPases the “ATPase binding domain.”



**Figure 3.8: Supplementary data: The MHC class II antibody is specific.**

Flow cytometry. HeLa cells were transfected with flag CIITA ATPase binding domain ( $\Delta 132-301$ ) deletion construct or flag CIITA. 72 hrs following transfection, cells were trypsinized, washed and incubated with PE-labeled IgG control antibody. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto. (i) HeLa cells transfected with CIITA (light gray line) and HeLa cells transfected with pcDNA (black line). (ii) HeLa cells transfected with CIITA ( $\Delta 132-301$ ) (light gray line) and HeLa cells transfected with pcDNA (black line).

Lack of the ATPase binding domain significantly increases CIITA half-life, indicating that the binding of 19S ATPases plays important roles in CIITA turnover. However, the CIITA that accumulates in the absence of the ATPase binding domain is transcriptionally inactive as overexpression of ATPase domain mutants fails to facilitate cell surface expression of MHC class II proteins. We have previously shown the 19S ATPase Sug1 is essential for CIITA recruitment to the MHC class II proximal promoter region and for MHC class II expression and have demonstrated critical roles for the 19S ATPases in regulating activating modifications to histones (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. 2009; Truax, Koues et al. 2009). Here we have identified a novel N-terminal ATPase binding domain within the PST domain that is crucial for 19S ATPase binding to CIITA, for MHC class II expression and for CIITA degradation. Together these observations suggest proteolytic and non-proteolytic roles for 19S ATPases in CIITA mediated MHC class II transcription.

Studies in yeast have demonstrated that physical interaction of 19S ATPase subunits with the activation domain of Gal4 leads to destabilization of Gal4 from the promoter (Ferdous, Sikder et al. 2007). This “stripping” activity of the ATPases, but not binding of a larger 19S complex, was rendered inactive when Gal4 was mono-ubiquitinated (Archer, Burdine et al. 2008), and suggests regulatory binding of a 19S complex to activation domains. Mutation analysis indicated that ubiquitination of lysine residues between the CIITA P/S/T domain and GBD domain is not required for CIITA –Sug1 interactions. These data suggest the possibility that ATPase binding in the nearby ATPase binding domain is indicative of association of a larger 19S complex whose function, not binding, is modulated by the ubiquitination status of local lysine residues.

One scenario in which the 26S could regulate CIITA in a bimodal fashion is as follows: Mono-ubiquitinated CIITA is recruited to the MHC class II promoter where, aided by 19S ATPase recruitment of chromatin remodeling enzymes, CIITA stably binds the enhanceosome complex and the 19S complex and drives MHC class II transcription. Although 19S ATPases are simultaneously localized to the MHC class II promoter, mono-ubiquitination protects CIITA from stripping by the 19S ATPases. Following poly-ubiquitination, protection from 19S stripping is lost and CIITA is pulled from the enhanceosome complex and degraded by a promoter assembled 26S proteasome. In this scenario if 19S binding to CIITA is blocked, as seen in ATPase binding mutants, CIITA exhibits enhanced stability as it is protected from the stripping and degradative activities of the 19S. Although protected, CIITA that does not bind the 19S ATPases is inactive; indicating the 19S ATPase binding domain of CIITA stabilizes CIITA association with the enhanceosome complex via the promoter localized 19S. Thus, the interactions that tether mono-ubiquitinated CIITA to the 19S proteasome initially drive CIITA transactivation and eventually target CIITA degradation.

## ACKNOWLEDGEMENTS

We thank Dr. Jenny Ting for kindly providing flag CIITA, flag CIITA 1-224, 1-335, 1-444, 1-949aa truncations, and flag CIITA ( $\Delta$ 132-301) plasmids and Dr. A. Wani for kindly providing the pGEX-GST Sug1 and Myc Sug1 constructs. Special thanks to K. Dudley for providing myc-S6a and myc-S7 constructs. This work was supported by grants from the American Cancer Society, the Georgia Cancer Coalition, the Georgia Research Alliance (to S.F. Greer) and by a Ph.D. fellowship from the Georgia State University Molecular Basis of Disease Area of Focus (to K.P. Bhat and A.D. Truax).

## CHAPTER 4

PHOSPHORYATION AND UBIQUITINATION OF DEGRON PROXIMAL RESIDUES IS  
ESSENTIAL FOR CIITA TRANSACTIVATION AND MHC CLASS II EXPRESSION

PUBLICATION: KAVITA PURNANDA BHAT<sup>†</sup>, AGNIESZKA DOROTA TRUAX<sup>†</sup>, AND  
SUSANNA FLETCHER GREER<sup>†</sup> AS SUBMITTED TO JOURNAL OF BIOLOGICAL  
CHEMISTRY

## SUMMARY

Major histocompatibility (MHC) class II molecules are cell surface glycoproteins that present extracellular antigens to CD4<sup>+</sup> T cells and are essential for initiation of the adaptive immune response. MHC class II expression requires recruitment of a master regulator, the class II transactivator (CIITA), to the MHC class II promoter. Post-translational modifications of CIITA play important roles in modulating CIITA mediated transcription of various genes in different cell types. We and others have previously linked regulation of CIITA to the ubiquitin proteasome system (UPS) by demonstrating that mono-ubiquitination of CIITA dramatically increases its transactivity whereas poly-ubiquitination leads to CIITA degradation. Here we have identified three degron proximal lysine residues, K315, K330 and K333, and a phosphorylation site, S280, located within the CIITA degron, that regulate CIITA ubiquitination, stability and MHC class II expression. Together, these findings contribute to the developing post-translational modification code for CIITA.

## BACKGROUND

MHC class II proteins present processed exogenous antigens on the cell surface to activate CD4<sup>+</sup> T cells (Matheux and Villard 2004) and are thus critical contributors to both cell and antibody mediated immune responses (Glimcher and Kara 1992). In addition to eliciting pathogen mediated immune responses, MHC class II molecules play important roles in anti-tumor immunity as tumor specific CD4<sup>+</sup> T cells recruit and activate CD8<sup>+</sup> T cells at tumor sites (Wang 2003). Because MHC class II is a critical regulator of both pathogen and tumor mediated adaptive immune responses, its expression is tightly regulated (Benoist and Mathis 1990), and this

regulation is primarily at the level of transcription. CIITA is a non-DNA binding co-factor required for initiation of MHC class II gene transcription (Masternak, Muhlethaler-Mottet et al. 2000). CIITA is expressed from three distinct promoters, (pI, pIII and pIV), which direct the synthesis of CIITA isoforms I, III and IV to ensure CIITA expression in different cell types under different conditions (Muhlethaler-Mottet, Otten et al. 1997). Isoform I (IFI) is expressed from pI in dendritic cells and macrophages; IF III is expressed from pIII in B cells and is upregulated in response to IFN- $\gamma$ ; and IF IV is expressed from pIV in all nucleated IFN- $\gamma$  induced cells (Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Linhoff et al. 1999). CIITA is not only essential for MHC Class II transcription, but also plays crucial roles in transcriptional regulation of additional diverse immune response genes including IL-4 (Sisk, Gourley et al. 2000), IL-10 (Yee, Yao et al. 2005), E-cathepsin (Yee, Yao et al. 2004), MMP-9 (Nozell, Ma et al. 2004), plexin (Wong, Brickey et al. 2003) and Fas ligand (Gourley and Chang 2001). As expected for a critical regulator of diverse inflammatory genes, dysregulated expression and activity of CIITA is implicated in disease including head and neck cancers (Meissner, Whiteside et al. 2009), small cell lung cancer (Yazawa, Kamma et al. 1999), Erwins sarcoma (Dagmar, Alfons et al. 2009), autoimmune Addison's disease (Skinningrud, Husebye et al. 2008), arteriosclerosis (Buttice, Miller et al. 2006), and many others. However, due to the complex domain structure and distinct roles of CIITA in transcriptional regulation of multiple genes in various cell types, CIITA post-translational regulation remains enigmatic.

Post-translational regulation of CIITA is a crucial regulatory point because functional CIITA requires multiple 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) including phosphorylation,



ubiquitination, and acetylation (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). Amongst these, phosphorylation of CIITA is critical for CIITA transactivity, nuclear localization, oligomerization, and for interactions with transcription factors and co-factors (Li, Harton et al. 2001; Sisk, Nickerson et al. 2003; Greer, Harton et al. 2004; Xu, Harton et al. 2008). CIITA ubiquitination is equally important as mono-ubiquitinated CIITA displays enhanced transactivity, association with the MHC class II enhanceosome complex, and MHC class II transcription (Greer, Zika et al. 2003). Poly-ubiquitinated CIITA has been shown to be degraded by the ubiquitin-proteasome pathway (Schnappauf, Hake et al. 2003). While ubiquitination is a known regulator of CIITA, sites of ubiquitination in CIITA remain to be identified, in part due to CIITA's large size and multiple potential ubiquitination sites of 42 lysine residues.

We identify here three lysine residues, K315, K330 and K333, proximal to the degron sequence in CIITA isoform III as sites of mono-ubiquitination. Mutating these lysine residues to arginine reduces CIITA mono-ubiquitination, protein stability, transactivation and MHC class II expression. We also identify a regulatory phosphorylation site at serine 280 within the CIITA degron as a gatekeeper of CIITA mono-ubiquitination. A mutant that combines a loss of phosphorylation at serine 280 with a loss of ubiquitination at the degron proximal lysine residues restores MHC class II expression beyond that of wild type CIITA, indicating that regulated mono-ubiquitination at these lysine residues is essential for CIITA stability and transactivity. These findings identify novel sites in CIITA isoform III that regulate transactivation and CIITA mediated MHC class II expression.

## MATERIALS AND METHODS

**Cell culture:** HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% FCS, 50U/ml of penicillin, 50µg/ml of streptomycin and 2mM of L-glutamine. The cells were maintained at 37°C with 5% CO<sub>2</sub>.

**Plasmids:** Full length Flag-CIITA, truncation mutants Flag-CIITA 1-335aa, pCDNA3, HA-Mono-Ubiquitin (HA-Mono-Ub) and HA-WT-Ubiquitin (HA-WT-Ub) plasmids were kindly provided by Dr. J. Ting. The HLA-DRA luciferase reporter construct was described previously (Bhat, Turner et al. 2008). The following site specific mutants: full length Flag-CIITA K1R (K315R), Flag-CIITA K2R (K330, 333R), Flag-CIITA K3R (K315, 330, 333R), Flag-CIITA K3R-S280A, Flag-CIITA S280A and truncation mutant Flag-CIITA 1-335aa K3R (K315, 330, 333R) were generated using QuickChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specific primers with desired mutations were designed using Stratagene's primer design tool and mutagenesis reactions were performed as per the manufacturer's protocol. Mutagenesis was confirmed by sequence analysis and expression analyzed by western blot.

*N-terminal fusion of Mono-ubiquitin to CIITA and CIITA K3R.* N-terminal fusions of mono-ubiquitin to full length Flag-CIITA or Flag-CIITA K3R were generated using HA-Mono-Ub and Flag-CIITA or Flag-CIITA K3R constructs and QuickChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). Unique restriction sites for AgeI were engineered between the Flag-tag and CIITA or CIITA K3R using specific primers. Unique restriction sites for AgeI were engineered upstream and downstream of the mono-Ub gene using specific primers. Insertion of restriction sites were confirmed by sequencing. Positive clones were subjected to restric-

tion (RE) digestion with AgeI for 4hrs at 37°C and were heat inactivated for 10 minutes at 65°C. Appropriately sized DNA fragments were gel purified using QIAquick gel extraction kit as per the manufacturer's protocol (Qiagen, Valencia, CA). The RE digested CIITA or CIITA K3R were ligated with RE digested fragment of mono-Ub over-night at 16°C and heat inactivated for 10 minutes at 65°C. The ligated mixture was transformed into Top 10 cells and plated on antibiotic selective plate. DNA isolated from selected colonies was sequenced for insertion of mono-Ub gene between the Flag-tag and CIITA or CIITA K3R. Directionality of the construct was confirmed by DNA sequencing.

**Transient transfection and luciferase reporter assays:** HeLa cells were plated at  $5 \times 10^4$  cells/well density (60% confluency) in 6-well plates and were incubated for 18 hrs at 37°C. Following adhesion, cells were co-transfected as indicated with HLA-DRA and pcDNA, Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R, Flag-Mono-Ub-CIITA or Flag-Mono-Ub-CIITA K3R plasmids using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Eighteen hrs following transfection, cells were lysed with 100µl of 1x cell culture lysis reagent (Promega, Madison, WI) supplemented with complete EDTA-free protease inhibitors (Roche). Following addition of cell lysis reagent, cells were scraped and the lysed cell suspension was centrifuged for 2 minutes at 12,000 rpm (Thermo electron 851, Thermo INC, Needham Heights, MA) at 4°C and luciferase assays were performed using Lmax II<sup>384</sup> (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. Luciferase readings were normalized to protein content in the lysates by Bradford Assay. Western blots were performed to confirm equal expression of expressed plasmids.

**RNA expression:** HeLa cells were plated at a cell density of  $8 \times 10^5$  cells/plate. Following adhesion, cells were transfected with pcDNA, Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R or Flag-CIITA S280A plasmids using Fugene 6. Cells were stimulated with interferon  $\gamma$  and 18 hrs post-stimulation, cells were harvested, washed with cold PBS, centrifuged at 3,000 rpm at 4°C for 5 minutes, and 9/10<sup>th</sup> of the cells were used to extract RNA. Total RNA was prepared with 1ml of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reconstituted in 30 $\mu$ l DEPC water (MP Biomedical, LLC, Aurora, OH) and stored at -80°C. The Omniscript reverse transcription kit (Qiagen) was used to reverse transcribe 1 $\mu$ g of RNA into cDNA. Gene specific antisense primers (Sigma, Saint Louis, MO) were used for reverse transcription (RT). PCR was performed using a Mastercycle 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 on the basis of standard curves generated for each gene, were run in triplicate reactions and were analyzed using the SDS 2.0 program. 1/10<sup>th</sup> of cells were lysed in 100  $\mu$ l of 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and Bradford assays were performed to determine total protein content in the lysates. Western blots confirmed equal expression of expressed plasmids.

**Ubiquitination assay:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10cm tissue culture plates. Following adhesion, cells were co-transfected with 1-224aa, 1-335aa truncation mutants of CIITA, Flag-CIITA, Flag-CIITA 1-335aa K3R, Flag-CIITA K3R or Flag-CIITA S280A and HA-Mono-Ub or HA-WT-Ub. Cells were treated with MG132, proteasome inhibitor, for 4hrs,

harvested, and lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged, normalized for protein concentration, pre-cleared with 50  $\mu$ l mouse IgG (Sigma-Aldrich) and immunoprecipitated with 30  $\mu$ l of anti Flag-agarose beads (Sigma-Aldrich). Immune complexes were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-HA monoclonal antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Milipore) to determine *in vivo* ubiquitination patterns of various CIITA constructs. Bradford assays were used to normalize for protein and equal loading was determined in non-immunoprecipitated lysates.

**Half-life assay:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10 cm tissue culture plates. Following adhesion, cells were transfected with 5  $\mu$ g of Flag-CIITA, Flag-CIITA K3R, Flag-CIITA S280A or Flag-CIITA K3R-S280A. Transfected cells were treated with 100  $\mu$ M cycloheximide (Sigma-Aldrich) for 0-20 hrs. Following cycloheximide treatment, cells were lysed in 1% Nonidet P-40 buffer supplemented with complete EDTA-free protease inhibitors (Roche) on ice. Lysates were centrifuged and normalized for protein concentration. As controls, HeLa cells were transfected with Flag-CIITA ( $\Delta$ 132-301) deletion construct or Flag-CIITA construct and treated with 100  $\mu$ M cycloheximide and MG132 (EMD Biosciences, San Diego, CA) for 8 hrs to block new protein synthesis and protein degradation. Proteins were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-Flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Milipore) to deter-

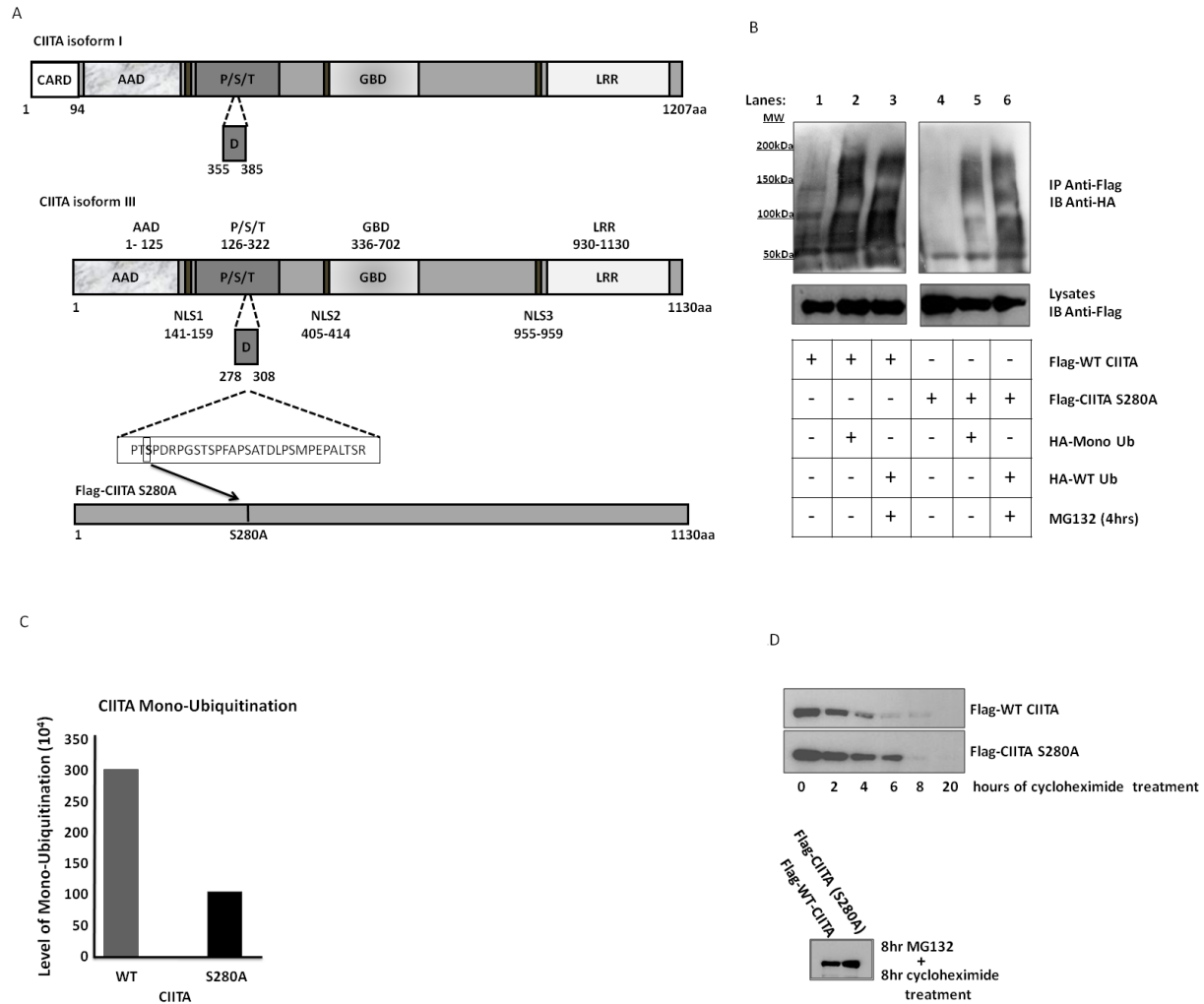
mine half-life of CIITA constructs. Proteins were normalized by Bradford assay and equal loading was determined in lysates.

**Flow cytometry:** HeLa cells were plated at a cell density of  $8 \times 10^5$  in 10cm tissue culture plates. Following adhesion, cells were transfected with Flag-CIITA, Flag-CIITA K1R, Flag-CIITA K2R, Flag-CIITA K3R, Flag-CIITA S280A or Flag-CIITA K3R-S280A. Seventy two hrs following transfection, cells were trypsinized and washed with PBS. Following the wash,  $9/10^{\text{th}}$  of the cells were resuspended in incubation buffer (0.5% bovine serum albumin in PBS w/v) and 10 $\mu$ g Phycoerythrin (PE)-labeled anti-human HLA-DR (clone L243, Biolegend, San Diego, CA) antibody or PE mouse IgG2a  $\kappa$  isotype control antibody (Biolegend) was added to the cell suspension and rotated at 4°C. Following antibody incubation, cells were washed twice with PBS, fixed with 2% paraformaldehyde and stored at 4°C. MHC class II cell surface expression was measured by FACS-Canto (Becton Dickinson, San Jose, CA) and analyzed using FlowJo. All samples were analyzed using 10,000 events per sample.  $1/10^{\text{th}}$  of the HeLa cells harvested for flow analysis were lysed in 1% Nonidet P-40 buffer supplemented with Complete EDTA-free protease inhibitors (Roche) on ice. Proteins were denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with anti-Flag antibodies (Sigma-Aldrich). HRP conjugates were detected with Immobilon Western Chemiluminescent substrate (Millipore) to determine expression of CIITA constructs. Proteins were normalized by Bradford assay and equal loading was determined in lysates.

## RESULTS

### **Mutating serine 280 in the CIITA degron reduces CIITA ubiquitination and enhances CIITA half-life.**

CIITA is large protein with four functional domains, an N-terminal acidic activation domain (AAD) required for CIITA transactivity, a proline/serine/threonine rich (P/S/T) domain required for binding to factors and cofactors, a GTP-binding domain (GBD), and a C-terminal leucine rich region (LRR) required for localization and oligomerization (Masternak, Muhlethaler-Mottet et al. 2000; Cressman, O'Connor et al. 2001; Tosi, Jabrane-Ferrat et al. 2002) (**figure 4.1A, top panel**). The P/S/T domain of CIITA isoform 1 (IF1) contains a degron (Drozina, Kohoutek et al. 2006), a sequence which typically targets proteins for ubiquitination at proximal lysine residues and degradation (Barbash, Egan et al. 2009). CIITA IF1 serine 357 lies within the degron sequence and is phosphorylated by ERK1/2 prior to IF1 ubiquitination (Drozina, Kohoutek et al. 2006). Our sequence analysis revealed a degron is also present in CIITA isoform III (IF3), so we first determined if the conserved serine at position 280 is important for CIITA IF3 ubiquitination and stability. CIITA IF3 serine 280 was mutated to alanine using site directed mutagenesis (**figure 4.1A, bottom panel**) and *in vivo* ubiquitination assays were performed. Expression of WT-CIITA or CIITA S280A with mono-ubiquitin in HeLa cells demonstrated that CIITA S280A was less mono-ubiquitinated than WT-CIITA (**figure 4.1B**). Densitometric quantitation revealed a 50% decrease in CIITA S280A mono-ubiquitination in comparison to that of WT-CIITA (**figure 4.1C**). Half-life assays indicated CIITA S280A is more stable than WT-CIITA (**figure 4.1D, top panel**) while transfection controls using a combined 8hr treatment of cycloheximide and the proteasome inhibitor MG132 equally reconstituted expression WT-CIITA and CIITA S280A (**figure 4.1D, bottom panel**).



**Figure 4.1: Mutating serine 280 in the degron of CIITA isoform III reduces CIITA ubiquitination and enhances CIITA half-life.**

**(A) Schematic representation of domain structure of CIITA.** CIITA is an 1130 amino acid protein with four functional domains: an N-terminal transcriptional acidic activation domain (AAD), a proline/serine/threonine rich (P/S/T) domain, a GTP-binding domain (GBD), and a C-terminal leucine rich region (LRR). CIITA has three nuclear localization sequences (NLS) dispersed through the length of the protein. The P/S/T rich region of CIITA contains a proteolytic signal sites termed a degron (D) (top panel). Site-directed mutagenesis was performed to mutate serine 280 to alanine (flag-CIITA S280A, bottom panel). **(B) Mutating serine 280 to alanine reduces CIITA ubiquitination.** Ubiquitination assay. HeLa cells were co-transfected with flag-WT-CIITA or flag-CIITA S280A and HA-Mono-Ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated as indicated with proteasome inhibitor MG132 for 4hrs prior to harvesting. Cells were harvested, lysed, precleared and immunoprecipitated (IP'd) with anti-flag antibody to pull down CIITA and bound proteins. Western blots were performed and IP'd samples were immunoblotted (IB) using anti-HA antibody to detect ubiquitinated CIITA. Lysate controls demonstrate expression of Flag-CIITA. Results shown are representative of 3 experiments. **(C) Densitometric quantitation.** Western blots of *in vivo* ubiquitination assays were quantified using Multi-Gauge V3.1 and graphed as shown. Results shown are representative of 3 experiments. **(D) Mutating serine 280 to alanine increases CIITA half life.** HeLa cells were transfected with flag-WT-CIITA or flag-CIITA S280A. Following transfection, cells were treated with cycloheximide for 0-20 hrs. Following cycloheximide treatment, cells were harvested and western blot analysis was performed to determine the half-life of transfected CIITA (top panel). As transfection and degradation controls, HeLa cells transfected with flag-WT-CIITA or flag-CIITA S280A were treated with cycloheximide and proteasome inhibitor MG132 for 8hrs (bottom panel). Results shown are representative of 3 experiments.

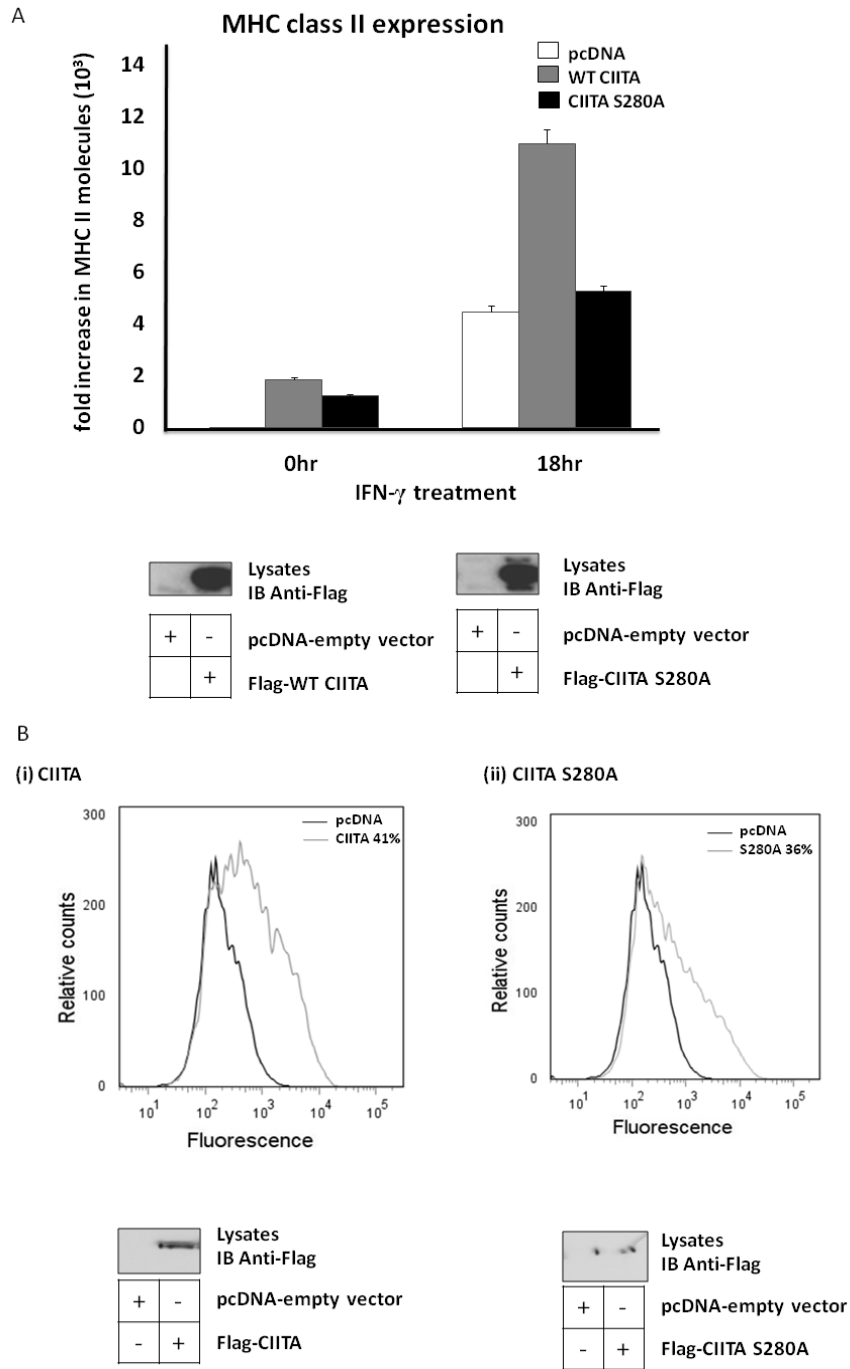


### **Despite its increased stability, CIITA S280A is less active than WT CIITA.**

We have recently identified a novel N-terminal ATPase binding domain in CIITA that lies within the P/S/T region of CIITA and encompasses a majority of the degron. Absence of this domain increases CIITA half-life, but dramatically decreases MHC class II surface expression indicating roles for the ATPase binding domain in regulating CIITA transactivation (Bhat, Truax et.al. 2009 in revision, *Journal of Immunology and Cell Biology*). As mutating serine 280 to alanine reduces CIITA mono-ubiquitination and enhances CIITA half-life, we next determined if this mutation affected CIITA mediated MHC class II transcription. MHC class II mRNA levels were measured in HeLa cells transfected with WT-CIITA or with CIITA S280A, and either untreated or stimulated with IFN- $\gamma$ . We observed that in unstimulated cells, expression of WT-CIITA resulted in increased MHC class II expression as compared to pcDNA or CIITA S280A transfected cells. Upon IFN- $\gamma$  stimulation, endogenous MHC class II transcript levels were increased, and expression of WT-CIITA further enhanced transcript levels. However, expression of the CIITA S280A did not significantly increase endogenous MHC class II transcript levels beyond that of IFN- $\gamma$  stimulation alone (**figure 4.2A**) despite equal expression of CIITA S280A in both experiments (**figure 4.2B**). MHC class II cell surface expression was similarly reduced by the CIITA serine 280 to alanine mutation in unstimulated HeLa cells (**figure 4.2C**).

### **CIITA is significantly ubiquitinated downstream of S280 and the degron sequence.**

Gene transcription involves highly coordinated and sequential post-translational modifications of activators with activator phosphorylation frequently preceding mono-ubiquitination.



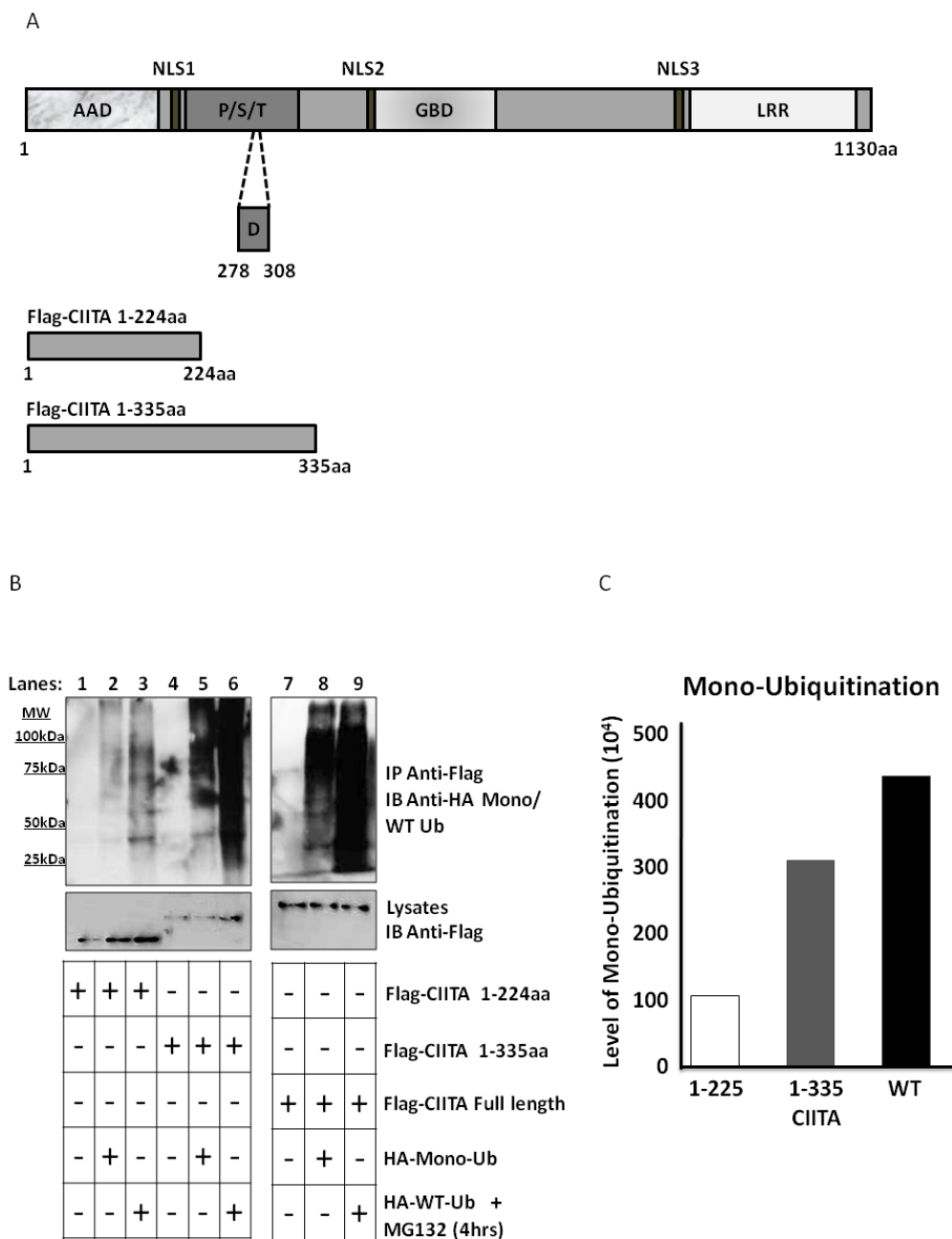
**Figure 4.2: CIITA S280A is less active than WT CIITA.**

**(A) Mutating CIITA serine 280 to alanine decreases MHC class II mRNA expression levels.** *mRNA quantification.* HeLa cells were transfected flag-CIITA or flag-CIITA S280A or pcDNA as indicated and were stimulated with IFN- $\gamma$  for 18hrs or left untreated. Cells were harvested, RNA was extracted and cDNA was prepared and quantified by Real-time PCR. Results shown are an average of 3 experiments (top panel). *Western blots.*  $1/10^{\text{th}}$  of the cell volume was lysed in NP40 to confirm expression of flag-CIITA and flag-CIITA S280A (bottom panels). **(B) Mutating CIITA serine 280 to alanine decreases MHC class II surface expression.** *Flow cytometry.* HeLa cells were transfected as indicated with flag-CIITA or flag-CIITA S280A mutant or pcDNA. 72hrs following transfection, cells were trypsinized, washed and incubated with PE-labeled anti-human HLA-DR antibody. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto.

Serine phosphorylation in the CIITA isoform I degron has been shown to precede CIITA mono-ubiquitination and subsequent poly-ubiquitination (Drozina, Kohoutek et al. 2006). To identify the lysine residues targeted for ubiquitination, we generated two CIITA truncation mutants: CIITA 1-224aa that lacked the entire degron and proximal downstream lysines, and CIITA 1-335aa that contained the entire degron and the lysine residues in close proximity to the degron (**figure 4.3A**) and compared them to ubiquitination of WT-CIITA (**figure 4.3B**). *In vivo* ubiquitination assays indicate low level mono-ubiquitination of CIITA 1-224aa compared to the robust monoubiquitination seen in CIITA1-335aa (**figure 4.3B**, compare lanes 2 and 5) and in WT-CIITA (**figure 4.3B**, compare lanes 2 and 8). Importantly, CIITA 1-335aa contains only 3 additional lysine residues more than CIITA 1-224aa; compared to WT-CIITA which contains an additional 35 lysine residues more than CIITA 1-224aa and 32 more lysine residues than CIITA truncation 1-335aa. As the difference of 3 lysine residues between the two CIITA truncations results in a substantial difference in their levels of mono-ubiquitination (**figure 4.3C**), these 3 lysine residues likely serve as sites for prominent CIITA mono-ubiquitination.

### **Mutating degron proximal lysines 315, 330 and 333 to arginine reduces CIITA mono-ubiquitination and half-life.**

Ubiquitination assay of CIITA truncations indicate residues located between amino acids 224 and 335 of CIITA may serve as sites for mono-ubiquitination. As lysine residues proximal to degrons are preferentially targeted for ubiquitination by E3 ligases (Barbash, Egan et al. 2009), we sought to further determine the ubiquitination status of residues K315, K330 and K333. When we first performed ubiquitination assay to determine the effect of lysine mutations

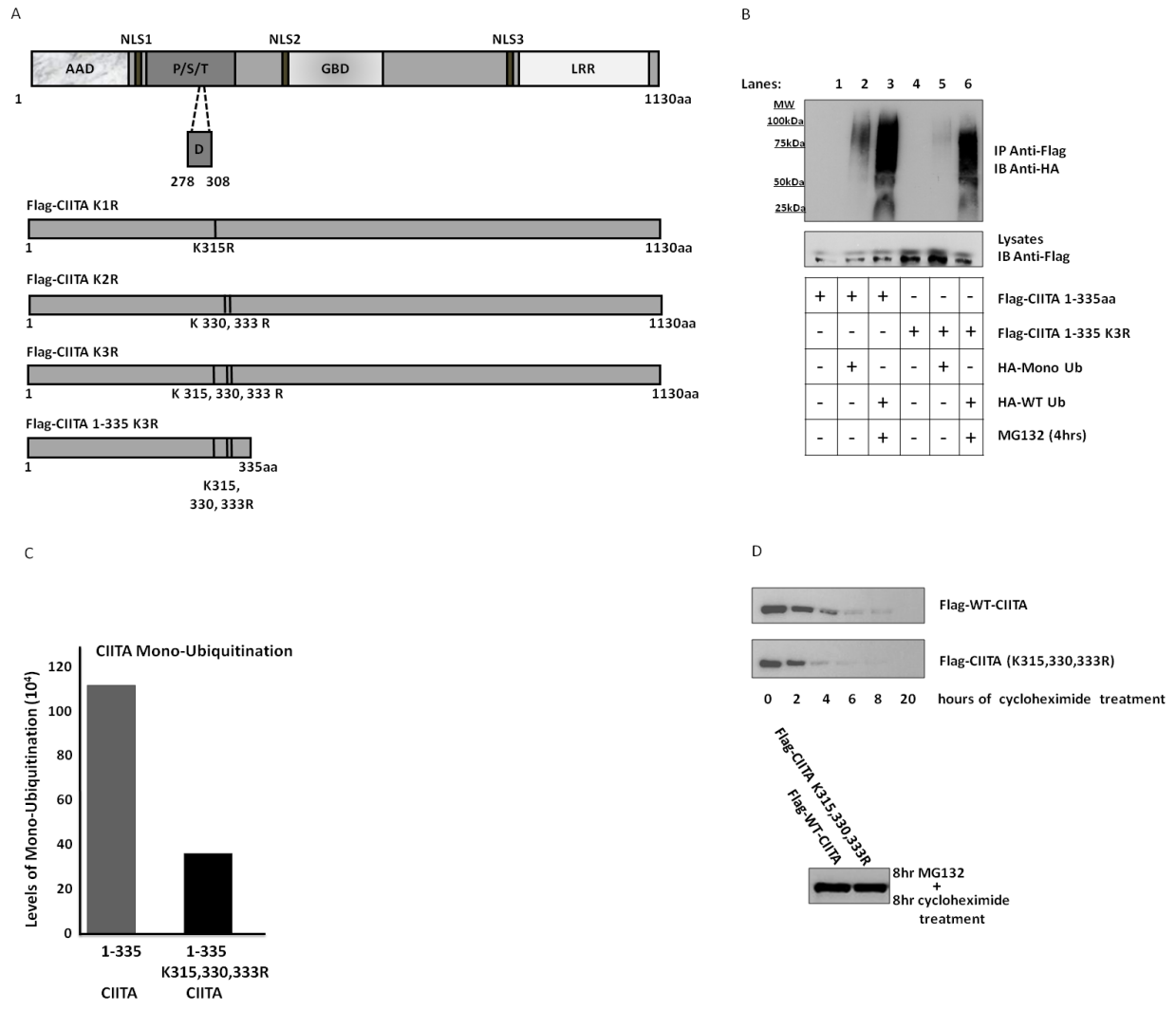


**Figure 4.3: CIITA is highly ubiquitinated downstream of the N-terminal degron.**

**(A) Schematic representation of CIITA truncation mutants.** Full length flag-CIITA was used to generate truncation constructs flag-CIITA 1-224aa and flag-CIITA 1-335aa. **(B) CIITA is ubiquitinated between residues 225 and 335.** Ubiquitination assay. HeLa cells were transfected with the 1-224aa or 1-335aa truncation mutants of CIITA or WT-CIITA, and HA-Mono-Ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated as indicated with the proteasome inhibitor MG132 for 4hrs prior to harvesting. Following lysis, cells were IP'd with anti-Flag antibody and IP'd samples were IB'd with anti-HA antibody (top panels). Lysates confirm expression of Flag-CIITA (bottom panels). Results shown are representative data of 3 experiments. **(C) Densitometric quantitation.** Western blots from *in vivo* ubiquitination assays were quantified using Multi-Gauge V3.1 and graphed. Results shown are representative data of 3 experiments.

on CIITA ubiquitination using full length CIITA (flag-CIITA K315,330,333R) (**figure 4.4A**), we observed a slight decrease in mono-ubiquitination (**Supplemental figure 4.7A-B**). The small difference can be attributed to the fact that full length CIITA has 39 additional lysine residues which may serve as ubiquitination sites (Greer, Zika et al. 2003) . When we performed ubiquitination assays comparing CIITA1-335 to the triple lysine mutant CIITA 1-335 K315, 330, 333R, we observed dramatic and quantifiable decrease in mono-ubiquitination (**figure 4.4B-C**). Together, these data indicate the three degron proximal lysine residues are targets for mono-ubiquitination, and highlight the fact that other lysine sites downstream of these lysines may serve as sites for mono-ubiquitination if lysines 315, 330, and 333 are unavailable for ubiquitination.

Mono-ubiquitination has been shown to protect target proteins from the “stripping” activity of 19S proteasomal ATPases, thus preventing transcription factor degradation and increasing transactivation potential (Archer, Burdine et al. 2008; Archer, Delahodde et al. 2008). If degron proximal lysine residues K315, K330 and K333 serve as sites for protective mono-ubiquitination, then mutating the three lysine residues to arginine may render them susceptible to rapid degradation and increased vulnerability to the “stripping” activity of 19S proteasomal ATPases. Indeed, cycloheximide chase assays indicate the CIITA K3R mutant has a shorter half-life than WT-CIITA (**figure 4.4D, top panels**). Transfection controls using a combined 8 hr treatment of cycloheximide and the proteasome inhibitor MG132 equally reconstituted expression WT-CIITA and CIITA K3R, further indication that CIITA degradation requires a functional 26S proteasome.



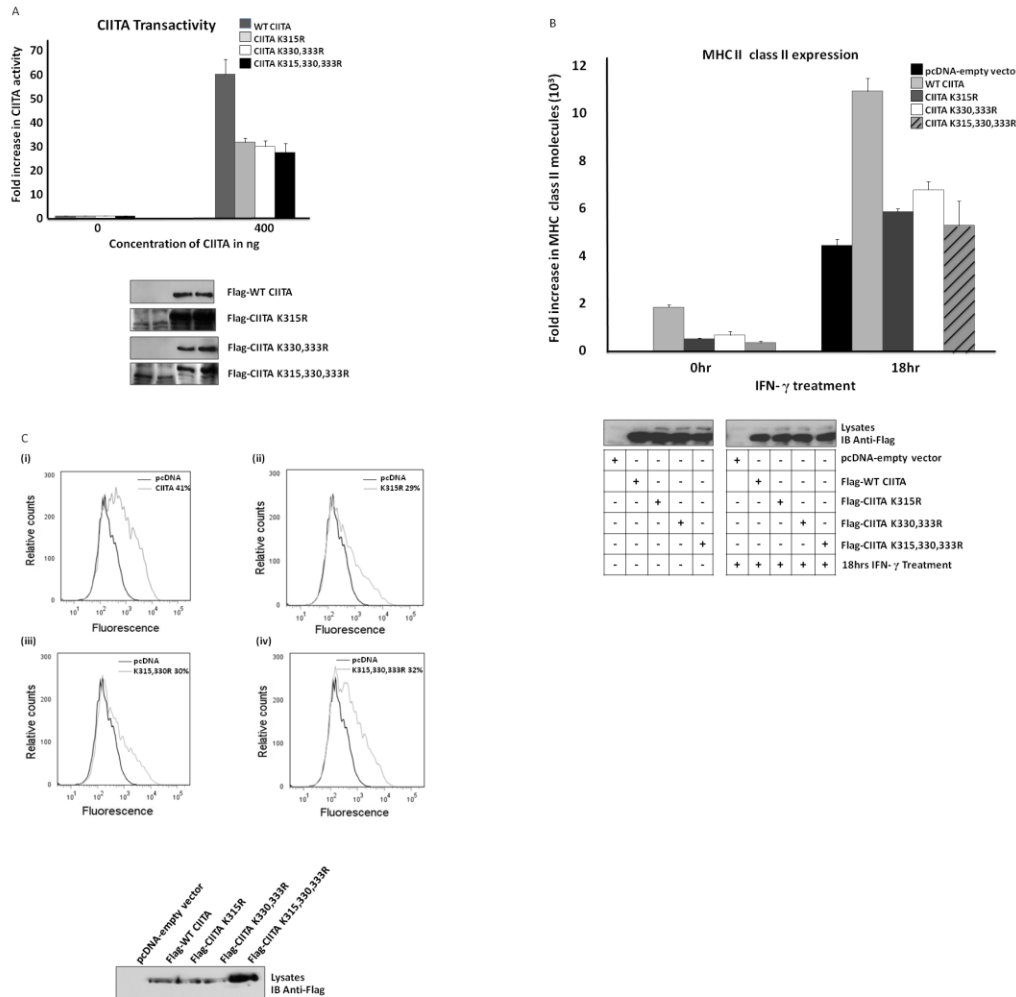
**Figure 4.4: Mutating CIITA lysines 315, 330 and 333 to arginine reduces CIITA mono-ubiquitination and half life.**

**(A) Schematic representation of CIITA lysine mutants.** Site-directed mutagenesis was performed to mutate lysine residues 315, 330 and 333 to arginine. Mutants generated were flag-CIITA K1R (K315R), flag-CIITA K2R (K330, 333R) and flag-CIITA K3R (K315, 330, 333R). **(B) Mutating lysine residues K315, K330 and K333 to arginine reduces CIITA mono-ubiquitination.** *Ubiquitination assay.* HeLa cells were transfected with flag-CIITA 1-335 or flag-CIITA 1-335 K3R, and HA-Mono-Ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated as indicated with the proteasome inhibitor MG132 for 4hrs prior to harvesting. Lysed cells were IP'd with anti-flag antibodies and IB'd with anti-HA antibodies (top). Lysate controls demonstrate expression of Flag-CIITA (bottom). Results shown are representative data of 3 experiments. **(C) Densitometric quantitation.** Western blots from ubiquitination assays were quantified using Multi-Gauge V3.1 and graphed. Results shown are representative data of 3 experiments. **(D) Mutating lysine residues K315, K330 and K333 to arginine decreases CIITA half-life.** HeLa cells were transfected with flag-CIITA or flag-CIITA K3R. Following transfection, cells were treated with cycloheximide for 0-20 hrs, harvested and IB'd to determine the half-life of transfected CIITA (top panel). As transfection and degradation controls, HeLa cells transfected with flag-WT-CIITA or flag-CIITA K3R were treated with cycloheximide and proteasome inhibitor MG132 for 8hrs (bottom panel). Results shown are representative of 3 experiments.

**Mutating CIITA lysines 315, 330 and 333 to arginine reduces CIITA transactivity, MHC class II mRNA levels and MHC class II surface expression.**

Mono-ubiquitination of CIITA has previously been shown to enhance CIITA transactivity and MHC class II expression (Greer, Zika et al. 2003). As mutating lysine 315, 330 and 333 to arginine reduces CIITA mono-ubiquitination and half-life, we next wanted to know if these mutations also affected CIITA mediated MHC class II transcription. To address this, we performed luciferase reporter assays to measure CIITA transactivity. Results from reporter assays indicated that expression of WT-CIITA dramatically increases transactivation from a MHC class II *HLA-DRA* reporter construct. However, expression of any of the CIITA lysine mutants: CIITA K315R, CIITA K330, 333R, and CIITA K315, 330, 333R resulted in dramatic decreases in CIITA transactivation (**figure 4.5A, top**). Western blots of transfection controls demonstrated expression of each of the indicated flag-tagged constructs (**figure 4.5A, bottom**).

We next tested if endogenous MHC class II transcript levels were similarly affected by the degon proximal lysine mutations. MHC class II mRNA levels were measured in HeLa cells transfected with WT-CIITA, CIITA K315R, CIITA K330,333R or CIITA K315,330,333R and either left untreated or stimulated with IFN- $\gamma$ . In unstimulated HeLa cells, expression of WT-CIITA resulted in an increase in MHC class II transcript levels as compared to cells transfected with any of the CIITA degon proximal lysine mutants (**figure 4.5B, top left panel**). When HeLa cells were stimulated with IFN- $\gamma$ , endogenous MHC class II transcript levels increased and were further enhanced by expression of WT-CIITA. However, expression of the degon proximal lysine mutants failed to significantly enhance MHC class II transcript levels beyond levels seen in cells stimulated with IFN- $\gamma$  and transfected with the pCDNA control (**figure 4.5B, top right panel**).



**Figure 4.5: Mutating CIITA lysine residues K315, K330 and K333 to arginine reduces CIITA transactivity, MHC class II mRNA levels, and MHC class II surface expression.**

**(A) Mutating CIITA lysine residues K315, K330 and K333 to arginine reduces CIITA transactivity.** *Luciferase reporter assays.* HeLa cells were transfected with flag-CIITA, flag-CIITA K1R, flag-CIITA K2R, flag-CIITA K3R or pcDNA and *HLA-DRA*-Luciferase reporter. Luciferase readings were normalized to total protein concentration. Results shown are an average of 3 experiments (top panel). **Expression of flag-CIITA, flag-CIITA K315R, flag-CIITA K330,333R and flag-CIITA K315,330,333R.** *Western blot.* Lysates IB'd with anti-flag antibodies confirm expression of flag-tagged constructs. Results shown are representative of 3 experiments (bottom panels). **(B) Mutating CIITA lysine residues K315, K330 and K333 to arginine reduces MHC class II mRNA levels.** *mRNA quantification.* HeLa cells were transfected with flag-CIITA, flag-CIITA K1R, flag-CIITA K2R, flag-CIITA K3R or pcDNA. Following transfection, cells were left untreated or were stimulated with IFN- $\gamma$  for 18hrs. Cells were harvested, RNA extracted, and cDNA was prepared and quantified by Real-time PCR. Results shown are an average of 3 experiments (top panel). **Expression of flag-CIITA, flag-CIITA K315R, flag-CIITA K330,333R and flag-CIITA K315,330,333R.** *Western blot.* 1/10<sup>th</sup> of the cell volume was lysed and IB'd with anti-flag antibodies to confirm expression of flag-tagged constructs. Results shown are representative of 3 experiments (bottom panels). **(C) Mutating CIITA lysine residues K315, K330 and K333 to arginine reduces MHC class II surface expression.** *Flow cytometry.* HeLa cells were transfected with flag-CIITA, flag-CIITA K315R, flag-CIITA K330,333R, flag-CIITA K315,330,333R or pcDNA. 72 hrs following transfection, cells were trypsinized, washed and incubated with PE-labeled anti-human HLA-DR antibody. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto. (i) HeLa cells transfected with flag-CIITA (light gray line) and HeLa cells transfected with pcDNA (black line). (ii) HeLa cells transfected with flag-CIITA K315R (light gray line) and HeLa cells transfected with pcDNA (black line). (iii) HeLa cells transfected with flag-CIITA K330,333R (light gray line) and HeLa cells transfected with pcDNA (black line). (iv) HeLa cells transfected with flag-CIITA K315,330,333R (light gray line) and HeLa cells transfected with pcDNA (black line).



As mutating the degron proximal lysine residues to arginine reduces CIITA mono-ubiquitination and also affects CIITA mediated MHC class II transcription and surface expression, we next genetically fused mono-ubiquitin to the N-terminus of CIITA and CIITA K3R constructs in order to overcome the lack of mono-ubiquitination at the degron proximal lysine residues in hopes of resuscitating CIITA activity in the lysine mutants. However, luciferase reporter assays using these fusion constructs demonstrated substantial decreases in CIITA transactivation when either WT CIITA (Mono-Ub-CIITA) or CIITA K315, 330, 333R (Mono-Ub-K315, 330, 333R) was fused to mono-ubiquitin (**supplementary figure 4.8A**). Western blot analysis of lysates isolated from cells transfected with these fusion-constructs demonstrated CIITA was rapidly degraded when fused with mono-Ub as indicated by the formation of a smear as compared to a single band for WT-CIITA (**supplementary figure 4.8B**). Minimally, these data indicate CIITA fragmentation, and likely rapid degradation, of ubiquitin-fused CIITA.

### **Mutating Serine 280 to alanine in the triple lysine mutant restores MHC class II surface expression.**

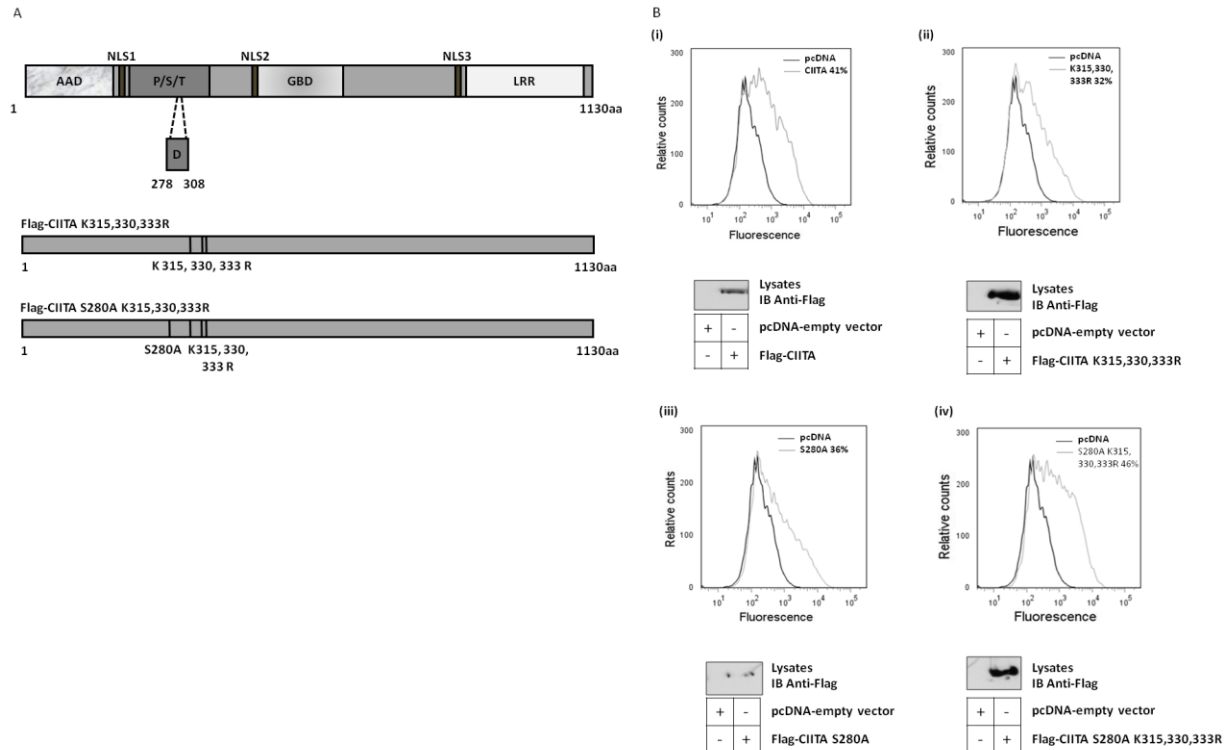
Although multiple sites in CIITA can be targeted for mono-ubiquitination, our results suggest that degron proximal lysine residues, K315, K330, and K333, are important regulatory targets for CIITA mono-ubiquitination and CIITA stability and for MHC class II expression. Serine 280 within the degron of CIITA regulates CIITA half life and plays important roles in regulating CIITA ubiquitination, mostly likely affecting access to degron proximal lysine residues. To determine the effects of combining the degron proximal serine and lysine mutations, we mutated serine 280 to alanine in CIITA K315, 330, 333R (**figure 4.6A**). The CIITA S280AK315,

330, 333R mutant restored MHC class II surface expression to a level beyond that seen in cells transfected with WT CIITA, indicating the importance of an initiating phosphorylation event to properly regulate ubiquitination and CIITA mediated MHC class II expression.

## DISCUSSION

CIITA, the master regulator of MHC Class II genes, has been implicated in disease development due to its function as a multi-potent transactivator of various critical immune genes (Sisk, Gourley et al. 2000; Gourley and Chang 2001; Wong, Brickey et al. 2003; Nozell, Ma et al. 2004; Yee, Yao et al. 2004; Yee, Yao et al. 2005). How CIITA is able to wear multiple hats and modulate this wide variety of genes in a cell and tissue specific manner remains under investigation. Post-translational modifications of CIITA are major players in modulating CIITA activity as they fine-tune CIITA transactivity in response to various cellular stimuli. Here we have sought to identify essential ubiquitination sites in CIITA and to link ubiquitination to a phosphorylation event.

The process of protein ubiquitination is a complex enzymatic cascade involving covalent attachment of a ubiquitin moiety to an internal lysine residue. Although E3 ligases can target different lysine residues for ubiquitination, it is known that lysine residues proximal to degron sites are preferentially targeted (Barbash, Egan et al. 2009).

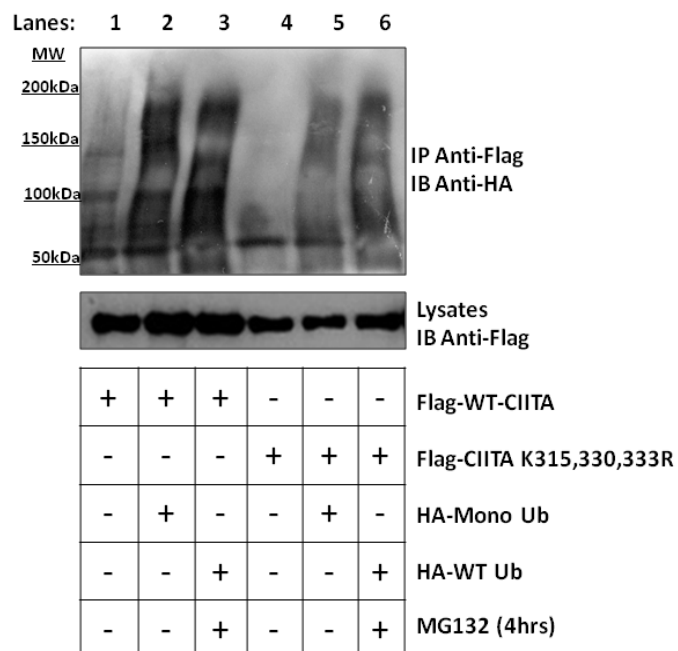


**Figure 4.6: Combining CIITA mutations of S280A with K315, 330, 333R rescues CIITA.**

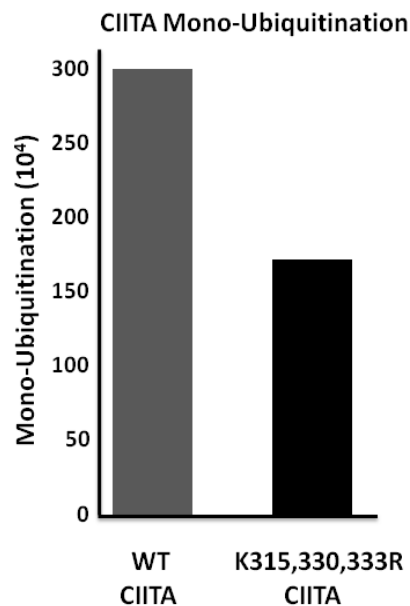
**(A) Site directed mutagenesis of serine 280 to alanine in the K315, 330, 333R lysine mutant of CIITA.** Generation of flag-CIITA S280AK315,330,330R. **(B) Mutating serine 280 to alanine in the triple lysine mutant stabilizes MHC class II surface expression.** *Flow cytometry.* HeLa cells were transfected with flag-CIITA, flag-CIITA K315,330,333R, flag-CIITA S280A, flag-CIITA S280AK315,330,333R or pcDNA. 72 hrs following transfection, cells were trypsinized, washed and incubated with PE-labeled anti-human HLA-DR antibody. Following antibody incubation, cells were fixed and PE cell surface staining was measured by FACS-Canto. (i) HeLa cells transfected with flag-CIITA (light gray line) and with pcDNA (black line). (ii) HeLa cells transfected with flag-CIITA K315,330,333R (light gray line) and with pcDNA (black line). (iii) HeLa cells transfected with flag-CIITA S280A (light gray line) and with pcDNA (black line). (iv) HeLa cells transfected with flag-CIITA S280AK315,330,333R (light gray line) and pcDNA (black line).

Studies have shown that mutation of threonine 286 in the degron sites of cyclin D1 leads to loss of phosphorylation dependent ubiquitination. This loss of ubiquitination results in accumulation of cyclin D1 and has been attributed to development of various cancers including endometrial cancer, ductal breast carcinoma, head and neck, prostate and lung cancer (Moreno-Bueno, Rodriguez-Perales et al. 2003). Furthermore, CIITA isoform I transactivity has been demonstrated to be dependent on ubiquitination that proceeds phosphorylation of S357 in its degron (Drozina, Kohoutek et al. 2006). In a recent study, we identified a novel N-terminal ATPase binding domain in CIITA that encompasses a majority of the degron (Bhat, Truax et al. 2009, in revision, *The Journal of Immunology and Cell Biology*). Absence of the ATPase binding domain increases CIITA half-life, but dramatically decreases MHC class II surface expression, indicating roles for the ATPase binding domain in regulating CIITA transactivation. These observations point to the fact that phosphorylation of residues in the degron site may be modulating CIITA transactivity via ubiquitination. Here we have identified a conserved serine residue within the degron of CIITA isoform III that regulates CIITA. Mutating serine 280 to alanine blocks phosphorylation, reduces CIITA ubiquitination, and stabilizes the protein. Despite increased accumulation of CIITA, the S280A mutation decreases CIITA mediated MHC class II expression. Previous studies have shown that mono-ubiquitination of CIITA enhances its transactivity and MHC class II expression (Greer, Zika et al. 2003). Together, these data indicate that phosphorylation at serine 280 is required for CIITA mono-ubiquitination and subsequent CIITA mediated MHC class II expression. Recent studies have identified serine residues in the CIITA degron and P/S/T domain that are targets of phosphorylation by ERK1/2 (Greer, Harton et al. 2004; Drozina, Kohoutek et al. 2006; Voong, Slater et al. 2008). Future studies will determine if S280 is also a target of ERK1/2 mediated phosphorylation.

A



B



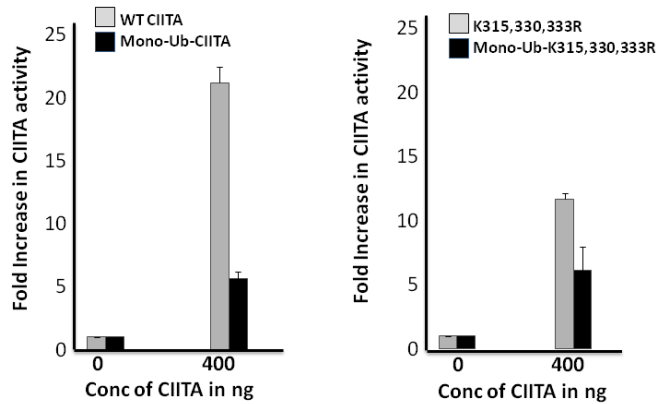
**Figure 4.7: Supplementary data: Mutating lysine residues K315, K330 and K333 to arginine in full length CIITA has modest effects on CIITA ubiquitination.**

**(A) Ubiquitination assay.** HeLa cells were co-transfected with the flag-CIITA or flag-CIITA K3R and HA-Mono-Ubiquitin (HA-Mono-Ub) or HA-WT-Ubiquitin (HA-WT-Ub). Cells were treated with MG132, proteasome inhibitor, for 4hours before harvesting. Cells were harvested, lysed in NP40, precleared and IP'd with anti-Flag antibody to pull down proteins attached to CIITA. Western blots were performed and the IP'd samples were IB using anti-HA antibody (for HA-Mono/WT-Ub). Lysates were run to control for proper expression of HA-Mono/WT-Ub and Flag-CIITA. The results are representative data of 3 experiments. **(B) Densitometry and quantitation of data in B.** Western blots from *in vivo* ubiquitination assays were quantified using Multi-Gauge V3.1 and graphed as shown. Results shown are representative of 3 experiments.

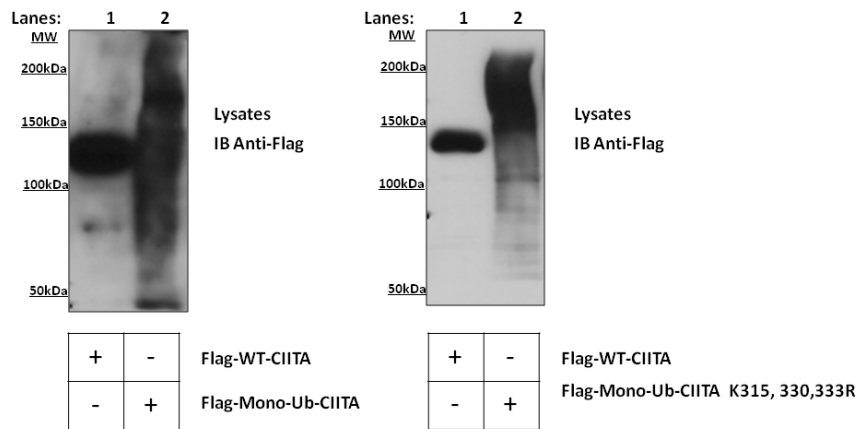
Previous studies have linked activator phosphorylation and downstream, de-gron proximal mono-ubiquitination (Drozina, Kohoutek et al. 2006; Barbash, Egan et al. 2009). E3 ubiquitin ligases have specificity to lysine residues in substrate proteins (Zheng, Schulman et al. 2002; Wu, Xu et al. 2003; Barbash, Egan et al. 2009) and lysine residues proximal to de-gron sequences are preferentially targeted for ubiquitination by E3 ligases (Barbash, Egan et al. 2009). We have identified three lysine residues in CIITA isoform III, K315, K330 and K333, proximal to the de-gron sequence as sites of mono-ubiquitination. Mutating these lysine residues to arginine reduces CIITA mono-ubiquitination and half-life, indicating that mono-ubiquitination at these residues stabilizes CIITA. Additionally, these lysine mutations reduce CIITA transactivity, leading to reductions in MHC class II transcript levels and MHC class II surface expression. In addition, we demonstrate the mono-ubiquitination events at the three lysine residues are interchangeable; CIITA K315R, CIITA K330, 333R, and CIITA K315, 330, 333R all have similar effects on CIITA transactivity, indicating that these sites may serve as alternate sites for ubiquitination due to steric hindrance.

Mutating CIITA serine 280 to alanine in the triple lysine mutant restores MHC class II cell surface expression levels in excess of levels modulated by WT CIITA. These data indicate mono-ubiquitination at these lysine residues is regulated by phosphorylation at serine 280 and that both events, phosphorylation and mono-ubiquitination, are crucial for CIITA stability and transactivity. Based on our observations, we propose that phosphorylation of S280 is recognized by a specific E3 ligase that binds CIITA and mono-ubiquitinates proximal lysine residues K315, K330 or K333. Mono-ubiquitination of these lysine residues protects them from the “stripping activity” of proteasomal ATPases, and thus protects CIITA from degradation, increases CIITA transactivation potential and upregulates MHC class II transcription.

A



B



**Figure 4.8: Supplementary data: Genetically fusing Mono-ubiquitin to WT or triple lysine mutant of CIITA reduces its transactivity due to rapid degradation of fusion products.**

**(A) Flag-mono-ubiquitin-CIITA and Flag-mono-ubiquitin-CIITAK315,330,333R are rapidly degraded.**

*Western blot.* HeLa cells were transfected with the flag-CIITA /flag-mono-ubiquitin-CIITA (left panel) or the flag-CIITA / flag-mono-Ubiquitin-CIITA K3R (right panel). Cells were harvested, lysed in NP40 and western blots were performed to check for expression of CIITA constructs.

**(B) Flag-mono-ubiquitin-CIITA and Flag-mono-ubiquitin-CIITAK315,330,333R are show lower transactivity as compared to flag-CIITA or flag- CIITAK315,330,333R.** *Luciferase reporter assays.* HeLa cells were co-transfected with flag-CIITA/flag-mono-ubiquitin-CIITA (left panel), flag-CIITA K315,330,333R /flag-mono-Ubiquitin-CIITA K315,330,333R (right panel) or pcDNA (empty vector) and HLA-DRA-Luciferase reporter constructs. Cells were harvested, lysed in cell lysis buffer and luciferase assay was performed using manufacturers' protocol. Luciferase readings were normalized to total protein concentration. Results are average of 2 experiments.

While previous studies have demonstrated ubiquitination to be a regulatory mechanism of CIITA transactivity, they have failed to map sites of ubiquitination in CIITA (Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003). This report identifies ubiquitination sites for CIITA that regulate CIITA mediated MHC class II expression. These findings will further our knowledge of the roles of post-translational modifications of CIITA in modulating CIITA activity and will provide novel tools for development of therapeutic strategies regarding the expression of CIITA and MHC class II.

#### ACKNOWLEDGEMENTS

We thank Dr. Jenny Ting for kindly providing full length Flag-CIITA, truncation mutants Flag-CIITA 1-335aa, pCDNA3, HA-Mono-Ubiquitin (HA-Mono-Ub) and HA-WT-Ubiquitin (HA-WT-Ub) plasmids. This work was supported by grants from the American Cancer Society, the Georgia Cancer Coalition, the Georgia Research Alliance (to S.F. Greer) and by a Ph.D. fellowship from the Georgia State University Molecular Basis of Disease Area of Focus (to K.P. Bhat and A.D. Truax).



## CHAPTER 5

### CONCLUSIONS

Eukaryotic transcription involves a complex interplay of multiple protein complexes which must be recruited to, and ultimately removed from, DNA. Though intensive studies have been undertaken to elucidate the mechanisms involved, how cells perform the tasks required to initiate, maintain and terminate transcription remains largely enigmatic. The ubiquitin proteasome system (UPS), long known to be the master regulator of protein degradation, plays crucial roles in a multitude of cellular activities including transcription. The UPS is known to regulate transcription by proteolytically degrading transcription factors, co-factors and chromatin remodeling enzymes; however, novel, non-proteolytic roles for the UPS in transcriptional regulation have only recently been investigated (Ciechanover, Shkedy et al. 1994; Hochstrasser 1996; Carrano, Eytan et al. 1999; Aviel, Winberg et al. 2000; Reinstein, Scheffner et al. 2000; Koepp, Schaefer et al. 2001; Haglund, Sigismund et al. 2003; Schnappauf, Hake et al. 2003; Coulombe, Rodier et al. 2004; Hegde 2004; Hernandez-Pigeon, Laurent et al. 2004; Kinyamu, Chen et al. 2005; Auld and Silver 2006; Duncan, Piper et al. 2006; Huang, Kirkpatrick et al. 2006; Barbash, Egan et al. 2009).

Initial observations indicating that the intact 26S proteasome, the 19S regulatory particle, and the 20S proteolytic core exist in both cytoplasmic and nuclear compartments in multiple cell types, including *Xenopus laevis* oocytes (Peters, Franke et al. 1994) demonstrated the 26S, 19S and 20S could exist separately and therefore might play independent roles in transcriptional regulation. To date, subunits of the 19S and/or 20S proteasome have been shown to associate

with an excess of 6400 genes (Sikder, Johnston et al. 2006). While the 26S proteasome is known to associate with RNA polymerase II (RNA Pol II) (Gillette, Gonzalez et al. 2004) and to be critical for efficient RNA Pol II mediated elongation (Ferdous, Gonzalez et al. 2001), specific inhibition of 19S activity decreases elongation while inhibition of 20S activity increases elongation, indicating a balance between these two subunits and their activities may be important for transcriptional regulation (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004). While the role of the 19S proteasome in yeast gene transcription has been robustly investigated, only recently have publications highlighted the significance of the 19S in regulating mammalian transcription. Highlights of these studies include observations that the 19S proteasomal ATPase Sug1 interacts with various mammalian transcription factors including viral transcription factor E1A, tumor suppressor p53, HIV-1 transactivator Tat and retinoic acid receptor, and via these interactions, increases transcription of target genes (Ferdous, Kodadek et al. 2002; Rasti, Grand et al. 2006; Zhu, Wani et al. 2007; Ferry, Gianni et al. 2009).

While the evidence for 19S involvement in mammalian transcription is enticing, it remains unclear whether or not 19S subunits play independent roles in recruiting and stabilizing transcription factors at responsive promoters. In order to more fully understand the roles of the proteasome system in regulating mammalian genes, we initially investigated the role of the 19S ATPase subunit Sug1 in regulating MHC class II transcription. We chose this model system because MHC class II molecules are cell surface glycoproteins that serve as major determinants of the immune response (Kvist and Levy 1993). Because MHC class II is a critical regulator of adaptive immune and anti-tumor responses (Glimcher and Kara 1992; Wang 2003), its expression is tightly regulated (Benoist and Mathis 1990). MHC class II expression is regulated primarily at the level of transcription by the master regulator, the class II transactivator, CIITA

(Masternak, Muhlethaler-Mottet et al. 2000). Although much research has been performed to understand the transcriptional mechanisms of CIITA, how CIITA is recruited, stabilized and degraded at MHC Class II promoters remains unclear. It was with this deficit of knowledge in CIITA and MHC class II regulation that we approached the hypothesis that 19S ATPases modulated mammalian transcription factor activity independent of proteolysis.

Our initial observations were that proteasome inhibition decreases CIITA transactivity and MHC class II expression without affecting CIITA expression, and indicated non-proteolytic roles for the proteasome in regulating CIITA mediated MHC class II transcription. The 19S ATPase Sug1 rapidly associates with the MHC class II proximal promoter within 4 hours of cytokine stimulation and association correlated with CIITA recruitment to the MHC class II promoter. Furthermore, CIITA and Sug1 co-precipitate, suggesting the existence of a regulatory interaction between the two proteins. Association of the 19S Sug1 subunit at the MHC class II promoter peaks between 4 and 24 hours following IFN- $\gamma$  stimulation, then declines and maintains reproducibly sustained binding for up to 95 hours. It is interesting to note that the  $\alpha 4$  subunit of the 20S core also associates with the MHC class II promoter following prolonged cytokine stimulation. Together, these observations indicate potential reassembly of the 26S proteasome at the less transcriptionally active promoter. CIITA association with the MHC class II promoter remains relatively stable up to 65 hours of IFN- $\gamma$  stimulation, after which the CIITA promoter association rapidly declines. These observations correlate promoter recruitment and assembly of a functional 26S proteasome with the loss of CIITA and are consistent with a role for the reconstituted 26S proteasome in mediating transcriptional termination by degrading promoter bound CIITA.

Chromatin immune precipitation (ChIP) assays indicate that the 19S proteasomal ATPase Sug1 and CIITA are recruited to the MHC class II proximal promoter region early during cytokine stimulation. In addition, we observed that these two proteins co-immunoprecipitate. Together, these studies indicate roles for Sug1 in regulating MHC class II transcription by controlling CIITA promoter association. When Sug1 expression is decreased via RNAi, the ability of CIITA to regulate MHC class II transcription and MHC class II transcript levels are also reduced. However, CIITA expression remains stable in the absence of Sug1, indicating selectivity in transcriptional systems regulated by Sug1. Despite sustained expression of CIITA, in the absence of Sug1, CIITA recruitment to the MHC class II promoter is diminished. Reporter assays indicate that proteolytic function of the 26S proteasome is also required for efficient transcriptional activation by CIITA, and therefore, for cytokine inducible MHC class II expression. Additionally, in Koues et.al., and Truax et.al., we demonstrated that additional 19S proteasomal ATPases bind the MHC class II promoter and play positive roles in regulating MHC class II transcription (Koues, Dudley et al. 2008; Koues, Dudley et al. 2009; Truax, Koues et al. 2009). In sum, these observations indicate that the proteasome functions both proteolytically and non-proteolytically at promoter and coding regions of MHC class II during different stages of transcription. Although these observations clearly indicate a non-proteolytic role for 19S proteasomal ATPases in CIITA mediated MHC class II gene expression, how CIITA is recruited, stabilized and degraded from the promoter remains is unknown.

CIITA is not only essential for MHC Class II transcription but also plays crucial roles in transcriptional regulation of additional diverse immune response genes including IL-4 (Sisk, Gourley et al. 2000), IL-10 (Yee, Yao et al. 2005), E-cathepsin (Yee, Yao et al. 2004), MMP-9 (Nozell, Ma et al. 2004), plexin (Wong, Brickey et al. 2003) and Fas ligand (Gourley and Chang

2001). Involvement of CIITA in transcriptional regulation of a diverse set of immune response genes clearly indicates that CIITA may play roles in disease development. To date, CIITA has been implicated in the development of diverse diseases including multiple tumor types (Yazawa, Kamma et al. 1999; Dagmar, Alfons et al. 2009; Meissner, Whiteside et al. 2009) and autoimmune conditions (Drozina, Kohoutek et al. 2005). Due to the role of CIITA in regulating the expression of multiple genes and in diverse disease development, intense studies have focused on understanding CIITA regulation. However, these studies are limited in their findings due to the complex domain structure and the distinct roles of CIITA in transcriptional regulation of multiple genes in various cell types. We and others have previously linked CIITA to the UPS through demonstration of CIITA mono-ubiquitination (Greer, Zika et al. 2003) and poly-ubiquitination (Schnappauf, Hake et al. 2003), and also through our previous observation of functional dependence of CIITA on 19S ATPases (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Agnieszka D. Truax 2009; Koues, Dudley et al. 2009). Continued study of the roles of 19S ATPases in modulating CIITA activity has helped us gain significant insight into CIITA regulation.

To this end, we mapped the association of 19S ATPases with CIITA. We observed that CIITA interacts with the C-terminal domain of the 19S ATPase Sug1 and that this interaction is independent of the ATPase activity of Sug1. We observed in co-immunoprecipitation experiments that while C-terminal domains of CIITA, including the GBD, NLS2, and 3, and LRR, are not important in interactions with Sug1; the P/S/T region is critical for CIITA association with Sug1. Deletion of part of the P/S/T domain does not affect CIITA transactivity (Chin, Li et al. 1997), the P/S/T has been shown to be essential for interactions with other transcription factors and cofactors (Fontes, Jiang et al. 1997; Fontes, Kanazawa et al. 1999) and deletion of the entire

P/S/T domain reduces CIITA transactivity (Chin, Li et al. 1997). Therefore, the functional importance of the P/S/T domain of CIITA remains under investigation (Harton and Ting 2000).

We show that the 19S ATPases Sug1, S7, and S6a, as representative members of the additional 19S heterodimers, interact within the proline rich P/S/T region. Based on these observations, we have termed the region required for CIITA association with 19S ATPases the “ATPase binding domain.”

To further understand the functional importance of the ATPase binding domain, we performed chase assays to determine CIITA stability upon loss of the 19S binding site. Cycloheximide chase assays suggested that lack of the ATPase binding domain significantly increases CIITA half-life, indicating that binding of 19S ATPases plays important roles in CIITA turnover. Although CIITA half-life is enhanced in the absence of the ATPase binding domain, flow cytometric studies demonstrate that MHC class II cell surface expression is diminished. These data indicate CIITA is transcriptionally inactive in the absence of the ATPase binding domain. Thus, the N-terminal ATPase binding domain that lies within the PST domain is crucial for 19S ATPase binding to CIITA, for MHC class II expression and for CIITA degradation. These observations further enumerate proteolytic and non-proteolytic roles for 19S ATPases in CIITA mediated MHC class II transcription.

Thus far, our studies establish that 19S subunits of the 26S proteasome regulate CIITA transactivity, stability, and MHC class II expression via binding of 19S proteasomal ATPases to the ATPase binding domain of CIITA. From previous studies in our lab (Greer, Zika et al. 2003) and others, we know that the subunits of the UPS work in concert with the ubiquitination status of proteins to determine protein fate and that mono-ubiquitination and non-canonical poly-ubiquitination of proteins is linked to transcription regulation (Bres, Kiernan et al. 2003; Greer,

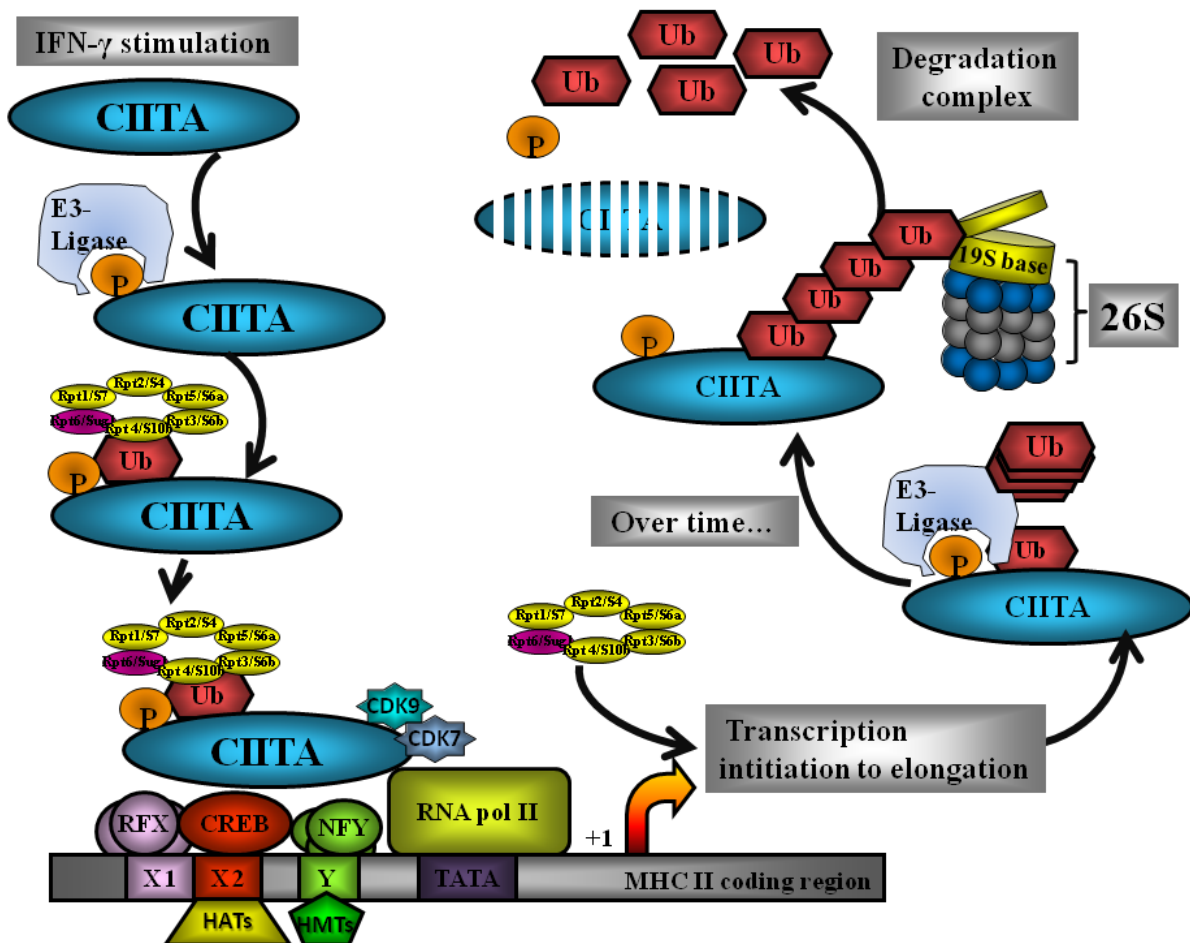
Zika et al. 2003; Gregory, Taniguchi et al. 2003; Li, Brooks et al. 2003; Kurosu and Peterlin 2004; van der Horst, de Vries-Smits et al. 2006). Previous studies have shown that transcription factors phosphorylation that precedes degron proximal mono-ubiquitination can play important roles in transcription factor transactivity and stability (Drozina, Kohoutek et al. 2006; Barbash, Egan et al. 2009). These observations indicate phosphorylation of residues in the degron site may modulate CIITA transactivity via ubiquitination. Studies have demonstrated that in CIITA isoform I phosphorylation of serine 357 in the degron precedes ubiquitination events that modulate transactivity (Drozina, Kohoutek et al. 2006). We identify here a conserved serine residue within the degron of CIITA isoform III that regulates CIITA ubiquitination, protein stability and MHC class II expression. Mutating serine 280 to alanine blocks phosphorylation, reduces CIITA ubiquitination, and stabilizes the protein. Despite increased accumulation of CIITA, the S280A mutation decreases CIITA mediated MHC class II expression. Previous studies have shown that mono-ubiquitination of CIITA enhances its transactivity and MHC class II expression (Greer, Zika et al. 2003). Together, these data indicate that phosphorylation at serine 280 is required for CIITA mono-ubiquitination and subsequent CIITA mediated MHC class II expression.

Although, ubiquitination of CIITA has been shown to modulate its transactivity, the sites of ubiquitination in CIITA have not been identified (Greer, Zika et al. 2003; Schnappauf, Hake et al. 2003). Here we have identified three degron proximal lysine residues in CIITA isoform III, K315, K330 and K333 that serve as sites for mono-ubiquitination. Mutating these lysine residues to arginine reduces CIITA mono-ubiquitination and half-life, indicating that mono-ubiquitination at these residues stabilizes CIITA. Additionally we observed that the lysine mutants show reduced CIITA transactivity, leading to reductions in MHC class II transcript levels and MHC class II cell surface expression. Thus, mono-ubiquitination at these lysine residues

plays important roles in regulating CIITA transactivity. In addition, our data demonstrate the mono-ubiquitination events at the three lysine residues are interchangeable; CIITA K315R, CIITA K330, 333R, and CIITA K315, 330, 333R all have similar effects on CIITA transactivity, indicating that these sites may serve as alternate sites for ubiquitination. Ours is the first report to identify ubiquitination sites for CIITA that regulate CIITA mediated MHC class II expression. Finally, mutation of CIITA serine 280 to alanine in the triple lysine mutant resulted in a restoration of MHC class II cell surface expression, indicating that mono-ubiquitination at these lysine residues is likely regulated by phosphorylation at serine 280, and that both events are crucial for CIITA stability and transactivity.

Based on our observations, we propose the following scenario in which the UPS regulates CIITA mediated MHC class II transcription: Upon cytokine stimulation, CIITA is phosphorylated at S280. Phosphorylation of S280 is recognized by a specific E3 ligase that binds CIITA and mono-ubiquitinates the proximal lysine residues K315, K330 or K333. Mono-ubiquitinated CIITA and 19S ATPases are recruited to the MHC class II promoter where, aided by 19S ATPases, CIITA orchestrates the assembly of PTEFb and other transcription factors including chromatin remodeling enzymes. CIITA stably binds the enhanceosome complex and the 19S complex and drives MHC class II transcription. Although 19S ATPases are simultaneously localized to the MHC class II promoter, mono-ubiquitination protects CIITA from stripping by the 19S ATPases, increases CIITA transactivation potential, and upregulates MHC class II transcription. The APIS complex of the 19S proteasome then disassociates from CIITA and proceeds to regulate transcription elongation. Following prolonged cytokine stimulation, CIITA is poly-ubiquitinated by E3 ligases, protection from 19S stripping is lost, CIITA is pulled from the enhanceosome complex, and CIITA is degraded by a promoter assembled 26S proteasome





**Figure 5.1: Ubiquitin and the 26S proteasome modulate CIITA transactivity and MHC class II expression**

Upon cytokine stimulation, CIITA is phosphorylated at S280 which allows mono-ubiquitination of lysines 315, 330, and 333. Mono-ubiquitinated CIITA and the 19S ATPases are recruited to the MHC class II promoter where, aided by 19S ATPases, CIITA orchestrates the assembly various transcription factors and cofactors. Once bound, mono-ubiquitinated CIITA stably binds both the enhanceosome complex and the 19S complex and drives MHC class II transcription. Although 19S ATPases are simultaneously localized to the MHC class II promoter, mono-ubiquitination protects CIITA from 19S ATPase mediated stripping. The APIS complex then disassociates from CIITA and proceeds to drive transcription elongation. Subsequent prolonged cytokine stimulation drives assembly of the 26S proteasome at the MHC class II promoter. Once CIITA is poly-ubiquitinated by E3 ligases, protection from 19S stripping is lost and CIITA is pulled from the enhanceosome complex and degraded by a promoter assembled 26S proteasome.

**(Figure 5.1).** Thus, the interactions that tether mono-ubiquitinated CIITA to the 19S proteasome initially drive CIITA transactivation and eventually target CIITA degradation.

These findings have enhanced our knowledge of the roles of UPS and of post-translational modifications of CIITA in modulating CIITA activity and MHC class II expression and thus provide novel tools for development of therapeutic strategies to manipulate the expression of CIITA and MHC class II. Our findings also bring to light an array of unanswered questions. It will be of interest to identify the kinases and E3 ligase(s) involved in serine 280 phosphorylation and lysine 315, 330 and 333 ubiquitination. Recent studies have identified serine residues in the CIITA degron and P/S/T domain that are targets of phosphorylation by ERK1/2 (Greer, Harton et al. 2004; Drozina, Kohoutek et al. 2006; Voong, Slater et al. 2008), therefore, future studies will determine if S280 is also a target of ERK1/2 mediated phosphorylation. Furthermore, understanding the signaling pathway that leads to the post-translational modifications that modulate CIITA transactivity will link posttranslational modifications of CIITA to the inflammatory events that make necessary increased MHC class II cell surface expression. Future studies will also address how the mono-ubiquitinated state of CIITA is maintained and identify deubiquitinating enzymes that target CIITA. CIITA is a multi-potent transactivator that plays roles in regulating the expression of multiple genes and in disease development.

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