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The Epigenetic Regulation of Cytokine Inducible Mammalian Transcription by the 26S Proteasome

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THE EPIGENETIC REGULATION OF CYTOKINE INDUCIBLE MAMMALIAN
TRANSCRIPTION BY THE 26S PROTEASOME

by

OLIVIA I KOUES

Under the Direction of Susanna F Greer, PhD

ABSTRACT

It is evident that components of the 26S proteasome function beyond protein degradation in the regulation of transcription. Studies in yeast implicate the 26S proteasome, specifically the 19S cap, in the epigenetic regulation of transcription. *Saccharomyces cerevisiae* 19S ATPases remodel chromatin by facilitating histone acetylation and methylation. However, it is unclear if the 19S ATPases play similar roles in mammalian cells. We previously found that the 19S ATPase Sug1 positively regulates transcription of the critical inflammatory gene MHC-II and that the MHC-II promoter fails to efficiently bind transcription factors upon Sug1 knockdown. MHC-II transcription is regulated by the critical coactivator CIITA. We now find that Sug1 is crucial for regulating histone H3 acetylation at the cytokine inducible MHC-II and CIITA promoters. Histone H3 acetylation is dramatically decreased upon Sug1 knockdown with a

preferential loss occurring at lysine 18. Research in yeast indicates that the ortholog of Sug1, Rpt6, acts as a mediator between the activating modifications of histone H2B ubiquitination and H3 methylation. Therefore, we characterized the role the 19S proteasome plays in regulating additional activating modifications. As with acetylation, Sug1 is necessary for proper histone H3K4 and H3R17 methylation at cytokine inducible promoters. In the absence of Sug1, histone H3K4me3 and H3R17me2 are substantially inhibited. Our observation that the loss of Sug1 has no significant effect on H3K36me3 implies that Sug1's regulation of histone modifications is localized to promoter regions as H3K4me3 but not H3K36me3 is clustered around gene promoters.

Here we show that multiple H3K4 histone methyltransferase subunits bind constitutively to the inducible MHC-II and CIITA promoters and that over-expressing one subunit significantly enhances promoter activity. Furthermore, we identified a critical subunit of the H3K4 methyltransferase complex that binds multiple histone modifying enzymes, but fails to bind the CIITA promoter in the absence of Sug1, implicating Sug1 in recruiting multi-enzyme complexes responsible for initiating transcription. Finally, Sug1 knockdown maintains gene silencing as elevated levels of H3K27 trimethylation are observed upon Sug1 knockdown. Together these studies strongly implicate the 19S proteasome in mediating the initial reorganization events to relax the repressive chromatin structure surrounding inducible genes.

INDEX WORDS: Transcriptional regulation, Epigenetics, Histone modifications, Histone remodeling enzymes, Major histocompatibility complex class II, Class II transactivator, 26S proteasome, 19S proteasome, Sug1

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OLIVIA I KOUES

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

2009

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Olivia I Koues
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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
August 2009

MY EUREKA MOMENT

Modern media often romanatizes the life of a scientist with images of a person making a great discovery with seemingly little effort. In reality, science is a profession filled with many more failures than successes. Yes there are times when a series of experiments turn out perfectly, providing a little more insight into some inticate process. However, most of the time science is just lots of reading, followed by some well-educated guesses, and painstakingly excuted experiments that return either no results or those that are completely opposite of what you predicted. In short, science involves much time, effort and sacrifice and would not be possible if not for the support of the “Snuggles”, those non-scientific folk around you. Therefore, I would like to dedicate this document to all of my Snuggles; my family and friends as well as my dogs. Hopefully the effort we all put into the last four and a half years will allow me to someday obtain My Eureka Moment.

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It is a pleasure to have this opportunity to acknowledge some of the people who have contributed to this research. I am grateful to Susanna Greer for her levelheaded personal style and never wavering support over the last several years. Her enthusiasm for science is definitely contagious. I would also like to thank Delon Barfuss for sharing his love of basic science and Teryl Frey for urging me to strive higher.

The idea of working in isolation is absolutely absurd. Therefore I would like to extend my heartfelt thanks to all of my labmates (too numerous to name) for their support and encouragement. We all face weeks characterized by things such as the frustration of bad data, the stress of exams or the anger of manuscript rejection. Yet, we've also had the opportunity to celebrate the successes, to make lasting friendships and along the way learn a little about gene regulation. We have shared the many ups and downs and I'm grateful I got to enjoy the rollercoaster ride that is research with all of you. Ladies and Gentlemen, I applaud you!

Thanks are also due to the Molecular Basis of Disease Program for their assistance with funding and the Howard Hughes Biotechnology Program for funding my undergraduate research assistant, Kyle Dudley. At times you were invaluable, at times you drove me crazy. Hopefully you've developed the desire to continue in the field.

My parents, Frank and Glenda, have provided both moral and material support during these long years. Also, I would like to mention my grandmother Evelyn, whom at the age of 98 still urges me to reach for the stars and my brother Mitchell. Given the ribbing I took from him over the years, it's surprising I didn't complete this degree just for spite. Finally, I hope that Heather MacDonald knows how much I have appreciated her dry sense of humor, her willingness to listen to my rants and whines, and most importantly her perspective.

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LIST OF ABBREVIATIONS

| | |
|---------|---|
| acH3 | Acetylated histone H3 |
| ADP | Adenosine diphosphate |
| AMP | Adenosine monophosphate |
| ATP | Adenosine triphosphate |
| BRG1 | Brahma-related gene 1 |
| CIITA | Class II transactivator |
| CARM1 | Coactivator-associated arginine methyltransferase 1 |
| CBP | CREB binding protein |
| ChIP | Chromatin immunoprecipitation |
| COMPASS | Complex of proteins associated with SET1 |
| CpG | Cytosine and guanine nucleotide islands |
| CREB | cAMP response element binding protein |
| DMEM | Dulbecco's modified eagle's medium |
| DTT | 1,4-dithiothreitol |
| GCN5 | General control of amino-acid synthesis 5 |
| HAT | Histone acetyltransferase |
| HC | Heavy Chain |
| HDAC | Histone Deacetylase |
| HMTase | Histone methyltransferase |
| HP1 | Heterochromatin protein 1 |
| H3K4me3 | Histone H3 trimethylated at lysine 4 |
| H3K9ac | Histone H3 acetylated at lysine 9 |

| | |
|---------------|---|
| H3K9me2 | Histone H3 dimethylated at lysine 9 |
| H3K18ac | Histone H3 acetylated at lysine 18 |
| H3K36me3 | Histone H3 trimethylated at lysine 36 |
| H3R17 | Histone H3 dimethylated at arginine 17 |
| HeLa | Human epithelial cells |
| IFN- γ | Interferon gamma |
| IRE | Interferon responsive element |
| IRF1 | Interferon regulatory factor 1 |
| JAK | Janus kinase |
| JmjC | Jumonji C |
| K | Lysine |
| MHC-I | Major histocompatibility complex class I |
| MHC-II | Major histocompatibility complex class II |
| mIgG | Mouse isotype control immunoglobulin |
| MLL | Mixed lineage leukemia |
| NFY | Nuclear Factor Y |
| pCAF | p300/CBP-associated factor |
| PCR | Polymerase chain reaction |
| R | Arginine |
| RFX | Regulatory Factor X |
| rIgG | Rabbit isotype control immunoglobulin |
| RPMI | Roswell Park Memorial Institute |

| | |
|-------|--|
| SAGA | Spt-Ada-Gcn5-acetyltransferase |
| siRNA | Short interfering RNA |
| SRC | Steroid receptor co-activator |
| STAT | Signal transducer and activator of transcription |
| ub | Ubiquitin |
| ubH2B | Histone H2B ubiquitinated at lysine 120 |
| USF-1 | Ubiquitous factor 1 |
| UTX | Ubiquitously transcribed tetratricopeptide repeat gene on X chromosome |
| 19S | Regulatory particle (cap) of the 26S proteasome |
| TAD | Transcriptional activation domain |

CHAPTER 1

INTRODUCTION

Translation, the ability of cells to make proteins, is fundamental to life. For translation to occur, tightly packed DNA must be available for transcription so that a RNA copy can be exported into the cytoplasm where it can be translated into protein (Carey and Smale 2000). For nuclei to accommodate the estimated 20,000-25,000 genes in the human genome (Human Genome Project 2008), DNA is packaged into a highly organized chromatin structure. The fundamental unit of chromatin is the nucleosome, which consists of the DNA double helix and histone proteins. Histones are small (~15-17kDa) proteins which provide a scaffold for DNA to wrap (Carey and Smale 2000; Lewin 2004). There are four histone proteins, H2A, H2B, H3 and H4, which form an octomer structure, consisting of a central H3/H4 tetramer flanked on either side by a H2A/H2B dimer (Figure 1-1a) (Luger, Mader et al. 1997; Richmond and Davey 2003). Approximately 146 base pairs of DNA wrap 1.67 turns around the histome octomer to form the intact nucleosome (Luger, Mader et al. 1997). A fifth protein, histone H1, is a linker histone (Widom 1998), which aides in the coiling of dinucleosomes to further compact the chromatin structure into 30 nm chromatin fibers that are themselves condensed, albeit in a poorly understood process (Widom 1998; Dorigo, Schalch et al. 2004). Chromatin is dynamic and can be found in multiple forms: the highly condensed, silent chromatin (heterchromatin), the inactive but poised for transcription chromatin and the transcriptionally accessible chromatin (euchromatin) (Figure 1-1b) (Lewin 2004).

A



B

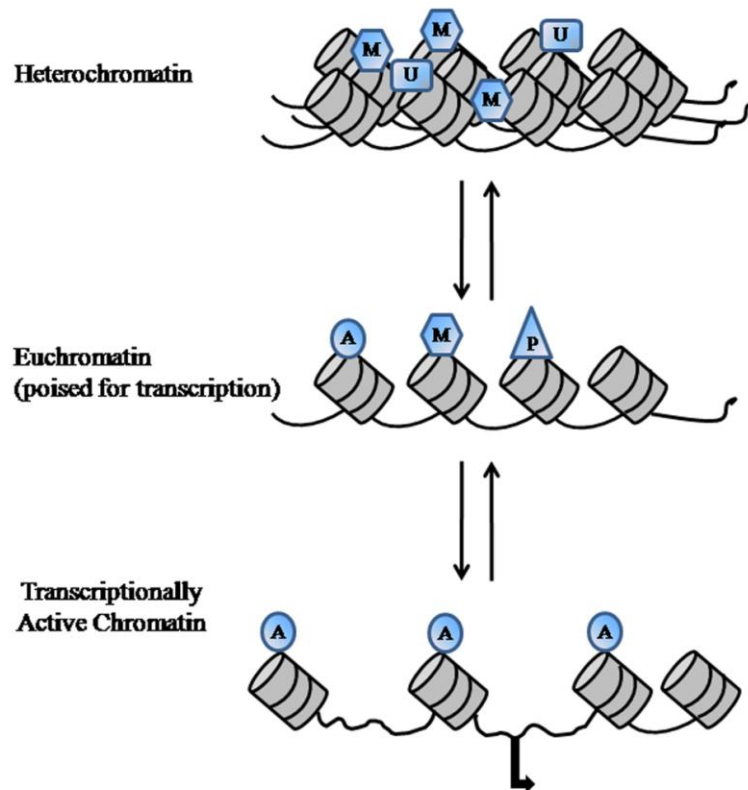


Figure 1-1. Chromatin structure & dynamics. (A) Histone octamer. The nucleosome histone core consists of a central H3/H4 tetramer flanked on either side by a H2A/H2B dimer (Luger, Mader et al. 1997; Richmond and Davey 2003). (B) Three states of chromatin. Chromatin is found in three forms: highly condensed, methylated (M) silent chromatin (heterochromatin), the methylated (M), phosphorylated (P) and acetylated (A) poised for transcription (euchromatin) and highly acetylated (A) actively transcribed chromatin (Lewin 2004).

THE EPIGENETIC CODE

The chromatin state is largely determined by an epigenetic code. Traditionally the element of this code was considered to be DNA methylation, occurring on cytosines of CpG dinucleotides. Indeed, this heritable modification is considered to be the predominant means of gene silencing and is associated with processes such as imprinting and X-chromosome inactivation (Constancia, Pickard et al. 1998; Schubeler, Lorincz et al. 2000; Bird 2002; Chow, Yen et al. 2005). However there now exists a second layer to the epigenetic code involving the ends of histone proteins that extend out of the core octamer structure of the nucleosome and are regions for post-translational modifications (Figure 1-2) (Carey and Smale 2000; Felsenfeld and Groudine 2003; Kouzarides 2007). Potential covalent modifications of histone tail regions include acetylation, phosphorylation, methylation and ubiquitination, (Carey and Smale 2000; Shilatifard 2006). The addition of various modifications loosens or tightens the chromatin structure, dictating gene expression patterns (Jenuwein and Allis 2001; Zhang and Reinberg 2001; Felsenfeld and Groudine 2003; Zhang 2003; Kouzarides 2007; Shahbazian and Grunstein 2007). The histone code hypothesis proposes that it is a combination of multiple histone modifications which determines the openness of the chromatin structure and thus the expressivity of the gene. Indeed, the various states of chromatin can be characterized by specific combinations of histone modifications.

Histone Acetylation

The most studied histone modification is acetylation, which relaxes the interactions between histone proteins and DNA. Most histone acetylation events occur at lysine residues located on histone H3 and H4 (table 1-1). Histone acetyltransferases (HATs) attach an acetyl

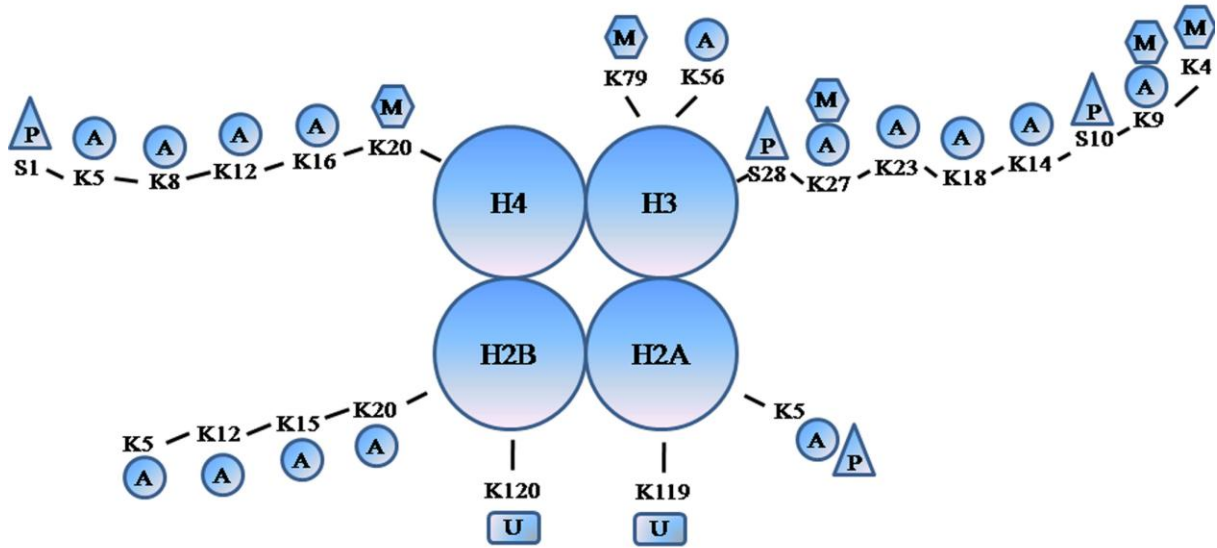


Figure 1-2. Histone post-translational modifications. The tail regions of histones proteins extend out of the nucleosome structure and are regions for post-translational modifications. Potential modifications include acetylation (A), phosphorylation (P), methylation (M) and ubiquitination (U) (Carey and Smale 2000; Shilatifard 2006).

Table 1-1. Common histone modifications

| Modification | Residue Modified | Enzyme | Reverse Enzyme | Effect on Transcription |
|-----------------------|---|---|--|--|
| Acetylation | H3K9 H3K14 H3K18 H4K8 | pCAF, GCN5 pCAF, CBP/p300 CBP/p300 CBP/p300 | HDAC1, HDAC2 HDAC1, HDAC2 HDAC1, HDAC2 HDAC1, HDAC2 | Permissive |
| Methylation | H3K4 H3R17 H3K36 H3K9 H3K27 | SET1, MLL CARM1 HYPB/SET2d G9a, SUV39H EZH2 | JMJD1A- JMJD1D ??? JDHM1b JMJD2A- JMJD2D UTX (JMJD3) | Permissive Repressive |
| Ubiquitination | H2AK119 H2BK120 | hPRC1L Bre1 | 2A-DUB Ubp8 | Repressive Permissive |

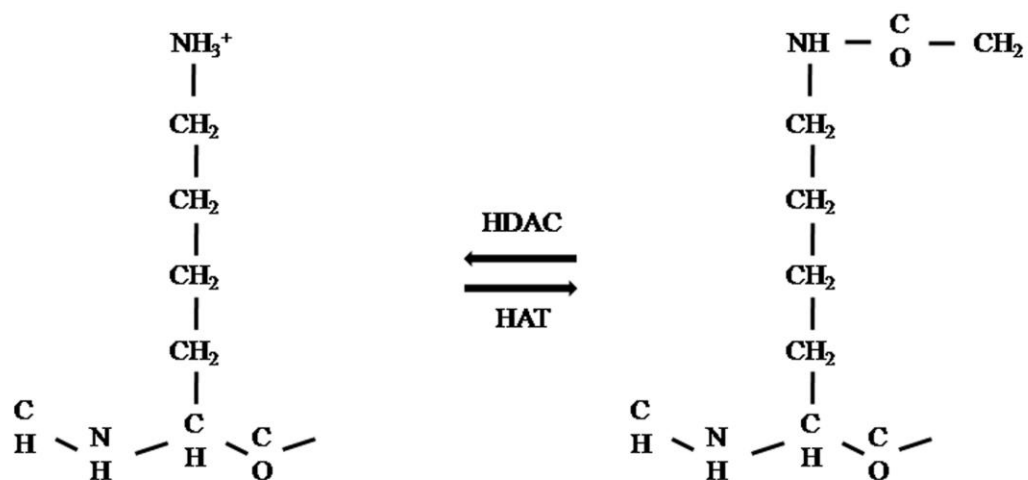
Note: Identified histone modifications with their potential forward and reverse enzymes (Roth, Denu et al. 2001; Santos-Rosa, Schneider et al. 2002; Schubeler, MacAlpine et al. 2004; Kim, Hake et al. 2005; Fodor, Kubicek et al. 2006; Shilatifard 2006; Tsukada, Fang et al. 2006; Kouzarides 2007; Lee, Tate et al. 2007; Lee, Norman et al. 2007; Lee, Villa et al. 2007).

group to the ϵ amino group of a histone lysine residue (Figure 1-3) (Carrozza, Utley et al. 2003). This modification is reversed by histone deacetylases (HDACs) which remove the acetyl group from the target residue (de Ruijter, van Gennip et al. 2003). Although promiscuous, there are a variety of known HATs, including general control of amino-acid synthesis 5 (GCN5), CREB binding protein (CBP), p300, p300/CBP-associated factor (pCAF) as well as a number of additional proteins found to have HAT activity, that are capable of acetylating various lysine residues in histone proteins (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996; Chan and La Thangue 2001; Roth, Denu et al. 2001). Acetylation neutralizes the positive charge associated with lysine, providing one potential mechanism by which the histone-DNA interaction is relaxed to allow transcriptional machinery access to bind the DNA (Agalioti, Chen et al. 2002; Shukla, Vaissiere et al. 2008). Since the modification is reversible, removal of the acetyl group by HDACs returns the chromatin to a more compact state Figure 1-3 (de Ruijter, van Gennip et al. 2003).

Histone Methylation

The role of histone methylation is less understood and more complex because these modifications are associated with either gene expression or gene repression depending on the position of the modification. Furthermore, the mechanisms as to how certain methylation events contribute to a more open or closed DNA/histone structure are poorly understood. Methylation also offers an additional level of control as multiple methyl groups can be added to target residues. Methylation occurs at both arginine (R) residues and lysine (K) residues. Methylation of arginine residues has been linked to gene activation (Bauer, Daujat et al. 2002), but methylation of lysine residues has been historically associated with gene inactivation

A



B

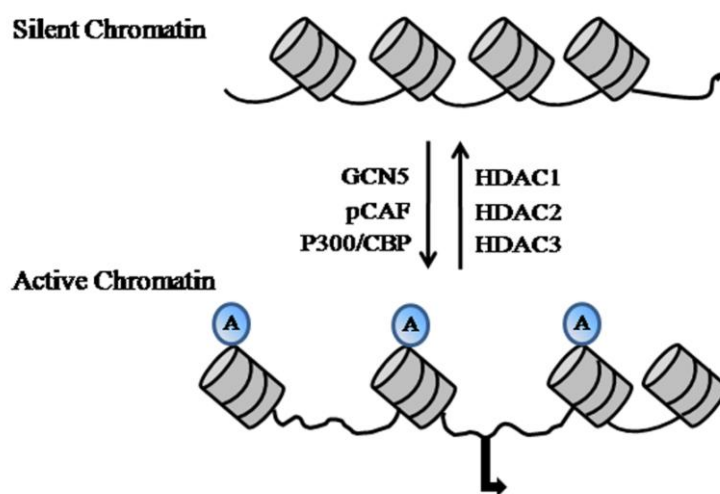


Figure 1-3. Histone acetylation. (A) Acetylation of lysine residues. Histone acetyltransferases attach an acetyl group to the ϵ amino group of a histone lysine residue, which can be removed by histone deacetylases. (B) Histone acetylation is associated with a open chromatin structure. Acetylation of lysine residues loosens the DNA/protein interaction to allow for active transcription. (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996; Chan and La Thangue 2001; Roth, Denu et al. 2001)

(Jenuwein and Allis 2001; Nakayama, Rice et al. 2001; Berger 2002; Lehnertz, Ueda et al. 2003; Shilatifard 2006). Indeed, heterochromatin protein 1 (HP1) specifically interacts with histone H3 dimethylated at lysine 9 (H3K9me₂) during chromatin silencing and heterochromatin formation (Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001; Jacobs and Khorasanizadeh 2002). Similarly, histone H3 trimethylated at lysine 27 (H3K27me₃) is associated with X chromosome inactivation, imprinting, stem cell maintenance and cancer (Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). However, H3K36 hypermethylation is thought to have a role in transcriptional memory by marking already transcribed regions of DNA and hypermethylation at H3K4 is associated with actively transcribed genes and is found primarily at actively transcribed promoters (Bernstein, Humphrey et al. 2002; Santos-Rosa, Schneider et al. 2002; Bannister, Schneider et al. 2005; Shilatifard 2006).

The mechanism by which histone methylation contributes to opening and maintaining a loose chromatin structure is poorly understood, although histone H3K4 hypermethylation has been found to inhibit H3K9 methylation and is associated with the demethylation of H3K27 to release chromatin from a silencing conformation (Wang, Cao et al. 2001; Agger, Cloos et al. 2007; Lee, Villa et al. 2007). Histone H3K36 methylation is thought to reestablish native chromatin conformation by recruiting histone deacetylases (HDACs) in the wake of elongating RNA polymerase II and thus prevents the inappropriate initiation of transcription (Carrozza, Li et al. 2005; Joshi and Struhl 2005; Lee and Shilatifard 2007).

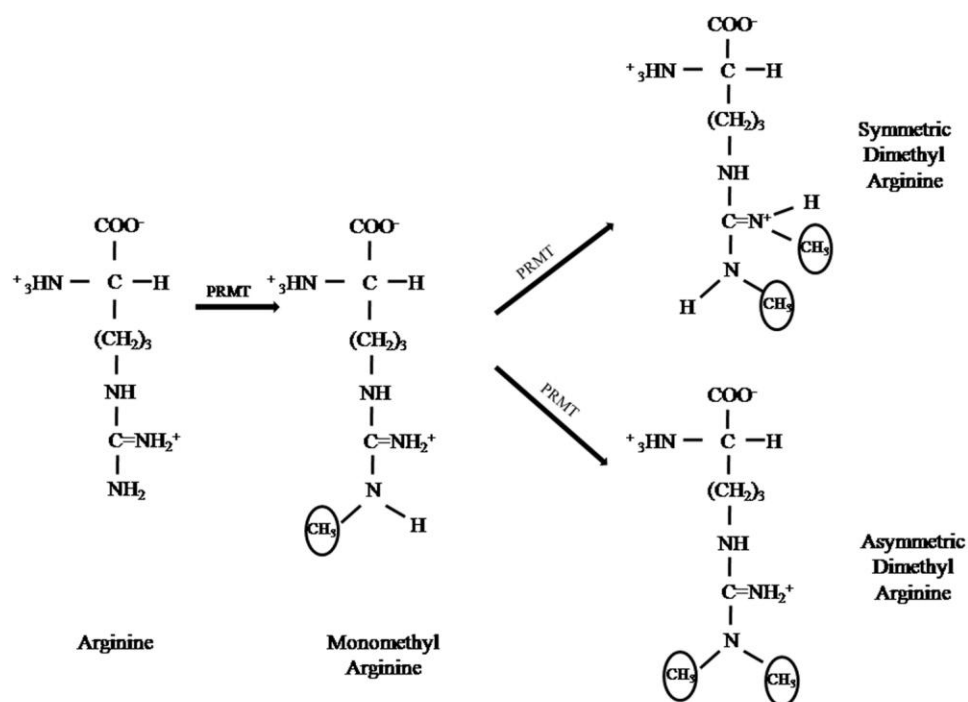
Methyl groups are added to target residues by histone methyltransferases (HMTases). There are two major groups of HMTases: protein arginine methyltransferases (PRMTs) and SET-domain or non-SET-domain containing lysine methyltransferases. PRMT enzymes attach methyl groups to arginine residues. There are three forms of arginine methylation: N^G-mono-

methylarginine, $N^G N^G$ – symmetric di-methylarginine and $N^G N^G$ – symmetric di-methylarginine (Figure 1-4a). Methylation of lysine residues can occur in mono-, di-, and trimethylated forms and SET-domain or non-SET domain enzymes attach methyl groups on the ϵ -nitrogen of lysine residues (Figure 1-4b) (Shilatifard 2006). Until recently histone methylation was considered an irreversible modification. However, evidence has now identified a variety of histone demethylases, the first of which was LSD1 that demethylates mono- and dimethyl H3K4 in a flavin adenine dinucleotide (FAD)-dependent oxidative reaction (Metzger, Wissmann et al. 2005; Wysocka, Milne et al. 2005). The inability of LSD1 to demethylate trimethyl H3K4 led to the discovery and characterization of the highly conserved Jumonji C (JmjC)-domain-containing proteins also capable of removing methyl groups from lysine residues (Fodor, Kubicek et al. 2006; Klose, Yamane et al. 2006; Tsukada, Fang et al. 2006; Whetstine, Nottke et al. 2006; Yamane, Toumazou et al. 2006; Lee, Norman et al. 2007). This recent discovery of histone demethylases provides yet another means of regulating the chromatin dynamics.

Histone Ubiquitination

Similar to methylation, histone ubiquitination can be either activating or silencing depending on the histone residue being modified. Initial reports indicated a role for histone H2A ubiquitination (ubH2A) in gene activation, but more recently ubH2A has been shown to interact with H3K27me3 and indicate gene silencing (de Napoles, Mermoud et al. 2004; Cao, Tsukada et al. 2005; Wei, Zhai et al. 2006; Lee, Norman et al. 2007) where as monoubiquitination of histone H2B at lysine 123 precedes activating histone methylation events (Sun and Allis 2002; Wood, Krogan et al. 2003; Wood, Schneider et al. 2003; Shilatifard 2006).

A



B

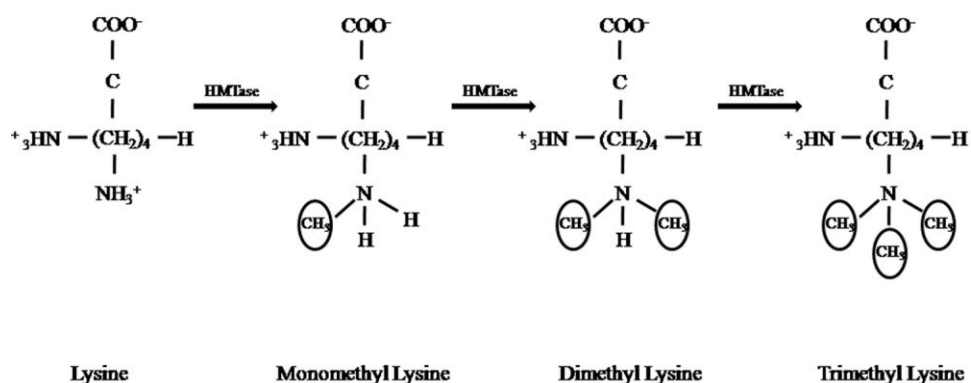


Figure 1-4. Histone methylation. (A) Arginine Methylation. PRMT enzymes attach methyl groups to arginine residues to obtain one of three forms of arginine methylation: N^G -mono-methylarginine, $N^G N^G$ - symmetric dimethylarginine and $N^G N^G$ - symmetric di-methylarginine. (B) Lysine Methylation. SET-domain or non-SET domain enzymes attach one, two, or three methyl groups on the ϵ -nitrogen of lysine residues (Shilatifard 2006).

Ubiquitin is attached to target lysine residues in a three step enzyme cascade (Figure 1-5) which begins when the C-terminus of ubiquitin is activated in an ATP-dependent step and covalently linked by a thioester bond to a cysteine site on an E1 ubiquitin-activating enzyme. The activated ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme and finally an E3 ubiquitin ligase attaches the activated ubiquitin to the targeted lysine residue (Ciechanover 1994; Hochstrasser 1996; Finley, Ciechanover et al. 2004; Kinyamu, Chen et al. 2005). Ubiquitin is removed by deubiquitinases, providing a the ability for ubiquitin to cycle on and off their target lysines to aid in gene regulation (Shilatifard 2006). Moreover, the removal of ubiquitin from H2B by Ubp8, a component of the HAT containing Spt-Ada-Gcn5-acetyltransferase (SAGA) complex suggested a role for deubiquitination in promoting a more transcriptionally accessible chromatin structure (Henry, Wyce et al. 2003). Recently, studies in mammalian cells have suggested that histone ubiquitination is highly dynamic with elevated levels ubH2B being localized not to promoter regions of genes but rather to coding sequence, suggesting a role of H2B ubiquitination in transcriptional elongation rather than initiation (Minsky, Shema et al. 2008).

Additional Histone Modifications

There are a variety of additional histone modifications that are not as well studied as those mentioned previously. Histone sumoylation is thought to be associated with transcriptional repression (Nathan, Sterner et al. 2003; Shiio and Eisenman 2003). Poly (ADP-ribosylated) histones are thought to function in the assembly and deposition of histone complexes onto DNA during replication (Boulikas 1990). Phosphorylation of histones is associated with active transcription, although the mechanism by which histone phosphorylation contributes to

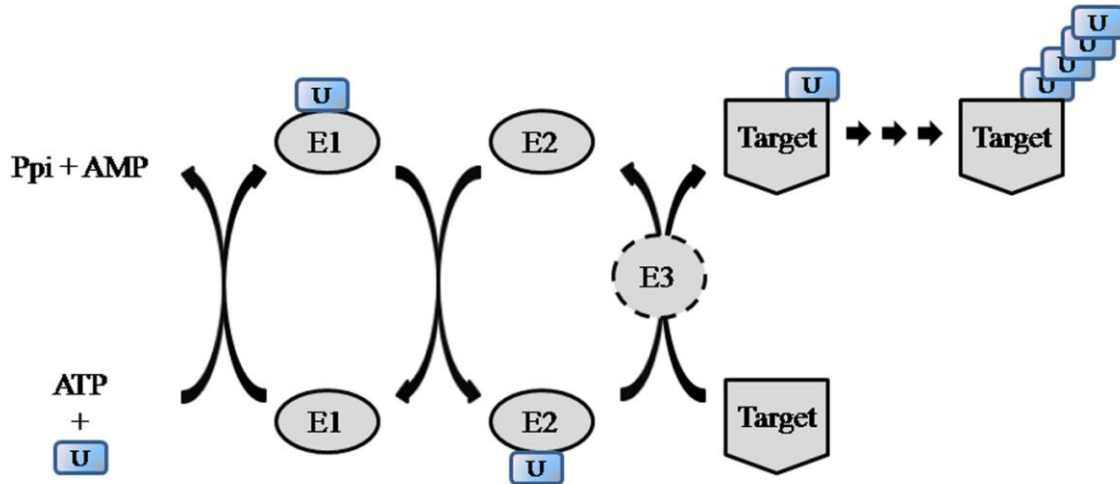


Figure 1-5. Enzyme cascade for ubiquitination. The C-terminus of ubiquitin is activated in an ATP-dependent step and covalently linked to a cysteine site on an E1 ubiquitin-activating enzyme. The activated ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme and either directly or via an E3 ubiquitin ligase to a lysine residue on the target protein (Ciechanover 1994; Hochstrasser 1996; Finley, Ciechanover et al. 2004; Kinyamu, Chen et al. 2005).

transcription is not well understood (Nowak and Corces 2000). However, it has been found that several histone acetyltransferases have enhanced HAT activity on phosphorylated substrates (Cheung, Tanner et al. 2000; Lo, Trievel et al. 2000). Phosphorylation of histone H3 at serine 10 is thought to be important for early elongation events as this modification occurs after transcription initiation but prior to recruitment of P-TEFb (Ivaldi, Karam et al. 2007).

Coordinating the Histone Code

The state of chromatin is dictated by the presence of numerous histone posttranslational modifications. Histone H3K27 and K9 methylation is associated with silenced chromatin, poised but inactive chromatin often is characterized by both activation associated modifications (H3K4me3) and inactivation associated modifications (H3K27me3) whereas transcriptionally active chromatin is characterized by histone acetylation (H3K9, H3K14, H3K8 etc) as well as activating histone methylation events (H3K4me3) (Jenuwein and Allis 2001; Lachner, O'Carroll et al. 2001; Zhang and Reinberg 2001; Bernstein, Humphrey et al. 2002; Santos-Rosa, Schneider et al. 2002; Schubeler, MacAlpine et al. 2004; Shilatifard 2006). However, how various histone modifying enzymes interact to dictate specific combinatorial patterns of histone modifications to open or close the chromatin structure is unclear. One of the few links established between chromatin modifications involves two well known chromatin remodelers: the coactivator-associated arginine methyltransferase 1 (CARM1) which mediates H3R17me2 and the HAT CBP/p300 known to mediate H3K18 acetylation (Ma, Baumann et al. 2001; Daujat, Bauer et al. 2002; Zika, Fauquier et al. 2005). At estrogen-responsive genes, fifteen minutes post estrogen stimulation CBP has been shown to associate with chromatin and H3K18 and H3K23 acetylation occur, which is closely followed by CARM1 binding and H3R17 methylation

(Daujat, Bauer et al. 2002). Similarly at interferon gamma (IFN- γ) inducible genes, unstable binding of CBP is quickly followed by CARM1 association. CARM1 then methylates not only H3R17, but CBP itself (Zika, Fauquier et al. 2005).

A second link has been established in *Saccharomyces cerevisiae* between histone H2B monoubiquitination and H3 methylation. A yeast study has shown H2B monoubiquitination to be a prerequisite for activating lysine methylation modifications (H3K4 and H3K79) on histone H3, a link that may involve components of the 19S proteasome and a histone methyltransferase complex (COMPASS) as well as the CCR4/NOT complex (Wood, Schneider et al. 2003; Ezhkova and Tansey 2004; Laribee, Shibata et al. 2007; Lee, Shukla et al. 2007). In addition subunits of COMPASS have been found to interact with UTX, a histone demethylase that removes methyl groups from histone H3K27, releasing chromatin from a repressive state (Lee, Villa et al. 2007). However how these various histone modifying enzymes interact to coordinate histone modifications to open the chromatin structure for full assembly of transcriptional machinery and transcription initiation remains unknown.

THE UBIQUITIN-PROTEASOME SYSTEM (UPS)

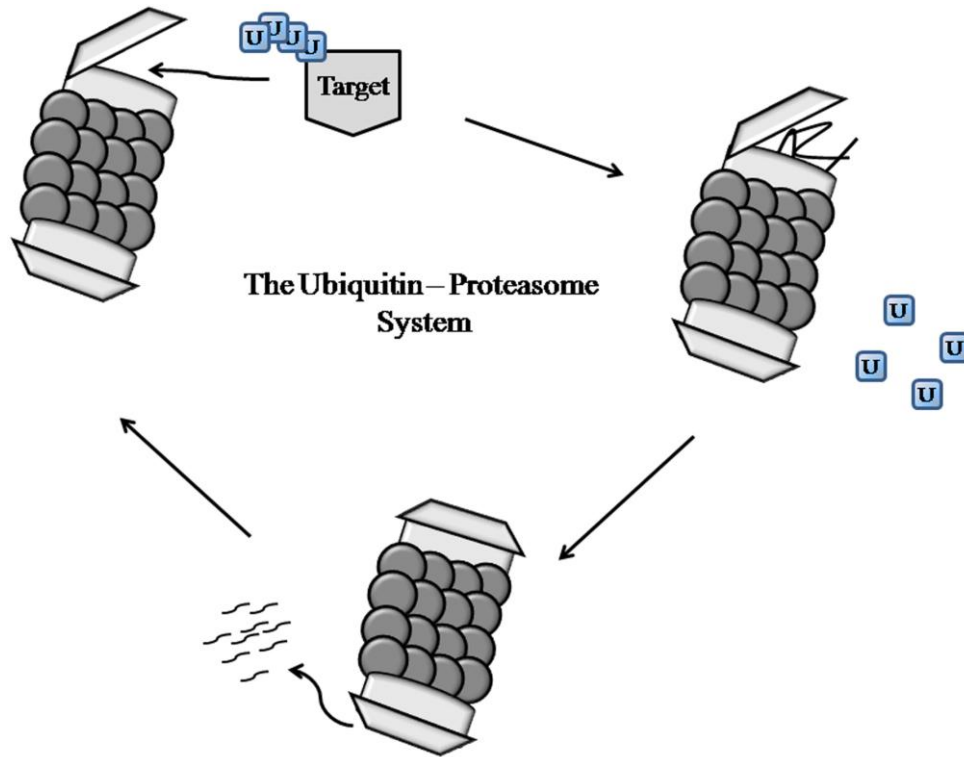
Proteasomes are large multi-subunit complexes that function to degrade both nuclear and cytoplasmic proteins (Coux, Tanaka et al. 1996; Ciechanover 1998; Brooks, Fuertes et al. 2000). A protein targeted for degradation is tagged with ubiquitin monomers via an enzyme cascade that terminates with an E3 ubiquitin ligase attaching ubiquitin onto a lysine residue of the target protein (Ciechanover 1994; Hochstrasser 1996; Finley, Ciechanover et al. 2004). As previously described, this three step enzyme cascade (Figure 1-5) involves the ATP-dependent activated ubiquitin attached to an E1 ubiquitin-activating enzyme being transferred to an E2 ubiquitin

conjugating enzyme. An E3 ubiquitin ligase then transfers the activated ubiquitin to a lysine residue on the target protein (Ciechanover 1994; Hochstrasser 1996; Finley, Ciechanover et al. 2004; Kinyamu, Chen et al. 2005). Polyubiquitination occurs when additional ubiquitin groups are sequentially added to the initial ubiquitin on the target protein (Thrower, Hoffman et al. 2000). Although there are various forms of polyubiquitin chain formation, polyubiquitination for protein degradation occurs via the covalent attachment between lysine 48 of Ub_n to glycine 76 on Ub_{n+1} (Chau, Tobias et al. 1989).

Subunits of the 26S Proteasome

Once a protein is tagged with a chain of four or more ubiquitin groups, it can then be recruited to the 26S proteasome for degradation (Figure 1-6a) (Ciechanover 1998; Thrower, Hoffman et al. 2000; Finley, Ciechanover et al. 2004). The evolutionally conserved 26S proteasome consists of two large complexes: the 20S catalytic core and the 19S regulatory particle (Figure 1-6b). The 20S catalytic core is a 700 kDa cylindrical structure, comprised of four seven member rings of repeating α and β subunits that functions to proteolytically cleave and degrade proteins into peptides (Baumeister, Walz et al. 1998). The α subunits lack catalytic activity but are important for the 20S core to interact with the 19S regulatory particle (Coux, Tanaka et al. 1996). The β subunits are proteolytic sites that degrade proteins via a variety of mechanisms (Kinyamu, Chen et al. 2005). The 20S core is activated by the binding of the 19S regulatory particle at both ends of the cylindrical structure (DeMartino and Slaughter 1999). The 19S regulatory particle consists of a lid, comprised of twelve non-ATPase proteins and a base comprised of six ATPase proteins (Glickman, Rubin et al. 1999). Each of the six ATPases belong to the family of ATPases associated with a variety of cellular activities (AAA

A



B

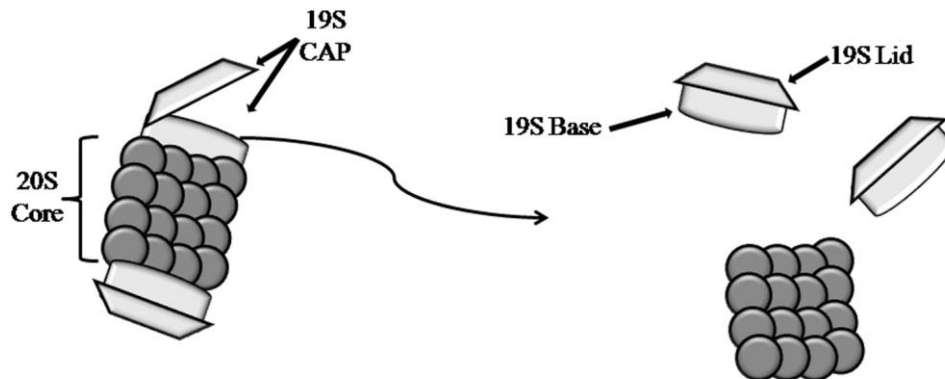


Figure 1-6. Proteasome mediated protein degradation. (A) The Ubiquitin Proteasome System (UPS). The UPS functions to recruit and degrade polyubiquitinated proteins. (B) Structure of the 26S Proteasome. The 26S proteasome is comprised of a 19S regulatory particle and a 20S core. The 19S regulatory particle (Cap) recruits polyubiquitinated proteins, cleaves off ubiquitin groups and unfolds/shuttles proteins to the 20S core (Coux, Tanaka et al. 1996; Strickland, Hakala et al. 2000). The 20S core is catalytic and proteolytically cleaves proteins into peptides (Baumeister, Walz et al. 1998).

proteins) (Glickman, Rubin et al. 1999). The 19S regulatory particle recruits targeted polyubiquitinated proteins and functions to cleave off the ubiquitin groups, unfold and shuttle the target protein to the 20S core for degradation (Coux, Tanaka et al. 1996; Strickland, Hakala et al. 2000).

The UPS and Transcription

The ubiquitin-proteasome system (UPS) has previously been linked to transcriptional activity (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003). The traditional role for the UPS in transcription involves the degradation of polyubiquitinated transcription factors and chromatin remodelers to terminate transcription (Ciechanover 1994; Hochstrasser 1996). However a novel role for the UPS in regulating transcription now exists, whereby ubiquitin has been found to be essential for transcription, independent of its role in tagging target proteins for degradation (Salghetti, Caudy et al. 2001; Conaway, Brower et al. 2002; Dennis and O'Malley 2005). The first evidence for this stemmed from the observation that the addition of a single ubiquitin molecule to the transcriptional activation domain (TAD) of VP16 is required for activation of VP16 target genes, a requirement that is overcome by the fusion of a single ubiquitin molecule to VP16 (Salghetti, Caudy et al. 2001; Kurosu and Peterlin 2004). Monoubiquitination has since been identified as a critical post-translational modification of a variety of transcription factors (Lonard, Nawaz et al. 2000; Greer, Zika et al. 2003).

More recently, research in yeast has indicated that the proteasome disassociates *in vivo* and both 20S core complexes and 19S regulatory particles Figure 1-6b are found in the nucleus as well as the cytoplasm of cells (Brooks, Fuertes et al. 2000). Reports of a non-proteolytic

function for the 19S proteasome soon followed (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002). *Saccharomyces cerevisiae* 19 proteasomes were found to be required for efficient transcription elongation by RNA polymerase II, with inactivation of a single 19S AAA ATPase, Sug1, but not inhibition of the 20S core, being sufficient to observe defects in elongation (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002). The 19S proteasome was found to disassociate further (Figure 1-7) into the six ATPases of the 19S base independent of the 20S (APIS) complex, forming an independent 19S subcomplex that associates with inducible yeast promoters (Gonzalez, Delahodde et al. 2002; Sun, Johnston et al. 2002). This discovery supported previous observations that 19S ATPases associate with activation domains aiding recruitment of transcription factors to TBP (Swaffield, Melcher et al. 1995) and specific alleles of Rpt6 (Sug1) were capable of rescuing a class of Gal4 activation domain mutants (Rubin, Coux et al. 1996).

However, it remained unclear how the APIS complex was promoting transcription. In 2002, Sun and Allis identified a lysine residue on yeast histone H2B (K123) that is post-translationally modified by a single ubiquitin, which is necessary for histone H3 methylation (Sun and Allis 2002). Two years later, Ezhkova and Tansey reported that this regulation is mediated by proteasomal ATPases, providing the first report implicating the 19S proteasome in regulating transcription at the level of epigenetics (Ezhkova and Tansey 2004). The cycling of ubiquitination/deubiquitination of histone H2B was found to be important for transcriptional activity in yeast, a process mediated by the Ubp8 deubiquitinase of the yeast SAGA (Spt-Ada-Gcn5-acetyltransferase) complex (Henry, Wyce et al. 2003) and the 19S proteasome was found to target the SAGA complex, which also contains HAT activity, to active promoters to promote a more open chromatin conformation (Lee, Ezhkova et al. 2005). Combined these studies

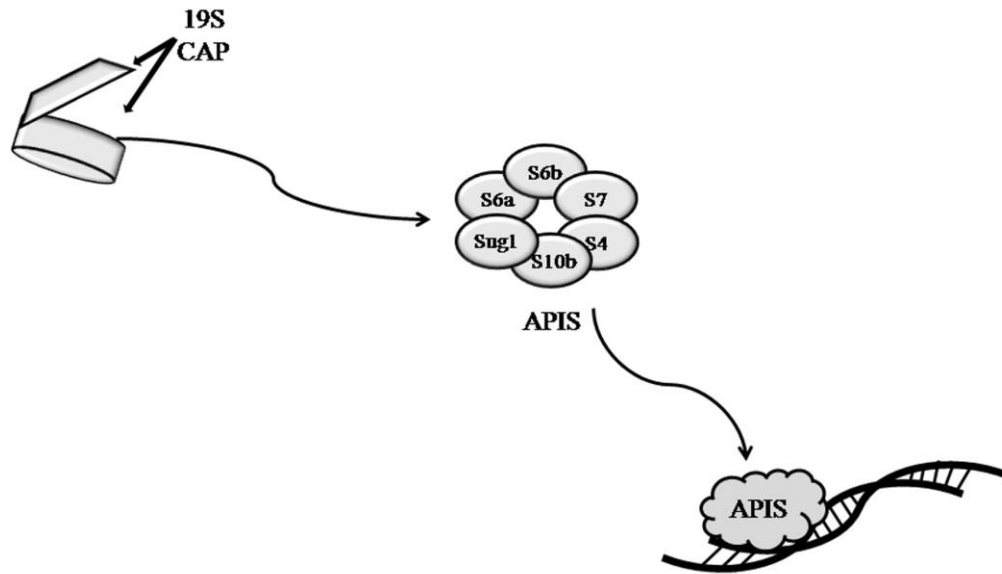


Figure 1-7. The 19S subcomplex. The 19S proteasome disassociates into the six ATPases of the 19S base independent of the 20S (APIS) complex, which is independently recruited to inducible yeast promoters (Gonzalez, Delahodde et al. 2002; Sun, Johnston et al. 2002).

suggested that the 19S ATPases function to reconfigure chromatin to allow for transcription to take place. The significance of these interactions is yet to be investigated in other organisms.

Studies have now shown that the APIS subcomplex associates with mammalian promoters suggesting that the APIS may have a conserved, non-proteolytic function in regulating transcription (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003; Yu and Kodadek 2007; Bhat, Turner et al. 2008). Sug1 is recruited to *p21^{waf1}* promoters in response to ultraviolet-induced DNA damage (Zhu, Wani et al. 2007), to the HIV-1 promoter to enhance Tat-dependent transcription (Lassot, Latreille et al. 2007), to major histocompatibility complex class II(MHC-II) promoters genes in response to cytokine stimulation (Bhat, Turner et al. 2008) and to retinoic acid (RA) induced genes via an interaction with Steroid receptor co-activator (SRC)-3 (Ferry, Gianni et al. 2009). Sug1 also associates with p53, E1A, class II transactivator (CIITA) and with several nuclear receptors (vom Baur, Zechel et al. 1996; Lee, Ezhkova et al. 2005; Bhat, Turner et al. 2008; Ferry, Gianni et al. 2009). However evidence of roles for the 19S in regulating mammalian chromatin structure has been lacking. Do the components of the 19 proteasome have a similar role in regulating transcription in mammalian cells, specifically cytokine inducible genes critical to eliciting an adaptive immune response?

MHC MOLECULES AND THE ADAPTIVE IMMUNE SYSTEM

The Immune System

The immune system is critical for survival in a less than sterile world as it functions to remove invading pathogens. The immune system is divided into two branches: innate immunity and adaptive immunity. Innate immunity is the first line of defense against invading pathogens.

Most components of the innate immune system are present prior to infection and serve to rapidly mount a localized non-specific response to any invading pathogen (Parham 2005; Kindt, Goldsby et al. 2007). The initial step of invasion involves a pathogen breaching the physical barriers (skin, mucosal membranes) as well as secondary barriers (the acidic nature of the stomach and perspiration) (Parham 2005; Kindt, Goldsby et al. 2007). Once a pathogen enters the host, a variety of mechanisms are present that recognize common molecular surface markers of pathogens (foreign to the host) and work to eliminate them.

The innate immune system also forms a bridge to the more specific, albeit delayed, adaptive immune system. Adaptive immunity differs from innate immunity in that the adaptive immune system is highly diverse and antigen specific with the ability to develop immunological memory which enables a more rapid, specific response when the host is re-challenged by the pathogen (Kindt, Goldsby et al. 2007). Cells of the adaptive immune system are called lymphocytes (B cells and T cells). B cells give rise to antibody producing plasma cells that work to clear free pathogen whereas T cells work to clear both intracellular and extracellular infections. There are three major types of T cells: T cytotoxic (T_c) cells, T helper (T_h) cells and T regulatory (T_{reg}) cells. Each cell type is distinguished by the membrane bound glycoproteins on their cell surface. Cytotoxic T cells are CD8 positive ($CD8^+$) cells that kill infected cells (Parham 2005; Kindt, Goldsby et al. 2007). Helper T cells are CD4 positive ($CD4^+$) cells that coordinate the immune response by activating additional lymphocytes and T_{reg} cells, a subset of T helper cells, are characterized by having both CD4 and CD25 glycoproteins on their cell surface and are negative regulators of an immune response as they suppress the immune system once an infection is eliminated (Parham 2005; Kindt, Goldsby et al. 2007). Unlike cells of the innate immune system, T cells must first be activated by the binding of appropriate antigen to

their T cell receptor. However, T cell receptors can only recognize antigen when presented in the context of major histocompatibility complex molecules (MHC) (Figure 1-8).

MHC Molecules

MHC molecules are cell surface glycoproteins that bind and present antigens to cells. There are two types of MHC molecules, MHC-I and MHC-II. MHC-I molecules are present on the surfaces of all cells and bind intracellularly derived peptides that activate CD8⁺ T lymphocytes. In contrast, MHC-II molecules bind extracellularly derived peptides to activate CD4⁺T lymphocytes (Glimcher and Kara 1992). MHC-II molecules are α/β heterodimeric glycoproteins whose expression is much more limited than MHC-I (Glimcher and Kara 1992). A subset of cells called antigen presenting cells (APCs), including activated macrophages, dendritic cells and B cells, constitutively express MHC-II. However, all nucleated cells can be induced to express MHC-II by inflammatory cytokine stimulation, such as interferon gamma (IFN- γ).

Once activated, CD4⁺T cells are then able to participate in both cell-mediated and antibody-mediated adaptive immune responses, allowing the body to focus an attack against the invading pathogens (Parham 2005). Repression of MHC-II expression results in deficiencies in identifying and removing pathogens and increases incidences of illness. Furthermore, highly elevated expression of MHC-II genes often leads to the binding of self peptides and results in the development of autoimmune diseases such as multiple sclerosis, diabetes, and arthritis. Dramatically reduced MHC-II expression leads to bare lymphocyte syndrome, which results in death in early childhood (Reith and Mach 2001; Parham 2005; Sospedra and Martin 2005). In addition, there is now evidence that MHC-II molecules also function in tumor suppression (Gerloni and Zanetti 2005). Therefore, studying the regulation of MHC-II transcription provides

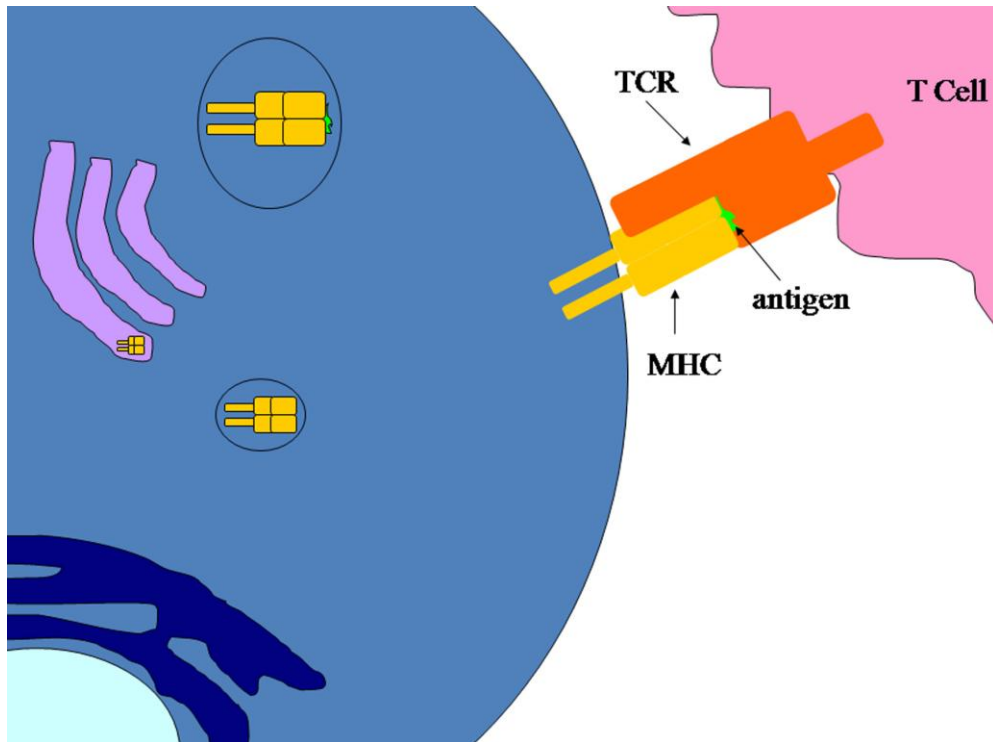


Figure 1-8. Activation of the adaptive immune response. Activation of lymphocytes requires that foreign pathogens be processed into small peptides (antigens) and complexed with additional major histocompatibility complex (MHC) molecules on the cell surface of cells.

novel information that will aid in enhancing our understanding of the immune response and provide new targets for developing therapies in combating these diseases.

Regulation of MHC-II Transcription

It is crucial that MHC-II expression be tightly regulated because aberrant expression is associated with a wide range of illnesses. Expression of class II transactivator (CIITA), the master regulator of MHC-II, mirrors that of MHC-II with constitutive expression found in APCs or inducible expression in most other cells types (Mach, Steimle et al. 1996; Ting and Trowsdale 2002; Boss and Jensen 2003). Prior to IFN- γ stimulation, *HLA-DRA* promoter histones are moderately acetylated, which correlates with the assembly of a basal enhanceosome complex (Beresford and Boss 2001; Adamski, Ma et al. 2004) which consists of regulatory factor X (RFX), nuclear factor Y (NF-Y) and cAMP response element binding protein (CREB) that constitutively associate with the X and Y elements of the MHC-II proximal promoter (Figure 1-9) (Steimle, Durand et al. 1995; Nagarajan, Louis-Pence et al. 1999). Following IFN- γ stimulation, CBP/p300 rapidly binds the MHC-II proximal promoter and results in increased histone acetylation to relax the chromatin structure surrounding the MHC-II promoter (Eberharter and Becker 2002; Freiman and Tjian 2003; Gorisch, Wachsmuth et al. 2005). Simultaneously, IFN- γ stimulation induces transcription of CIITA at the cytokine inducible promoter IV (pIV). CIITA is subsequently expressed and recruited via direct interaction with basal enhanceosome components to the MHC-II proximal promoter (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005). CIITA recruitment correlates with recruitment of basal transcriptional machinery including TBP, pTEFb and TAF_{II}32 as well as enhanced binding of various coactivators and histone modifying enzymes to fully open the chromatin structure and

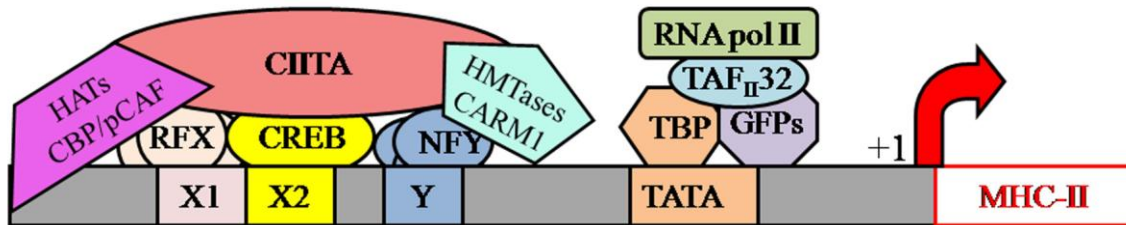


Figure 1-9. The MHC-II proximal promoter. The proximal promoter of MHC-II HLA-DRA genes contain conserved cis acting elements including an X and Y box. CIITA is recruited to the MHC-II promoter via direct interaction with basal enhanceosome components, regulatory factor X (RFX), nuclear factor Y (NF-Y) and cAMP response element binding protein (CREB), which bind the X and Y boxes, respectively (Steimle, Durand et al. 1995; Nagarajan, Louis-Pence et al. 1999). In addition, various HATS (CBP/p300, pCAF, and SRC-1) as well as an HMTase (CARM1) interact with the MHC-II promoter and CIITA (Kretsovali, Agaloti et al. 1998; Fontes, Kanazawa et al. 1999; Zika, Fauquier et al. 2005; Zika and Ting 2005).

initiate transcription (Fontes, Jiang et al. 1997; Mahanta, Scholl et al. 1997; Muhlethaler-Mottet, Otten et al. 1997; Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999; Kanazawa, Okamoto et al. 2000; Spilianakis, Papamatheakis et al. 2000; Wright and Ting 2006).

Chromatin Structure of MHC-II Promoters

Extensive studies have now established an epigenetic map (table 1-2) for the proximal promoter of the MHC-II gene *HLA-DRA* that corresponds to the initial transcriptional events described above (Beresford and Boss 2001; Gomez, Majumder et al. 2005; Zika, Fauquier et al. 2005; Gialitakis, Kretsovali et al. 2006). Initial epigenetic studies showed that histone H3 and histone H4 are acetylated at the active MHC-II promoter and that this acetylation is enhanced upon CIITA recruitment (Beresford and Boss 2001; Zika, Fauquier et al. 2005). Furthermore it appears that multiple histone acetyltransferases (HATs) are required to open the chromatin structure because cells with mutated CIITA that are unable to recruit the HAT CBP/p300, still show various levels of acetylated histones in the MHC-II region of the chromatin (Beresford and Boss 2001). Indeed, a variety of HATs are now known to interact with the MHC-II promoter and CIITA, including CBP/p300, pCAF and SRC-1 (Zika and Ting 2005). It is now clear that active expression of MHC-II is associated with histone H3 acetylation at K9, K14, K18, K27 and histone H4 acetylation at K8 (Gomez, Majumder et al. 2005). In addition, several histone methylation events are associated with active transcription of MHC-II, including the CARM1 mediated dimethylation of histone H3R17 and the trimethylation of histone H3 at K4 and K36 (Zika, Fauquier et al. 2005; Chou and Tomasi 2008). However, it is not understood how these modifications coordinate to allow for MHC-II expression. One of the few enzymes associated with a particular modification is the histone arginine methyltransferase CARM 1, which has been

Table 1-2. Identified histone modifications at the MHC-II *HLA-DRA* proximal promoter

| Modification | Residue Modified | Effect on Transcription |
|---------------------|---|--|
| Acetylation | H3K9 H3K14 H3K18 H3K27 H4K8 | Permissive |
| Methylation | H3K4 H3R17 H3K36 H3K9 H3K27 | Permissive Repressive |

Note: Active expression of MHC-II is associated with histone H3 acetylation at K9, K14, K18, K27 and histone H4 acetylation at K8 (Gomez, Majumder et al. 2005) as well as several histone H3 methylation events, including the dimethylation of histone H3R17 and the trimethylation of histone H3 at K4 and K36 (Zika, Fauquier et al. 2005; Chou and Tomasi 2008).

shown to rapidly bind the MHC-II promoter upon IFN- γ stimulation and mediate H3R17 methylation (Zika, Fauquier et al. 2005). The histone modifying enzymes that mediate the other MHC-II histone modifications and how they coordinate to open the chromatin structure for full enhanceosome formation and transcription initiation remains unknown.

Regulation of Class II Transactivator (CIITA)

As stated previously, CIITA expression correlates with MHC-II expression with cells that lack MHC-II expression also lacking CIITA expression. Thus, CIITA transcription is regulated in a cell-type specific manner by four distinct promoters. Promoter I drives CIITA expression in dendritic cells. The function of promoter II remains unknown. Promoter III primarily functions in the constitutive expression of CIITA in B cells and promoter IV is responsible for IFN- γ induced expression of CIITA in all nucleated cells (Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Linhoff et al. 1999). CIITA pIV is well characterized with three *cis*-acting elements: an IFN- γ activated sequence (GAS), an E-box and an IFN response element (IRE) site (Figure 1-10). IFN- γ initiates the JAK/STAT pathway by triggering the phosphorylation and nuclear translocation of signal transducer and activator of transcription 1 (STAT1) homodimers, which drive expression of interferon regulatory factor 1 (IRF1). STAT1 and subsequently IRF1 are then recruited to the c-myc associated, ubiquitous factor 1 (USF-1) occupied CIITA pIV, to bind the GAS element and IRE respectively (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002). CIITA transcripts are then observed 2 hours post IFN- γ stimulation coinciding with IRF1 binding (Morris, Beresford et al. 2002; Wright and Ting 2006). Although fewer studies have been done to epigenetically map CIITA pIV, it is clear that there are increases in acetylation of histone H3 and H4 upon IFN- γ stimulation (Morris, Beresford et al. 2002) with

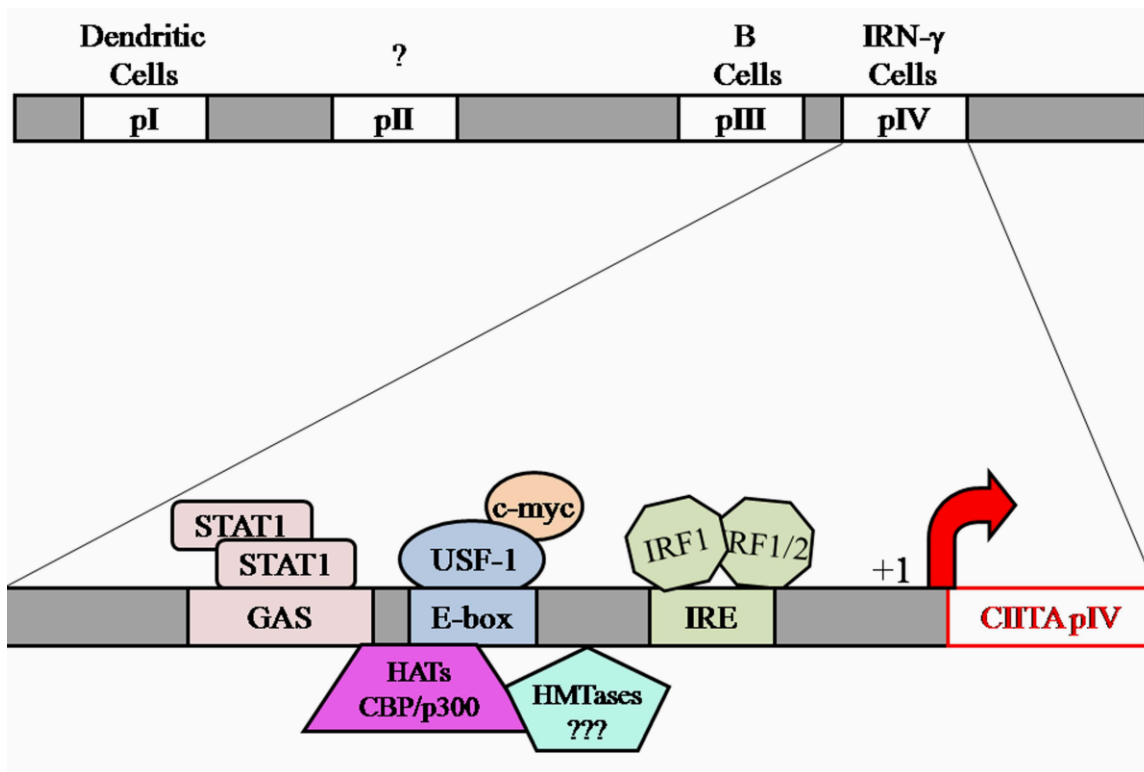


Figure 1-10. CIITA pIV. CIITA pIV consists of three *cis*-acting elements: an IFN- γ activated sequence (GAS) that binds STAT1 homodimers, an E-box that binds USF-1 and an IFN response element (IRE) site that binds IRF-1. (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002).

specific enhancement observed at H3K9, H3K18 and H4K8 (Table 1-3) (Ni, Karaskov et al. 2005). In addition, trimethylation of H3K4, which correlates with active transcription, is observed upon prolonged cytokine stimulation (Ni, Karaskov et al. 2005). Only two histone modifying enzymes are known to be recruited to CIITA pIV: the HAT CBP /p300 and the ATP dependent chromatin remodeler BRG1 which binds even prior to STAT1 recruitment (Pattenden, Klose et al. 2002; Ni, Karaskov et al. 2005). Unfortunately, a more detailed characterization of CIITA pIV's chromatin structure is lacking.

SUMMARY

For cell survival, information encoded in DNA must be transcribed and translated. Thus, the highly organized, packaged chromatin structure must undergo conformational changes to loosen DNA-protein interactions to allow for gene expression (Carey and Smale 2000). This conformational change utilizes various histone modifying enzymes that post-translationally phosphorylate, acetylate, ubiquitinate, and methylate specific histone tail residues, which is critical to allow transcription factors and other machinery access to the DNA. In yeast the 19S proteasome has been implicated as functioning as a transcriptional co-activator with the ability to promote histone modifications by targeting histone modifying enzymes to inducibly activated genes. Although the 19S proteasome is recruited to some mammalian promoters, a similar mechanism for the 19S in regulating the chromatin structure has not been isolated in higher eukaryotes.

MHC-II molecules are crucial to the adaptive immune systems as they present antigenic peptides to activate CD4⁺T lymphocytes (Marsman, Jordens et al. 2005). The fact that most cells

Table 1-3. Identified histone modifications at CIITA pIV

| Modification | Residue Modified | Effect on Transcription |
|---------------------|--------------------------------|--------------------------------|
| Acetylation | H3K9 H3K18 H3K27 H4K8 | Permissive |
| Methylation | H3K4 | Permissive |

Note: Active expression of CIITA pIV is associated with histone acetylation at H3K9, H3K18 and H4K8 as well as the trimethylation of H3K4 (Ni, Karaskov et al. 2005).

can be induced to express MHC-II by inflammatory cytokine stimulation (Parham 2005; Kindt, Goldsby et al. 2007) and our lab has observed inducible recruitment of the 19S ATPase Sug1 to the MHC-II promoter (Bhat, Turner et al. 2008), make this system a good model for studying the 19S proteasome's potential contribution to the epigenetic regulation of mammalian genes. Studying the role of the proteasome in regulating chromatin remodeling to allow MHC-II expression will not only provide novel information about global epigenetic regulation of transcription, but will specifically provide vital information about the regulation of MHC-II expression which is of utmost importance in developing treatments for diseases associated with aberrant expression of MHC-II genes.

CHAPTER 2REGULATION OF ACETYLATION AT THE MAJOR HISTOCOMPATIBILITY
COMPLEX CLASS II PROXIMAL PROMOTER BY THE
19S PROTEASOMAL ATPASE SUG1

PUBLICATION: OLIVIA I. KOUES, R. KYLE DUDLEY, AGNIESZKA D. TRUAX,
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NOTE: I PERFORMED ALL OF THE EXPERIMENTS IN THIS CHAPTER
INDEPENDENTLY WITH THE FOLLOWING EXCEPTIONS, HALF-LIFE
EXPERIMENTS WERE DONE BY AGNIESZKA D. TRUAX , CO-
IMMUNOPRECIPITATION BLOTS SUITABLE FOR PUBLICATION WERE
PERFORMED IN CONJUNCTION WITH R. KYLE DUDLEY; REPLICATES OF
TIME COURSE CHROMATIN IMMUNOPRECIPITATIONS WERE DONE WITH
THE AID OF AGNIESZKA D. TRUAX, DAWSON GERHARDT, KAVITA P. BHAT
AND SAM MCNEAL.

SUMMARY

Recent studies have made evident the fact that the 19S regulatory component of the proteasome has functions that extend beyond degradation, particularly in the regulation of transcription. Although 19S ATPases facilitate chromatin remodeling and acetylation events in yeast, it is unclear if they play similar roles in mammalian cells. We have recently shown that the 19S ATPase Sug1 positively regulates transcription of the critical inflammatory gene MHC-II by stabilizing enhanceosome assembly at the proximal promoter. We now show that Sug1 is crucial for regulating histone H3 acetylation at the MHC-II proximal promoter. Sug1 binds to acetylated histone H3 and, in the absence of Sug1, histone H3 acetylation is dramatically decreased at the proximal promoter with a preferential loss of acetylation at H3 lysine 18. Sug1 also binds to the MHC-II histone acetyltransferase CBP and is critical for recruitment of CBP to the MHC-II proximal promoter. Our current study strongly implicates the 19S ATPase Sug1 in modifying histones to initiate MHC-II transcription and provides novel insights into the role of the proteasome in the regulation of mammalian transcription.

BACKGROUND

Major histocompatibility class II (MHC-II) molecules are cell surface glycoproteins which bind and present processed antigenic peptides to CD4⁺ T lymphocytes to initiate immune system protection against invading pathogens and tumors (Parham 2005). Tight regulation of MHC-II expression is crucial to maintain a functional immune system and to limit the opportunity for the development of autoimmune diseases (Gerloni and Zanetti 2005; Parham 2005). MHC-II is expressed constitutively on antigen presenting cells and can be inducibly expressed on most nucleated cells by interferon gamma (IFN- γ) (7, 20). Constitutive and IFN- γ

inducible MHC-II expression is regulated at the level of transcription by a series of elements in the MHC-II promoter. Nuclear Factor Y (NFY), Regulatory Factor X (RFX), and cAMP response element binding protein (CREB) bind respectively to the Y and X elements of the MHC-II proximal promoter forming a multi-protein enhanceosome complex, which is necessary but not sufficient for transcription initiation. Once the enhanceosome is assembled on the MHC-II promoter, the Class II Transactivator (CIITA) can be recruited. CIITA binding stabilizes the enhanceosome complex and recruits basal transcriptional components, including the CDK7 subunit of TFIIF and the CDK9 subunit of P-TEFb, which phosphorylate Pol II and initiate the switch to an elongation complex (Mach, Steimle et al. 1996; Masternak, Barras et al. 1998; Kanazawa, Okamoto et al. 2000; Boss and Jensen 2003; Spilianakis, Kretsovali et al. 2003). CIITA is also known to interact with a variety of transcriptional cofactors including multiple histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) (Fontes, Kanazawa et al. 1999; Beresford and Boss 2001; Zika, Greer et al. 2003; Wright and Ting 2006). Although much is known about the requirement of these basal and inducible transcription factors for MHC-II expression, less is known regarding the importance of epigenetic modifications required to open the chromatin structure and allow these transcription factors to bind.

Integral to eukaryotic chromatin structure are nucleosomes, which consist of an octameric histone protein core. There are four histone proteins in the octamer, H2A, H2B, H3 and H4, around which DNA winds to create an environment of reduced DNA accessibility. The N-terminal ends of histone proteins extend out of the nucleosome and are regions for post-translational modifications (Carey and Smale 2000). Potential covalent modifications of histone N-terminal tail regions include acetylation, phosphorylation, and methylation, which can occur at the approximately 30 amino acid residues in these tails (Carey and Smale 2000; Jenuwein and

Allis 2001; Liang, Lin et al. 2004; Shilatifard 2006). It is now widely accepted that it is the complex combination of these modifications that opens the chromatin structure, creates docking sites for the recruitment of effector proteins, and thereby aids in determining the expressivity of the gene (Jenuwein and Allis 2001; Martens, Verlaan et al. 2003; Fish, Matouk et al. 2005; Francis, Chakrabarti et al. 2005).

The most studied histone modification is acetylation of lysine residues in the N-terminal tails of H3 and H4, which counteracts the compact nature of chromatin by relaxing the interaction between histone proteins and DNA (Eberharter and Becker 2002; Freiman and Tjian 2003; Gorisch, Wachsmuth et al. 2005). Epigenetic studies of the IFN- γ inducible MHC-II promoter in HeLa cells have shown that prior to IFN- γ stimulation, histone H3 and histone H4 are acetylated at low to moderate levels which coordinates with the assembly of the RFX, NFY and CREB components of the enhanceosome complex (Beresford and Boss 2001). Following IFN- γ stimulation in HeLa cells, CIITA is expressed and bound to the proximal promoter, which recruits basal transcriptional machinery and enhances recruitment of HATs, thus increasing acetylation and allowing transcription of the MHC-II gene *HLA-DRA* (Beresford and Boss 2001; Wright and Ting 2006). The CREB-binding protein (CBP)/p300 is a transcriptional co-activator with potent HAT activity which is capable of acetylating all four core histones (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996). CBP is thought to have multiple functions on the MHC-II promoter, both as a HAT that acetylates histones H3 and H4 and as an integrator that links CIITA and CREB (Chan and La Thangue 2001). In HeLa cells, CBP binding to the MHC-II proximal promoter is increased within two hours of IFN- γ stimulation, preceding CIITA promoter binding by several hours (Zika, Fauquier et al. 2005). In IFN- γ inducible endothelial cells, CBP is associated with the MHC-II promoter at low levels prior to IFN- γ stimulation and

association increases rapidly upon IFN- γ stimulation (Adamski, Ma et al. 2004). When CBP promoter binding is blocked, constitutive levels of MHC-II promoter acetylation are inhibited in unstimulated endothelial cells, indicating a closed chromatin conformation due to decreased CBP binding and promoter histone hypoacetylation that is independent of CIITA expression (Adamski, Ma et al. 2004).

Although active expression of MHC-II genes is associated with robust histone H3 acetylation, it is less understood how this and other modifications coordinate gene expression (Masternak, Peyraud et al. 2003; Spilianakis, Kretsovali et al. 2003; Gomez, Majumder et al. 2005; Zika, Fauquier et al. 2005; Rybtsova, Leimgruber et al. 2007). It is well established that many of the dramatic changes in acetylation occurring on histones H3 and H4 at the activated MHC-II promoter are CIITA dependent. That CIITA is required for MHC-II gene expression indicates that CIITA promoter binding likely directs HAT activity towards histone H3 and opens up a large portion of the promoter for transcriptional activation (Beresford and Boss 2001). However, as to the initial acetylation events that precede CIITA binding, only limited information is available on how histone modifying enzymes are initially recruited to the MHC-II promoter in order to open the chromatin structure for CIITA binding, full enhanceosome formation, and transcription initiation and elongation.

The 26S proteasome is a large, multi-subunit complex that functions to degrade polyubiquitinated proteins and has been determined to play an emerging role in transcriptional regulation (Baumeister, Walz et al. 1998; Ciechanover 1998; Muratani and Tansey 2003; Lee, Ezhkova et al. 2005; Sulahian, Johnston et al. 2006). The 26S proteasome consists of two macromolecular complexes: the 20S catalytic core and the 19S regulatory complex. The 20S catalytic core degrades proteins into peptides in an energy independent manner (Baumeister,

Walz et al. 1998; Voges, Zwickl et al. 1999). The 19S regulatory complex regulates assembly of the 26S proteasome, recruits polyubiquitinated proteins to the proteasome and shuttles the targeted proteins to the 20S core for degradation (Coux, Tanaka et al. 1996; Glickman, Rubin et al. 1999; Strickland, Hakala et al. 2000). The mammalian 19S consists of a lid, comprised of eight non-ATPase proteins, and a base comprised of six ATPase (S4, S6a, S6b, S7, S8 (Sug1) and S10b) and three non-ATPase (S1, S2 and S5a) proteins (Glickman, Rubin et al. 1999). Observations that *Saccharomyces cerevisiae* 19S ATPases associate with the Gal4 activator, are recruited to the *GAL* promoter (Gonzalez, Delahodde et al. 2002) and are critical for efficient transcriptional elongation by RNA polymerase II (Ferdous, Gonzalez et al. 2001) firmly established a role for the 19S proteasome in yeast transcription. Specific alleles of Rpt6, the yeast ortholog of Sug1, rescue a class of Gal4 activation domain mutants (Rubin, Coux et al. 1996), recruits transcription factors to TBP (Swaffield, Melcher et al. 1995), and is associated with actively transcribing genes (Gonzalez, Delahodde et al. 2002). Rpt6 also has recently been shown to link histone ubiquitination and histone methylation, which precede acetylation as important steps in initiating transcriptional elongation (Sun and Allis 2002; Ezhkova and Tansey 2004; Schubeler, MacAlpine et al. 2004; Pokholok, Harbison et al. 2005). Reports in yeast that the 19S proteasome enhances recruitment of the histone acetyltransferase complex SAGA (Spt-Ada-Gcn5-acetyltransferase) to promoters and that Rpt6 associates with SAGA and is required for SAGA targeting further imply that this 19S ATPase is also important for targeting histone acetyltransferases to promoters (Ferdous, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Lee, Ezhkova et al. 2005; Daniel and Grant 2007). In fact, the physical and functional interactions between 19S ATPases and chromatin structure seem to be quite widespread in yeast

as is evidenced by several recent reports (Auld, Brown et al. 2006; Sikder, Johnston et al. 2006; Sulahian, Sikder et al. 2006; Laribee, Shibata et al. 2007).

The ubiquitin-proteasome pathway has also been linked to the regulation of transcription in mammalian cells (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003; Yu and Kodadek 2007; Bhat, Turner et al. 2008). These emerging roles for the 19S ATPases in regulating transcription highlight the importance of understanding the biochemical function of Sug1 at mammalian promoters. Sug1 is recruited to $p21^{waf1}$ promoters in response to ultraviolet-induced DNA damage (Zhu, Wani et al. 2007) as well as to the HIV-1 promoter to enhance Tat-dependent transcription (Lassot, Latreille et al. 2007). Sug1 also associates with p53, E1A and with several nuclear receptors (vom Baur, Zechel et al. 1996; Lee, Ezhkova et al. 2005) however evidence of roles for the 19S in regulating mammalian chromatin structure has been lacking. We have previously shown that Sug1 is rapidly recruited to the MHC-II promoter upon IFN- γ stimulation and that decreased expression of Sug1 results in reduced CIITA recruitment to the MHC-II proximal promoter and decreased MHC-II gene expression (Bhat, Turner et al. 2008). However, the function of Sug1 in CIITA recruitment and the potential for interactions between Sug1 and histone modifications in mammalian cells remain to be investigated. We therefore analyzed the role played by Sug1 in regulating histone H3 acetylation at *HLA-DRA*. Data presented here offer new insights into MHC-II transcription and establish a role for Sug1 in the deposition of histone acetylation modifications at the MHC-II proximal promoter. We show that Sug1 associates *in vivo* with acetylated histone H3 and that decreased expression of Sug1 and additional 19S ATPases decreases MHC-II promoter specific H3 acetylation. We also show that recruitment of the HAT CBP to the MHC-II promoter is blocked in the absence of Sug1. Taken together, these studies offer novel information into the regulation

of MHC-II transcription and expand our knowledge of the role the proteasome plays in mediating the epigenetic regulation of mammalian genes.

MATERIALS AND METHODS

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

Antibodies: Histone H3, acetylated histone H3, acetylated histone H3 K9, and rabbit IgG isotype control antibodies were from Upstate (Lake Placid, NY). Acetylated H3 K18, p53, and Myc antibodies were from Abcam (Cambridge, MA). S5a, S6a, S7 and alpha 4 antibodies were from BIOMOL International, L.P. (Plymouth Meeting, PA). Sug1 antibody was from Novus Biologicals (Littleton, CO), CIITA antibody was from Rockland (Rockland, Gilbertsville, PA) and CBP antibody was from Santa Cruz (Santa Cruz, CA).

Plasmids: The Myc Sug1 and Myc Sug1 ATPase mutant constructs were a generous gift from Dr. A. A. Wani (Zhu, Wani et al. 2007).

Co-immunoprecipitations: HeLa cells were plated at a cell density of 8×10^5 in 10cm tissue culture plates. Following adhesion, cells were transfected with 5µg of the indicated plasmids using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours following transfection, cells were harvested and lysed in RIPA buffer (1M Tris pH 8.0, 5% DOC, 10% Nonidet P-40, 5M NaCl, 10% SDS, 5 mM EDTA, 1M DTT) supplemented with Complete EDTA-free protease inhibitors (Roche, Indianapolis, IN) on ice and then precleared with 30µl IgG beads (Sigma, Saint Louis, MO). Cell lysates were

immunoprecipitated (IP) overnight with 5 μ g anti-acetylated histone H3 (Upstate), anti-CBP (Santa Cruz Biotechnology), rabbit IgG isotype control (Upstate) or Myc conjugated beads (Sigma). Immune complexes were isolated by incubating lysates with 50 μ l protein G beads (Pierce, Rockland, IL) for 2 hours. Immunoprecipitated proteins were denatured with Leamml buffer (BioRad, Hercules, CA) and subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Associations were detected by immunoblotting (IB) with Myc HRP conjugated antibody (Abcam). Horseradish peroxidase was detected with Supersignal West Pico Chemiluminescent substrate (Pierce). Bradford assays were used to normalize for protein and equal loading was verified by immunoblot.

siRNA constructs and transient transfections: Short interfering RNA (siRNA) duplexes were used for transient knockdown of 19S ATPases Sug1, S6a and S7. siRNA sequences were designed with a GC content of 35-55% containing dTdT overhangs and were blasted using NCBI nucleotide BLAST. The target sequences of Sug1 and S7 siRNA used were 5'-AAGGTACATCCTGAAGGTAAA-3' and 5'-AACTGCGAGAAGTAGTTGAAA-3', respectively (Qiagen, Valencia, CA). S6a siRNA was predesigned siRNA directed against human PSMC3 (Qiagen). siRNA for Lamin protein was used as positive control siRNA (Qiagen) and scrambled sequence siRNA was used as negative control siRNA (Qiagen). HeLa cells were transfected with scrambled sequence control siRNA (Qiagen; Santa Cruz Biotechnology) or ATPase specific siRNA (Qiagen) and were treated with IFN- γ as indicated. Cells were lysed in NP40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) supplemented with Complete EDTA-free protease inhibitors (Roche) and knock down efficiency and specificity was assessed by western blotting for ATPase expression as described above.

Histone Extractions: HeLa cells were treated with Sug1 siRNA (Qiagen) or scrambled sequence control siRNA. Forty-eight hours after siRNA transfection, 10% of the total cell volume was lysed with 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with protease inhibitor and analyzed by western blot for ATPase knockdown as described above. The rest of the cell volume was lysed in triton extraction solution (0.4M sodium butyrate, 10 % triton-X-100, 2% NaN₃) supplemented with Complete EDTA-free protease inhibitors (Roche) at 4°C. Histone proteins were isolated by incubation with 0.2N HCl at 4°C for 4 hours. Lysate samples were normalized for protein concentration, supplemented with 5% (v/v) β-mercaptoethanol, denatured with Leammli buffer (BioRad) and separated by SDS-PAGE. Gels were transferred to nitrocellulose and IB using polyclonal acetylated histone H3 or acetylated histone H3 K18 primary antibodies (Upstate) and HRP conjugated rabbit secondary antibody (Santa Cruz). Horseradish peroxidase was detected with Supersignal West Pico Chemiluminescent substrate (Pierce). Bradford assays were used to normalize for protein.

Chromatin immunoprecipitations (ChIPs): ChIP assays were performed as previously described (Greer, Zika et al. 2003). Briefly, HeLa cells were stimulated with 500 U/ml IFN-γ (Peprotech, Rocky Hill, NJ), 5 mM HDAC inhibitor sodium butyrate (Upstate), 5.2 μM proteasome inhibitor MG132 (EMD Biosciences, San Diego, CA) or 10μM proteasome inhibitor Lactacystin (BostonBiochem, Cambridge, MA) as indicated. Cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature. 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, dH₂O) with protease inhibitor for 30 minutes on ice and were sonicated at constant pulse to generate an average of 500-750 bp sheared DNA.

Sonicated lysates were precleared with salmon-sperm coated agarose beads (Upstate) and half of the lysate was IP with 10 μ g of polyAb against CIITA (Rockland), acetylated H3 (Upstate), acetylated H3 K18 (Abcam), acetylated H3 K9 (Upstate), S6a (Biomol), S7 (Biomol), S5a (Biomol), alpha 4 (Biomol) or CBP (Santa Cruz) overnight at 4°C. The remaining half of the lysate was used as a control and was IP with isotype control antibody (Upstate). Following an additional 2 hour IP with 50 μ l 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, dH₂O), High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH₂O), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH₂O) and 1xTE buffer and were eluted with SDS elution buffer (1% SDS, 0.1M NaHCO₃, dH₂O). Following elution, crosslinks were reversed overnight with 5M NaCl at 65°C and IP DNA was isolated using phenol:chloroform:isoamyl alcohol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using primers spanning, the W-X-Y box of the MHC class II *HLA-DRA* promoter, MHC class II *HLA-DRA* exon III (Chou and Tomasi 2008) MHC class II *HLA-DRA* exon V (Chou and Tomasi 2008) the GAPDH promoter and CIITA pIV (Morris, Beresford et al. 2002). Sequences for primers and probe are located in Table 2-1. Values graphed were calculated based on standard curves generated. *Chromatin immunoprecipitation in ATPase knockdowns:* HeLa cells were transfected with ATPase specific siRNA (Qiagen) or control siRNA (Qiagen). Cells were treated with 5mM sodium butyrate (Upstate) and 500U/ml IFN- γ as indicated and 10% of the total cell volume was lysed with 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with protease inhibitor and analyzed by western blot for ATPase knockdown as described above. The remaining fraction of cells was

Table 2-1. Primer and probe sequences for real time PCR

| Gene | Sequence |
|--------------------------|---|
| MHC-II proximal promoter | sense primer: 5'-TCCAATGAACGGAGTATCTTGTG T-3' antisense primer: 5'-TGAGATGACGCATCTGTTGCT-3' probe: 5'-6 FAM-CTGGACCCTTTGCAAGAACCCTTCCC-TAMRA-3' |
| MHC-II exon III | sense primer: 5'-GTGGCTTCGAAATGGAAAACC-3' antisense primer: 5'-AAAAGGTGGTCTTCCCTGGG-3' probe: 5'-6 FAM-TCACCACAGGAGTGTGAGAGACAGTCTTCC-TAMRA-3' |
| MHC-II exon V | sense primer: 5'-GCAATGCCTCTGGAATAAAACAT-3' antisense primer: 5'-GAAACCTTAAACAATAAGTACACAAGAGATG-3' probe: 5'-6 FAM-CAGGAGTCTGTCTCTGCTATGGAATGCC-3' |
| CIITA pIV | sense primer: 5'-GCCACTGTGAGGAACCGACT-3' antisense primer: 5'-TGGAGCAACCAAGCACCTACT-3' probe: 5'-6 FAM-CAGGGACCTCTGGATGCCCA-TAMRA-3' |
| GAPDH promoter | sense primer: 5'-AATGAATGGGCAGCCGTTA-3' antisense primer: 5'-TAGCCTCGCTCCACCTGACT-3' probe: 5'-6 FAM-CCTGCCGGTGACTAACCCCTGCGCTCCT-TAMRA-3' |

Note: ChIP isolated DNA was analyzed by real-time PCR using primers spanning, the MHC class II *HLA-DRA* promoter, MHC class II *HLA-DRA* exon III, MHC class II *HLA-DRA* exon V, CIITA pIV or the GAPDH promoter.

subjected to ChIP assay. *Chromatin immunoprecipitation with ATPase overexpression:* HeLa cells were transfected with 5µg of the indicated plasmids using Fugene 6 (Roche) according to the manufacturer's protocol. Cells were treated with 500U/ml IFN-γ as indicated and 10% of the total cell volume was lysed with 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with protease inhibitor and analyzed by western blot for Sug1 overexpression as described above. The remaining fraction of cells was subjected to ChIP assay.

***In vivo* Proteolytic Activity:** HeLa cells were transfected with Sug1 specific siRNA (Qiagen) or control siRNA (Qiagen). Cells were treated with 5.2µM MG132 proteasome inhibitor (EMD Biosciences) and 100µM cycloheximide (Sigma) as indicated. Total cell volume was lysed with 1% Nonidet P-40 buffer with protease inhibitor and analyzed by western blot for ATPase knockdown efficiency and p53 degradation as described above.

RESULTS

Sug1 associates with histone H3 and acetylated histone H3 *in vivo*. The 19S proteasome recruits polyubiquitinated proteins to the 20S catalytic core for degradation. Research in yeast has also suggested that the 19S proteasome functions independent of protein degradation to regulate transcription by aiding in the opening of the chromatin structure (Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Lassot, Latreille et al. 2007). In yeast the 19S proteasome associates with the activated Gal1-10 promoter (Gonzalez, Delahodde et al. 2002; Lee, Ezhkova et al. 2005) and in mammalian cells, components of the 19S proteasome are recruited to *p21^{waf1}* promoters in response to ultraviolet-induced DNA damage

and to MHC-II *HLA-DRA* promoters in response to IFN- γ stimulation (Zhu, Wani et al. 2007; Bhat, Turner et al. 2008). Furthermore, the yeast 19S ATPase Rpt6 has been implicated in regulating histone modifications, including histone acetylation and methylation (Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005). We therefore sought to determine whether the mammalian ortholog of Rpt6, Sug1, plays a role in mediating posttranslational histone modifications in mammalian cells. To determine whether Sug1 associates with histone H3 *in vivo*, co-immunoprecipitations were performed in HeLa (human epithelial) cells transfected with Myc-tagged Sug1 (Fig.1). Polyclonal antibody was used to IP endogenous acetylated histone H3. Sug1 and acetylated histone H3 precipitated together (Fig.1, upper panel, lane 3). Control samples were immunoprecipitated with Myc beads (Figure 2-1, upper panel, lane 1) or an isotype control (Figure 2-1, upper panel, lane 2). Equal loading was confirmed by immunoblot analysis of lysates (Figure 2-1, lower panel).

Sug1 knockdown decreases MHC-II promoter specific histone H3 acetylation. We have previously shown that the 19S ATPase Sug1 is recruited to the MHC-II proximal promoter (Bhat, Turner et al. 2008). In the absence of Sug1, IFN- γ stimulation fails to recruit CIITA to the MHC-II promoter and results in a dramatic loss of MHC-II gene expression (Bhat, Turner et al. 2008). The molecular mechanism responsible for the loss of CIITA promoter binding in Sug1 deficient cells remains unknown. To investigate potential roles for Sug1 in regulating chromatin modifications responsible for stabilizing and/or recruiting CIITA to the MHC-II proximal promoter, we utilized a siRNA duplex to specifically knockdown endogenous Sug1 expression in HeLa cells and then performed ChIP experiments to detect endogenous levels of H3 acetylation at the MHC-II proximal promoter (Figure 2-2). siRNA-mediated knockdown of Sug1 resulted in

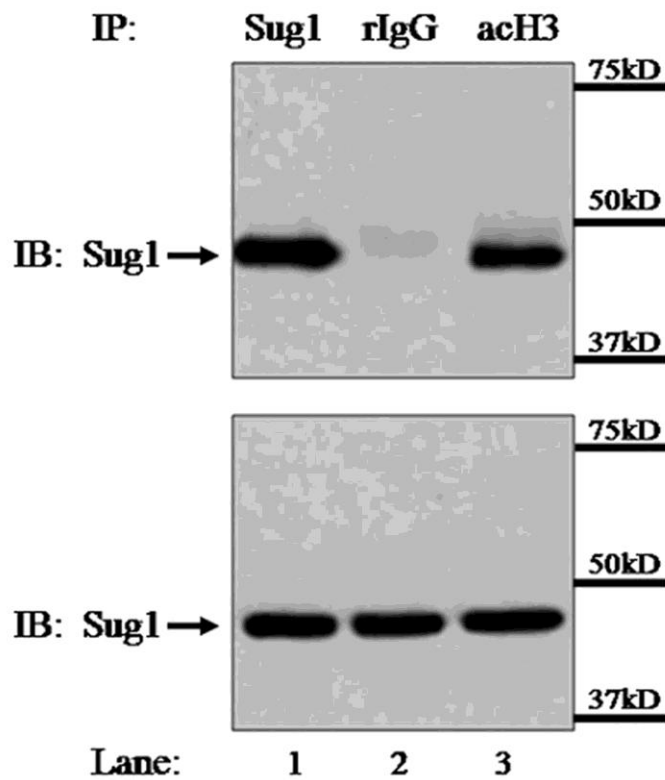
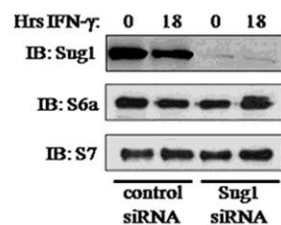
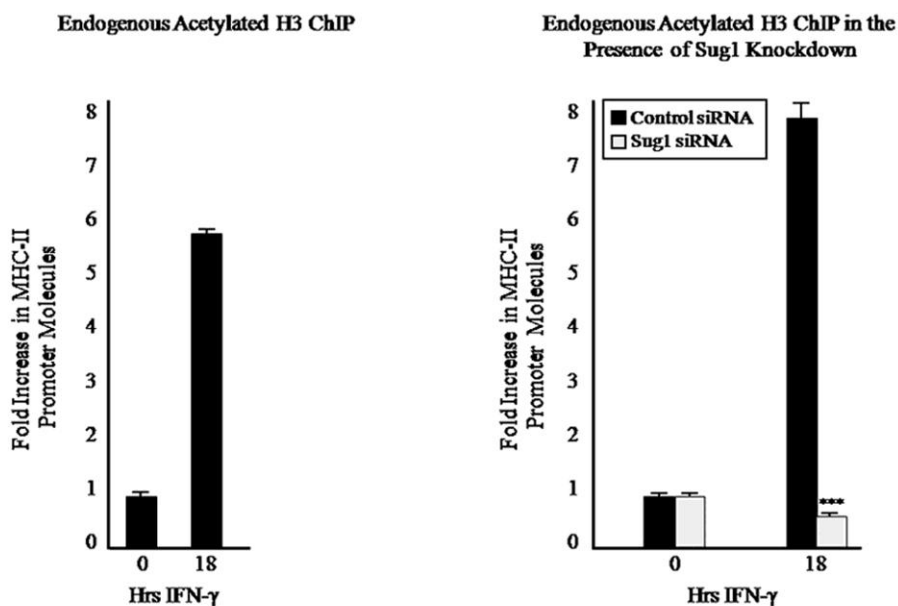


Figure 2-1. Sug1 associates with acetylated histone H3 *in vivo*. HeLa cells transfected with Myc tagged Sug1 were lysed and immunoprecipitated (IP) with polyclonal antibody against acetylated histone H3 (top panel, lane 3). Control samples were IP with Sug1 antibody (top panel, lane 1) and isotype control IgG (top panel, lane 2). IP and lysate control samples (bottom panel) were immunoblotted (IB) for Myc. Results reported are representative data of 3 experiments.

A



B



C

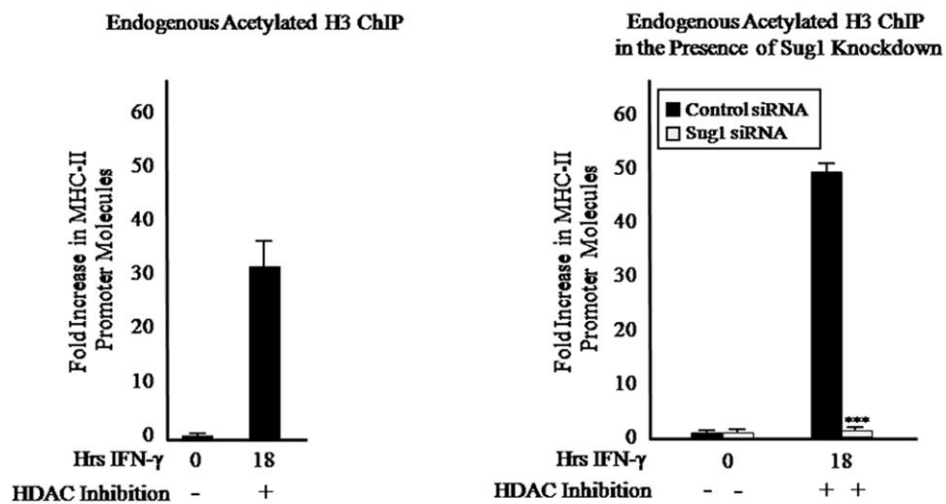


Figure 2-2. Sug1 knockdown decreases MHC-II promoter histone H3 acetylation. (A) Sug1 siRNA specifically and efficiently decreases Sug1 protein expression in the presence or absence of IFN- γ stimulation. HeLa cells transfected with control or Sug1 specific siRNA were harvested and subjected to Western blot analysis of endogenous 19S ATPases Sug1, S6a and S7 expression. Western blot shows >90% knockdown of Sug1 and stable expression of S6a and S7. (B) Sug1 knockdown decreases histone H3 acetylation at the MHC-II proximal promoter. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours or in HeLa cells transfected with control or Sug1 siRNA and stimulated with IFN- γ for 0-18 hours. Lysates were immunoprecipitated (IP) with control or acetylated histone H3 antibody. Associated DNA was isolated and analyzed via real-time PCR using primers spanning the W-X-Y box of the MHC-II *HLA-DRA* promoter. Real-time PCR values were normalized to the total amount of *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-II promoter DNA relative to unstimulated acetylated histone H3 IP samples. Control values were 1.2 ± 0.3 . Control and IP values represent mean \pm SEM of (n = 3) independent experiments. (C) Sug1 knockdown decreases histone H3 acetylation at the MHC-II proximal promoter in the presence of HDAC inhibition. ChIP assays were carried out in untransfected or siRNA transfected HeLa cells and were treated with HDAC inhibitors and stimulated with IFN- γ for 0-18 hrs. Lysates were IP with control antibody or with antibody to endogenous acetylated histone H3 and associated DNA was isolated and analyzed via real-time PCR as in B. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated acetylated histone H3 IP samples. Control values were 2.7 ± 0.8 . Control and IP values represent mean \pm SEM of (n = 3) independent experiments. *** P < 0.001 vs control siRNA.

an approximate 90% decrease in endogenous Sug1 expression (Figure 2-2a, top panel), but did not impact expression of additional proteasomal ATPases, S6a and S7, (Figure 2-2a, bottom panels). Initial ChIP mapping experiments demonstrated that constitutive levels of histone acetylation at the MHC-II promoter are enhanced upon IFN- γ stimulation (Figure 2-2b, left panel). When siRNA was used to knockdown Sug1 expression, dramatic effects on MHC-II promoter acetylation were observed. Upon IFN- γ stimulation, H3 acetylation was enhanced in control siRNA treated cells (Figure 2-2b, right panel, black bars), but was inhibited in Sug1 siRNA treated cells (Figure 2-2b, right panel, grey bars). Histone deacetylase (HDAC) enzymes were inhibited by pre-treating cells with HDAC inhibitor, which resulted in histone H3 hyperacetylation (compare Figure 2-2c, left panel to Figure 2-2b, left panel). Even in the presence of HDAC inhibition and IFN- γ induced H3 acetylation, H3 acetylation was dramatically decreased in the absence of Sug1 (Figure 2-2c, right panel, grey bars) as compared to control siRNA treated cells (Figure 2-2c, right panel, black bars). These experiments indicate that Sug1 plays a critical role in regulating MHC-II transcription by regulating key epigenetic events at the proximal promoter.

To determine if the loss of MHC-II promoter acetylation at histone H3 is indicative of a global decrease in levels of acetylated histone H3, siRNA was used to knockdown Sug1 expression in HeLa cells. Histones were acid extracted and lysates were immunoblotted for acetylated histone H3. Although Sug1 was sufficiently knocked down (Figure 2-3a, lower panel), levels of acetylated histone H3 were unaffected by the loss of Sug1 (Figure 2-3a, upper panel). Serially diluted lysates verified that H3 acetylation is indeed maintained in the Sug1 deficient cells (Figure 2-4). To account for potential loss of histone H3 at the MHC-II promoter upon IFN- γ stimulation and/or Sug1 knockdown, ChIP assays were repeated utilizing an antibody

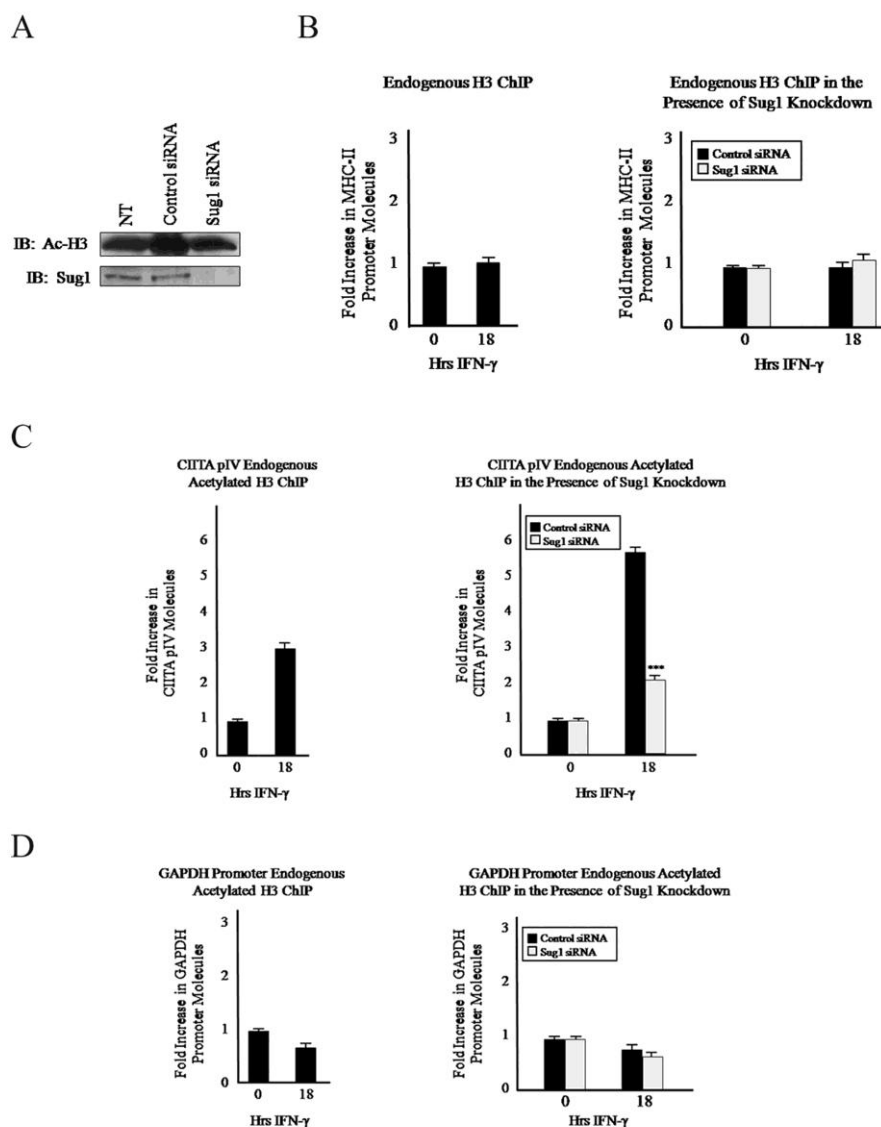


Figure 2-3. Sug1 knockdown decreases histone H3 acetylation in a promoter specific manner. (A) Global histone H3 acetylation is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control siRNA duplexes, or with Sug1 specific siRNA duplexes. Lysates were immunoblotted (IB) for acetylated histone H3 (upper panel) or for endogenous Sug1 (lower panel). Results reported are representative data of 3 independent experiments. (B) Sug1 knockdown does not impact levels of histone H3 at the MHC-II proximal promoter. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours (left) or in HeLa cells transfected with scrambled siRNA control or Sug1 specific siRNA duplexes and 24 hours later stimulated with IFN- γ for 0-18 hours (right). Lysates were immunoprecipitated (IP) with control antibody or antibody to endogenous histone H3. Associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2B. IP values are presented as fold increase in the MHC-793 II promoter DNA relative to unstimulated histone H3 IP samples. Control IP values were 1.15 ± 0.15 . Control and histone H3 IP values represent mean \pm SEM of ($n = 2-3$) independent experiments. (C) Sug1 knockdown does not impact levels of acetylated histone H3 at the GAPDH promoter. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours (left) or in HeLa cells transfected with scrambled control or Sug1 specific siRNA duplexes and 24 hours later stimulated with IFN- γ for 0-18 hours (right). Lysates were IP with control antibody or antibody to endogenous histone H3. Associated DNA was isolated and analyzed via real-time PCR using primers spanning the GAPDH promoter. Real-time PCR values were normalized to the total amount of GAPDH promoter DNA added to the reaction (input). Input values represent 5% of the total cell lysate. IP values are presented as fold increase in GAPDH promoter DNA relative to unstimulated acetylated histone H3 IP samples. Control IP values were 0.75 ± 0.25 . Control and histone H3 IP values represent mean \pm SEM of ($n = 3$) independent experiments.

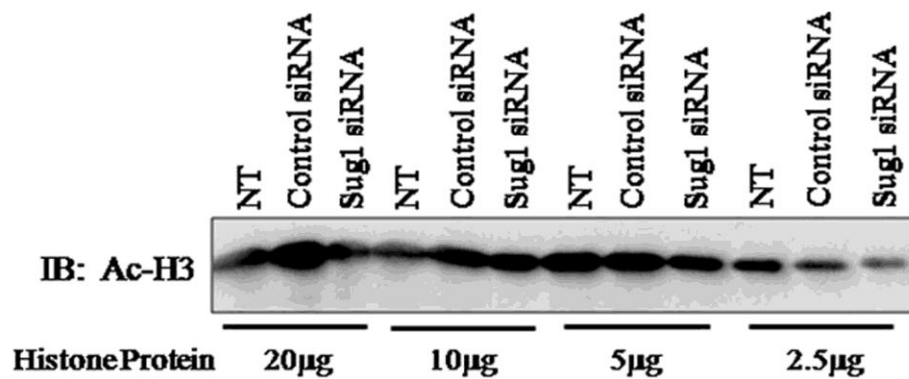


Figure 2-4. Global histone H3 acetylation is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control siRNA duplexes, or with Sug1 specific siRNA duplexes. Histones were subjected to acid extraction. Lysates were serially diluted and immunoblotted (IB) for acetylated histone H3. Results reported are representative data of 2 independent experiments.

specific for endogenous histone H3. Neither IFN- γ treatment (Figure 2-3b, left panel) nor IFN- γ treatment combination with Sug1 siRNA transfection (Figure 2-3b, right panel) affected levels of histone H3 at the MHC-II proximal promoter. To ascertain if additional IFN- γ inducible promoters are also epigenetically regulated by Sug1, ChIP experiments were performed to detect endogenous levels of H3 acetylation at the IFN- γ inducible CIITA promoter, pIV, in the absence of Sug1. ChIP mapping experiments demonstrated that levels of histone H3 acetylation at CIITA pIV are enhanced upon IFN- γ stimulation (Fig.2-3c, left panel). Consistent with ChIP studies indicating Sug1 association with CIITA pIV (data not shown), Sug1 knockdown resulted in reduced CIITA pIV acetylation (Fig.2-3c, right panel, grey vs black bars). We have previously shown that Sug1 knockdown decreases MHC-II transcript levels but not CIITA transcript levels (Bhat, Turner et al. 2008), suggesting that despite binding multiple inducible promoters, Sug1 binding may differentially regulate specific genes. To further determine if Sug1 is specifically regulating H3 acetylation at inducible promoters, we performed ChIP experiments to detect endogenous levels of H3 acetylation at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, in the absence of Sug1. ChIP mapping experiments demonstrated that levels of histone acetylation at the GAPDH promoter were comparable to previously published results (Beresford and Boss 2001; Morris, Beresford et al. 2002), indicating that IFN- γ stimulation results in only a marginal change in histone acetylation (Fig.3d, left panel). Furthermore, siRNA-mediated Sug1 knockdown had no effect on GAPDH promoter acetylation (Fig.3d, right panel, grey vs. black bars). These studies strongly implicate the Sug1 19S ATPase in the specific epigenetic regulation of transcription at inducible genes.

Inhibition of proteasomal activity does not affect MHC-II promoter acetylation.

Sug1, a component of the 19S proteasome, works with the other 19S ATPases (S4, S6a, S6b, S7,

and S10b) to recruit polyubiquitinated proteins for degradation (Ciechanover 1994; Glickman, Rubin et al. 1999). To determine the impact Sug1 knockdown has on proteasome function, we assayed *in vivo* proteolytic activity in HeLa cells transfected with Sug1 specific or control siRNA over a time course of cycloheximide treatment to prevent *de novo* translation. Because it is well established that p53 is polyubiquitinated and degraded via the 26S proteasome (Athanasios, Hu et al. 1999; McVean, Xiao et al. 2000; Alarcon-Vargas and Ronai 2002; Zhu, Wani et al. 2007), cells were lysed and analyzed for expression of this relatively short-lived protein. Consistent with published reports (Buschmann, Potapova et al. 2001; Wesierska-Gadek, Schloffer et al. 2002), control siRNA transfected cells showed a loss in p53 expression by 90 minutes post cycloheximide treatment (Figure 2-5a, top panel). As a control, one set of control siRNA transfected cells were pretreated with proteasome inhibitor MG132, which resulted in p53 accumulation upon cycloheximide treatment (Figure 2-5a, bottom panel). Not surprisingly, Sug1 knockdown impacted proteasome activity. Although p53 accumulation was not as drastic as in MG132 treated samples, cells transfected with Sug1 siRNA also showed elevated p53 expression upon cycloheximide treatment (Figure 2-5a, middle panel). To determine if non-ATPase components of the 19S proteasome as well as components of the 20S core are also associated with the MHC-II proximal promoter, ChIP assays were performed to detect the 19S non-ATPase S5a and the 20S Alpha 4 subunit. The 19S non-ATPase S5a associated with the MHC-II proximal promoter and this low association was enhanced and maintained upon cytokine stimulation (Figure 2-5b). The Alpha 4 subunit of the 20S catalytic core also showed low level binding upon cytokine stimulation that appeared to dissipate by 18 hrs post stimulation (Figure 2-5c). The binding of these additional subunits to the MHC-II proximal promoter might indicate a role for the intact proteasome in regulating epigenetics at the MHC-II promoter. Therefore, we

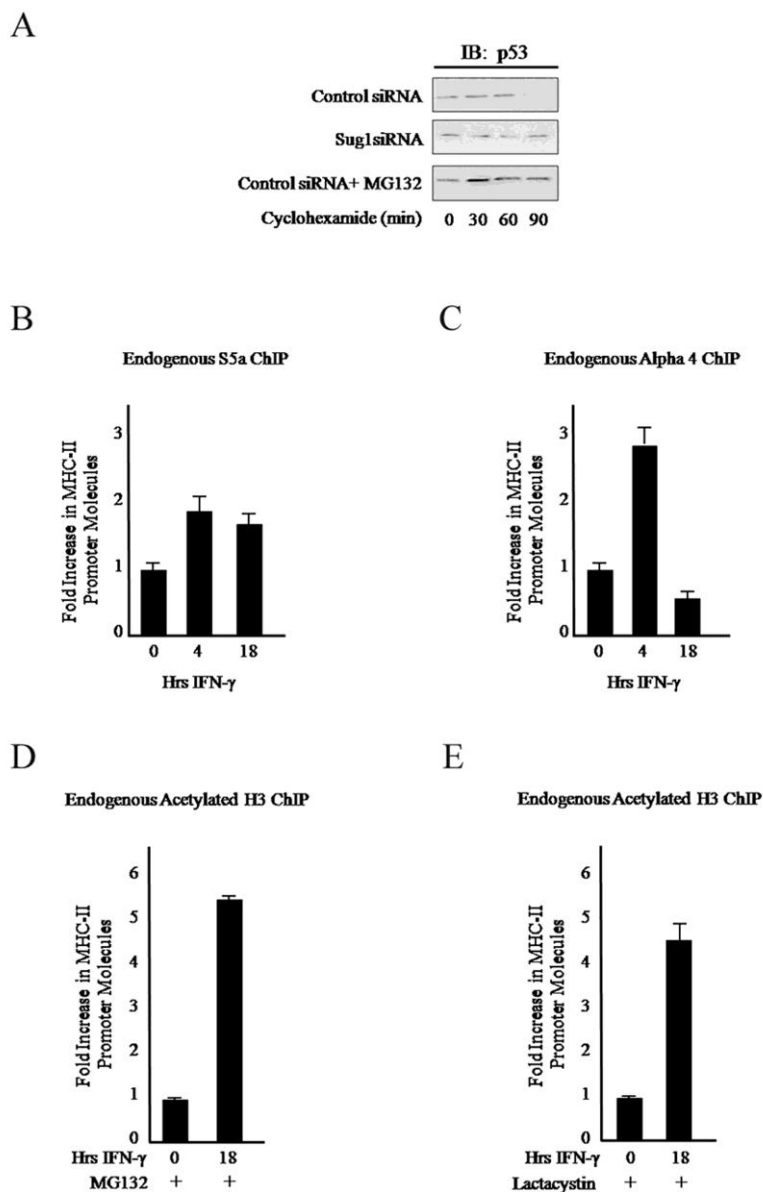


Figure 2-5. Sug1 dependent regulation of MHC-II promoter histone acetylation is proteolysis independent.

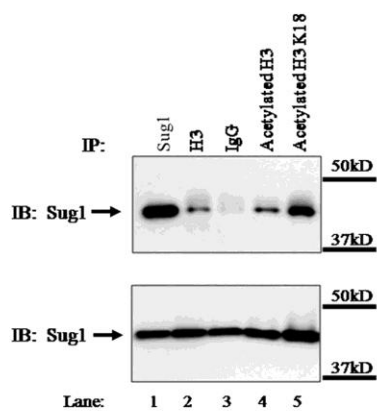
(A) Sug1 is critical for degradation mediated by the 26S proteasome. HeLa cells were transfected with Sug1 specific or control siRNA and were treated with proteasome inhibitor MG132 as indicated. Following cycloheximide treatment, cells were lysed and lysates analyzed by western blot for p53 degradation. Results reported are representative data of 3 independent experiments. (B-C) 19S non-ATPase S5a and 20S core protein alpha 4 are recruited to the MHC-II proximal promoter upon IFN- γ stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours. Lysates were IP with control antibody or antibody to endogenous S5a (B) or alpha 4 (C) and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated S5a or alpha 4 IP samples. Control IP values were 0.8 ± 0.1 . Control and IP values represent mean \pm SEM of (n=2) independent experiments. (D-E) MHC-II proximal promoter histone H3 acetylation is unaffected by proteasome inhibition. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours and treated with proteasome inhibitors MG132 (D) or lactacystin (E) for 4 hours prior to harvesting. Lysates were immunoprecipitated (IP) with control antibody or antibody to endogenous acetylated histone H3 and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated acetylated histone H3 IP samples. Control IP values were 1.2 ± 0.25 . Control and acetylated histone H3 IP values represent mean \pm SEM of (n = 2-3) independent experiments.

sought to ascertain if the loss of histone acetylation observed in Sug1 deficient cells was due in part to lack of proteasome function. Cells were treated with proteasome inhibitors MG132 or lactacystin and ChIP experiments were performed to detect endogenous levels of H3 acetylation at the MHC-II proximal promoter. MG132 treated HeLa cells (Figure 2-5d) or lactacystin treated HeLa cells (Figure 2-5e) stimulated with IFN- γ showed levels of H3 acetylation that were comparable to those observed in untreated cells (Figure 2-2b, left panel). These experiments emphasize that although Sug1 function is required for proteolysis mediated by the 26S proteasome, the role played by Sug1 in regulating MHC-II proximal promoter acetylation is independent of proteolysis.

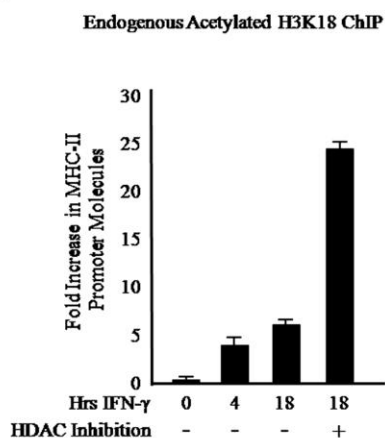
Sug1 knockdown decreases lysine 18 acetylation at the MHC-II proximal promoter.

Acetylation occurs on lysine residues in histone tails. Epigenetic mapping of the MHC-II promoter has shown that lysines (K) 9, K14, K18 and K27 of histone H3 are acetylated (Gomez, Majumder et al. 2005; Rybtsova, Leimgruber et al. 2007). Acetylation of H3 K9, K18 and K27 are CIITA dependent, whereas H3 K14 acetylation is also found constitutively in B cells lacking functional CIITA (RJ2.2.5). H3 K18 acetylation is robustly elevated at the activated MHC-II proximal promoter (Gomez, Majumder et al. 2005). To determine whether Sug1 preferentially associates with acetylated H3 K18 *in vivo*, co-immunoprecipitations were performed in HeLa cells transfected with Myc-tagged Sug1 (Figure 2-6a). Polyclonal antibody was used to IP endogenous histone H3, acetylated H3 or acetylated H3 K18. As expected, Sug1 and histone H3 as well as acetylated histone H3 precipitated together (Figure 2-6a, upper panel, lanes 2 and 4). Enhanced binding was observed in cells IP with acetylated H3 K18 (Figure 2-6a, upper panel, lane 5). Control samples were immunoprecipitated with Myc beads (Figure 2-6a, upper panel, lane 1) or an isotype control (Figure 2-6a, upper panel, lane 3). Equal loading was confirmed by

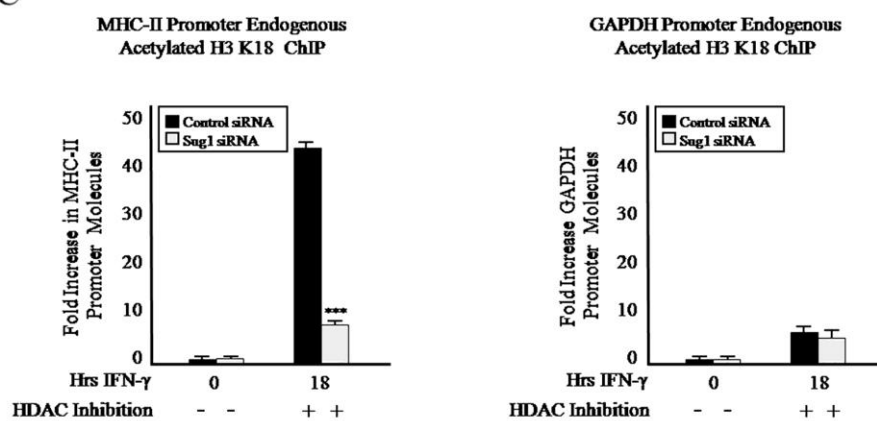
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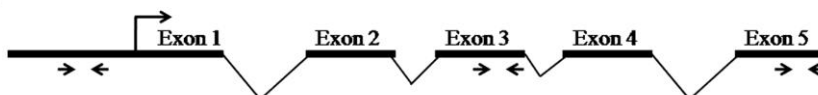
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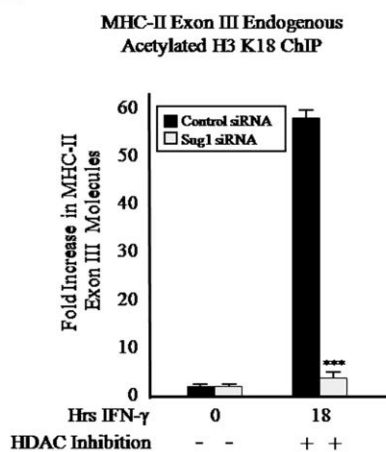
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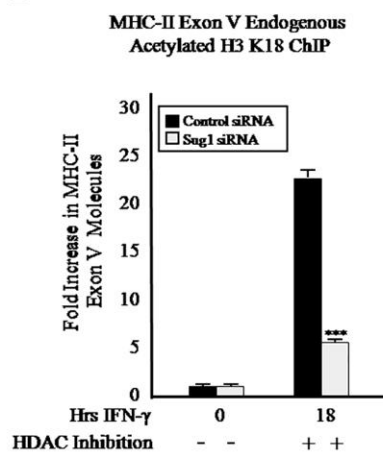


Figure 2-6. Sug1 knockdown decreases histone H3 lysine 18 acetylation at the MHC-II proximal promoter.

(A) Sug1 associates with acetylated H3 K18. HeLa cells transfected with Myc-tagged Sug1 were lysed and IP with polyclonal antibody against histone H3, acetylated histone H3 or acetylated H3 K18. Control samples were IP with Myc beads and isotype control IgG. IP and lysate control samples were immunoblotted (IB) for Myc. Results reported are representative data of 3 experiments. (B) Histone H3 K18 acetylation at the MHC-II promoter is enhanced upon IFN- γ stimulation and HDAC inhibition. ChIP assays were carried out in HeLa cells treated with IFN- γ for 0-18 hrs or HDAC inhibitor (20 hours) and IFN- γ (18 hrs). Lysates were IP with control or acetylated histone H3 K18 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in MHC-II promoter DNA relative to unstimulated acetylated H3 K18 IP samples. Control IP values were 0.8 ± 0.2 . Control and IP values represent mean \pm SEM of (n = 3) independent experiments. (C) Sug1 knockdown specifically decreases H3 K18 acetylation at the MHC-II proximal promoter. HeLa cells transfected with control or Sug1 siRNA were treated with HDAC inhibitor (20 hrs) and stimulated with IFN- γ for 0-18 hrs. Lysates were IP with control or acetylated H3 K18 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. Data are presented as fold increase in the MHC-II promoter DNA (left) or GAPDH promoter DNA (right) relative to unstimulated IP samples. Control IP values were 1.0 ± 0.4 . Control and IP values represent mean \pm SEM of (n = 2-4) independent experiments. (D) Real-time PCR primer locations in the MHC-II gene. (E-F) Sug1 knockdown decreases H3 K18 acetylation within the MHC-II coding region. ChIP assays were performed as in C. Isolated DNA was analyzed via real-time PCR using primers and probes specific for MHC-II exon III (E) or exon V (F). Data are presented as fold increase in MHC-II exon DNA relative to unstimulated acetylated H3 K18 IP samples. Control values were 0.9 ± 0.3 . Control and IP values represent mean \pm SEM of (n = 3) independent experiments. *** P < 0.001

immunoblot analysis of lysates (Figure 2-6, lower panel). To evaluate H3 K18 acetylation levels at the MHC-II proximal promoter, HeLa cells were stimulated with IFN- γ , immunoprecipitated (IP) with antibody to endogenous H3 K18, and analyzed by real-time PCR with primers spanning the MHC-II *HLA-DRA* proximal promoter. ChIP assays showed elevated levels of H3 K18 acetylation at the MHC-II promoter upon IFN- γ stimulation that were substantially enhanced upon HDAC inhibition (Figure 2-6b). While cells transfected with control siRNA showed a ~40 fold increase in histone H3 acetylation upon IFN- γ stimulation and HDAC inhibition (Figure 2-6c, left panel, black bars), similarly treated cells that were transfected with Sug1 specific siRNA showed dramatically decreased histone H3 K18 acetylation at the MHC-II promoter (Figure 2-6, left panel, grey bars). To further determine if Sug1 is specifically regulating H3 K18 acetylation at the MHC-II proximal promoter, we performed ChIP experiments to detect endogenous levels of H3 K18 acetylation at the GAPDH promoter. In the absence of Sug1, GAPDH promoter H3 K18 acetylation levels (Figure 2-6c, right panel, grey bars) were comparable to those in cells treated with control siRNA (Figure 2-6c, right panel, black bars). Because H3 K18 acetylation appears to be preferentially impacted by Sug1 knockdown, we sought to determine the extent with which this interaction extends into the coding sequence of MHC-II genes. Therefore we repeated real-time PCR using primers and probes specific for regions within MHC-II exon III and MHC-II exon V (Figure 2-6d) on the ChIP studies for acetylated H3 K18 in Sug1 specific or control siRNA transfected cells. Upon cytokine stimulation and in the presence of HDAC inhibition, H3 K18 acetylation was diminished within both exon III (Figure 2-6e, grey bars) and exon V (Figure 2-6f, grey bars) in Sug1 knockdown cells as compared to control samples (Figure 2-6e-f, black bars).

To determine if the loss of MHC-II promoter H3 K18 acetylation is indicative of a global decrease in levels of histone H3 K18 acetylation, siRNA was used to knockdown Sug1 expression in HeLa cells. Histones were acid extracted and lysates were immunoblotted for acetylated H3 K18. Although Sug1 was sufficiently knocked down (Figure 2-7a, lower panel), levels of acetylated H3 K18 were unaffected by the loss of Sug1 (Figure 2-7a, upper panel). Serial dilutions of lysates verified that H3 K18 acetylation is indeed maintained in the Sug1 deficient cells (Figure 2-8). To verify that this phenomenon is lysine residue specific, ChIP studies were performed in Sug1 knockdown cells using endogenous antibody against acetylated histone H3 lysine 9. HeLa cells stimulated with IFN- γ showed elevated levels of H3 K9 acetylation at the MHC-II promoter that were enhanced upon treatment with HDAC inhibitors (Figure 2-7b). H3 K9 acetylation was not decreased by Sug1 siRNA transfection (Figure 2-7c, grey bars) as compared to control transfected cells (Figure 2-7c, black bars).

Sug1 knockdown decreases recruitment of the HAT CBP to the MHC-II promoter.

An open chromatin conformation is required for the initiation of transcription. This restructuring event involves the recruitment of histone acetyltransferase (HAT) enzymes which add acetyl groups to lysine residues of the N-terminal tail region of histones and loosen protein-DNA interactions. One of the most studied HATs in yeast is GCN5, which is the catalytic subunit of the SAGA complex. The SAGA complex associates with various transcriptional activators *in vivo* and is recruited to promoters where GCN5 is able to acetylate nearby histones (Roth, Denu et al. 2001; Bhaumik, Raha et al. 2004). Proteasomal ATPases interact with the SAGA complex in yeast and decreasing expression of the 19S ATPases reduces global histone acetylation and SAGA recruitment to actively transcribing promoters (Lee, Ezhkova et al. 2005; Daniel and Grant 2007). We therefore sought to determine if the Sug1 ATPase associates with HATs in

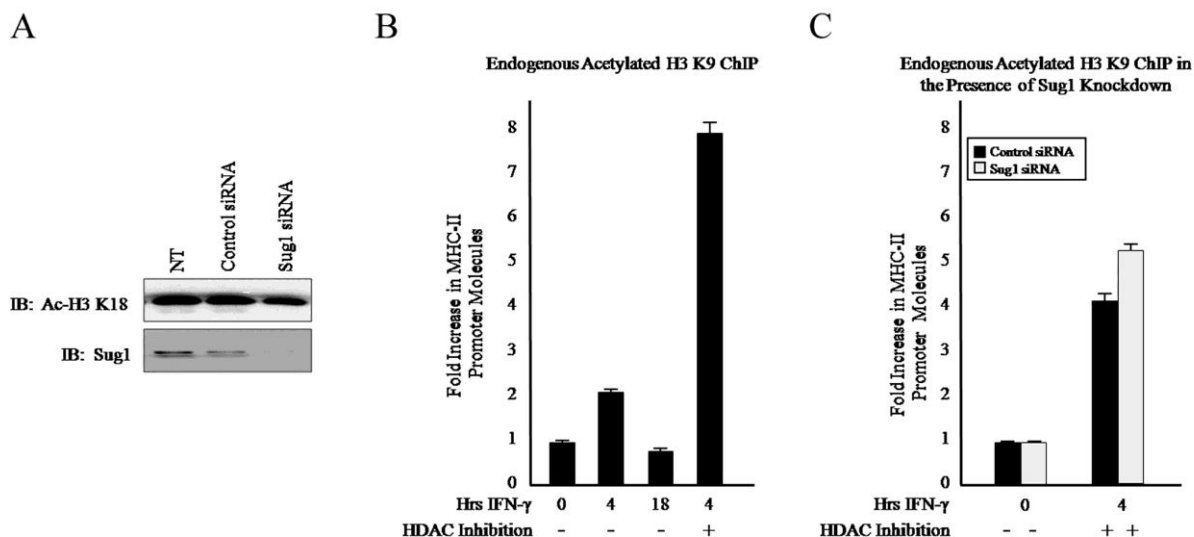


Figure 2-7. Sug1 knockdown decreases histone H3 lysine 18 acetylation in a promoter specific manner. (A) Global histone H3 K18 acetylation is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either control or Sug1 siRNA. Histones were subjected to acid extraction. Lysates were immunoblotted (IB) for acetylated H3 K18 (upper panel) or for endogenous Sug1 (lower panel). Results reported are representative data of 2 independent experiments. (B) Histone H3 K9 acetylation at the MHC-II proximal promoter is enhanced upon IFN- γ stimulation and HDAC inhibition. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours in combination with HDAC inhibitor (20 hours) as indicated. Lysates were IP with control or acetylated histone H3 K9 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated acetylated H3 K9 IP samples. Control values were 1.15 ± 0.1 . Control and IP values represent mean \pm SEM of ($n = 3$) independent experiments. (C) H3 K9 acetylation at the MHC-II proximal promoter is unaffected by loss of Sug1 expression. HeLa cells were transfected with control or Sug1 siRNA and 24 hours later were treated with HDAC inhibitor and stimulated with IFN- γ for 0-4 hours. Lysates were IP with control or acetylated H3 K9 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. Data are presented as fold increase in the MHC-II promoter DNA relative to unstimulated acetylated H3 K9 IP samples. Control values were 1.6 ± 0.27 . Control and IP values represent mean \pm SEM of ($n = 4$) independent experiments.

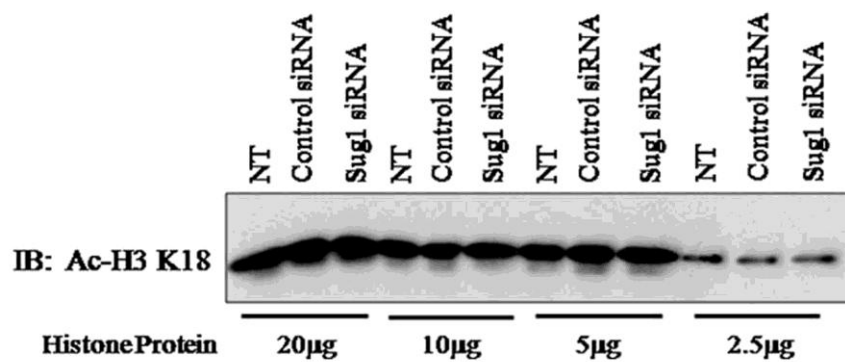


Figure 2-8. Global histone H3 K18 acetylation is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control siRNA duplexes, or with Sug1 specific siRNA duplexes. Histones were subjected to acid extraction. Lysates were serially diluted and immunoblotted (IB) for acetylated H3 K18. Results reported are representative data of 2 independent experiments.

mammalian cells. Sug1 deficient cells show dramatically decreased levels of histone H3 lysine 18 acetylation (Figure 2-6c, left panel and 2-6e-f), a modification that can be mediated by the HAT CBP (Schiltz, Mizzen et al. 1999; Agalioti, Chen et al. 2002). To determine whether Sug1 associates with CBP *in vivo*, co-immunoprecipitations were performed in HeLa cells transfected with Myc-tagged Sug1, Myc-tagged ATPase mutant Sug1 or Myc control plasmid (Figure 2-9a). Polyclonal antibody was used to IP endogenous CBP and associations were detected by immunoblotting the samples with Myc antibody. Sug1 and CBP precipitated together (Figure 2-9a, upper panel, lane 3) whereas the empty Myc-plasmid showed no association with CBP (Figure 2-9a, upper panel, lane 2). This interaction appeared independent of ATPase activity, as over-expressed ATPase mutant Sug1 also associated with CBP (Figure 2-9a, upper panel, lane 4). A positive control sample was immunoprecipitated with Myc beads (Figure 2-9a, upper panel, lane 1). Equal loading was confirmed by immunoblot analysis of lysates (Figure 2-9a, lower panels).

To determine the role played by Sug1 in recruiting CBP to the MHC-II proximal promoter, we performed ChIP experiments to detect endogenous levels of CBP at the *HLA-DRA* proximal promoter. Initial ChIP studies confirmed endogenous levels of CBP recruitment that rapidly increase upon IFN- γ stimulation in HeLa cells (Kretsovali, Agalioti et al. 1998; Fontes, Kanazawa et al. 1999; Zhu and Ting 2001; Zika, Fauquier et al. 2005). CBP interacts with CREB, a component of the MHC-II enhanceosome complex and thus can be found associated with the promoter region in unstimulated HeLa cells and in stimulated cells prior to CIITA recruitment (Figure 2-9b-c). CBP binding is enhanced upon prolonged cytokine stimulation when CIITA is also bound (Figure 2-9b-c). To determine if the decreased acetylation of histone H3 at the MHC-II *HLA-DRA* promoter in the absence of Sug1 is due to decreased recruitment of

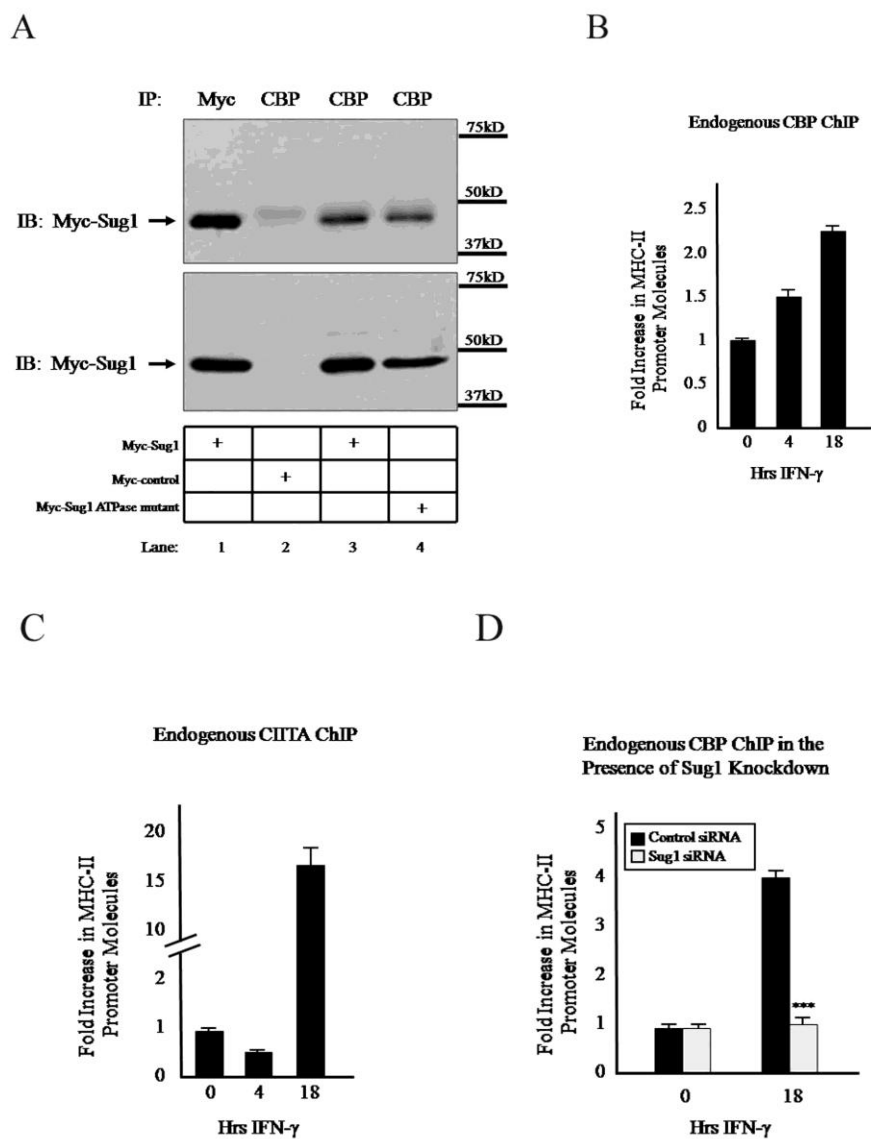


Figure 2-9. Sug1 knockdown decreases HAT recruitment to the MHC-II promoter. (A) Sug1 associates with CBP in HeLa cells. HeLa cells were transfected with Myc-tagged Sug1, Myc-tagged ATPase mutant Sug1, or Myc-control plasmid as indicated. Cells were lysed and immunoprecipitated (IP) with polyclonal antibody against CBP (lanes 2-4). A control sample was IP with Myc beads (lane 1). IP and lysate control (lower panel) samples were immunoblotted (IB) for Myc. Results reported are representative data of 3 experiments. (B-C) CBP association with the MHC-II proximal promoter precedes CIITA and is enhanced upon IFN- γ stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours. Lysates were IP with control or endogenous CBP antibody (B) or CIITA (C). Associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in MHC-II promoter DNA relative to unstimulated CBP or CIITA IP samples. Control values were 1 ± 0.4 . Control and IP values represent mean \pm SEM of ($n = 2-4$) independent experiments. (D) Sug1 knockdown decreases CBP association with the MHC-II proximal promoter. HeLa cells were transfected with control or Sug1 specific siRNA and stimulated with IFN- γ for 0-18 hours. Lysates were IP with control or CBP antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. Data are presented as fold increase in the MHC-II promoter DNA relative to unstimulated CBP IP samples. Control values were 1.2 ± 0.3 . Control and IP values represent mean \pm SEM of ($n = 3$) independent experiments. *** $P < 0.001$ vs control siRNA.

CBP, we performed ChIP experiments in the presence of siRNA-mediated knockdown of Sug1. Cells transfected with control siRNA showed a 3 fold increase in CBP recruitment following IFN- γ stimulation (Figure 2-9d, black bars), while cells transfected with Sug1 specific siRNA lacked this enhanced IFN- γ dependent recruitment of CBP to the MHC-II promoter (Figure 2-9d, grey bars).

ATPase activity is not required for Sug1 mediated MHC-II chromatin remodeling.

Because the Sug1 ATPase mutant associated with CBP (Figure 2-9a, upper panel, lane 4), we sought this Sug1 ATPase mutant had on H3 K18 acetylation. ChIP assays for endogenous acetylated H3 K18 at the MHC-II *HLA-DRA* proximal promoter were performed in HeLa cells transfected with either Myc-tagged Sug1 or Myc-tagged ATPase mutant Sug1 and stimulated with IFN- γ . Although the absence of wild type levels of Sug1 results in almost a complete loss of the H3 K18 acetylation necessary for chromatin remodeling at the MHC-II proximal promoter (Figure 2-6c, left panel, grey bars), overexpressing a single 19S ATPase (Sug1) showed only a marginal increase in the levels of H3 K18 acetylation observed at the MHC-II proximal promoter (Figure 2-10, grey bars vs black bars). Consistent with CBP binding to ATPase mutant Sug1 (Figure 2-9a, upper panel, lane 4), the lack of Sug1 ATPase activity did not impact H3 K18 acetylation (Figure 2-10, white bars).

Additional 19S ATPases also play a role in remodeling MHC-II chromatin.

Overexpressing Sug1 did not result in substantial enhancement of histone H3 acetylation at the MHC-II proximal promoter (Figure 2-10). Therefore, we sought to determine the contribution of additional 19S ATPases to histone H3 acetylation. First, ChIP assays were used to determine that both S6a (Figure 2-11) and S7 (Figure 2-11b) are recruited to the MHC-II promoter upon cytokine stimulation, preceding CIITA recruitment (Figure 2-9c). Next, specific siRNAs were

Endogenous Acetylated H3K18 ChIP

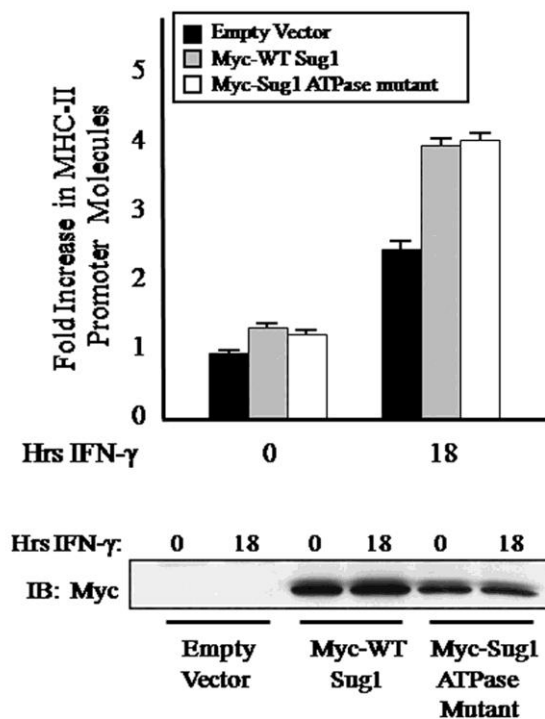


Figure 2-10. ATPase activity is not required for Sug1 mediated MHC-II chromatin remodeling. HeLa cells were transfected with Myc-tagged Sug1, Myc-tagged ATPase mutant Sug1, or Myc-control plasmid as indicated and treated with IFN- γ for 0-18 hours. 10% of the total cell volume was lysed and analyzed by western blot for Sug1 overexpression (lower panel) as described in Figure 2-1. The remaining fraction of cells was subjected to ChIP assay. Lysates were immunoprecipitated (IP) with control or acetylated H3 K18 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. Data are presented as fold increase in the MHC-II promoter DNA relative to unstimulated empty vector transfected acetylated H3 K18 IP samples. Control values were 1.0 ± 0.1 . Control and IP values represent mean \pm SEM of (n = 4) independent experiments.

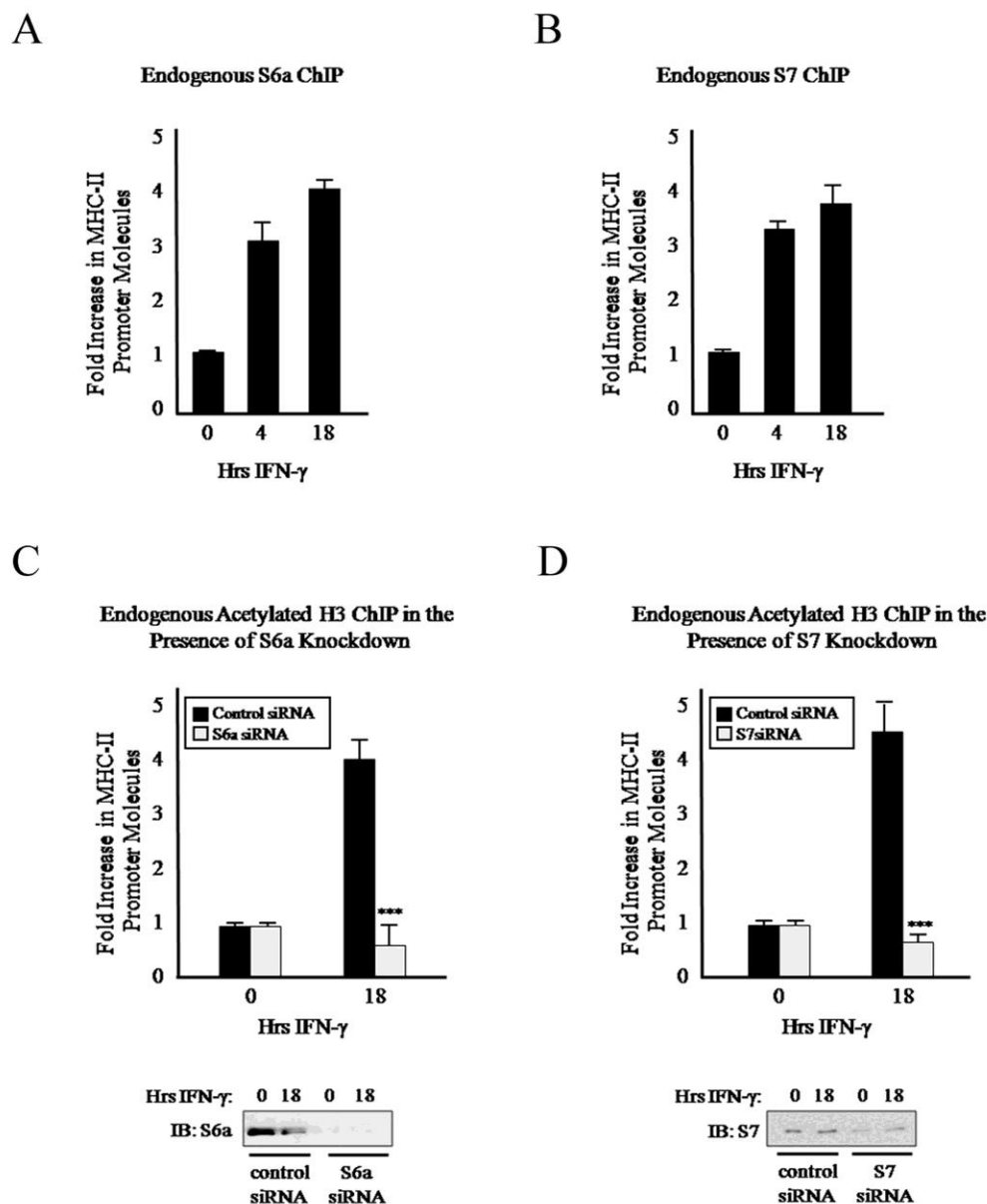


Figure 2-11. Additional 19S ATPases also mediate MHC-II promoter histone acetylation. (A-B) 19S ATPases S6a and S7 are recruited to the MHC-II promoter upon IFN- γ stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0-18 hours. Lysates were immunoprecipitated (IP) with control, S6a (A) or S7 (B) antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated S6a or S7 IP samples. Control values were 1.2 ± 0.2 . Control and IP values represent mean \pm SEM of (n =2-4) independent experiments. (C-D) S6a and S7 knockdowns diminish H3 acetylation at the MHC-II proximal promoter. ChIP assays were carried out in HeLa cells transfected with S6a, S7 or control siRNA and stimulated with IFN- γ for 0-18 hours. 10% of the total cell volume was lysed and analyzed by western blot for S6a or S7 knockdown (lower panels). The remaining fractions of cells were subjected to ChIP assay. Lysates were IP with control or acetylated H3 antibody and associated DNA was isolated and analyzed via real-time PCR as in Figure 2-2b. IP values are presented as fold increase in the MHC-II promoter DNA relative to unstimulated acetylated histone H3 IP samples. Control values were 0.85 ± 0.4 . Control and IP values represent mean \pm SEM of (n = 2) independent experiments. *** P < 0.001 vs control siRNA.

generated to knockdown 19S ATPases, S6a (Figure 2-11c, lower panel) and S7 (Figure 2-11d, lower panel). Similar to experiments in Sug1 knockdown cells (Figure 2-2b, right panel), acetylated H3 ChIP assays performed in S6a siRNA (Figure 2-11c, upper panel, grey bars) or S7 siRNA (Figure 2-11d, upper panel, grey bars) transfected cells exhibited impaired H3 acetylation at the MHC-II promoter as compared to control siRNA transfected cells (Figure 2-11c-d, lower panels, black bars).

DISCUSSION

Our results have provided novel evidence that the 19S proteasome plays an important role in mediating the epigenetic regulation of MHC-II transcription. Several pieces of data above argue that the 19S ATPase Sug1 recruits the histone acetyltransferase CBP and potentially additional histone modifying enzymes, to the MHC-II proximal promoter to enhance activation induced promoter acetylation and to establish a necessary platform for CIITA binding, additional HAT recruitment and robust histone H3 acetylation. We have previously demonstrated that following IFN- γ stimulation, Sug1 rapidly binds the MHC-II proximal promoter (Bhat, Turner et al. 2008). We now show that Sug1 associates *in vivo* with histone H3 and acetylated histone H3 and that when Sug1 is knocked down, levels of histone H3 acetylation are markedly reduced at the activated MHC-II proximal promoter. Despite our observations of several proteasomal subunits binding to the MHC-II promoter, the effects of Sug1 are independent of proteasome proteolytic function as proteasomal inhibition does not impact MHC-II promoter H3 acetylation. Furthermore, the effects observed on histone H3 acetylation by Sug1 knockdown are specific as whole cell lysates show no global change in levels of acetylated histone H3 upon Sug1 knockdown. The effects of Sug1 are also lysine specific as histone H3 lysine 18 acetylation is

diminished by Sug1 knockdown at the activated MHC-II promoter whereas H3 lysine 9 acetylation remains unaffected. The impact of Sug1 on MHC-II transcription is substantial and extends beyond the proximal promoter as exons III and V also show markedly decreased histone H3 lysine 18 acetylation in the absence of Sug1. These observations correlate with decreased binding of the HAT CBP to the MHC-II promoter, data consistent with experiments that indicate *in vivo* association between Sug1 and CBP.

The seminal discovery that 19S ATPases are recruited to activated yeast promoters in the absence of proteolytic components of the proteasome first suggested a non-proteolytic role for the 19S proteasome in transcriptional regulation (Gonzalez, Delahodde et al. 2002). We have recently demonstrated a role in mammalian transcription for the 19S as a positive regulator of MHC-II transcription initiation. Studies by Bhat et al. indicate that the 19S ATPase Sug1 is recruited to the MHC-II proximal promoter prior to CIITA, that knocking down Sug1 decreases MHC-II but not CIITA expression, and that in the absence of Sug1, markedly reduced levels of CIITA are recruited to the MHC-II *HLA-DRA* proximal promoter (Bhat, Turner et al. 2008). Research in yeast has recently indicated novel roles for the ortholog of Sug1, Rpt 6, in epigenetically regulating transcription. The ATPase activity of Rpt 6 is required to target the *Saccharomyces cerevisiae* SAGA histone acetyltransferase complex to a DNA bound activator and to globally regulate acetylation of histone H3 (Lee, Ezhkova et al. 2005; Daniel and Grant 2007). Our results illustrate similar regulatory activity in mammalian cells and indicate that in the absence of wild type levels of Sug1, necessary chromatin remodeling does not occur at the MHC-II proximal promoter, even in the presence of sustained HDAC inhibition. As in our study, in yeast cells ATP hydrolysis is less important for SAGA binding to promoters but is required for optimal dissociation of SAGA from the 19S (Lee, Ezhkova et al. 2005). It will be

useful to next determine the role played by energy utilization in the actions of the 19S and Sug1 on chromatin at the MHC-II proximal promoter.

Levels of histone H3 K18 acetylation are substantially reduced and the MHC-II promoter is rendered hypoacetylated in the absence of Sug1. H3 K18 acetylation occurs at the MHC-II proximal promoter within 4 hours of IFN- γ treatment and is sustained for 48 hours (Gomez, Majumder et al. 2005). The rapid occurrence of H3 K18 acetylation correlates with our demonstrated CBP promoter recruitment and CBP can generate this modification *in vitro* (Schiltz, Mizzen et al. 1999). Previous studies have shown that the association of CIITA with the MHC-II proximal promoter correlates with dramatic increases in histone H3 and H4 acetylation (Beresford and Boss 2001; Masternak, Peyraud et al. 2003). As CIITA binding to the MHC-II promoter is reduced in the absence of Sug1, it is not surprising that this specific CIITA driven modification would also be targeted. What is unexpected is the dramatic loss of MHC-II proximal promoter histone H3 acetylation in the absence of Sug1. The hypoacetylated state of the MHC-II promoter following IFN- γ stimulation and HDAC inhibition indicates that even the moderate levels of acetylation normally seen prior to CIITA expression are blocked in Sug1 knockdown cells. That CBP is the likely target of Sug1 is supported by data demonstrating CBP binding to the MHC-II promoter prior to CIITA and by a failure to increase CBP binding upon IFN- γ stimulation in the Sug1 knockdown cells. Our data do not however rule out the interesting possibility that Sug1 interacts with additional HATs to regulate histone modifications at the MHC-II promoter prior to, or in addition to, interactions with CBP. Future studies will enable determination of the full extent of the mechanisms by which Sug1 and additional 19S ATPases regulate HAT recruitment to the MHC-II promoter.

The finding that levels of H3 acetylation at the MHC-II proximal promoter are dramatically reduced in the absence of Sug1 suggests a model whereby Sug1 controls histone modifications associated with IFN- γ induced *HLA-DRA* gene activation. An interesting recent report suggests that histone modifications introduced at the MHC-II proximal promoter are likely important in activities such as promoter clearance, transcriptional elongation and transcriptional memory rather than transcription initiation (Rybtsova, Leimgruber et al. 2007). If so, Sug1 and the 19S ATPases may ultimately provide spatial and temporal regulation of mammalian transcription by controlling transcription elongation through the recruitment of histone modifying activators. It remains to be determined if additional histone modifying enzymes interact with 19S ATPases to coordinate MHC-II transcription. A complex series of specific histone modifications occurs to open the MHC-II chromatin structure and allow CIITA binding, but what links these modifying enzymes to each other and to other co-factors is not known. Active expression of MHC-II is associated with histone H3 acetylation, histone H4 acetylation, histone H3 methylation, and histone H2B ubiquitination (Masternak, Peyraud et al. 2003; Spilianakis, Kretsovali et al. 2003; Gomez, Majumder et al. 2005; Zika, Fauquier et al. 2005; Rybtsova, Leimgruber et al. 2007). Multiple possible scenarios exist for Sug1 and the 19S functioning in mediating these histone modifications at the MHC-II promoter. Similar to the role played by the 19S in targeting SAGA to yeast promoters, Sug1 may mediate promoter recruitment of CBP and additional HATs, like the elongator complex, that move along chromatin by binding elongating Pol II (Hawkes, Otero et al. 2002; Kim, Lane et al. 2002; Close, Hawkes et al. 2006). Indeed, ATPases of the 19S have been demonstrated to bind to coding sequence of yeast genes and to be required for transcriptional elongation (Ferdous, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Rasti, Grand et al. 2006; Laribee, Shibata et al. 2007). Our demonstration of decreased

histone H3 lysine 18 acetylation in exons III and V of *HLA-DRA* support a similar role for Sug1 in elongation in mammalian cells. The yeast ortholog of Sug1, Rpt 6, is recruited to promoters by the ubiquitination of histone H2B and is necessary for methylation of histone H3 (Ezhkova and Tansey 2004). Evidence that mammalian H3 acetylation is coupled to prior H2B ubiquitination and H3 K4 methylation provide the interesting possibility that Sug1 and the 19S will recruit histone modifying enzymes linked to these modifications as well (Bernstein, Kamal et al. 2005; Pavri, Zhu et al. 2006). Our observations that 19S ATPases S6a and S7 also bind MHC-II promoters and modulate MHC-II histone H3 acetylation provide evidence that multiple 19S ATPases play important roles in regulating mammalian transcription. Our demonstration that the 19S non-ATPase S5a and 20S subunit Alpha 4 also bind the MHC-II proximal promoter suggest that, despite the lack of a requirement for proteasome activity in the Sug1 dependent transcriptional regulation of *HLA-DRA* genes, additional components of the 26S proteasome are present at this promoter. These results support findings in the yeast literature that an intact, albeit proteolytically inactive, proteasome binds yeast promoters (Morris, Kaiser et al. 2003; Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005; Collins and Tansey 2006). However, the observation that binding of non-ATPase proteasome components is relatively low as compared to 19S ATPases does not rule out the possibility that APIS (19S ATPase proteins independent of 20S) structures also bind the MHC-II promoter as unique complexes, a finding which has also been supported by the yeast literature (Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004; Sikder, Johnston et al. 2006; Sulahian, Sikder et al. 2006).

Ours is the first report demonstrating a role for the 19S proteasome and the Sug1 ATPase in aiding in the transition to an open chromatin structure in a mammalian system and suggest an evolutionarily conserved role for the 19S in histone modifications. The finding that Sug1

modulates CBP promoter recruitment and acetylation of histones at the MHC-II proximal promoter advances our knowledge of 19S function in histone modifications. Evidence that promoter acetylation is dramatically reduced in the absence of Sug1 implicate Sug1 as being deeply involved in a mammalian histone modification pathway. Chromatin must be opened before transcription factors can bind and initiate transcription and it is a complex set of histone modifications that allow for this opening. A full understanding of the contributions of Sug1 and the 19S to the epigenetic regulation of MHC-II transcription will require further studies into the molecular interactions occurring at this and other promoters.

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CHAPTER 3THE 19S PROTEASOME POSITIVELY REGULATES HISTONE METHYLATION AT
CYTOKINE INDUCIBLE GENES

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NOTE: I PERFORMED ALL OF THE EXPERIMENTS IN THIS CHAPTER
INDEPENDENTLY WITH THE FOLLOWING EXCEPTIONS: REPLICATIONS OF
CO-IMMUNOPRECIPITATIONS WERE DONE BY R. KYLE DUDLEY OR NINAD
T. MEHTA AND H3K4Me3 CHROMATIN IMMUNOPRECIPITATIONS WERE
DONE IN CONJUNCTION WITH R. KYLE DUDLEY.

SUMMARY

Studies indicate that the 19S proteasome functions in the epigenetic regulation of transcription. We have shown that as in yeast, components of the 19S proteasome are crucial for regulating inducible histone acetylation events in mammalian cells. The 19S ATPase Sug1 binds to histone acetyltransferases and to acetylated histone H3 and, in the absence of Sug1, histone H3 acetylation is dramatically decreased at mammalian promoters. Research in yeast further indicates that the ortholog of Sug1, Rpt6, is a link between ubiquitination of histone H2B and H3 lysine 4 trimethylation (H3K4me3). To characterize the role the 19S proteasome plays in regulating additional activating modifications, we examined the methylation and ubiquitination status of histones at inducible mammalian genes. We find that Sug1 is crucial for regulating histone H3K4me3 and H3R17me2 at the cytokine inducible MHC-II and CIITA promoters. In the absence of Sug1, histone H3K4me3 and H3R17me2 are dramatically decreased, but the loss of Sug1 has no significant effect on H3K36me3 or H2BK120ub. Our observation that a subunit of hCompass interacts with additional activating histone modifying enzymes, but fails to bind the CIITA promoter in the absence of Sug1, strongly implicates Sug1 in recruiting enzyme complexes responsible for initiating mammalian transcription.

BACKGROUND

Components of the large macromolecular 26S proteasome have recently been identified as playing novel roles in regulating transcription independent of the intact proteasome's primary function of degrading polyubiquitinated proteins (Baumeister, Walz et al. 1998; Ciechanover 1998; Muratani and Tansey 2003; Lee, Ezhkova et al. 2005; Sulahian, Sikder et al. 2006). The 26S proteasome consists of two subunits: a 19S regulatory particle and a 20S catalytic core.

When associated with the 20S core, the 19S regulatory particle regulates assembly of the 26S proteasome, recruits polyubiquitinated proteins and shuttles targeted proteins to the 20S core for degradation (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998; Glickman, Rubin et al. 1999; Voges, Zwickl et al. 1999; Strickland, Hakala et al. 2000; Rabl, Smith et al. 2008). The 19S consists of a lid which is comprised of eight non-ATPase proteins, and a base which is comprised of six ATPase (S4, S6a, S6b, S7, Sug1(S8) and S10b) and three non-ATPase (S1, S2 and S5a) proteins (Glickman, Rubin et al. 1999). Work in yeast has established a role for ATPase components of the 19S as transcriptional regulators. Yeast 19S ATPases can associate with actively transcribing genes (Rubin, Coux et al. 1996; Chang, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Archer, Burdine et al. 2008), facilitate recruitment of transcription factors (Swaffield, Melcher et al. 1995), are necessary for efficient transcriptional elongation (Ferdous, Gonzalez et al. 2001) and are important for targeting histone modifying enzymes to promoters (Ferdous, Gonzalez et al. 2001; Gonzalez, Delahodde et al. 2002; Lee, Ezhkova et al. 2005; Daniel and Grant 2007). More recent studies have implicated roles for the 19S as a regulator of transcription in mammalian cells as well (Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). We have recently shown that decreased expression of endogenous Sug1 results in impaired recruitment of the class II transactivator (CIITA), the master regulator, to the major histocompatibility class II (MHC-II) proximal promoter and in reduced MHC-II expression (Bhat, Turner et al. 2008).

Despite these advances, evidence of roles for the 19S in regulating mammalian chromatin structure remains limited. We have recently shown that components of the 19S proteasome positively regulate acetylation of the chromatin structure of the above mentioned critical inflammatory genes, MHC-II and CIITA. MHC-II molecules are cell surface glycoproteins

which perform crucial roles in initiating adaptive immune responses against invading pathogens and tumors by binding and presenting exogenously derived antigenic peptides to CD4⁺ T lymphocytes (Parham 2005). MHC-II proteins are constitutively expressed on antigen presenting cells and are inducibly expressed on nucleated cells by the inflammatory cytokine interferon gamma (IFN- γ) (7, 20). In order to limit opportunistic infection, autoimmune disease and tumor growth, MHC-II expression is tightly regulated at the level of transcription (Gerloni and Zanetti 2005; Parham 2005). Following IFN- γ stimulation, transcription of the MHC-II master regulator CIITA is induced at CIITA promoter IV (pIV). CIITA is subsequently expressed and recruited to the MHC-II proximal promoter (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005). These events correlate with recruitment of basal transcriptional machinery and histone acetyltransferases (HATs) to the MHC-II proximal promoter (Beresford and Boss 2001; Wright and Ting 2006). Furthermore, extensive studies have established an epigenetic map for the MHC-II gene *HLA-DRA* at the proximal promoter that corresponds to these initial transcriptional events (Beresford and Boss 2001; Gomez, Majumder et al. 2005; Zika, Fauquier et al. 2005; Gialitakis, Kretsovali et al. 2006). Prior to IFN- γ stimulation, *HLA-DRA* promoter histone H3 and histone H4 are low to moderately acetylated, which correlates with the assembly of an enhanceosome complex at the MHC-II proximal promoter (Beresford and Boss 2001; Adamski, Ma et al. 2004). Following IFN- γ stimulation, the CREB-binding protein (CBP/p300) rapidly binds the MHC-II proximal promoter and results in increased histone acetylation at lysine residues. The relaxation of chromatin that follows allows for CIITA binding, full enhanceosome formation and transcription initiation and elongation (Eberharther and Becker 2002; Freiman and Tjian 2003; Gorisch, Wachsmuth et al. 2005). Our recent studies demonstrate that 19S ATPases play important roles in regulating activating acetylation events at

the MHC-II promoter. We identified a role for the 19S ATPase Sug1 (PSMC5) in regulating K18 acetylation at histone H3 that correlates with a loss of the initial stable binding of the histone acetyltransferase CBP in the presence of reduced Sug1 expression (Koues, Dudley et al. 2008).

The evidence above has now prompted further studies into the contributions of Sug1 and the 19S in the full epigenetic regulation of MHC-II transcription. It is well accepted that specific histone acetylation, methylation, phosphorylation and ubiquitination events alter the transcriptional status of chromatin. Less is known as to how this “histone code” is organized and how sequential crosstalk between these histone modifications results in the subsequent recruitment and/or activation of additional remodeling enzymes to allow chromatin reorganization. Here we analyze the role of the 19S proteasome ATPase Sug1 in regulating activating methylation events at these inducible mammalian genes and the previously proposed potential for H2B ubiquitination to initiate these remodeling events. We report that Sug1 associates *in vivo* with histone H3 trimethylated at lysine 4 (H3K4me3) and that knocking down Sug1 blocks H3K4me3 at the promoters of the inflammatory genes MHC-II *HLA-DRA* and *CIITA* pIV. The effects observed on histone H3 methylation are residue specific and are localized to the promoter as histone H3 trimethylated at lysine 36 (H3K36me3), which is found throughout the MHC-II coding sequence, is not impacted by Sug1 knockdown while histone H3 dimethylated at arginine 17 (H3R17me2) is dramatically impaired at both the MHC-II promoter and at *CIITA* pIV. Furthermore, we have mapped the ubiquitination of histone H2B at both the MHC-II promoter and at *CIITA* pIV and have found Sug1 knockdown to have no significant impact on this activation associated modification. Moreover, the lack of H3K4me3 correlates with impaired recruitment of critical hCompass subunits, the multisubunit histone

methyltransferase required for efficient H3K4 trimethylation. These results reveal that Sug1 positively regulates activating histone H3 methylation events at inducible mammalian genes.

MATERIALS AND METHODS

Cell Lines: HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 10% fetal calf serum, 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide. Raji B cells (Burkitt's lymphoma-derived cell line) from ATCC were maintained in Roswell Park Memorial Institute (RPMI) Media (Mediatech) supplemented with 10% fetal calf serum, 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies: Histone H3 (06-755), histone H2B (07-371), acetylated histone H3 (06-599), rabbit IgG isotype control (12-370) and mouse IgG isotype control (12-371) antibodies were from Upstate (Lake Placid, NY). Trimethylated H3K4 (ab8580), trimethylated H3K36 (ab9050), dimethylated H3R17 (ab8284), CARM1 (ab51742), WDR5 (ab22512), Ash2L (ab50699) and Myc-HRP (ab1326), hBre1 (ab32629) antibodies were from Abcam (Cambridge, MA). Monoubiquitinated H2B antibody (MM-0029) was from MediMabs (Montreal, Canada). Sug1 antibody (NB100-345) was from Novus Biologicals (Littleton, CO). CBP antibody (sc-369) and IRF1 antibody (sc-497) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). CIITA antibody (100-401-192) was from Rockland Immunochemicals Inc. (Gilbertsville, PA). Flag-HRP antibody (A8592) was from Sigma (Saint Louis, MO). HRP conjugated mouse antibody (W4021) was from Promega (Madison, WI) and HRP conjugated rabbit antibody (1858415) was from Pierce (Rockland, IL).

Plasmids: Flag-CIITA, *HLA-DRA*-Luc, pcDNA3 plasmids were previously described (Bhat, Turner et al. 2008) *Renilla* luciferase control vector (E2231) was from Promega (Madison, WI). Myc-Sug1, Myc-ATPase mutant Sug1 and myc-control plasmids were generously provided by A. A. Wani (Zhu, Wani et al. 2007).

Co-immunoprecipitations: *Over-expression co-immunoprecipitations:* HeLa cells, plated at a density of 8×10^5 in 10cm tissue culture plates, were transfected with 5 μ g of the indicated plasmids using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Twenty-four hours post transfection, cells were lysed in RIPA buffer (1M Tris pH 8.0, 5% DOC, 10% Nonidet P-40, 5M NaCl, 10% SDS, 5 mM EDTA, 1M DTT) supplemented with Complete EDTA-free protease inhibitors (Roche, Indianapolis, IN) on ice and then precleared with 30 μ l IgG beads (Sigma). Cell lysates were immunoprecipitated (IP) overnight with 5 μ g anti-H3K4me3 (Abcam), anti-CARM1 (Abcam), anti-CBP (Santa Cruz) rabbit IgG isotype control (Upstate), Flag conjugated beads (Sigma) or Myc conjugated beads (Sigma). Immune complexes were isolated by incubating lysates with 50 μ l protein G beads (Pierce) for 2 hours. Immunoprecipitated proteins were denatured with Laemmli buffer (BioRad, Hercules, CA) and subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Associations were detected by immunoblotting (IB) with Myc HRP conjugated antibody (Abcam) or Flag HRP conjugated antibody (Sigma). Horseradish peroxidase was detected with Supersignal West Pico Chemiluminescent substrate (Pierce). Bradford assays were used to normalize for protein and equal loading was verified by immunoblot. *Endogenous co-immunoprecipitations:* Raji cells were lysed in RIPA buffer supplemented with Complete EDTA-free protease inhibitors (Roche) on ice and then precleared with 30 μ l IgG beads (Sigma). Cell lysates were IP overnight with 5 μ g anti-H3K4me3 (Upstate), anti-Sug1 (Novus Biologicals or rabbit IgG isotype control

(Upstate). Immune complexes were isolated by incubating lysates with 50 μ l protein G beads (Pierce) for 2 hours. Immunoprecipitated proteins were denatured with Laemmli buffer (BioRad) and subjected to SDS-PAGE. Associations were detected by IB with monoclonal Sug1 antibody (Novus Biologicals) and HRP conjugated mouse antibody (Promega) or polyclonal CARM1 antibody (Abcam) and HRP conjugated rabbit antibody (Pierce). Horseradish peroxidase was detected with Supersignal West Pico Chemiluminescent substrate (Pierce). Bradford assays were used to normalize for protein and equal loading was verified by immunoblot.

siRNA constructs and transient transfections: Short interfering RNA (siRNA) duplexes previously described (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008) were used for transient knockdown of the Sug1 19S ATPase. siRNA sequence was designed with a GC content of 35-55% containing dTdT overhangs and were compared to the NCBI BLAST nucleotide database. The target sequence of Sug1 was 5'-AAGGTACATCCTGAAGGTAAA-3' (Qiagen, Valencia, CA). HeLa cells were transfected with Allstar scrambled sequence control siRNA (Qiagen) or Sug1 ATPase specific siRNA (Qiagen) and were treated with IFN- γ as indicated. Cells were lysed in NP40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) supplemented with Complete EDTA-free protease inhibitors (Roche) and knock down efficiency and specificity was assessed by western blotting for ATPase expression as described above. siRNA sequence for human ubiquitin ligase hBre1 was kindly provided by Dr. Moshe Oren.

Luciferase reporter assays: HeLa cells were plated in 6-well plates at a density of 5×10^4 cells/well and transfected with 0.7 μ g of control or Sug1 siRNA using the RNAi transfection reagent (Qiagen) according to manufacturer's instructions. Twenty-four hours post siRNA transfection, CIITA, *HLA-DRA-Luc*, *Renilla*, Sug1, ATPase mutant Sug1 and pcDNA3

plasmids were transfected as indicated using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Twenty-four hours following DNA transfection, cells were subjected to dual-luciferase assay (Promega) according to the manufacturer's instructions.

Histone immunoblotting: HeLa cells were transfected with Sug1 siRNA (Qiagen) or scrambled sequence control siRNA using RNAiFect transfection reagent (Qiagen) according to manufacturer's protocol. Forty-eight hours following siRNA transfection, 10% of the total cell volume was lysed with 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with Complete EDTA-free protease inhibitors (Roche) and analyzed by western blot for ATPase knockdown as described above. The rest of the cell volume was lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, dH₂O) supplemented with Complete EDTA-free protease inhibitors (Roche) at 4°C and subjected to sonication to shear the chromatin. Lysate samples were normalized for protein concentration, denatured with Leammli buffer (BioRad) and separated by SDS-PAGE. Gels were transferred to nitrocellulose and subjected to IB using polyclonal H3K4me3 (Abcam), H3K36me3 (Abcam), H3 (Upstate), H3R17me2 (Abcam), H2BK120ub (MediMabs), H2B (Upstate) and either HRP conjugated rabbit secondary antibody (Pierce) or HRP conjugated mouse secondary antibody (Promega). Horseradish peroxidase was detected with Supersignal West Pico Chemiluminescent substrate (Pierce).

Chromatin immunoprecipitations (ChIPs): ChIP assays were performed as previously described (Greer, Zika et al. 2003). Briefly, HeLa cells were stimulated with 500 U/ml IFN- γ (Peprotech, Rocky Hill, NJ) as indicated. Cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature. Crosslinking was halted 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, dH₂O) with Complete EDTA-free protease inhibitors (Roche) for 20 minutes on ice and were sonicated at constant pulse to generate an average of 500-750 bp sheared DNA. Sonicated lysates were precleared with salmon-sperm coated agarose beads (Upstate) and half of the lysate was IP with 5µg of polyclonal antibody against, H3K4me3 (Abcam), H3K36me3 (Abcam), H3 (Upstate), H3R17me2 (Abcam), CARM1 (Abcam), H2BK120ub (MediMabs), H2B (Upstate), Sug1 (Novus Biologicals), WDR5 (Abcam) and Ash2L (Abcam) overnight at 4°C. The remaining portion of lysate was IP with isotype control antibody (Upstate). Following an additional 2 hour IP with 60µl 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, dH₂O), High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH₂O), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH₂O) and 1xTE buffer and were eluted with SDS elution buffer (1% SDS, 0.1M NaHCO₃, dH₂O). Following elution, crosslinks were reversed overnight with 5M NaCl at 65°C and IP and control DNA was isolated using phenol:chloroform:isoamyl alcohol mix (Invitrogen) as per the manufacturer's instructions. Isolated DNA was analyzed by real-time PCR using previously published probes and primers spanning the W-X-Y box of the MHC-II *HLA-DRA* promoter, MHC-II *HLA-DRA* exon III, MHC-II *HLA-DRA* exon V, GAPDH promoter (Koues, Dudley et al. 2008) and CIITA pIV. CIITA pIV primer and probe sequences were as follows: sense primer 5'-CAGTTGGGATGCCACTTCTGA-3', antisense primer 5'-TGGAGCAACCAAGCACCTACT-3' and probe 5'-6FAM-AAGCACGTGGTGGCC-TAMRA-3'. Values graphed were calculated based on standard curves generated. *Chromatin immunoprecipitation in ATPase knockdowns:* HeLa cells were transfected with ATPase specific siRNA (Qiagen) or control siRNA (Qiagen)

using RNAiFect transfection reagent (Qiagen) according to manufacturer's protocol. Cells were treated with 500U/ml IFN- γ as indicated and 10% of the total cell volume was lysed with 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with Complete EDTA-free protease inhibitors (Roche) and analyzed via western blot for ATPase knockdown verification. The remaining fraction of cells was subjected to ChIP assay.

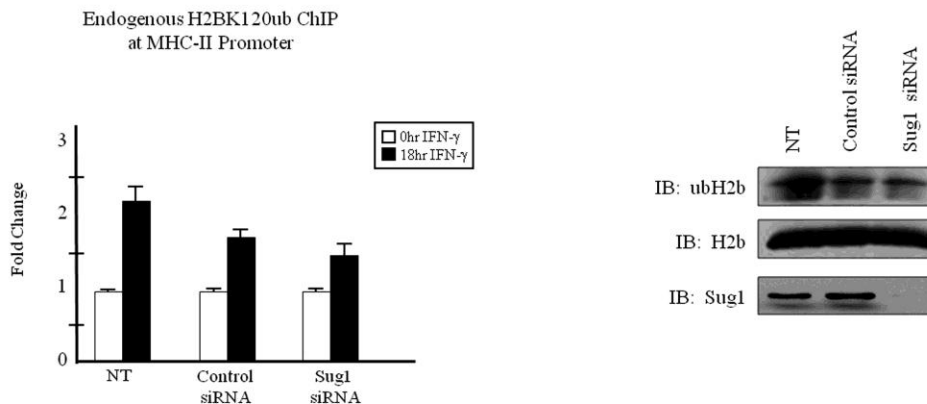
RESULTS

Sug1 is not critical for histone H2B ubiquitination at lysine 120. The 19S proteasome is recruited to active promoters and functions to regulate transcription by aiding in the opening of chromatin structure (Gonzalez, Delahodde et al. 2002; Lee, Ezhkova et al. 2005; Lassot, Latreille et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). In yeast cells, 19S ATPases associate with the chromatin remodeling Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, are required for efficient histone H3 acetylation, and have been implicated in regulating histone methylation modifications (Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005; Laribee, Shibata et al. 2007). In mammalian systems, components of the 19S proteasome are recruited to inducible promoters (Zhu, Wani et al. 2007; Bhat, Turner et al. 2008) and we have previously shown that 19S ATPases positively regulate histone acetylation at cytokine inducible promoters (Koues, Dudley et al. 2008). In yeast, recruitment of 19S ATPases require prior histone H2B monoubiquitination and inactivating mutations of these ATPases prevent subsequent H3K4 methylation (Ezhkova and Tansey 2004). Until recently, studying H2B ubiquitination in higher eukaryotes has been difficult due to the lack of a suitable antibody recognizing ubH2B. However, a recent monoclonal antibody recognizing histone H2B ubiquitinated at lysine 120 (ubH2B) has provided evidence that ubH2B is found associated with robustly expressed genes

and extends into coding sequence (Minsky, Shema et al. 2008). Consistent with this, we observed that levels of ubH2B at the MHC-II *HLA-DRA* gene were initially low and were enhanced upon prolonged IFN- γ stimulation (data not shown). MHC-II expression is likewise delayed due to the need to synthesize and bind CIITA and levels of ubH2B mirror the established pattern of MHC-II expression (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005). To investigate contributions of the 19S ATPases to ubH2B, we utilized RNA interference to knockdown endogenous Sug1 expression in HeLa cells (Figure 3-1a, right panel) and then performed ChIP experiments to detect endogenous levels of ubH2B at the MHC-II proximal promoter (Figure 3-1a, left panel). In contrast to the dramatic loss in histone acetylation previously observed in Sug1 knockdown cells (Koues, Dudley et al. 2008), no significant decrease in ubH2B levels were found at the MHC-II promoter (Figure 3-1a, left panel) or within the MHC-II coding sequence (data not shown) in Sug1 siRNA treated cells as compared to control siRNA treated cells or untransfected cells (Figure 3-1a, left panel).

As robust expression of MHC-II genes is delayed in HeLa cells due to the requirement for transcription, translation and recruitment of CIITA (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005), we sought to determine the levels of ubH2B of the more rapidly expressed CIITA pIV. Indeed, a more rapid increase in ubH2B was observed upon IFN- γ stimulation (Figure 3-1b, left panel). However, siRNA-mediated Sug1 knockdown did not significantly impact ubH2B at CIITA pIV (Figure 3-1b, right panel). Furthermore, diminished expression of Sug1 did not impact ubH2B in whole cell lysates (Figure 3-1a, right panel; Figure 3-2). Taken together these experiments suggest that the Sug1 ATPase is not a requirement for H2B ubiquitination but do not rule out a potential role, similar to that observed in yeast (Ezhkova and Tansey 2004), for H2B ubiquitination in recruiting 19S ATPases to

A



B

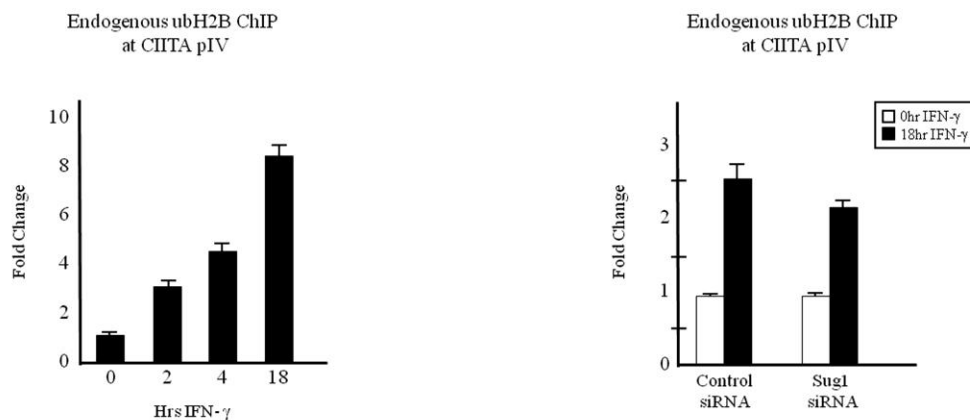


Figure 3-1. Sug1 is not critical for histone H2B ubiquitination at lysine 120. (A) Sug1 knockdown does not significantly impact ubH2B levels. HeLa cells untransfected or transfected with either control or Sug1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were immunoprecipitated (IP) with control or ubiquitinated histone H2B antibody. Associated DNA was isolated and analyzed via real-time PCR using primers specific for the MHC-II promoter. IP values were normalized to unmodified histone levels and presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.5 ± 0.3 . Values represent mean \pm SEM of (n = 3) independent experiments. (B) Sug1 siRNA efficiently decreases Sug1 protein expression, but does not impact global histone H2B ubiquitination. HeLa cells were transfected with control or Sug1 specific siRNA, harvested and subjected to Western blot analysis for endogenous Sug1, global histone H2B and global monoubiquitinated H2B. Results reported are representative data of 3 independent experiments. (C) Sug1 knockdown does not significantly impact ubH2B levels at CIITA pIV. HeLa cells untransfected (left panel) or transfected with control or Sug1 specific siRNA (right panel) were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control or ubiquitinated histone H2B antibody. Associated CIITA pIV DNA was isolated and analyzed via real-time PCR as described in (A). Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.1 ± 0.1 . Values represent mean \pm SEM of (n = 3) independent experiments.

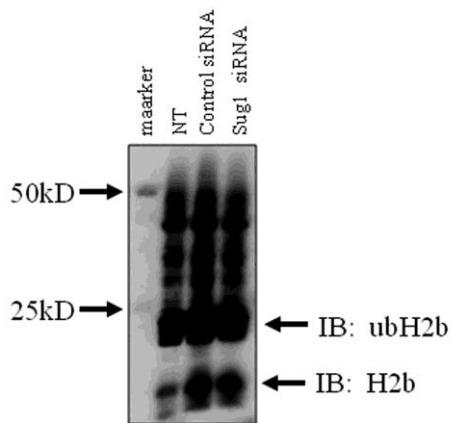
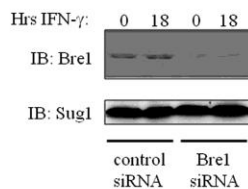


Figure 3-2. H2B polyubiquitination is unaffected by Sug1 knockdown. Global histone H2B polyubiquitination is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control siRNA duplexes, or with Sug1 specific siRNA duplexes. Lysates were immunoblotted (IB) for ubH2B. Results reported are representative data of 3 independent experiments.

inducible gene promoters. To determine if 19S recruitment is dependent on H2B ubiquitination, RNAi was used to knockdown the H2B ubiquitin ligase, hBre1 (Figure 3-3a). Chromatin immunoprecipitations in these Bre1 knockdown cells showed reduced H2B ubiquitination (Figure 3-3b). However, the loss of H2B ubiquitination did not impact Sug1 binding to CIITA pIV (Figure 3-3c).

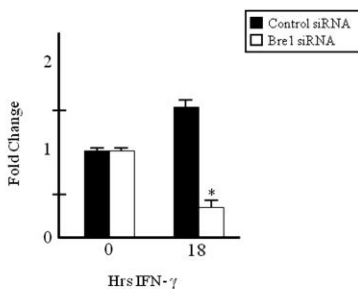
Sug1 inhibits histone H3 arginine 17 dimethylation. We have recently shown that Sug1 plays a critical role in stabilizing MHC-II promoter recruitment of the histone acetyltransferase CBP and in acetylating histone H3 at lysine 18 (Koues, Dudley et al. 2008). Previous studies have indicated a link between the HAT CBP which can acetylate histone H3 at lysine 18 and the coactivator-associated arginine methyltransferase 1 (CARM1) which mediates H3 arginine 17 dimethylation (H3R17me₂) (Ma, Baumann et al. 2001; Daujat, Bauer et al. 2002; Zika, Fauquier et al. 2005). At the MHC-II promoter, CBP and CARM1 bind in parallel and CARM1 dependent methylation of CBP likely stabilizes association of CBP with the promoter to allow for histone acetylation and chromatin opening (Zika, Fauquier et al. 2005). Therefore, we sought to determine if the 19S proteasome also plays a role in CARM1 mediated events. CARM1 is recruited to the *HLA-DRA* promoter rapidly upon cytokine stimulation (Figure 3-4a) preceding CIITA recruitment by several hours (Figure 3-5) (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005; Koues, Dudley et al. 2008). Furthermore, endogenous CARM1 associated with immunoprecipitated endogenous Sug1 in Raji (Burkitt's lymphoma-derived) B cells (Figure 3-4b, upper panel, lane 3). Similarly, Myc-Sug1 associated with immunoprecipitated endogenous CARM1 (Figure 3-6) in unstimulated HeLa cells which lack CIITA expression.

A



B

Endogenous H2BK120ub ChIP at CIITA pIV



C

Endogenous Sug1 ChIP at CIITA pIV

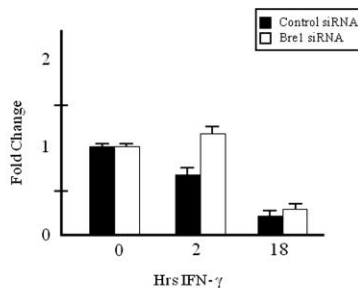


Figure 3-3. H2B ubiquitination at lysine 120 is not critical for Sug1 recruitment to CIITA pIV. (A) hBre1 siRNA efficiently decreases hBre1 protein expression. HeLa cells (untreated or stimulated with IFN- γ) were transfected with control or hBre1 specific siRNA, harvested and subjected to Western blot analysis for endogenous hBre1 (top) and endogenous Sug1 (bottom). Western blot shows >80% knockdown of hBre1. (B) hBre1 siRNA efficiently decreases CIITA pIV H2B ubiquitination. HeLa cells transfected with scrambled control or hBre1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with isotype control or ubiquitinated histone H2B antibody. Associated CIITA pIV DNA was isolated and analyzed via real-time PCR as in Figure 1. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.1 ± 0.1 . Values represent mean \pm SEM of (n = 3) independent experiments. (C) H2B ubiquitination at lysine 120 is not critical for Sug1 recruitment. HeLa cells transfected with scrambled control or hBre1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control or endogenous Sug1 antibody. Associated DNA was isolated and analyzed via real-time PCR using primers spanning CIITA pIV. Real-time PCR values were normalized to the total amount of CIITA pIV DNA added to the reaction (input). Input values represent 5% of the total cell lysate. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control IP values were 0.5 ± 0.1 . Values represent mean \pm SEM of (n = 2) independent experiments.

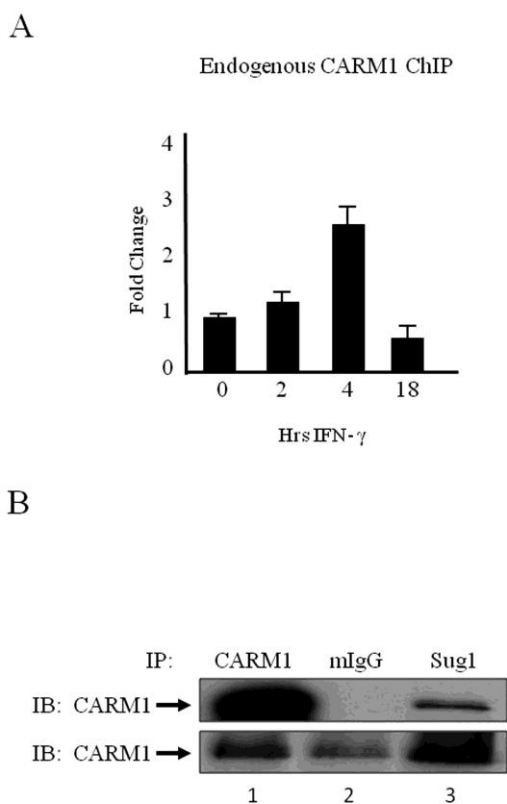


Figure 3-4. CARM1 associates with Sug1. (A) CARM1 associates with the MHC-II proximal promoter. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or endogenous CARM1 antibody. Associated MHC-II DNA was isolated and analyzed via real-time PCR as described in figure 2. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.5 ± 0.2 . Values represent mean \pm SEM of (n = 3) independent experiments. (B) Sug1 associates with CARM1. Raji cells were lysed and immunoprecipitated (IP) with endogenous Sug1 (top panel, lane 3). Control samples were IP with endogenous CARM1 antibody (top panel, lane 1) and isotype control IgG (top panel, lane 2). IP and lysate control samples (bottom panel) were immunoblotted (IB) for CARM1. Results reported are representative data of 3 experiments.

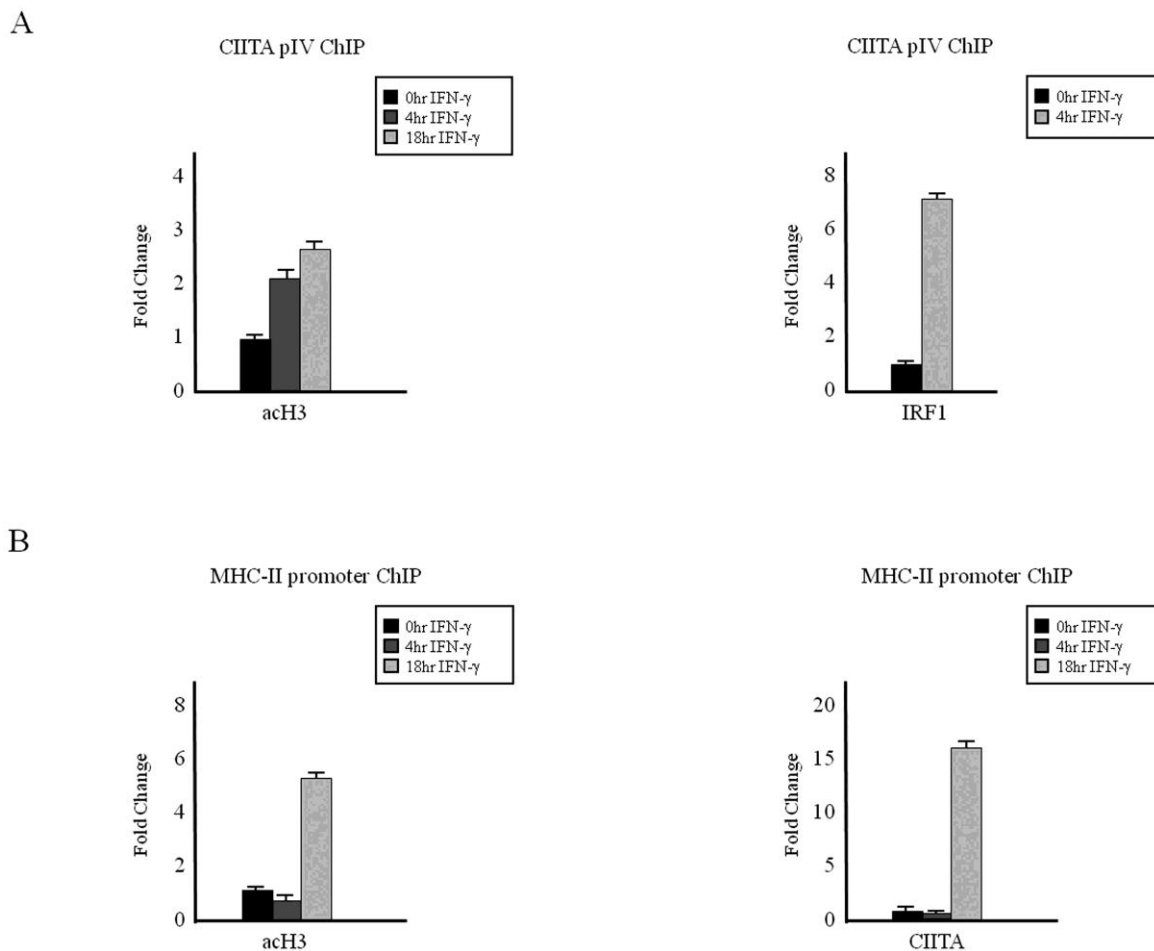


Figure 3-5. CIITA pIV and the MHC-II proximal promoter are differentially activated upon cytokine stimulation. (A) CIITA pIV is activated rapidly upon cytokine stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ as indicated. Lysates were IP with control, endogenous acetylated H3, or endogenous IRF1 antibody. Associated DNA was isolated and analyzed via real-time PCR. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.5 ± 0.2 . Values represent mean \pm SEM of ($n = 3$) independent experiments. (B) The MHC-II proximal promoter is activated upon prolonged cytokine stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ as indicated. Lysates were IP with control, endogenous acetylated H3, or endogenous CIITA antibody. Associated DNA was isolated and analyzed via real-time PCR. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.4 ± 0.1 . Values represent mean \pm SEM of ($n = 3$) independent experiments.

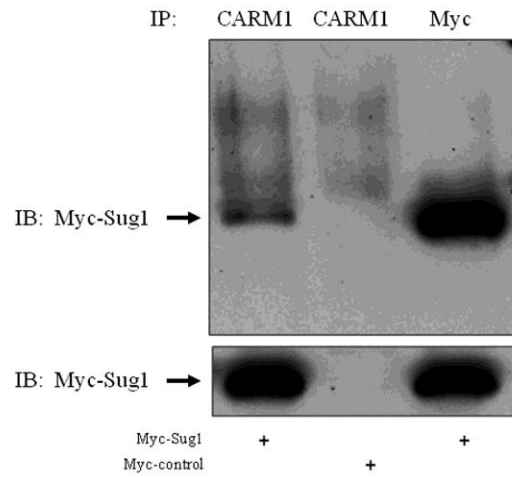


Figure 3-6. CARM1 associates with Sug1 in the absence of CIITA. Unstimulated HeLa cells transfected with Myc-Sug1 or empty vector were lysed and IP with polyclonal antibody against CARM1 (top panel, lane 3). Control samples were IP with myc beads (top panel, lane 3). IP and lysate control samples (bottom panel) were IB for Myc. Results reported are representative data of 3 experiments.

CARM1 is known to specifically enhance transcriptional activation by methylating histone H3 at arginine 17 rapidly upon cytokine stimulation at MHC-II promoters (Bauer, Daujat et al. 2002). Chromatin immunoprecipitation experiments confirm that levels of H3R17me2 parallel CARM1 recruitment to the MHC-II promoter (data not shown). Therefore, we sought to determine the pattern of H3R17me2 at the MHC-II *HLA-DRA* promoter under Sug1 knockdown conditions (Figure 3-7a). Sug1 siRNA transfected HeLa cells lacked IFN- γ enhancement of H3R17me2 levels at the MHC-II proximal promoter (Figure 3-7a, white bars) as compared to similarly treated control siRNA transfected cells (Figure 3-7a, black bars). To determine if Sug1 knockdown influences H3R17me2 at additional genes, chromatin immunoprecipitations were performed and isolated DNA was subjected to real-time PCR using primers specific for the cytokine inducible *CIITA* pIV as well as *GAPDH*. A loss of *CIITA* pIV H3R17me2 was observed upon siRNA-mediated Sug1 knockdown (Figure 3-7b), but *GAPDH* promoter H3R17me2 levels were unaffected (Figure 3-7c). To further ensure that the loss of H3R17me2 observed at the two cytokine inducible promoters is not indicative of a total loss of H3R17me2 in the cells, whole cell lysates were immunoblotted for endogenous levels of H3R17me2 (Fig.4D). Although levels of Sug1 were efficiently diminished (Figure 3-7d, bottom panel), cells treated with Sug1 specific siRNA showed H3R17me2 levels equal to those observed in control siRNA treated and untreated cells (Figure 3-7d, top panel). Taken together, these experiments strongly implicate the 19S ATPase Sug1 in mediating methylation events at cytokine inducible genes.

Sug1 regulation of histone H3 lysine methylation is localized to the MHC-II *HLA-DRA* promoter. We have previously shown that 19S ATPases are recruited to the MHC-II proximal promoter (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). In the absence of the

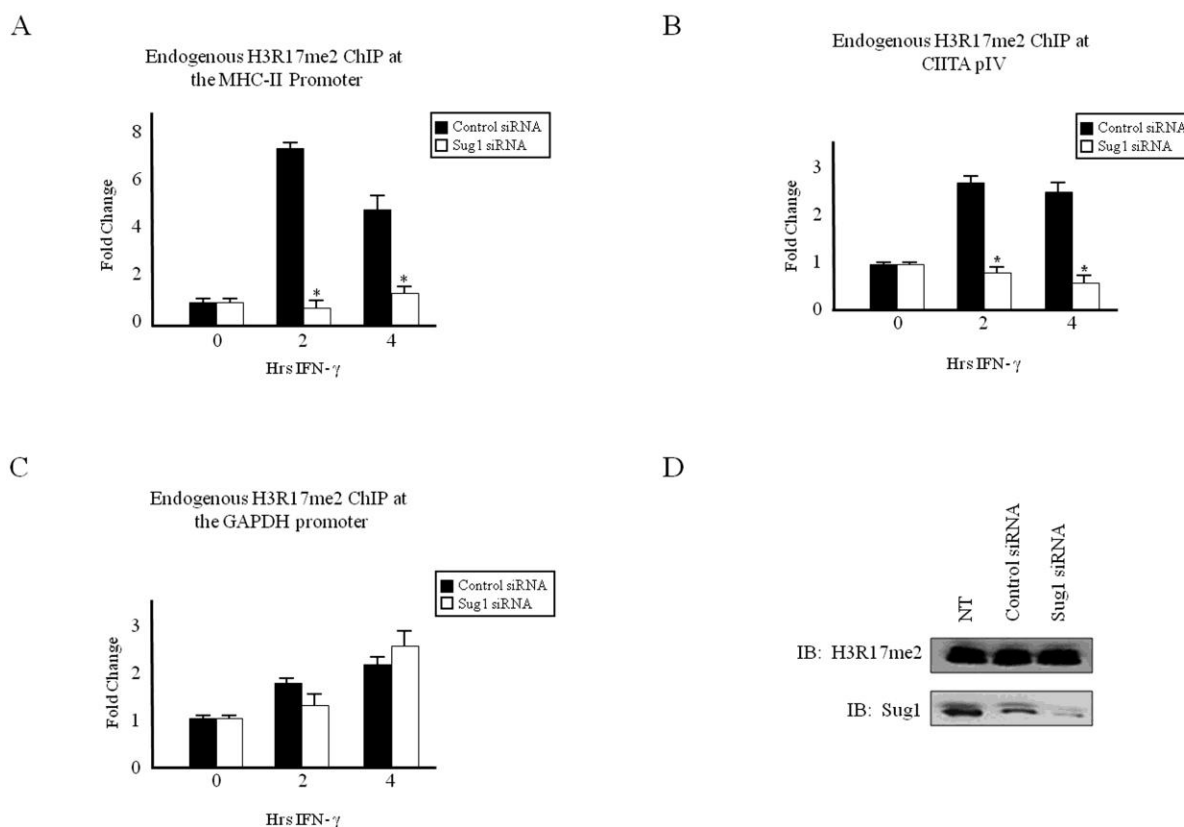


Figure 3-7. Sug1 knockdown prevents inducible histone H3 arginine 17 dimethylation. (A-C) Sug1 knockdown decreases H3R17me2 at IFN- γ inducible promoters. HeLa cells transfected with scrambled control or Sug1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with isotype control or H3R17me2 antibody. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 1 using primers specific for the MHC-II promoter (A), CIITA pIV (B) or the GAPDH promoter (C). Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.8 ± 0.1 . Values represent mean \pm SEM of (n = 3) independent experiments. *P < 0.05 vs control siRNA. (D) Global histone H3R17me2 is unaffected by Sug1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control or Sug1 specific siRNA. Lysates were immunoblotted (IB) for H3R17me2 (upper panel) or for endogenous Sug1 (lower panel). Results reported are representative data of 3 independent experiments.

19S ATPase Sug1, histone H3 is hypoacetylated and IFN- γ stimulation fails to recruit CIITA to the MHC-II promoter, resulting in a dramatic loss of MHC-II gene expression (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). In yeast, components of the 19S proteasome have been identified as a link between histone ubiquitination and activating histone lysine methylation events (Ezhkova and Tansey 2004; Laribee, Shibata et al. 2007). Previous studies have shown that at MHC-II genes, trimethylation of histone H3 lysine 4 (H3K4me3) overlaps spatially and temporally with histone H3 acetylation events (Gomez, Majumder et al. 2005; Rybtsova, Leimgruber et al. 2007; Chou and Tomasi 2008). Therefore we sought to determine if Sug1 plays a role in mediating posttranslational histone lysine methylation modifications in mammalian cells. To investigate contributions of the 19S ATPases to activating H3K4me3 and H3K36me3, we utilized RNA interference to knockdown endogenous Sug1 expression in HeLa cells and then performed ChIP experiments to detect endogenous levels of these two modifications at the MHC-II *HLA-DRA* gene (Figure 3-8). Consistent with previous reports (Chou and Tomasi 2008), ChIP mapping experiments demonstrated that IFN- γ inducible levels of H3K4me3 are localized to the MHC-II promoter whereas H3K36me3 levels are localized to MHC-II coding sequence (data not shown). RNAi-mediated knockdown of Sug1 negatively impacted H3K4me3 levels at the MHC-II promoter (Figure 3-8b). In contrast, H3K36me3 levels localized to MHC-II coding sequence were not significantly impacted by the Sug1 knockdown (Figure 3-8c). As an additional control for Sug1 siRNA specificity, reconstituting Sug1 expression in cells transfected with Sug1 siRNA, restores *HLA-DRA* promoter activity (Figure 3-9).

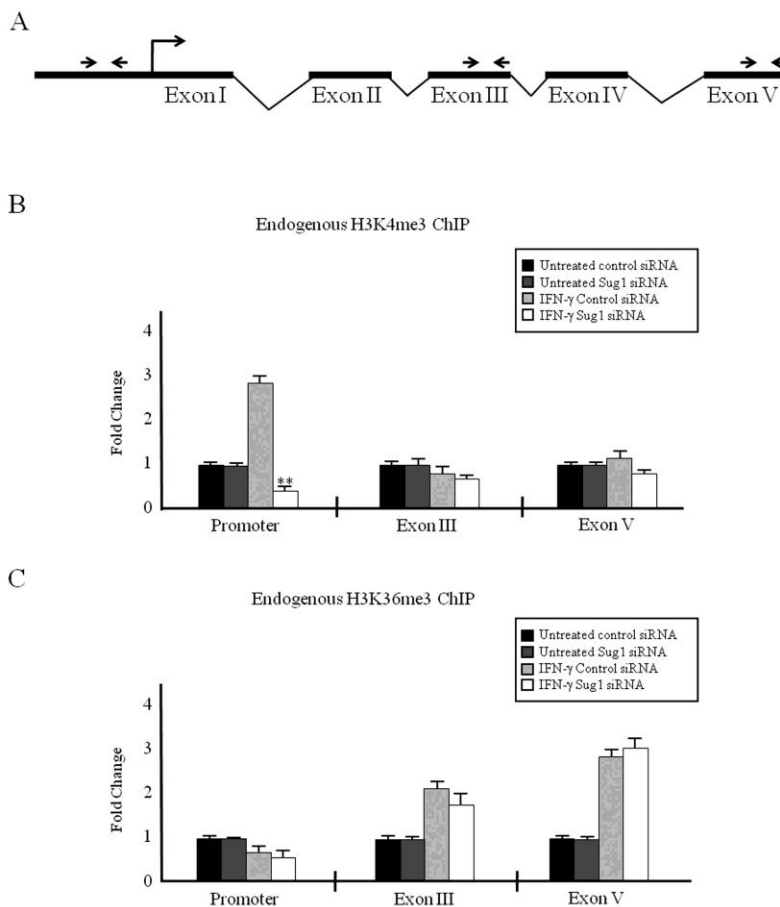


Figure 3-8. Sug1 knockdown decreases histone H3 trimethylation at lysine 4. (A) Location of MHC-II primer/probe sets. (B-C) Sug1 preferentially targets H3K4me3 and effects are localized at the MHC-II genes proximal promoter. HeLa cells transfected with scrambled control or Sug1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control, H3K4me3 (B), or H3K36me3 (C) antibody. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 1 using primers and probes specific for the MHC-II promoter, exon III, and exon V. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.2 ± 0.2 . Values represent mean \pm SEM of (n = 3) independent experiments. ** P < 0.01 vs control siRNA.

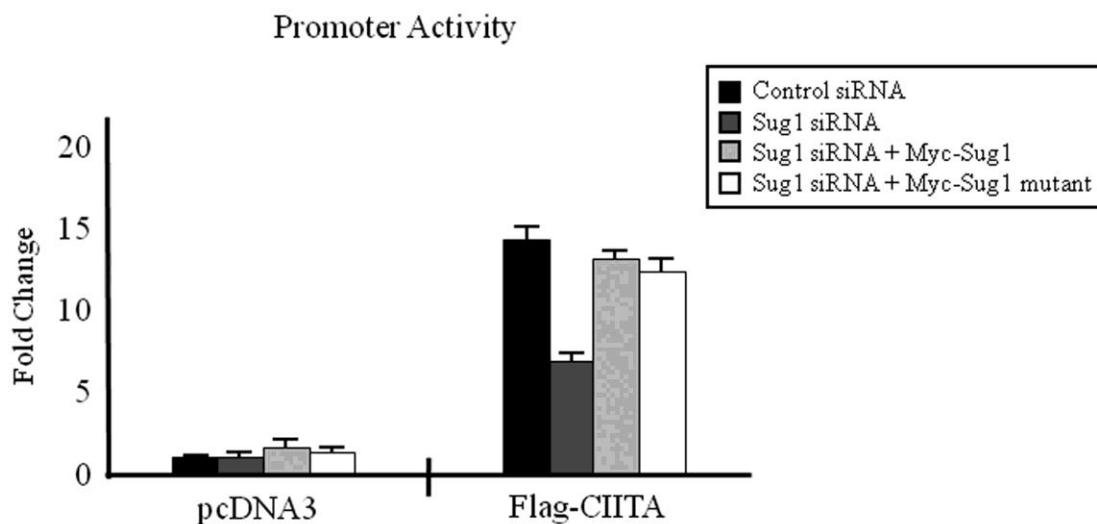


Figure 3-9. Overexpressing Myc-Sug1 restores promoter activity in Sug1 knockdown cells. HeLa cells were transfected with control or Sug1 siRNA and were co-transfected with *HLA-DRA-Luc*, Renilla, pcDNA3, CIITA, Sug1 or ATPase mutant Sug1 as indicated. Luciferase activity is reported as fold activation relative to that of the reporter alone. Luciferase readings were normalized to Renilla activity. Luciferase assays were performed in triplicate and values represent mean \pm SEM of (n = 3) independent experiments.

Sug1 knockdown decreases CIITA pIV promoter specific histone H3 trimethylation at lysine 4. We have previously observed that Sug1 positively regulates histone acetylation (Koues, Dudley et al. 2008) and H3R17me2 (Figure 3-7b) at the IFN- γ inducible CIITA pIV. To determine if a similar regulatory pattern occurs with H3K4me3, chromatin immunoprecipitation experiments were performed to determine levels of H3K4me3 at CIITA pIV upon treatment with Sug1 specific siRNA. Consistent with published reports (Ni, Karaskov et al. 2005), H3K4me3 levels do not accumulate at CIITA pIV until prolonged IFN- γ stimulation (Figure 3-10a). However, similar to observations at the MHC-II promoter, RNAi-mediated Sug1 knockdown has a negative effect on inducible H3K4me3 levels at CIITA pIV (Figure 3-10b). To verify that the loss of H3K4me3 is due to diminished Sug1 expression and not loss of proteasomal function, chromatin immunoprecipitation experiments were also performed in the presence of proteasome inhibitor MG132 (Figure 3-10b). Proteasomal inhibition did not impact H3K4me3 levels at CIITA pIV. Levels of H3K4me3 in cells treated with MG132 (Figure 3-10b) were comparable to those in untreated samples. Furthermore, Sug1 knockdown showed no impact on H3K4me3 levels at the constitutively active GAPDH promoter (Figure 3-10c). To ensure that the loss of H3K4me3 observed at these two cytokine inducible promoters is not indicative of a global decrease in H3K4me3, whole cell lysates were immunoblotted for endogenous levels of H3K4me3 (Figure 3-10d). Although levels of Sug1 were efficiently diminished (Figure 3-10d, bottompanel), cells treated with Sug1 specific siRNA showed levels of total H3 and trimethylation at both lysine 4 and lysine 36 that were equivalent to those observed in control siRNA treated cells (Figure 3-10d). These studies strongly emphasize a specific role for the

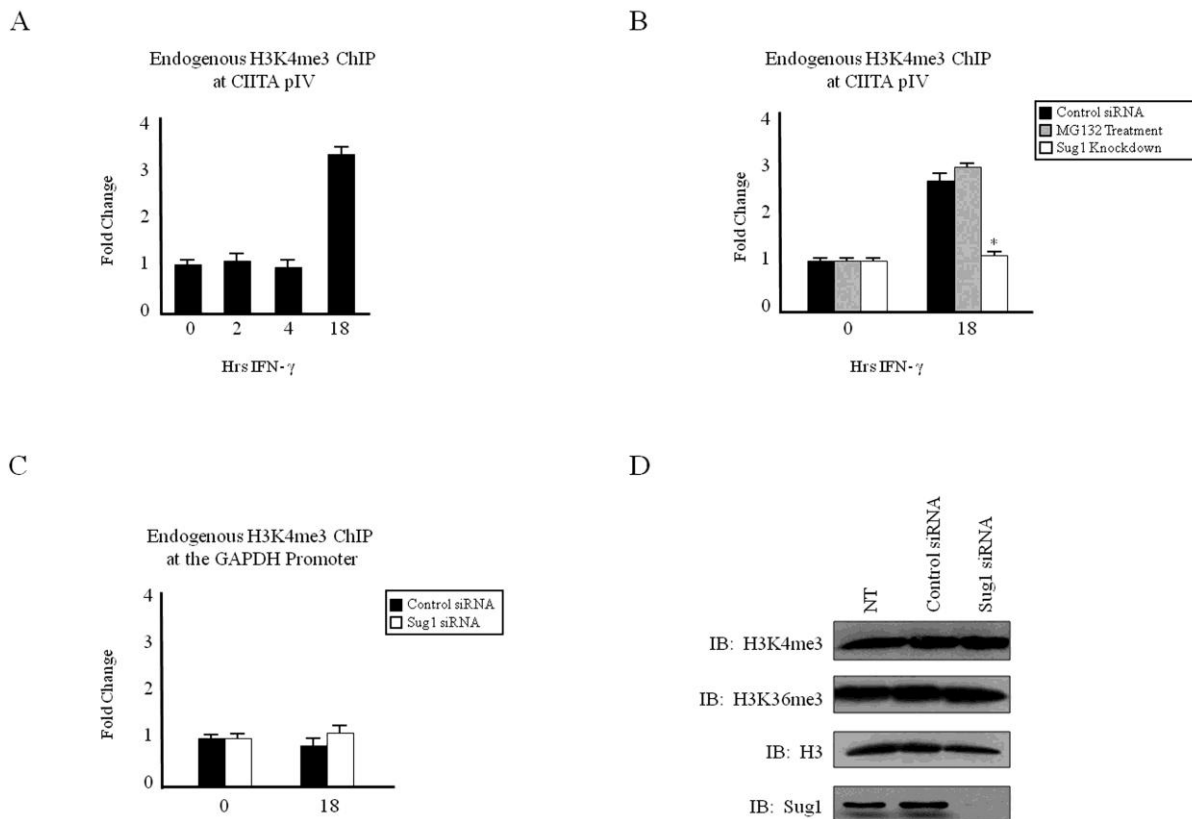


Figure 3-10. Suggest 1 knockdown decreases histone H3K4 trimethylation in a promoter specific manner. (A) Levels of H3K4me3 at CIITA pIV are enhanced upon prolonged IFN- γ stimulation. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or H3K4me3 antibody. Associated CIITA pIV DNA was isolated and analyzed via real-time PCR as described in Figure 1. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.4 ± 0.4 . Values represent mean \pm SEM of ($n = 4$) independent experiments. (B-C) Suggest 1 knockdown, not proteasomal inhibition, decreases histone H3K4me3 at CIITA pIV. HeLa cells transfected with scrambled control or Suggest 1 specific siRNA were stimulated with IFN- γ and treated with proteasomal inhibitor MG132 as indicated and subjected to ChIP assay. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 1 using primers and probes specific CIITA pIV (B) and the GAPDH promoter (C). Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.1 ± 0.1 . Values represent mean \pm SEM of ($n = 3$) independent experiments. * $P < 0.05$ vs control siRNA. (D) Global histone H3K4me3 is unaffected by Suggest 1 knockdown. HeLa cells were left untreated, or were transfected with either scrambled control or Suggest 1 specific siRNA as indicated. Lysates were immunoblotted (IB) for H3K4me3, H3K36me3, H3 or for endogenous Suggest 1. Results reported are representative data of 3 independent experiments.

Sug1 ATPase in regulating promoter specific activating histone methylation events at cytokine inducible genes independent of proteasomal degradation.

Sug1 is critical for recruitment of a hCompass subunit. We have previously shown that Sug1 positively regulates CBP mediated histone H3 lysine 18 acetylation (Koues, Dudley et al. 2008) and now show that Sug1 also positively regulates CARM1 mediated H3R17me2 as well as SET1 mediated H3K4me3 at the IFN- γ inducible promoters. The H3K4 methyltransferase SET1 is recruited to DNA as part of a large complex of proteins, hCompass (Lee, Tate et al. 2007; Wu, Wang et al. 2008). Chromatin immunoprecipitations show that a common subunit of hCompass, WDR5 (Figure 3-11a) binds constitutively to CIITA pIV with moderate enhancement observed early upon cytokine stimulation. Previous studies have demonstrated association of CBP with immunoprecipitated MLL (mixed lineage leukemia)-like methyltransferases (Demers, Chaturvedi et al. 2007). To determine if WDR5 similarly interacts with additional histone modifying enzymes, co-immunoprecipitation experiments were performed in unstimulated HeLa cells overexpressing the hCompass subunit WDR5 (Figure 3-11b). Both the HAT CBP (Figure 3-11b, lane 3) and arginine HMTase CARM1 (Figure 3-11c, lane 4) associate with Flag-WDR5. Although endogenous WDR5 expression is unaltered in Sug1 knockdown cells (Figure 3-11c), IFN- γ inducible WDR5 recruitment to CIITA pIV, but not the GAPDH promoter, is inhibited (Figure 3-11d). Similarly Ash2L, an additional hCompass subunit, fails to bind to CIITA pIV upon Sug1 knockdown (data not shown). Taken together, these results suggest that the 19S proteasome may play a role in targeting stable hCompass association and multi-enzyme recruitment at cytokine inducible promoters.

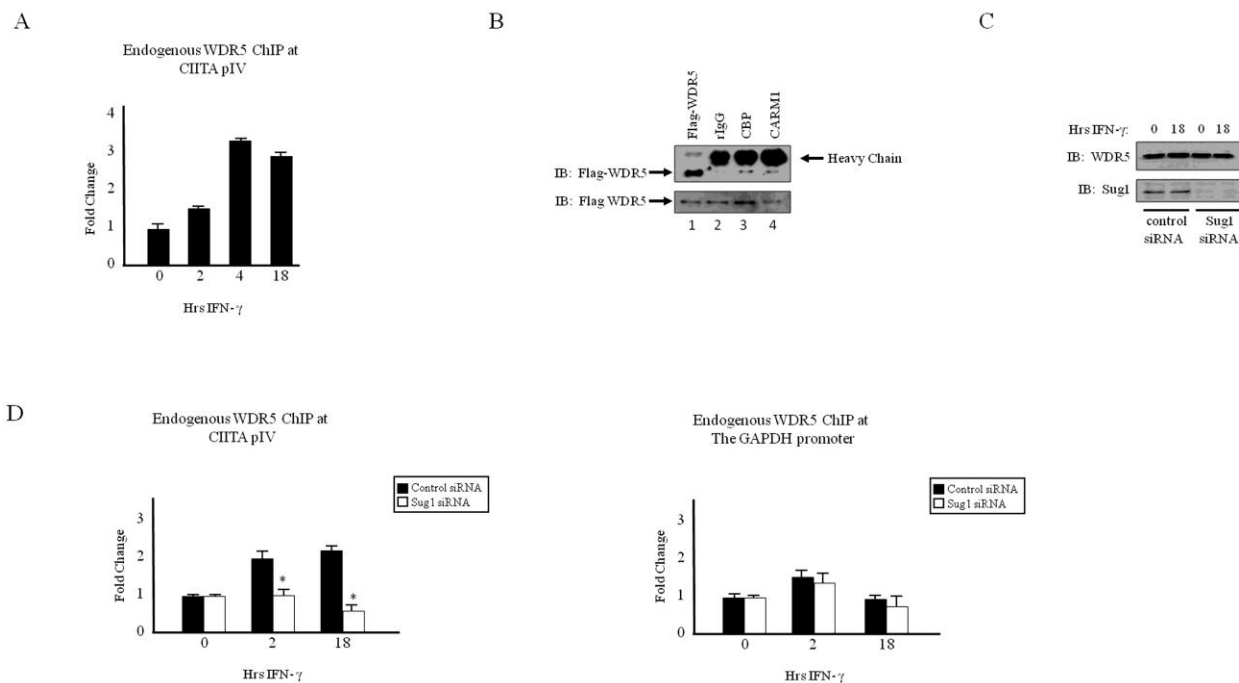


Figure 3-11. Sug1 is critical for recruitment of a hCompass subunit. (A) Common hCompass subunit WDR5 associates with CIITA pIV. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or endogenous WDR5 antibody. Associated CIITA pIV DNA was isolated and analyzed via real-time PCR as described in figure 2. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control values were 0.2 ± 0.2 . Values represent mean \pm SEM of ($n = 2$) independent experiments. (B) WDR5 associates with CBP and CARM1. Unstimulated HeLa cells transfected with Flag-WDR5 were lysed and immunoprecipitated (IP) with endogenous CBP (top panel, lane 3) or endogenous CARM1 (top panel, lane 4). Control samples were IP with Flag-beads (top panel, lane 1) and isotype control IgG (top panel, lane 2). IP and lysate control samples (bottom panel) were immunoblotted (IB) for Flag. Results reported are representative data of 3 experiments. (C) Sug1 knockdown does not impact endogenous WDR5 expression. HeLa cells (untreated or stimulated with IFN- γ) were transfected with control or Sug1 specific siRNA, harvested and subjected to Western blot analysis for endogenous WDR5 (top) and endogenous Sug1 (bottom). (D) Sug1 knockdown decreases WDR5 association with CIITA pIV. HeLa cells transfected with scrambled control or Sug1 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control or endogenous WDR5 antibody. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 2 using primers and probes specific for CIITA pIV (left) and the GAPDH promoter (right). Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control IP values were 0.1 ± 0.1 . Values represent mean \pm SEM of ($n = 2$) independent experiments. * $P < 0.05$ vs control siRNA.

DISCUSSION

Previous studies from our laboratory indicate that 19S ATPases regulate transcription at inducible mammalian genes by recruiting histone acetyltransferases and activators to activated promoters. We have demonstrated that following IFN- γ stimulation, 19S ATPases rapidly bind the MHC-II proximal promoter and that diminished expression of the ATPase Sug1 substantially inhibits MHC-II promoter acetylation and decreases recruitment of the transactivator CIITA, resulting in diminished MHC-II expression (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008). Results shown here further this argument and indicate that the 19S proteasome participates in the cascade of regulatory histone modifications that occurs following stimulation of mammalian gene transcription. We showed recently that the loss of association of the HAT CBP with the MHC-II proximal promoter correlates with the dramatic inhibition of H3K18 acetylation (Koues, Dudley et al. 2008). Others have shown in yeast that the 19S complex is important for targeting HATs to promoters (Lee, Ezhkova et al. 2005) and that mutations in 19S ATPases specifically disrupt H3K4 and -K79 methylation (Ezhkova and Tansey 2004). We now demonstrate that Sug1 knockdown impairs the additional activating HMTase mediated modification of H3R17 at both the MHC-II promoter and CIITA pIV. These data indicate an inability of CARM1, the HMTase responsible for the H3R17me2 modification, to bind inducible promoters in the absence of Sug1. Despite multiple attempts, we were unable to test this hypothesis as we failed to successfully perform chromatin immunoprecipitation experiments to isolate endogenous CARM1 at MHC-II promoter sequences in siRNA transfected cells. However, given the substantial evidence that CBP and CARM1 crosstalk on chromatin to link H3 acetylation and arginine methylation (Ma, Baumann et al. 2001; Daujat, Bauer et al. 2002; Zika, Fauquier et al. 2005), it is not surprising that CARM1 mediated H3R17me2 is regulated by Sug1.

The roles of histone methylation in regulating transcription are complex as this modification is associated with both gene expression and gene silencing. H3K36 methylation is found within actively transcribed genes and is thought to have a role in transcriptional termination and/or early RNA processing (Krogan, Kim et al. 2003; Xiao, Hall et al. 2003; Bannister, Schneider et al. 2005). In addition, histone H3K4 hypermethylation has been found at active genes and may inhibit methylated H3K9 gene silencing, perhaps allowing for acetylation to occur (Wang, Cao et al. 2001; Santos-Rosa, Schneider et al. 2002; Schneider, Bannister et al. 2004; Francis, Chakrabarti et al. 2005). Here we show that when Sug1 is knocked down, levels of histone H3K4me3 are markedly reduced at the cytokine induced MHC-II proximal promoter and CIITA pIV. Sug1 appears to mediate H3K4me3 at inducible promoters as H3K4me3 at the GAPDH promoter is not impacted by Sug1 knockdown and whole cell lysates show no global change in levels of H3K4me3. Furthermore, the effects observed on histone H3 methylation are localized to the promoter as H3K36me3, which is found throughout the MHC-II coding sequence, is not impacted by Sug1 knockdown. Importantly, the effects of Sug1 are independent of proteasome proteolytic function, as proteasomal inhibition does not impact promoter H3K4me3. Additionally as a control for siRNA specificity, reconstituting Sug1 expression in Sug1 siRNA transfected cells restores promoter activity.

Promoter methylation of H3K4 is thought to promote activating acetylation events at promoters; our results therefore link the 19S proteasome and its ATPase components to transcriptional regulation by demonstrating preferential promoter regulation of activating histone modifications by the 19S ATPase Sug1. As further evidence, we show that WDR5, a component of the Set1 containing histone methyltransferase hCompass, associates with two additional histone modifying enzymes, CBP and CARM1. Moreover, WDR5 recruitment to CIITA pIV is

inhibited when endogenous Sug1 expression is diminished. The novelty in our study results from our observation that the effects of 19S ATPases on mammalian transcription extend beyond recruitment and regulation of enzymes responsible for lysine residue modifications. Our data argues that upon gene activation, 19S ATPases reorganize chromatin to allow multiple specific enzymes access to their target residues in a promoter specific fashion. We propose that one way in which 19S ATPases facilitate elongation is promoting binding of multiple enzymes responsible for activating modifications to histone H3.

Studies in yeast have identified 19S ATPases to be a link between H2B monoubiquitination and activating lysine methylation modifications (Wood, Schneider et al. 2003; Ezhkova and Tansey 2004; Laribee, Shibata et al. 2007; Lee, Shukla et al. 2007). The effects of ubiquitination of histones are complex with reports indicating roles for histone ubiquitination in both gene inactivation and activation. Indeed, ubiquitination of histone H2A has recently been shown to precede gene silencing (de Napoles, Mermoud et al. 2004; Cao, Tsukada et al. 2005) while ubiquitination of histone H2B is associated with robustly transcribed regions of DNA (Henry, Wyce et al. 2003; Minsky, Shema et al. 2008). We are the first to map the ubiquitination of histone H2B at both the MHC-II promoter and at CIITA pIV. Our observations that a decrease in endogenous levels of Sug1 prevents efficient H3K4me₃, but not H2B ubiquitination, first suggested that similar links of H2B ubiquitination and activating lysine modifications exist in higher eukaryotes and that recruitment of mammalian 19S ATPases to chromatin might require ubiquitination of H2B. However, the 19S ATPase Sug1 was efficiently recruited to CIITA pIV in the presence of reduced H2B ubiquitination, implying that an additional mechanism/s exists in mammalian cells to initiate recruitment of 19S ATPases following transcription stimulation.

Ours is the first report demonstrating a role for the 19S proteasomal ATPases in regulating the initial activating methylation events at inducible mammalian promoters. Our observations that in the absence of Sug1, activating methylation and acetylation modifications to histone H3 do not occur while H2B ubiquitination does, strongly implicate the 19S ATPases in targeting histone modifying enzymes to activated mammalian genes and indicate specificity in not only the enzymes recruited, but in the residues targeted.

ACKNOWLEDGEMENTS. This work was supported by funding from the Molecular Basis of Disease Area of Focus at Georgia State University (in support of O.I.K.), the HHMI Biotechnology Scholars Program (in support of R.K.D.), and grants from the American Cancer Society; the Georgia Cancer Coalition; and the Georgia Research Alliance (to S.F.G.).

CHAPTER 4THE 19S PROTEASOME PROMOTES THE RELAXATION OF THE CHROMATIN
STRUCTURE AT CYTOKINE INDUCIBLE GENES

NOTE: I PERFORMED ALL OF THE EXPERIMENTS IN THIS CHAPTER, EXCEPT FOR
DUPLICATION OF OF THE LUCIFERASE REPORTER ASSAYS DONE BY NINAD
T. MEHTA AND JEANNE K BROOKS

SUMMARY

Studies indicate that the 19S proteasome contributes to chromatin reorganization, independent of the role the proteasome plays in protein degradation. We have previously shown that components of the 19S proteasome are critical for regulating inducible histone activation events in mammalian cells. The 19S ATPase Sug1 binds to histone remodeling enzymes and, in the absence of Sug1 a subset of activating epigenetic modifications including histone H3 acetylation, H3 lysine 4 trimethylation and H3 arginine 17 dimethylation are inhibited at cytokine inducible MHC-II and CIITA promoters. Furthermore, we observed that a subunit of histone H3 lysine 4 methyltransferase MLL/COMPASS binds activating histone modifying enzymes in addition to the HMTase Set1, implicating Sug1 in recruiting multiple enzyme complexes required to for initiate mammalian transcription. Here we show that multiple subunits of MLL/COMPASS bind constitutively to the inducible MHC-II and CIITA promoters and that over-expressing a single MLL/COMPASS subunit significantly enhances promoter activity. In addition, we show that H3 lysine 27 trimethylation is elevated in the presence of diminished Sug1, suggesting that the 19S proteasome plays a role in the initial reorganization of events enabling the relaxation of the repressive chromatin structure surrounding inducible promoters.

BACKGROUND

Critical for immune system protection against invading pathogens and tumor formation are major histocompatibility class II (MHC-II) molecules, which are cell surface glycoproteins that function to present antigenic peptides to CD4⁺ T lymphocytes of the adaptive immune system (Gerloni and Zanetti 2005; Parham 2005). MHC-II molecules are expressed constitutively in only a small subset of cells referred to as antigen presenting cells (APCs).

However the interferon gamma (IFN- γ) cytokine can induce MHC-II expression in all nucleated cells (Masternak, Barras et al. 1998; Masternak, Muhlethaler-Mottet et al. 2000). Constitutive and inducible MHC-II expression is regulated at the level of transcription by a series of well studied elements within the MHC-II proximal promoter. Nuclear Factor Y (NFY), Regulatory Factor X (RFX), and cAMP response element binding protein (CREB) bind to the Y and X elements of the MHC-II promoter forming a multi-protein enhanceosome complex (Steimle, Durand et al. 1995; Masternak, Barras et al. 1998; Nagarajan, Louis-Plence et al. 1999). Once assembled the enhanceosome recruits the master regulator, the Class II Transactivator (CIITA)(Masternak, Muhlethaler-Mottet et al. 2000).

CIITA expression mirrors that of MHC-II expression with cells deficient in CIITA expression being deficient in MHC-II expression as well. Transcription of CIITA is driven by one of four distinct promoters in a cell-type specific manner. Promoter I (pI) and III (pIII) drive constitutive CIITA expression in APCs, dendritic and B cells, respectively. The function of promoter II (pII) remains unclear whereas the well characterized CIITA promoter IV (pIV) is responsible for IFN- γ inducible expression in all other nucleated cells (Muhlethaler-Mottet, Otten et al. 1997; Piskurich, Linhoff et al. 1999). Rapidly upon cytokine stimulation, the phosphorylation and nuclear translocation of signal transducer and activator of transcription 1 (STAT1) via the Janus kinase (JAK)/STAT pathway is triggered. Activated STAT1 homodimers are recruited to the interferon regulatory factor 1 (IRF1) gene to initiate transcription. STAT1 and subsequently IRF1 bind to the IFN- γ activated sequence (GAS) and IFN response element (IRE) of CIITA pIV, joining the constitutively expressed ubiquitous factor 1 (USF-1) which binds the E box (Muhlethaler-Mottet, Di Berardino et al. 1998; Morris, Beresford et al. 2002). CIITA transcripts are observed 2 hours post IFN- γ stimulation (Morris, Beresford et al. 2002; Wright

and Ting 2006). Thus inducible MHC-II expression is delayed due to the time it takes to synthesize and bind CIITA (Beresford and Boss 2001; Spilianakis, Kretsovali et al. 2003; Zika, Fauquier et al. 2005). Once recruited (4-6 hours post IFN- γ stimulation) CIITA binding stabilizes the enhanceosome complex and recruits basal transcriptional components, including the CDK7 subunit of TFIID and the CDK9 subunit of P-TEFb that phosphorylate Pol II to initiate the switch to an elongation complex (Mach, Steimle et al. 1996; Masternak, Barras et al. 1998; Kanazawa, Okamoto et al. 2000; Boss and Jensen 2003; Spilianakis, Kretsovali et al. 2003). Although much is known about the requirement of transcription factors at these two cytokine inducible promoters, considerably less is understood about the histone modifying enzymes that bind these promoters and regulate access to the DNA.

It is now well accepted that histone acetylation of lysine residues relaxes the interaction between histone proteins and DNA to promote gene transcription (Eberharther and Becker 2002; Freiman and Tjian 2003; Gorisch, Wachsmuth et al. 2005). Epigenetic studies of the inducible CIITA and MHC-II promoters in HeLa cells have shown that prior to IFN- γ stimulation, histone H3 and histone H4 lysines are acetylated at low levels which correlates with the binding of constitutively expressed transcription factors, RFX, NFY and CREB at the MHC-II promoter and USF-1 at CIITA pIV (Beresford and Boss 2001; Ni, Karaskov et al. 2005). Following IFN- γ stimulation in HeLa cells, several well known histone acetyltransferases, including CREB binding protein (CBP)/p300 and p300/CBP-associated factor (pCAF), are recruited and levels of histone lysine acetylation are dramatically enhanced to fully open the chromatin structure allowing for CIITA and subsequently MHC-II to be expressed (Beresford and Boss 2001; Wright and Ting 2006).

The requirement for histone methylation in opening the chromatin structure is both less understood and more complex as multiple methyl groups can be added to either arginine (R) residues or lysine (K) residues. Methylation of arginine residues is associated with gene activation (Bauer, Daujat et al. 2002), but methylation of lysine residues has been historically associated with gene inactivation as methylation was thought to be both silencing and irreversible (Jenuwein and Allis 2001; Nakayama, Rice et al. 2001; Berger 2002; Lehnertz, Ueda et al. 2003; Plath, Fang et al. 2003; Shilatifard 2006; Sparmann and van Lohuizen 2006). Some of the first histone methylating events identified were silencing in nature. Histone H3 dimethylated at lysine 9 (H3K9me₂) interacts with heterochromatin protein 1 to facilitate chromatin silencing (Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001; Jacobs and Khorasanizadeh 2002) and histone H3 trimethylated at lysine 27 (H3K27me₃) has been associated with a variety of processes including X chromosome inactivation and imprinting (Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). However with the discovery of histone demethylases, lysine methylation is now known to be dynamic and certain methylation events are intrinsic for gene expression as modifications such as the di- and trimethylation (hypermethylation) of H3 at lysine (K) 4 and K36 are found at actively transcribed genes (Bernstein, Humphrey et al. 2002; Santos-Rosa, Schneider et al. 2002; Bannister, Schneider et al. 2005; Shilatifard 2006; Tsukada, Fang et al. 2006; Whetstine, Nottke et al. 2006). Histone H3K36 methylation is localized within gene coding sequence and functions in transcriptional memory to prevent inappropriate transcription initiation by recruiting histone deacetylases (HDACs) in the wake of elongating RNA polymerase II (Carrozza, Li et al. 2005; Joshi and Struhl 2005; Lee and Shilatifard 2007). In addition histone H3K4 hypermethylation has been found to inhibit H3K9 methylation and is associated with the demethylation of H3K27 to release chromatin from a silencing conformation (Wang,

Cao et al. 2001; Agger, Cloos et al. 2007; Lee, Villa et al. 2007). Epigenetic studies have shown that upon prolonged cytokine stimulation both the MHC-II proximal promoter (Gomez, Majumder et al. 2005; Chou and Tomasi 2008) and CIITA pIV (Ni, Karaskov et al. 2005) are trimethylated at H3K4 (H3K4me₃). Unfortunately the mechanisms by which histone methylation contributes to an open chromatin structure remain poorly understood.

H3K4 methylation is mediated by mixed lineage leukemia (MLL)/complex of proteins associated with Set I (COMPASS) - like complexes, which can monomethylate, dimethylate or trimethylate histone H3 at lysine 4 (Hughes, Rozenblatt-Rosen et al. 2004; Yokoyama, Wang et al. 2004). MLL/COMPASS-like complexes contain several well characterized, common subunits, Ash2L, WDR5 and RbBP5, which are conserved from yeast to humans (Dou, Milne et al. 2006; Steward, Lee et al. 2006; Lee, Tate et al. 2007). These subunits are thought to constitute a MLL/COMPASS subcomplex that forms a platform to mediate the Set1 enzyme and H3K4 substrate interaction (Crawford and Hess 2006; Cho, Hong et al. 2007). WDR5 is necessary for proper complex formation and stabilizes Ash2L association (Dou, Milne et al. 2006; Seward, Cubberley et al. 2007). Ash2L and RbBP5 are required for proper Set1 enzymatic activity (Dou, Milne et al. 2006; Steward, Lee et al. 2006).

As previously stated H3K4me₃ is highly associated with active transcription and is induced by IFN- γ at both CIITA pIV and the MHC-II proximal promoter (Gomez, Majumder et al. 2005; Ni, Karaskov et al. 2005; Chou and Tomasi 2008; Koues, Dudley et al. submitted 2009). Promoter H3K4me₃ is associated with a release from a repressive state by MLL/COMPASS associated histone demethylase, UTX (Lee, Villa et al. 2007), and is functionally linked to activating acetylation events at promoters (Nightingale, Gendreizig et al. 2007). Previously we have demonstrated that the 19S proteasome and its ATPase components

preferentially regulate activating histone acetylation, H3R17me2 and H3K4me3 at cytokine inducible promoters (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009).

Furthermore, we have identified an association between WDR5, a component of the MLL/COMPASS complex, and two additional histone modifying enzymes, CBP and co-activator associated arginine methyltransferase (CARM1). Moreover, inducible WDR5 recruitment to CIITA pIV is inhibited when endogenous Sug1 expression is diminished (Koues, Dudley et al. submitted 2009), suggesting a role for the 19S proteasome in promoting an open chromatin structure by regulating multiple enzyme associations with inducible promoters.

Here we show that common MLL/COMPASS subunits, Ash2L, RbBP5 and WDR5 are recruited to both the MHC-II promoter as well as CIITA pIV and overexpressing a single subunit significantly enhances promoter activity in overexpression-reporter assays. Conversely, although knockdown of a single subunit decreases promoter specific H3K4me3, it does not impact promoter activity in similar reporter assays. Interestingly, although WDR5 co-immunoprecipitates with a histone acetyltransferase (Koues, Dudley et al. submitted 2009), there was no observable change in levels of histone H3 lysine 18 acetylation. Furthermore, knockdown of Sug1 promotes a more silenced chromatin structure as is evidenced by elevated H3K27me3 levels at cytokine inducible promoters upon Sug1 knockdown. Together these results implicate the 19S proteasome in the initial reorganization of chromatin to relax the repressive histone environment surrounding cytokine inducible promoters.

MATERIALS AND METHODS

Cell Lines: HeLa cells (human epithelial) from ATCC (Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Inc., Herndon, VA) that was

supplemented with 10% fetal calf serum, 5mM L-glutamine and 5mM penicillin-streptomycin at 37°C with 5% carbon dioxide.

Antibodies: Trimethylated H3K4, acetylated H3K18 and WDR5 antibodies were from Abcam (Cambridge, MA). Ash2L and RbBP5 antibodies were from Bethyl Laboratories (Montgomery, TX). Rabbit IgG isotype control and mouse IgG isotype control antibodies were from Upstate (Lake Placid, NY). Sug1 antibody was from Novus Biologicals (Littleton, CO). HRP conjugated mouse antibody (W4021) was from Promega (Madison, WI) and HRP conjugated rabbit antibody (1858415) was from Pierce (Rockland, IL).

Plasmids: Flag-CIITA, *HLA-DRA*-Luc, pcDNA3 plasmids were previously described (Bhat, Turner et al. 2008) *Renilla* luciferase control vector (E2231) was from Promega (Madison, WI). Myc-Sug1, was generously provided by A. A. Wani (Zhu, Wani et al. 2007). Flag-WDR5, Flag-Ash2L and Flag-RbBP5 were graciously provided by A. Shilatifard (Wu, Wang et al. 2008).

siRNA constructs and transient transfections: Short interfering RNA (siRNA) duplexes previously described (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008) were used for transient knockdown of the Sug1 19S ATPase. Pooled siRNA duplexes for WDR5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HeLa cells were transfected with Allstar scrambled sequence control siRNA (Qiagen) or specific siRNA were treated with IFN- γ as indicated. Cells were lysed in NP40 lysis buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) supplemented with Complete EDTA-free protease inhibitors (Roche) and knockdown efficiency was assessed by immunoblotting as described above.

Luciferase reporter assays: *Overexpression luciferase assays.* HeLa cells were plated in 6-well plates at a density of 5×10^4 cells/well were transfected with CIITA, *HLA-DRA*-Luc, *Renilla*, pcDNA3 and hCompass subunit plasmids as indicated using Fugene 6 (Roche,

Indianapolis, IN) according to the manufacturer's instructions. Twenty-four hours post transfection cells were subjected to dual-luciferase assay (Promega) according to the manufacturer's protocol. *Knockdown luciferase assays.* HeLa cells were plated in 6-well plates at a density of 5×10^4 cells/well and transfected with 1.0 μg of control or WDR5 siRNA using the RNAi transfection reagent (Qiagen) according to manufacturer's instructions. Twenty-four hours post siRNA transfection, CIITA, *HLA-DRA-Luc*, *Renilla* and pcDNA3 plasmids were transfected as indicated using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Twenty-four hours post DNA transfection cells were subjected to dual-luciferase assay (Promega) according to the manufacturer's instructions.

Chromatin immunoprecipitations (ChIPs): ChIP assays were performed as previously described (Greer, Zika et al. 2003). In brief, HeLa cells were stimulated with 500 U/ml IFN- γ (Peprotech, Rocky Hill, NJ) as indicated. Crosslinked cells were lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, dH₂O) with Complete EDTA-free protease inhibitors (Roche) for 20 minutes on ice and sonicated to generate an average of 500-750 bp sheared DNA. Sonicated samples were precleared with salmon-sperm coated agarose beads (Upstate) and half of the lysate was IP with 5 μg of polyclonal antibody against, H3K4me3 (Abcam), H3K18ac (Abcam), WDR5 (Abcam), Ash2L (Bethyl Laboratories), RbBP5 (Bethyl Laboratories) overnight at 4°C. The remaining lysate was IP with isotype control antibody (Upstate). Following a 2 hour incubation with 60 μl 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, dH₂O), High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.0, 500mM NaCl, dH₂O), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH 8.0, dH₂O) and 1xTE buffer and were eluted with SDS elution

buffer (1% SDS, 0.1M NaHCO₃, dH₂O). Following elution, crosslinks were reversed with 5M NaCl at 65°C and IP and control DNA was isolated using phenol:chloroform:isoamyl alcohol mix (Invitrogen) as per the manufacturer's protocol. Isolated DNA was analyzed by real-time PCR using probes and primers spanning the W-X-Y box of the MHC-II *HLA-DRA* promoter and CIITA pIV (Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009). Values graphed were calculated based on standard curves generated. *Chromatin immunoprecipitation in siRNA knockdown cells:* HeLa cells were transfected with specific siRNA (Qiagen, Santa Cruz) or control siRNA (Qiagen) using RNAiFect transfection reagent (Qiagen) according to manufacturer's instructions. Cells were treated with 500U/ml IFN- γ as indicated. 10% of the total cell volume was lysed in 1% Nonidet P-40 buffer (1M Tris pH 8.0, 1M KCl, 10% NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH₂O) with Complete EDTA-free protease inhibitors (Roche) and analyzed via immunoblot for knockdown verification. The remaining fraction of cells was subjected to ChIP assay.

RESULTS

MLL/COMPASS subunits constitutively associate with cytokine inducible promoters. We have previously shown that Sug1 positively regulates the trimethylation of histone H3 at lysine 4 (H3K4me₃), a common activating methylation event, at the MHC-II proximal promoter and CIITA pIV, potentially by stabilizing the association of MLL/COMPASS subunits (Koues, Dudley et al. submitted 2009). H3K4me₃ is mediated by histone methyltransferase (HMTase) enzymes, which are typically recruited to DNA as part of a larger complex of proteins MLL/COMPASS (Hughes, Rozenblatt-Rosen et al. 2004; Yokoyama, Wang et al. 2004; Lee, Tate et al. 2007; Wu, Wang et al. 2008). We have previously observed that

WDR5 constitutively binds to CIITA pIV with a moderate enhancement in recruitment, which is lost upon Sug1 knockdown, observed upon cytokine stimulation (Koues, Dudley et al. submitted 2009). To expand on the potential for MLL/COMPASS recruitment to CIITA pIV, we performed chromatin immunoprecipitations to determine if additional subunits are also recruited to CIITA pIV (Figure 4-1a-b). RbBP5 (Figure 4-1a) and Ash2L (Figure 4-1b) also associate with CIITA pIV. To determine if MLL/COMPASS subunits are recruited to the MHC-II proximal promoter, similar chromatin immunoprecipitations were performed for three common MLL/COMPASS subunits (Figure 4-1c-e). Similar to observations at CIITA pIV, all three MLL/COMPASS subunits RbBP5 (Figure 4-1c), Ash2L (Figure 4-1d) and WDR5 (Figure 4-1e), bind constitutively to the MHC-II promoter, suggesting that indeed MLL/COMPASS mediates H3K4me3 at cytokine inducible genes.

Overexpression of a single MLL/COMPASS subunit enhances CIITA transactivity.

To characterize the impact MLL/COMPASS has on the MHC-II proximal promoter, we performed luciferase reporter assays in unstimulated HeLa cells in which the MHC-II *HLA-DRA* promoter is fused to the luciferase gene, CIITA and various MLL/COMPASS subunits, RbBP5 (Figure 4-2a), Ash2L (Figure 4-2b), and WDR5 (Figure 4-2c) are overexpressed. In cells transfected with CIITA alone, there is a 15-20 fold increase in *HLA-DRA* promoter activity (Figure 4-2a-c, gray bars). Interestingly, overexpressing a single MLL/COMPASS subunit (Figure 4-2a-c, white bars) significantly enhances CIITA's ability to drive the *HLA-DRA* promoter. In contrast performing the same luciferase reporter assay in cells in which a single MLL/COMPASS subunit was knocked down (Figure 4-3a) showed no changes in CIITA's ability to drive the *HLA-DRA* promoter. HeLa cells transfected with WDR5 siRNA showed

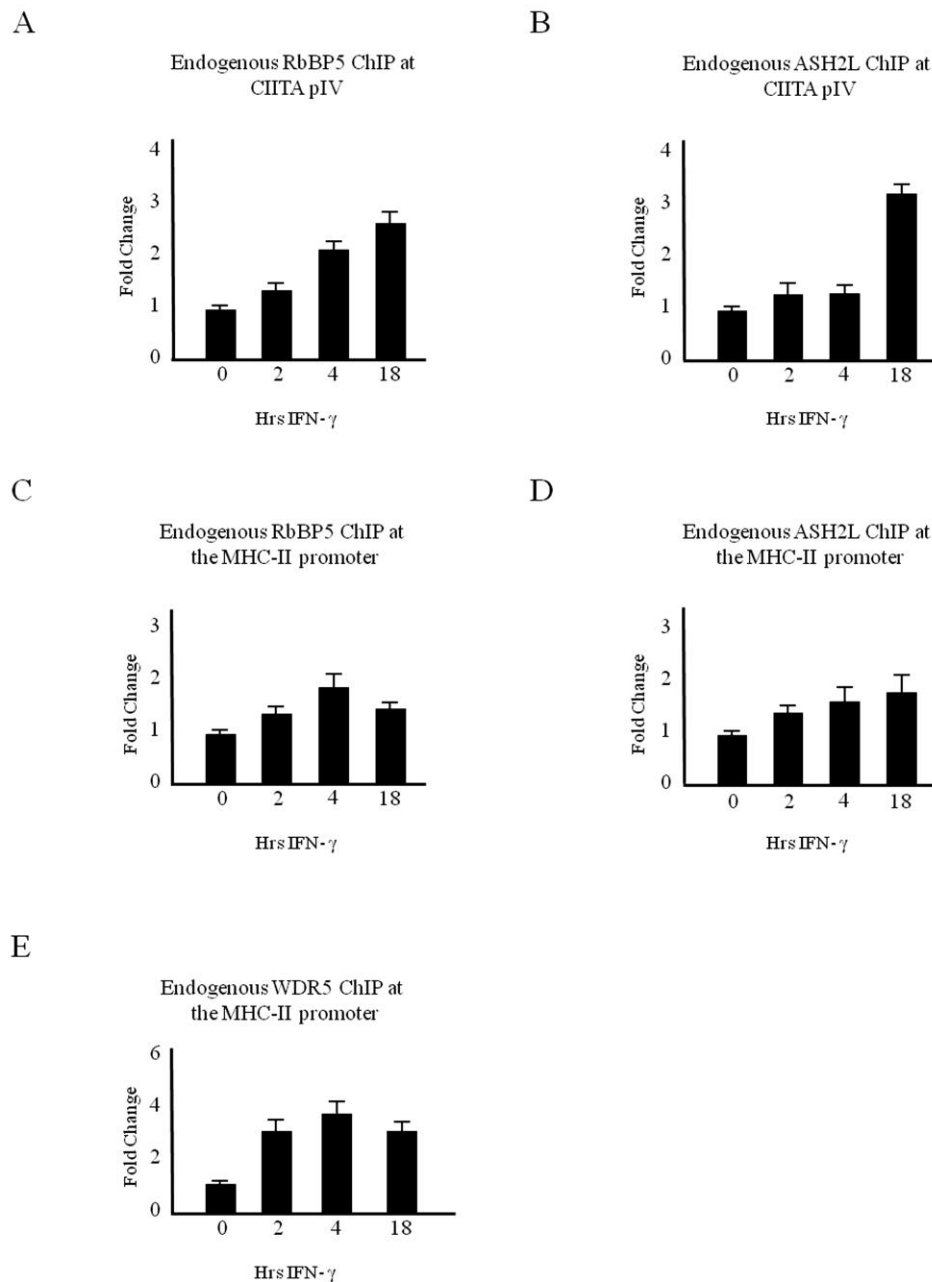
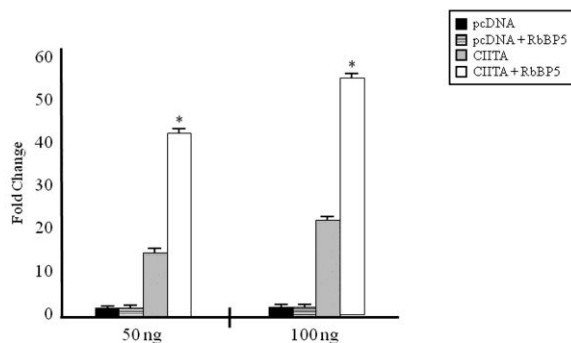
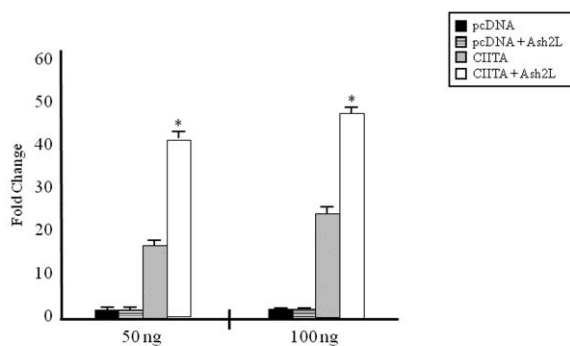


Figure 4-1. Subunits of MLL/COMPASS associate with cytokine inducible genes. (A-B) Subunits of MLL/COMPASS associate with CIITA pIV. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or endogenous RbBP5 (A) or Ash2L (B) antibody. Associated DNA was isolated and analyzed via real-time PCR using primers and probe spanning CIITA pIV. (C-E) Subunits of MLL/COMPASS associate with the MHC-II proximal promoter. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or endogenous RbBP5 (C), Ash2L (D) or WDR5 (E) antibody. Associated DNA was isolated and analyzed via real-time PCR using primers and probe spanning the MHC-II proximal promoter. Real-time PCR values were normalized to the total amount of DNA added to the reaction (input). Input values represent 5% of the total cell lysate. Data are presented as fold changes in bound DNA relative to unstimulated samples. Isotype control IP values were 0.2 ± 0.2 . Values represent mean \pm SEM of (n = 2) independent experiments.

A

HLA-DR promoter activity with RbBP5 overexpression

B

HLA-DR promoter activity with ASH2L overexpression

C

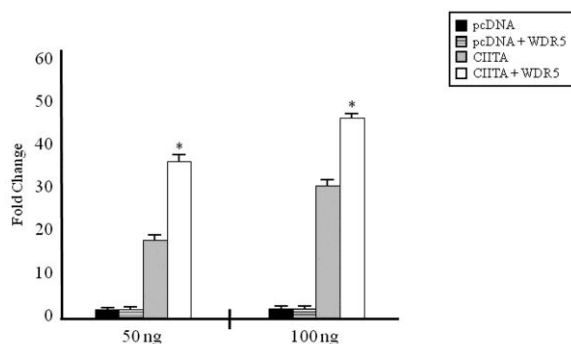
HLA-DR promoter activity with WDR5 overexpression

Figure 4-2. Overexpressing a single MLL/COMPASS subunit enhances CIITA transactivity. HeLa cells were transfected with *HLA-DRA-Luc*, *Renilla*, pcDNA3, CIITA, and RbBP5 (A), Ash2L (B), WDR5(C). Luciferase activity is reported as fold activation relative to that of the reporter alone. Luciferase readings were normalized to *Renilla* activity. Luciferase assays were performed in triplicate and values represent mean \pm SEM of (n = 3) independent experiments. * $p < 0.01$ over CIITA alone

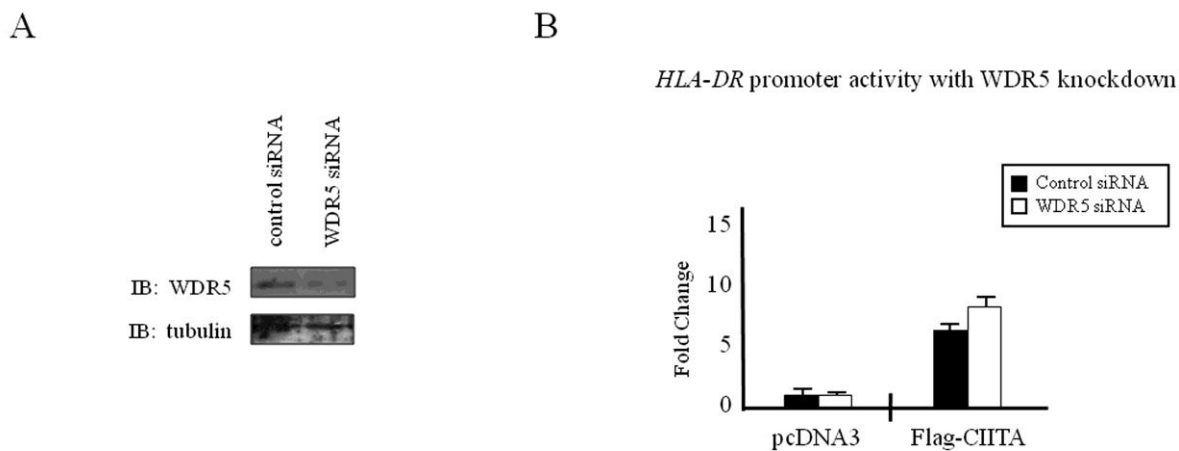


Figure 4-3. Knockdown of a MLL/COMPASS subunit does not influence CIITA transactivity. (A) WDR5 siRNA reduces levels of endogenous WDR5. HeLa cells were transfected with control or WDR5 specific siRNA, harvested and subjected to Western blot analysis for endogenous WDR5 (top) and endogenous tubulin (bottom). (B) WDR5 knockdown does not impact CIITA's ability to drive the MHC-II *HLA-DRA* promoter. HeLa cells were transfected with control or WDR5 siRNA and subsequently co-transfected with *HLA-DRA*-Luc, *Renilla*, pcDNA3, CIITA as indicated. Luciferase activity is reported as fold activation relative to that of the reporter alone. Luciferase readings were normalized to *Renilla* activity. Luciferase assays were performed in triplicate and values represent mean \pm SEM of (n = 3) independent experiments.

levels of CIITA transactivity (Figure 4-3b, white bars) comparable to those in control siRNA transfected cells (Figure 4-3b, black bars).

MLL/COMPASS trimethylates H3K4 at cytokine inducible genes. We have now observed that various MLL/COMPASS subunits are recruited to both the MHC-II proximal promoter as well as CIITA pIV (Figure 4-1). To determine if MLL/COMPASS contributes to the trimethylation of histone H3 lysine 4 at these genes, ChIP assays were performed in HeLa cells transfected with WDR5 siRNA (Figure 4-4). Knockdown of WDR5 resulted in a loss in both basal and inducible H3K4me3 at the MHC-II proximal promoter (Figure 4-4a) and CIITA pIV (Figure 4-4b).

WDR5 does not contribute to histone H3K18 acetylation. The ATPases of the 19S regulator, which serves as the cap of the 26S proteasome, have been implicated in mediating a subset (H3K18ac, H3R17me2 and H3K4me3) of histone activating modifications, independent of the canonical protein degradation role of the proteasome (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009). Previously we have observed that WDR5 associates with histone modifying enzymes known to be able to mediate this subset of activating modifications, CREB binding protein (CBP) and coactivator -associated arginine methyltransferase 1 (CARM1) (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009), in addition to the HMTase SET1 (Lee, Tate et al. 2007; Wu, Wang et al. 2008). To characterize WDR5's role in mediating additional histone modifications, we performed chromatin immunoprecipitations in cells transfected with WDR5 siRNA (Figure 4-5). Since, CBP is capable of mediating H3K18ac (Schiltz, Mizzen et al. 1999; Agalioti, Chen et al. 2002), we determined by ChIP assay, the levels of H3K18ac at the MHC-II proximal promoter (Figure 4-5a) and CIITA pIV (Figure 4-5b) in WDR5 knockdown cells. Levels of H3K18 acetylation in WDR5 knockdown cells (Figure 4-5a-

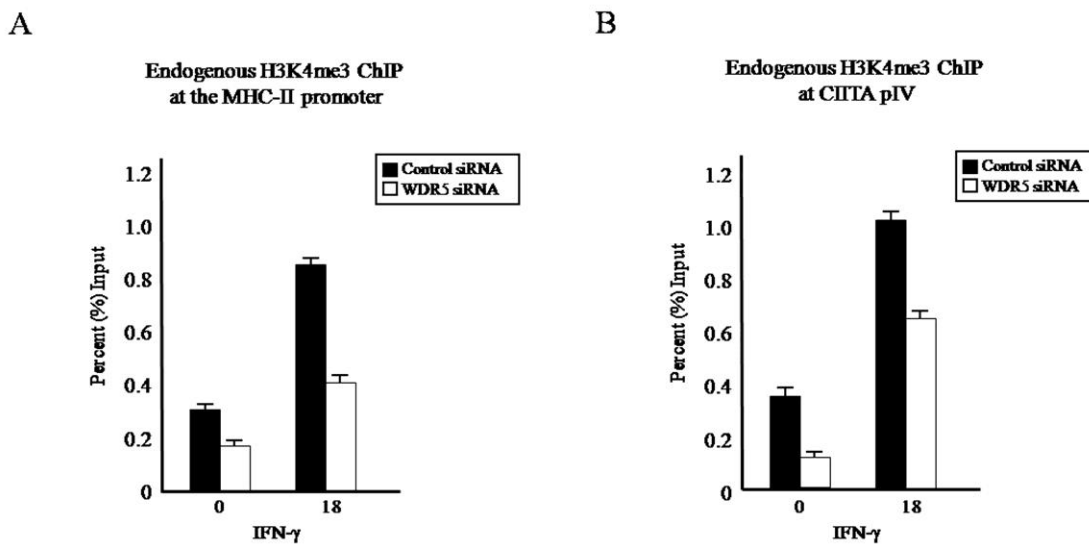


Figure 4-4. Knockdown of a MLL/COMPASS subunit decreases H3K4me3. (A-B) WDR5 knockdown decreases H3K4me3. HeLa cells transfected with scrambled control or WDR5 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control or endogenous H3K4me3 antibody. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 2 using primers and probes specific for the MHC-II promoter (A) or CIITA pIV (B). Data are presented as percent input. Isotype control values were 0.1 ± 0.1 . Values represent mean \pm SEM of (n = 2) independent experiments.

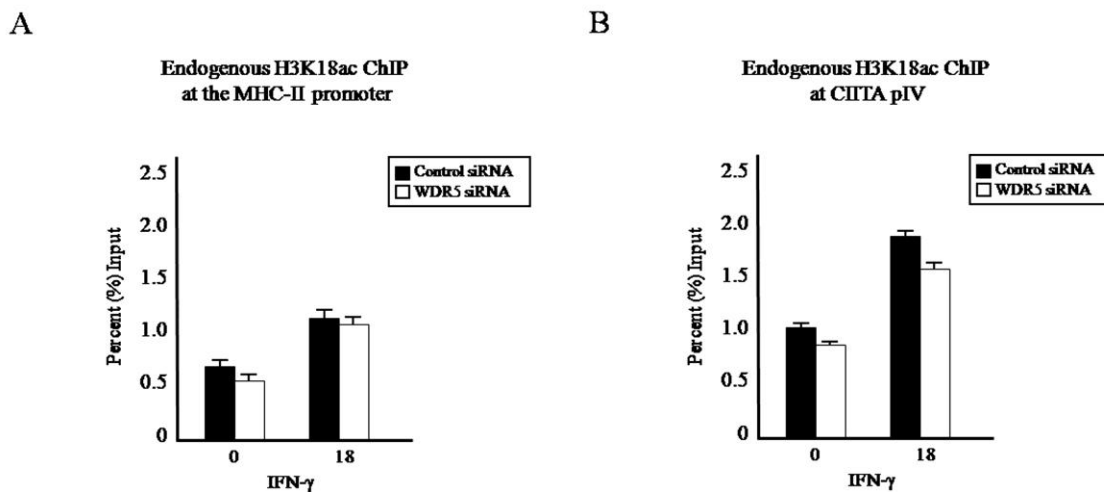


Figure 4-5. Knockdown of a MLL/COMPASS subunit does not impact H3K18 acetylation. (A-B) HeLa cells transfected with scrambled control or WDR5 specific siRNA were stimulated with IFN- γ and subjected to ChIP assay. Lysates were IP with control or endogenous H3K18ac antibody. Associated DNA was isolated and analyzed via real-time PCR as described in Figure 4-4 using primers and probes specific for the MHC-II promoter (A) or CIITA pIV (B). Data are presented as percent input. Isotype control values were 0.1 ± 0.1 . Values represent mean \pm SEM of (n = 2) independent experiments.

b, white bars) were comparable to those in control siRNA transfected cells (Figure 4-5a-b, black bars). Although we had previously observed a common binding partner of three histone modifying enzymes known to be capable of mediating a subset of activating events that are also impacted by knockdown of a 19S ATPase Sug1, knockdown of WDR5 did not mirror that of Sug1 knockdown as there was no impact on histone H3K18 acetylation.

H3K27me3 is enhanced upon Sug1 knockdown. Evidence now suggests that demethylation of silencing modifications is intimately linked to the deposition of histone activating events to promote the relaxation of the repressive nature of chromatin. One such instance of this was the identification of the H3K27me3 histone demethylase UTX, which is found complexed to MLL/COMPASS subunits (Agger, Cloos et al. 2007; Lee, Villa et al. 2007). Therefore, we sought to determine the degree of chromatin silencing in Sug1 knockdown cells. Chromatin immunoprecipitations in HeLa cells transfected with Sug1 siRNA (Figure 4-6b-c, white bars) show that H3K27me3 levels are elevated over similarly treated control siRNA transfected cells (Figure 4-6b-c, black bars). Together these data strongly suggests that the lack of Sug1 promotes a more closed chromatin structure.

DISCUSSION

Previously we have demonstrated that the 19S proteasome regulates activating histone H3K4 trimethylation at cytokine inducible promoters, potentially through an interaction with the MLL/COMPASS subunit WDR5 as a loss of recruitment of WDR5 is observed upon Sug1 knockdown (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009). Now we have shown that multiple common MLL/COMPASS subunits, Ash2L, RbBP5 and WDR5 constitutively bind and inducibly recruited to both the MHC-II promoter and CIITA pIV. The

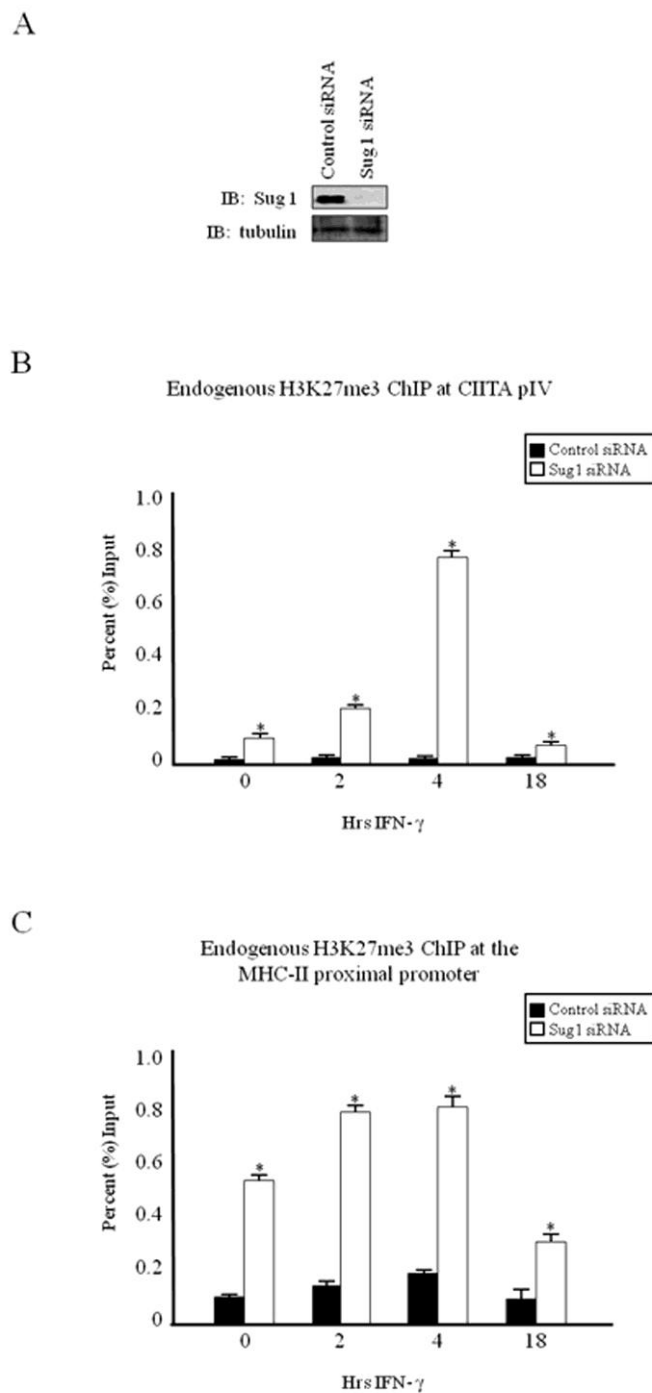


Figure 4-6. H3K27me3 is enhanced upon Sug1 knockdown. (A) Sug1 siRNA efficiently decreases endogenous Sug1. HeLa cells were transfected with control or Sug1 specific siRNA, harvested and subjected to Western blot analysis for endogenous Sug1 (top) and endogenous tubulin (bottom). (B-C) H3K27me3 is elevated at cytokine inducible genes upon diminished Sug1 expression. ChIP assays were carried out in HeLa cells stimulated with IFN- γ for 0 to 18 hours. Lysates were IP with control or endogenous H3K27me3 antibody. Associated DNA was isolated and analyzed as in Figure 4-1 using primers and probe spanning CIITA pIV (B) and the MHC-II proximal promoter (C). Data are presented as percent input. Isotype control values were 0.005 ± 0.005 . Values represent mean \pm SEM of (n = 3) independent experiments. * p < 0.05 as compared to control siRNA transfected samples

timecourse for recruitment of individual subunits varies slightly, likely due to differences in antibodies. However, there remains the possibility that MLL/COMPASS is assembled on promoters rather than binding as a single complex. Using a reporter assay, overexpressing a single MLL/COMPASS subunit significantly enhances promoter activity implicating these subunits as strong co-activators of the MHC-II promoter. However knocking down a single subunit does not impact promoter activity in similar reporter assays. MLL/COMPASS functions in hypermethylating H3K4 and thus it is unsurprising that plasmids in an overexpression system, which are not found in native chromatin structure, are not preferentially impacted by knockdown of a single HMTase subunit. Consistent with this, we observed a loss of H3K4me3 at the MHC-II promoter and CIITA pIV when endogenous WDR5 is diminished.

We also found that WDR5, a component of the MLL/COMPASS complex, associates with CBP and CARM1, both of which perform histone modifying events targeted by the 19S proteasome, H3K18ac and H3R17me2, respectively. In addition, we have observed a loss in inducible WDR5 binding to CIITA pIV when endogenous Sug1 is diminished (Koues, Dudley et al. submitted 2009). This insinuated that the the 19S proteasome may play a role in stabilizing a large multi-enzyme histone remodeling complex through an interaction with WDR5 at cytokine inducible genes. Therefore, we sought to determine if knockdown of WDR5 mirrors that of Sug1 knockdown which results in a loss of H3K18ac in addition to H3K4me3 (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009). However, knockdown of endogenous WDR5 did not result in a corresponding loss in H3K18ac.

We have shown that in mammalian cells, 19S ATPases are crucial for regulating a subset of inducible histone activation modifications; H3K18ac, H3R17me2 and H3K4me3 (Koues, Dudley et al. 2008; Koues, Dudley et al. submitted 2009). Although we have previously

observed a common binding partner, WDR5, of histone modifying enzymes known to be capable of mediating these modifications, knockdown of WDR5 did not mirror that of Sug1 knockdown as there was no impact on histone H3K18 acetylation upon WDR5 knockdown. Evidence now suggests that demethylation of silencing modifications is intimately linked to the deposition of histone activating events to promote the relaxation of the repressive nature of chromatin (Agger, Cloos et al. 2007; Benevolenskaya 2007; Lee, Norman et al. 2007; Lee, Villa et al. 2007). This same common MLL/COMPASS subunit is also associated with the histone demethylase UTX (Lee, Villa et al. 2007). Here we show that the 19S ATPase Sug1 functions to release chromatin from a repressive state. Previously we have observed that WDR5 binding to CIITA pIV is inhibited, implying that UTX recruitment may also be lost upon diminished Sug1 expression (Koues, Dudley et al. submitted 2009). Consistent with this hypothesis, H3K27me3 levels are elevated in Sug1 siRNA transfected cells. Further studies are required to characterize the molecular interactions between 19S ATPases, UTX and silenced chromatin.

Ours is the first report implicating the 19S proteasomal ATPases in regulating the initial chromatin remodeling events at cytokine inducible promoters that releases surrounding chromatin from a more condensed, repressive state. Our observations that in the absence of Sug1, silencing methylation of histone H3 is not only elevated, but also persistent, strongly implicate the 19S ATPases in chromatin reorganization in response to stimuli. A full understanding of the contributions of Sug1 and the other 19S ATPases into the initial epigenetic regulation and chromatin reorganization at cytokine inducible genes requires further studies into the molecular interactions occurring at these promoters.

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CHAPTER 5

CONCLUSIONS

To accommodate the over 20,000 genes in the human genome (Human Genome Project 2008), DNA is highly organized and tightly packed into nuclei (Lewin 2004). DNA is wrapped around histone proteins (H2A, H2B, H3 and H4) which are then condensed into chromatin (Luger, Mader et al. 1997; Carey and Smale 2000; Richmond and Davey 2003; Lewin 2004). In any given cell, there are groups of genes constitutively silenced, those that are constitutively expressed, and finally those that are inducibly expressed when necessary (Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001; Agalioti, Chen et al. 2002; Jacobs and Khorasanizadeh 2002; Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006; Shukla, Vaissiere et al. 2008). Thus chromatin needs to be quite dynamic for genes to be up or down regulated in response to various stimuli. One of the fundamental obstacles of this process is the nature of the chromatin structure.

The various stages of chromatin are characterized by the presence of particular post-translational modifications located on the tails of histones. Heterochromatin is methylated predominantly on histone H3 at lysines 9 and 27 (Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001; Jacobs and Khorasanizadeh 2002; Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). Euchromatin is highly acetylated on histones H3 and H4 and poised for transcription chromatin is characterized by a combination of activating and silencing modifications (Jenuwein and Allis 2001; Eberharter and Becker 2002; Schubeler, MacAlpine et al. 2004; Gorisch, Wachsmuth et al. 2005). The addition and removal of these modifications are mediated by a group of histone modifying enzymes, including histone acetyltransferases, histone

deacetylases, histone methyltransferases and histone demethylases. The coordination of these enzymes and the histone code they regulate remains an area of intense investigation.

The ubiquitin-proteasome system (UPS) has been implicated as a regulator of transcriptional activity for some time as the UPS is known to terminate transcription by degrading transcription factors and chromatin remodelers (Ciechanover 1994; Hochstrasser 1996; Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003). More recently, a novel role for the UPS in regulating transcription has emerged in which components of the proteasome have been found to be essential for transcription, independent of protein degradation (Salghetti, Caudy et al. 2001; Conaway, Brower et al. 2002; Dennis and O'Malley 2005). The 20S core complexes and 19S regulatory particles of the 26S proteasome were identified as existing as individual complexes in the nucleus as well as the cytoplasm of cells (Brooks, Fuertes et al. 2000). Furthermore, 19S proteasomes were found to disassociate with the six ATPases of the 19S forming an independent 19S subcomplex that associates with transcription factor activation domains and is recruited to inducible yeast promoters (Swaffield, Melcher et al. 1995; Rubin, Coux et al. 1996; Gonzalez, Delahodde et al. 2002; Sun, Johnston et al. 2002). At the level of chromatin remodeling, the 19S proteasome was found to enhance promoter targeting of the large histone remodeling complex, SAGA (Spt-Ada-Gcn5-acetyltransferase) that contains both histone acetylase and histone deubiquitinase activity (Henry, Wyce et al. 2003; Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005). However, it remained unclear if the 19S proteasome's non-proteolytic role in regulating transcription was present in higher eukaryotes.

Studies by Bhat et al. indicated that the 19S ATPase Sug1 is recruited to the MHC-II proximal promoter prior to the master regulator CIITA and that in the absence of Sug1, markedly

reduced levels of CIITA are recruited to the MHC-II *HLA-DRA* proximal promoter (Bhat, Turner et al. 2008). Yeast studies have implicated the ortholog of Sug1, Rpt 6, in epigenetically regulating transcription (Lee, Ezhkova et al. 2005; Daniel and Grant 2007). Although there remained a lack of evidence for the 19S proteasome's role in chromatin remodeling in higher eukaryotes, the 19S proteasome was found to associate with a variety of inducible mammalian promoters suggesting this might indeed be the case (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003; Lassot, Latreille et al. 2007; Yu and Kodadek 2007; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Ferry, Gianni et al. 2009).

To this end, we found that the 19S ATPase Sug1 associates with histone H3 and acetylated histone H3. Furthermore when Sug1 is knocked down, levels of histone H3 acetylation are markedly diminished at the cytokine inducible MHC-II and CIITA promoters in a proteolytic independent manner as proteasomal inhibition does not impact H3 acetylation. Moreover, the effects of Sug1 are lysine specific with a preferential loss of histone H3 acetylation occurring on lysine 18 while lysine 9 acetylation remains elevated in the presence of diminished Sug1 expression. This loss of H3K18 acetylation correlates with the observed loss of CBP recruitment, a histone acetyltransferase known to bind the MHC-II promoter. Furthermore bulk histone acetylation was unaffected by Sug1 knockdown suggesting that the effects observed on histone H3 acetylation by Sug1 were promoter specific.

The initial observations indicated that the 19S ATPase Sug1 plays a role in mediating promoter acetylation at cytokine inducible genes. The function of histone methylation in regulating transcription is more complex than acetylation as this modification is associated with both gene expression and gene silencing (Wang, Cao et al. 2001; Santos-Rosa, Schneider et al. 2002; Krogan, Kim et al. 2003; Xiao, Hall et al. 2003; Schneider, Bannister et al. 2004;

Bannister, Schneider et al. 2005; Francis, Chakrabarti et al. 2005). Histone H3 dimethylation at lysine 9 (H3K9me₂) and trimethylation at lysine 27 (H3K27me₃) are associated with a closed chromatin structure (Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001; Jacobs and Khorasanizadeh 2002; Plath, Fang et al. 2003; Sparmann and van Lohuizen 2006). Interestingly, recent findings indicate that methylation may prove to be as relevant for the activation of gene transcription as acetylation. Trimethylation at H3K4 is associated with actively transcribed genes and the demethylation of H3K27 to release chromatin from a silencing conformation (Wang, Cao et al. 2001; Agger, Cloos et al. 2007; Lee, Villa et al. 2007). H3K36 hypermethylation marks recently transcribed regions of DNA to prevent spontaneous transcription initiation with coding sequences (Carrozza, Li et al. 2005; Joshi and Struhl 2005; Lee and Shilatifard 2007).

Others have shown in yeast that mutations in 19S ATPases specifically disrupt H3K4 and -K79 methylation (Ezhkova and Tansey 2004). Similarly, we were able to demonstrate that Sug1 knockdown impairs the additional activating dimethylation of H3R17 and trimethylation of H3K4 at both the MHC-II promoter and CIITA pIV. Consistent with roles for Sug1 in mediating inducible chromatin modifications at specific genes, levels of H3R17me₂ and H3K4me₃ at the GAPDH promoter and in whole cell lysates were not impacted by Sug1 knockdown. Lysine methylation modifications are restricted in their localization on DNA; H3K4me₃ is found clustered around gene promoters whereas H3K36me₃ is found throughout gene coding regions (Bannister, Schneider et al. 2005; Chou and Tomasi 2008). Therefore the spatial distribution of histone lysine methylation allowed us to localize the effects of Sug1 chromatin reorganization to inducible gene promoters as H3K4me₃ but not H3K36me₃ levels were impacted at MHC-II genes by Sug1 knockdown.

Initially our data suggested that the 19S ATPase Sug1 helps target CBP to the MHC-II proximal promoter to enhance promoter acetylation, loosen the chromatin structure and establish a platform for additional histone remodeling enzymes and transcription factors to bind. As H3K4me3 levels were also inhibited in Sug1 knockdown cells, we investigated the histone methyltransferase that targets H3K4, the large, multi-protein MLL/COMPASS complex. Similar to the previously observed loss in CBP binding to the MHC-II promoter, we found a loss in MLL/COMPASS subunit, WDR5, recruitment to CIITA pIV when endogenous Sug1 is diminished. Furthermore, the loss in H3R17me2 indicated a similar loss of the arginine methyltransferase CARM1 binding to inducible promoters in the absence of Sug1. We were unfortunately unable to test this as we failed in multiple attempts to perform chromatin immunoprecipitation experiments to isolate endogenous CARM1 in siRNA transfected cells. Nevertheless, it is unsurprising that CARM1 mediated H3R17me2 is also regulated by Sug1 as substantial evidence supports the notion that CBP and CARM1 crosstalk on chromatin to link H3 acetylation and arginine methylation (Ma, Baumann et al. 2001; Daujat, Bauer et al. 2002; Zika, Fauquier et al. 2005). Combined these results argue that upon gene induction, 19S ATPases reorganize chromatin to allow multiple enzymes access to their target histone residues in a promoter specific fashion.

Importantly, the effects of Sug1 on chromatin remodeling are independent of proteasome proteolytic function as proteasomal inhibition does not impact either histone H3 acetylation or H3K4 methylation. As a further control for siRNA specificity, reconstituting Sug1 expression in Sug1 siRNA transfected cells restores promoter activity. Previously studies by Bhat et al. showed that the 19S ATPase Sug1 is recruited to the MHC-II proximal promoter prior to CIITA (Bhat, Turner et al. 2008). Our data now suggests that multiple 19S ATPases play roles in

regulating mammalian epigenetics as both S6a and S7, in addition to Sug1, bind MHC-II promoters and are required for efficient histone H3 acetylation. Although proteasome activity is not a requirement for transcriptional regulation of histone acetylation, we found that additional proteasome subunits, the 19S non-ATPase S5a and 20S subunit Alpha 4, also bind MHC-II promoters implicating the eventual recruitment of intact 26S proteasomes to these promoters. These results support findings in the yeast literature that an intact, non-proteolytic proteasome binds promoters (Morris, Kaiser et al. 2003; Ezhkova and Tansey 2004; Lee, Ezhkova et al. 2005; Collins and Tansey 2006). However, the observed binding of non-ATPase proteasomal components was relatively low and does not rule out the potential for recruitment of an independent 19S ATPase subcomplex, a finding also reported in yeast (Ferdous, Kodadek et al. 2002; Gillette, Gonzalez et al. 2004; Sikder, Johnston et al. 2006; Sulahian, Sikder et al. 2006).

Despite the growing evidence indicating non-proteolytic roles for the 19S proteasome in regulating mammalian transcription (Makino, Yoshida et al. 1999; Ferdous, Kodadek et al. 2002; Rasti, Grand et al. 2006; Lassot, Latreille et al. 2007; Zhu, Wani et al. 2007; Bhat, Turner et al. 2008; Koues, Dudley et al. 2008; Ferry, Gianni et al. 2009), the mechanism by which the 19S proteasome or a 19S subcomplex is recruited to inducible mammalian genes remains unclear. Studies in yeast have identified H2B monoubiquitination to be a prerequisite for 19S ATPase recruitment and subsequent activating lysine methylation (Wood, Schneider et al. 2003; Ezhkova and Tansey 2004; Laribee, Shibata et al. 2007; Lee, Shukla et al. 2007). As with methylation, the effects of histone ubiquitination are complex with reports indicating roles in both gene inactivation and activation. Although more difficult to study due to the transient nature of the ubiquitination modification and lack of suitable reagents, ubiquitinated histone H2B has been found to be associated with robustly transcribed regions of DNA (Henry, Wyce et al. 2003;

Minsky, Shema et al. 2008). Using a new H2BK120ub antibody, we were able to map the ubiquitination pattern of histone H2B at both the MHC-II promoter and at CIITA pIV and found that this pattern was not effected by Sug1 knockdown, suggesting that recruitment of mammalian 19S ATPases , as in yeast, might also require ubiquitination of H2B. Yet in the presence of reduced H2B ubiquitination, the 19S ATPase Sug1 is recruited to CIITA pIV. This implies that H2B ubiquitination may not be a prerequisite for 19S recruitment and suggests that additional mechanisms may exist in mammalian cells.

Although proteasome activity does not appear to be required for Sug1 mediated chromatin remodeling, it remains unknown if the enzymatic activity of the 19S ATPases are necessary. The ATPase activity of the 19S proteasome is required for optimal global *in vivo* histone H3 acetylation and H3K4 dimethylation in *Saccharomyces cerevisiae* and mutations within the ATPase domain disrupts targeting of the HAT containing SAGA complex to DNA (Lee, Ezhkova et al. 2005; Daniel and Grant 2007). Our observations that in the absence of endogenous Sug1 chromatin remodeling is inhibited at cytokine inducible genes, indicated that similar regulatory activity may exist in mammalian cells. However, we were able to reconstitute promoter activity in Sug1 siRNA transfected cells by overexpressing an ATPase mutant Sug1, suggesting that ATPase enzymatic activity may not be required for the initial recruitment of histone modifying enzymes. This is consistent with our observations that the Sug1 ATPase mutant efficiently associates with CBP and overexpression of the mutant has no effect on histone acetylation at the MHC-II promoter. Likewise, it has been proposed that in yeast cells ATP hydrolysis is less important for SAGA targeting to DNA but may be required for optimal dissociation of SAGA from the 19S proteasome (Lee, Ezhkova et al. 2005). Further studies are

needed to characterize the requirement of energy in the form of ATP hydrolysis for the role the 19S proteasome plays in chromatin reorganization at cytokine inducible promoters.

We have demonstrated that the 19S proteasome regulates histone H3K4 trimethylation at cytokine inducible promoters, potentially through an interaction with the MLL/COMPASS subunit WDR5 as a loss of recruitment of WDR5 is observed upon Sug1 knockdown. For the first time, we have shown that multiple common MLL/COMPASS subunits, Ash2L, RbBP5 and WDR5 bind constitutively to both the MHC-II promoter and CIITA pIV, joining the host of other histone modifying enzymes known to interact with these promoters (Spilianakis, Papamatheakis et al. 2000; Pattenden, Klose et al. 2002; Zika, Greer et al. 2003; Ni, Karaskov et al. 2005; Zika, Fauquier et al. 2005; Wright and Ting 2006). Reporter assays implicate these subunits as strong co-activators as overexpressing just a single MLL/COMPASS subunit significantly enhances MHC-II *HLA-DRA* promoter activity. Unsurprisingly, knocking down just one subunit does not impact promoter activity in the same reporter assay due to the lack of proper chromatin structure associated with plasmids. MLL/COMPASS functions to hypermethylate H3K4 and we observed that knockdown of WDR5, a subunit critical for MLL/COMPASS formation and HMTase activity, resulted in a loss of H3K4me3 at the inducible MHC-II and CIITA promoters, furthering our observations of a novel interaction between MLL/COMPASS and cytokine inducible genes.

We have observed a loss in WDR5 binding to CIITA pIV when endogenous Sug1 is diminished. In addition, we found that WDR5 associates with CBP and CARM1, which mediate H3K18ac and H3R17me2, respectively. This provided a link between all three events, H3K4me3, H3K18ac and H3R17me2, identified as targets of the 19S proteasome and suggested that the 19S proteasome may interact with WDR5 to stabilize the association of a large multi-enzyme histone remodeling complex with cytokine inducible promoters. Alas, knockdown of

WDR5 did not mirror that of Sug1 knockdown as Sug1, but not WDR5, siRNA resulted in a loss of H3K18ac in addition to H3K4me3. Nevertheless, WDR5 is also associated with the histone demethylase UTX and research now suggests that demethylation of H3K27me3 silencing is associated with the deposition of histone activating events (Lee, Villa et al. 2007). We have shown that recruitment of MLL/COMPASS subunits is associated with the release of the repressive nature of chromatin at cytokine inducible genes. Moreover, loss of Sug1 inhibits recruitment of WDR5 which correlates with the observed enhancement of H3K27me3 silencing, potentially through a loss in UTX association as well. The maintenance of a closed chromatin structure in Sug1 knockdown cells demonstrates a novel role for the 19S ATPases in regulating the initial events that reorganizes chromatin in response to stimuli (Figure 5-1). Further molecular investigations into the initial histone changes at these promoters are required for the full characterization of epigenetic regulation by the 19S proteasome.

In conclusion, we have shown that in mammalian cells 19S ATPases are essential for regulating a subset of inducible histone activation modifications; H3K18ac, H3R17me2 and H3K4me3. Although our study looked solely at cytokine inducible genes, studies have now shown that the APIS subcomplex associates with a variety of inducible mammalian promoters suggesting that the APIS may have a conserved, non-proteolytic function in regulating chromatin remodeling (Conaway, Brower et al. 2002; Ferdous, Kodadek et al. 2002; Greer, Zika et al. 2003; Yu and Kodadek 2007; Bhat, Turner et al. 2008). 19S ATPases are recruited to *p21^{waf1}* promoters in response to ultraviolet-induced DNA damage (Zhu, Wani et al. 2007), to the HIV-1 promoter to enhance Tat-dependent transcription (Lassot, Latreille et al. 2007) and to retinoic acid (RA) induced genes via an interaction with Steroid receptor co-activator (SRC)-3

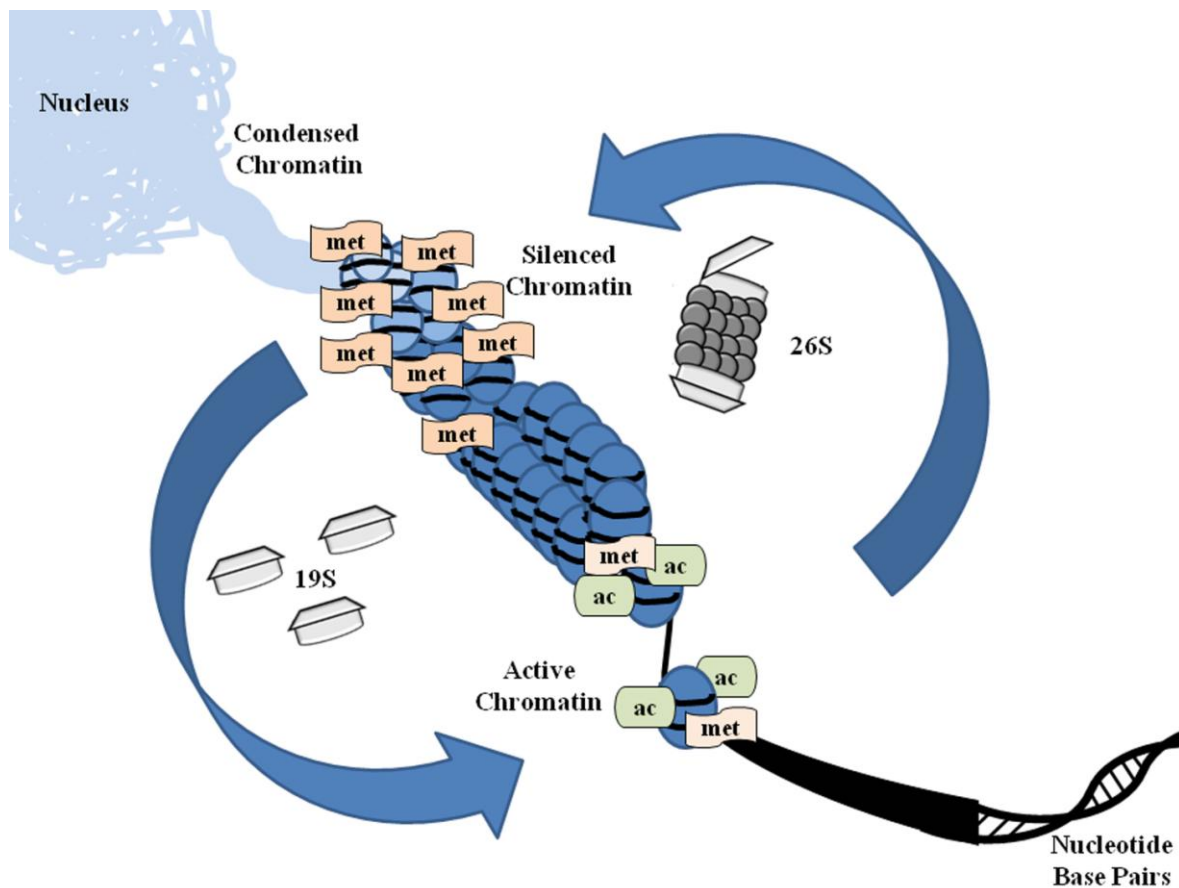


Figure 5-1. The 19S proteasome promotes the switch from a closed to open chromatin conformation in inducible gene expression. The 19S proteasome binds to heavily methylated (met) silenced promoters of inducible genes and is required for recruitment of histone modifying enzymes that acetylate (ac) and methylate histones to open chromatin and allow transcription factors access to DNA. Potentially the 19S proteasome associates with the 20S catalytic core, forming a functional 26S proteasome that helps return recently transcribed genes to their native conformation.

(Ferry, Gianni et al. 2009). Therefore it is likely that components of the 19 proteasome play a similar role in regulating chromatin structure at a variety of inducible genes.

Moreover, we showed that the 19S ATPases contribute to chromatin structure independent of the proteasome's proteolytic function and that ATP hydrolysis may not be central to their interaction with chromatin remodelers. How these 19S ATPases are functioning to regulate chromatin reorganization remains a mystery. Although we observed a common binding partner of the various histone modifying enzymes known to mediate events targeted by the 19S proteasome, knockdown of the binding partner did not mirror that of Sug1 knockdown. In addition, we were not able to determine the mechanism by which the 19S proteasome gets recruited to induced promoters. Evidence suggests that removal of silencing modifications is intimately linked to the deposition of histone activating events to reorder chromatin structure.

We found that levels of the silencing modification H3K27me3 are elevated and prolonged in the presence of diminished Sug1 expression at early timepoints post induction. A proposed model for these findings is shown in Figure 5-1. It is likely that the 19S ATPases are working to promote the relaxation of the repressive chromatin structure. Consequently, further studies into the early events that occur at inducible promoters are required for a full understanding of the contributions of the 19S proteasome to the epigenetic regulation of mammalian transcription.

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