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Role of Post-Translational Protein Modifications in Regulating HIV-1 and Mammalian Transcription

Author
Ali, Ibraheem Irfan

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Role of Post-Translational Protein Modifications in Regulating HIV-1 and Mammalian Transcription.

by

Ibraheem Irfan Ali

DISSERTATION

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Dedication

I want to dedicate this thesis to my mother, my family and those that give me inspiration.

I first need to thank Irfan Ayub Ali, my dad. The summer before this all started mom, Monica Lourido-Ali, passed away after her 10 year battle with cancer. I want to thank you for everything you have been through these last few years. I know navigating the challenges and changes in our lives has been really hard for you, but I will always admire the peace and philosophies you bring to the spaces we share. Seeing you happy, active, and on what seems like a perpetual vacation (the photos of all your travels makes me a bit jealous :P), will continue to make me smile and roll my eyes. You are so cool. Thanks for being such an important part to the most interesting family in the world.

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In this document I will provide a brief introduction and outline that will provide context for my studies in Chapter I, followed by the publications towards which I contributed substantially that are peer-reviewed literature reviews or original research in Chapters II-V. Followed by a chapter of conclusions and outlooks based on this body of work in Chapter VI. The citations for chapters II-V are as follows:

Jeng M.Y., **Ali I.**, Ott M. 2015. Manipulation of the host protein acetylation network by human immunodeficiency virus type-1. *Crit. Rev. Biochem. Mol. Biol.* 50, 314-325.

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Ali I. *et al*. 2018 Crosstalk between RNA Pol II C-Terminal Domain Acetylation and Phosphorylation via RPRD Proteins. BioRxiv doi: https://doi.org/10.1101/442491 (*Under Review, Molecular Cell*)

Role of Post-Translational Protein Modifications in Regulating HIV-1 and Mammalian Transcription

Ibraheem Irfan Ali

Abstract

The molecular gatekeepers of nearly all gene expression in living cells are the proteins that function in the process of transcription. Transcription occurs when a cell must respond to a signal. These signals can be in the form of metabolic responses, signals for growth or differentiation, signals to defend against stress or pathogenic invasion, to name a few. The fundamentals of transcription have been extensively studied in bacterial systems and model organisms, but technical limitations have hindered their studies in mammalian and human systems. Recent developments in mass spectrometric methodologies, next-generation sequencing and techniques to study difficult-to-detect post-translational protein modifications are extensively reviewed here to highlight an important regulatory network through which gene expression is regulated. In addition, I present two vignettes: the first, a study of the regulatory mechanisms of monomethylation of the HIV-1 Tat protein in regulating HIV-1 gene expression and latency; the second, a study investigating the role of acetylation in regulating RNA Polymerase II protein modifications and gene expression in mammalian systems. Together, these studies combine new mass spectrometric techniques, modification-specific antibodies, protein purification methods, and next generation sequencing to better understand the role of these modifications in regulating the transcriptional response in mammalian systems. These findings can be applied to better understand mechanisms that regulate HIV-1 viral latency, along with fundamentally shifting the field of mammalian transcription by pinpointing unique modes of regulation only found in higher eukaryotes relevant to HIV-1 infection and cancer.

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Chapter I: Introduction

DNA, RNA, Protein, Post-Translational Modification?

Transcription is a key step in the central dogma of gene expression in living systems. In its simplest form, cellular information is stored in the form of DNA. The information encoded in DNA can be transcribed into a message in the form of mRNA, then the mRNA is translated into a protein capable of performing actions for the cell. The structure of a protein determines its function, and a protein's structure is determined by the sequence of amino-acids and their higher-order organization. Functioning proteins interact with a variety of partners to mediate cellular functions and establish homeostasis, a kind-of ~zen~ state in which cell exist. Cells must constantly manage many different signals: fluctuating nutrient conditions; signals for proliferation and adaptation; signals against stress, damage or invasion. Often the interpretation of these signals can be observed in live cells by looking at the behavior of specific proteins in the cell and specifically, many protein behaviors are marked with specific post-translational modifications (PTMs) that occur in functional regions of the relevant proteins. Importantly, these PTMs modify the structure or charge of the target protein in order to execute context-specific responses to signals to change their homeostasis.

Changes in protein PTMs occur much faster than the production of proteins. This allows for rapid responses to changes in the cellular environment. This allows for a cell to manage an immediate physical response, and simultaneously prepare for follow up responses by activating the transcription of key genetic pathways. PTMs act as handles on proteins by changing the charge or bulkiness of amino acid residues on proteins. These handles can be used to move proteins, change their interacting partners, or influence their stability in the cell. Therefore, regulating the placement, removal and interpretation of PTMs is extremely important for cells to maintain their homeostasis.

Proteins that place modifications are generally referred to as “writers,” proteins that interpret and recognize the modifications are called “readers,” and the proteins that remove the modifications are referred to as “erasers.” Together, between dynamic phases of writing, reading and erasing; modifications can be placed, interpreted and removed to carefully control cellular signaling pathways. Furthermore, many environmental cues emerge in the form of small molecules, generally referred to as metabolites, Writers and erasers of cellular PTMs use some of these metabolites as essential co-factors that regulate their enzymatic function (Lin and Caroll, 2018). Therefore a surplus or deficiency in a key metabolite could potentially lead to changes in the way a cell responds to the environment.

There are a number of very well studied PTMs but for the purposes of this discussion I think it will be important to name four modifications: phosphorylation, acetylation, methylation and ubiquitination. Protein phosphorylation is induced by a family of proteins called kinases, and removed by phosphatases. Kinases are energy (ATP) dependent enzymes that add a negatively charged phosphate group to a hydroxyl-containing amino acid, releasing energy in the process. The energy released from this chemical reaction can be harnessed to activate molecular processes, change protein structure or localization, among other things. Phosphatases remove the phosphate group, effectively recycling the protein or making way for other relevant modifications to influence the protein domain. There are many kinases and phosphatases in the cell and these proteins often have highly specific targets. Pharmacologists have taken advantage of this specificity to produce highly specific small molecule inhibitors to treat diseases such as cancer, which is regulated in-part by the disruption of specific cellular pathways (Hunter, 1995).

Acetylation describes the transfer of an acetyl-group to the amine at the ε-position of a lysine residue. Writing of acetylation is regulated by lysine- or histone-acetyltranferases (KDACs aka, HDACs). Acetylation neutralizes the lysine’s positive charge and creates an amide group which can induce very stable hydrogen bonding with specific reader proteins. Removal of

acetylation is regulated by deacetylases. These proteins will be thoroughly discussed in **Chapter IV**. There are fewer of acetylation modulating proteins in cells relative to kinases and phosphatases, but they tend to have broad specificity and can simultaneously influence several cellular pathways and molecular functions. The regulation of protein acetylation has been closely associated with regulation of aging, metabolism and cancer. Furthermore, the way acetylation regulates protein pathways is evolutionarily conserved in eukaryotic organisms, and can be studied using the array of model-organisms scientists have used in the last century (Verdin and Ott, 2015). As a consequence, targeting protein acetylation is of great interest to pharmaceutical companies and healthcare providers, as they can propose long-term treatment courses for nearly impossible-to-treat diseases under the guise of “reducing morbidity” and creating hope in an aging, dying population.

Protein methylation is a special case as it has some species specific functions and does not change the charge, but changes the bulkiness and hydrophobicity of lysine residues. Methylation occurs on lysine or arginine residues. There are residue specific methyltransferases (lysine methyltransferases - KMTs; protein arginine methyltransferases- PRMTs). KMTs can catalyze mono-, di-, or tri-methylation of lysine residues; PRMTs catalyze mono- or di-methylate arginine residues in a symmetric, or asymmetric pattern. Methylation of proteins is recognized by specific protein domains, and also known to alter specific protein-DNA interactions. Focusing primarily on KMTs, these proteins have more specific sets of targets and are more diverse than protein acetyltranferases, but also remain difficult to target, as technical limitations have made it difficult to study these modifications until recently. Lysine methylation is removed by my favorite subfamily of proteins, lysine-specific demethylases (LSDs). Protein acetylation and methylation are best studied in the nucleus of the cell and are known to regulate chromatin, the organization of DNA with histones and other regulatory proteins in this subcellular compartment (Lee et al., 2005).

Ubiquitination describes the addition of the “ubiquitin” moiety. This is a ~8.5kd protein that can be covalently attached by its C-terminal glycine to lysine (or other) residues. Ubiquitin can serve as a subunit of a larger polyubiquitin chain, as there are several lysine residues in the ubiquitin protein upon which an additional ubiquitin group can be added. Ubiquitylation is tightly regulated by layers of enzymes (E1, E2 and E3 ligases) that can independently regulate the recognition, initial catalysis, and polymeraization of the modifications. Ubiquitin is best known to regulate the degradation of proteins, but can also regulate localization, translation or function of proteins in different ways (Komander, 2009). Ubiquitin is removed by deubiquitinating enzymes (DUBs) and recognized by ubiquitin binding domains (UBDs).

The modifications described above are typically studied in isolation, or for individual proteins. Recent studies have taken combinatorial approaches to understanding PTMs using either mass spectrometry or modification-specific antibodies in combination with functional assays. These works have shed light on the interconnected nature of PTMs when influencing protein function. For example, phosphorylation can expose a regulatory region in a protein normally folded to prevent activation. This regulatory region could contain a lysine residue upon which a methyl group can lead to the suppression of acetylation at an adjacent position. This may lead to ubiquitin mediated protein degradation in order to suppress its function. With this example alone, you can see how metabolites influencing each modification could push the function of a protein in many different directions.

This document will discuss the importance of post-translational modifications for the mammalian transcriptional response and provide a conceptual reflection on the stochastic and dynamic nature of these modifications. I will report experiments conducted in two model systems: first, in the context of HIV-1 infection, then second, in the context of RNA Polymerase II response to signals or genetic perturbation. Together, these studies will build a picture of the transcription cycle with specific focus on observations unique to mammalian systems with the hope I can shed some light on HIV-1 latency and therapeutics, mammalian organismal

development and cancer progression. To better understand how PTMs can regulate a process as important as transcription I will provide a brief overview of the various layers of transcription regulation.

A Master of Immune Evasion

The challenges surrounding the eradication of HIV-1 is one of the most beautiful illustrations of the complexities around transcription regulation and their role in disease. As of 2016, the World Health Organization estimates that there are approximately 36.7 million people infected with HIV-1, with nearly 5,000 new infections per year. This disease is particularly dangerous because, shortly after infection, the virus goes through a long incubation period. This leaves some infected individuals unaware of the infection for years despite still being contagious. Fortunately, in wealthy countries, kits to detect HIV-1 infection are readily available; and the development of (Highly Active Anti-Retroviral Therapy) HAART has reduced HIV-1 from a terminal disease to a manageable chronic disease, greatly limiting the disease spread. Despite these developments, there is no cure for HIV-1, as there is a pool of "latent" virus silently residing within the DNA of infected host cells, invisible to HAART, and capable of spontaneous reactivation and thus re-establishment of infection. Aside from HIV-1 vaccine development projects, investigations behind the mechanism behind the control of latent HIV-1 are thought to be the next step towards a functional cure for this devastating disease.

HIV-1 latency is established shortly after infection is initiated. The best evidence for this comes from studies of the vertical transmission of HIV-1 from mother to child during birth. The classical example of this is the "Mississippi baby" who contracted HIV-1 from her mother, but thirty hours after birth was treated with intense antiretroviral therapy. While the infant tested positive for HIV-1 at 30-hours after birth and throughout her first month of life, she was found to be HIV-1 negative at 29 days. The baby remained free from detectable infection until her 24th month, after several months without anti-retroviral therapy (ART) (Persaud et al., 2013). While the infant remained free from infection for longer than had ever been reported without ART, the

pool of latent virus had already been established, and her infection resurfaced. In a typical patient, stopping anti-retroviral therapy leads to the re-establishment of infection on the time-scale of weeks (Dahabieh et al., 2015). As a consequence, HIV-1 infection remains an infection for life.

During the HIV-1 life cycle, the virus begins by fusing with the plasma membrane of a CD4+ T cell. It deposits the viral nucleocapsid into the cell cytoplasm where the protein complex disassembles and becomes exposed to the contents of the cell. At this stage, the HIV-1 reverse-transcriptase enzyme converts the single-stranded viral RNA genome into a double stranded DNA intermediate. This DNA intermediate is organized into a complex with the viral integrase protein, histones and other host factors to translocate into the nucleus and integrate into the human genome (Ali et al., 2016). In order to successfully replicate within a cell, the virus must be able to express the genes that its genome encodes. This can generally be accomplished by two means. The first is to integrate into a region of the human genome where gene expression is very robust (Schroder et al., 2002). This would allow host transcription factors attracted to specific genes to accidentally transcribe through the viral gene and initiate low levels of viral gene expression and kick start the viral stage of transcription. However, the probability of landing in the right place for this is unreasonably low, especially considering cells in a mature human are largely quiescent.

Naïve CD4+ T cells that are infected by HIV-1 are not like most cells. These cells are typically circulating throughout the body, occasionally landing in lymph nodes where they are informed of the cellular immune state via antigen presenting cells. Antigen presenting cells, such as macrophages and dendritic cells, present protein fragments they have generated from surveilling their environment to T cells and other lymphocytes. If the specialized, and antigen-specific, T cell receptor is compatible with the antigen presented, it will activate and proliferate to initiate an arm of the “adaptive” immune response. If the T cell receptor is not compatible, then the T cell will continue along. Since T cells are constantly on-the-move and interpreting

their environment, one must keep in mind that they will encounter many metabolic fluctuations and signals that must be kept in check in order to minimize unnecessary activation.

Through sexual transmission, HIV-1 is first taken in by dendritic cells and macrophages (Granelli-Piperno et al., 1998). Replication in these cells is very inefficient, but the virus can stably reside on the surface of these cells until they encounter a CD4+ T cell with the appropriate receptors to transfer the infection. In addition, some fraction of HIV-1 is able to stably integrate into macrophages or dendritic cells, even if they cannot sustain productive infection (Kumar et al., 2014). This leads me to the second strategy of viral infection. Where the proviral DNA ends up in a suboptimal region of the host cell where it can initiate both an acute and chronic program for infection (Jordan et al., 2001). The decision between acute versus chronic infection is made based on stochastic fluctuations in viral transcription factor expression, and host transcription (Dar et al., 2012; Weinberger et al., 2005).

In these suboptimal regions the virus must rely more heavily on its promoter, the Long Terminal Repeat, for gene activation. The HIV-1 promoter contains several conserved transcription factor binding sites that have co-evolved with human transcription machinery. This includes transcription factor binding sites for SP1, NF-κB, C/EBP, AP-1 and NFAT (Churchill et al., 2015). Various fluctuations in signaling for these transcription factors can lead to viral gene expression, as most clearly evidenced by J-Lat cell lines produced in the laboratory of Dr. Eric Verdin which are designed to reactivate in response to particular stimuli such as TNFα mediated NF-κB activation (Jordan et al., 2003). Again, the virus runs into another roadblock, these pathways will not always be active in the cell, even if the cells are constantly handling signals from its environment.

In order to get around this barrier, HIV-1 encodes for its own transcription activator, the **T**rans**a**ctivator of **t**ranscription (Tat) protein. Tat is a potent transcriptional activator and modulator of the host transcriptional response. Comparison of Tat deficient, and Tat competent, HIV-1 lentiviral expression cassettes demonstrates that Tat upregulates LTR gene expression

100-1000 fold (Jordan et al., 2003). Furthermore, Tat interacts with a variety of key transcription regulators including SIRT1, KMT7, p300, P-TEFb, SMYD2 (Ott et al., 2011). Tat also has a strong affinity to RNA, where it probably binds all over the genome via nascent transcripts produced from transcription. Furthermore, expression of other viral proteins causes the downregulation of various cell surface receptors including CD4, MHC-1, CD28, CCR5 and WW through direct and indirect mechanisms. In addition to transcriptional effects, HIV-1 Tat is quite toxic to cells and generally induces apoptosis if it occurs in too high of concentrations - probably a consequence of being a sticky protein that disrupts normal host protein complexes. This rearrangement of the cellular transcriptional program, and disruption of immune defenses is an important step in shifting the cell into a viral replication factory and its eventual demise.

In addition to this, HIV-1 evades the immune response using additional strategies. The HIV-1 virion uses components of the host cell membrane. The rapid mutation rate during reverse transcription causes point mutations in viral cell surface receptors, Env and gp120, making the proteins hard to consistently detect if antibodies are from a different viral clone. Furthermore gp120 non-specifically binds to B-cells to induce their activation and inducing random antibody responses. For these reasons, among others, is why it is so difficult to successfully produce vaccines against this virus. Only broadly-neutralizing antibody responses have been found to be effective but these can take years to develop and do not always work (Burton et al., 2012). All this goes to show that the virus is pretty damn good at evading the immune response. These challenges, however pale in comparison to the challenges surrounding viral latency.

One would imagine that a transcriptionally stunted HIV-1 virion is a good thing for the host. If the virus can only partially complete the viral life cycle, then the cell is intact and infection doesn't spread. However, today we are going to be savvy evolutionary biologists, and see that this partially functional virus is a huge advantage for the virus. With transcription of some fraction of virus generally silenced, the virus is able to live alongside the host without

disrupting host function. On random occasion, the cellular environment will favor replication and tilt the scales, viral replication may resume in the cell and reestablish infection, even if antiretroviral therapy is completely effective in its suppression of active viral replication. As a consequence, patients must take ART for the rest of their lives, incurable.

Chapter II will cover how HIV-1 manipulates aspects of the host acetylation machinery in order to create an environment suitable for viral infection. **Chapter III** is original research where the HIV-1 Tat protein and an HIV-1 reporter virus are used to demonstrate the role protein mono-methlyation has in regulating the HIV-1 transcriptional response. Together these materials outline current developments ongoing in HIV-1 research. It demonstrates the relevance of acetylation and methylation and their metabolites in regulating HIV-1 transcription, and discusses ongoing strategies to develop a functional cure for the disease.

The Gatekeepers of Cell Fate Decisions

HIV-1 has served as an early model for the understanding of eukaryotic transcription. As a brief recap: The chromatin environment, or degree to which the DNA is compacted in the region, affects whether or not the gene is accessible for activation. Chromatin can be remodeled in order to activate or suppress transcription. This can be induced by specific differentiation programs or the recruitment of transcription activators. Once the chromatin is accessible, the central driver of mammalian transcription RNA Polymerase II (Pol II), is recruited to the promoters of genes and is primed for transcription initiation. At many genes, Pol II can initiate, but then needs an additional signal in order to transition into productive transcription elongation and the production of an mRNA (one example). During transcription elongation, there are co-transcriptional events that ensure the mRNA is stable and communicating the proper information. Finally, the processed mRNA can be brought to ribosomes where proteins can be produced and the protein product can begin its life. In this section, I will briefly elaborate on these topics which will provide context for Chapters IV, V and VI.

The remodeling of the chromatin environment is a critical step during cellular differentiation and organismal development. Differentiation into different cell types requires exposing or hiding certain genomic regions. This is achieved by a balance between chromatin remodeler protein complexes such as the **B**RG1/BRM **A**ssociated **F**actor (BAF) and the **P**olybromo-associated BAF (PBAF) complexes (which are also known as SWI/SNF) and the Polycomb Repressive Complexes which are constantly invading the genome to repress aberrant or selfish-gene activation (Hodges et al., 2016). This balance leads to the expression of specific gene subsets based on the accessibility of the genetic material at doses appropriate for the differentiation of certain cell types. At a more granular level, it has been shown that positioning of a single nucleosome can play an important role in repressing HIV-1 to support viral latency. BAF/PBAF play important roles in activating and suppressing HIV-1 expression by moving this nucleosome, a process that is facilitated by the activities of an important acetyl-lysine reader protein and kinase BRD4 (Conrad et al., 2017).

If the promoter of a gene is accessible, recruitment of the **p**re-**i**nitiation **c**omplex (PIC) is an essential step in activating transcription. The PIC is a large multisubunit complex that recruits Pol II to gene promoters. This complex is composed of several **g**eneral **t**ranscription **f**actors (GTFs) including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH along with components of the Mediator complex, and Pol II (Taatjes, 2010). At this stage RNA Pol II is in a naïve state where its regulatory domain found on its C-terminus (CTD) remains in an unmodified state. The Pol II CTD is an intrinsically disordered protein domain which is composed of a repeated heptapeptide sequence with a consensus of $Y_1S_2P_3T_4S_5P_6S_7$. This domain is largely conserved across species, but often contains some degree of species specific differences in the number of repeats and the degree to which they diverge from the consensus sequence (Eick and Geyer, 2013). All the consensus residues of the CTD consensus sequence can be modified either through phosphorylation or proline-isomerization. Importantly, for transcription initiation to occur, Pol II must dissociate from the PIC and get loaded onto the promoter to initiate transcription. In

most eukaryotes this is marked by the phosphorylation of serine residues at positions 5 and 7 (Tietjen et al., 2010). Overall, different Pol II modifications are associated with specific transcriptional events.

Another key step in transcription is promoter-proximal pausing. Pol II pausing occurs after transcription initiation and the production of a nascent RNA transcript. Pausing has been shown to be important in many highly expressed genes and specifically regulates a certain subset of signal responsive genetic processes in higher eukaryotes (Adelman and Lis, 2012). Pausing is induced by the presence of **N**egative **El**ongation **F**actors (NELF) and **D**RB-**S**ensitivity **I**nducible **F**actor (DSIF) which effectively block transcription elongation from occurring. The transition between transcription initiation and elongation for HIV-1 is marked by the recruitment of the super-elongation complex which is composed of several important proteins and kinases that allow for the displacement of NELF and activation of DSIF. The SEC contains critical positive transcription elongation factors like P-TEFb, ELL, and AFF proteins. The recruitment of these proteins allows for the phosphorylation NELF and DSIF along with serine residues at position 2 of the Pol II CTD. Together these events allow for productive transcription elongation, and several co-transcriptional events that are important for the production of the final RNA product (Luo et al., 2012).

For the context of this body of work it is important we pay special attention to the molecular events that occur in the Pol II CTD. Phosphorylation of key residues on this domain is associated with molecular events that are generally conserved across eukaryotic organisms. For example S_5 phosphorylation is important for the recruitment of factors important for 5' methylguanosine capping and the recruitment of transcription elongation factors. Phosphorylation of S_2 induces the recruitment of splicing and polyadenylation factors important for mRNA processing and stability. The importance of CTD PTMs will be discussed in detail in Chapter V.

There are many proteins, molecules, nutrients and signals that must synergize in order to produce a proper transcriptional response. Mass spectromentric advances and modification-specific antibodies have highlighted that a huge number of the important components involved in transcription are modified post-translationally, especially by protein acetylation (Choudhary et al., 2009; Lundby et al., 2012; Weinert et al., 2018). Many of these modifications are not yet understood and they pose a critical gap in the scientific literature, especially as pharmaceutical companies continue to look towards the use of HDAC/KDAC or methyltransferase inhibitors as therapies. Furthermore, acetylation and methylation are tightly linked to metabolic intermediates and therefore could be affected by macro and micronutrient availabilities explaining their role in cancer, ageing and other disease states (Choudhary et al., 2014). Through this body of work I hope to demonstrate the importance and interconnected nature between transcription and post-translational modifications such as acetylation and methylation.

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Chapter II: Manipulation of the host protein acetylation network by HIV type 1

Mark Y. Jeng, Ibraheem Ali, and Melanie Ott

Gladstone Institute of Virology and Immunology, San Francisco, CA 94158, USA
Department of Medicine, University of California, San Francisco, CA 94158, USA

Address for correspondence: Melanie Ott, Gladstone Institute of Virology and Immunology, 1650 Owens Street, San Francisco, CA 94158. Tel.: +1 (415) 734-4807. Fax: +1-415-355-0855. E-mail:

mott@gladstone.ucsf.edu

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Abstract

Over the last 15 years, protein acetylation has emerged as a globally important post-translational modification that fine-tunes major cellular processes in many life forms. This dynamic regulatory system is critical both for complex eukaryotic cells and for the viruses that infect them. HIV-1 accesses the host acetylation network by interacting with several key enzymes, thereby promoting infection at multiple steps during the viral life cycle. Inhibitors of host histone deacetylases and bromodomain-containing proteins are now being pursued as therapeutic strategies to enhance current antiretroviral treatment. As more acetylation-targeting compounds are reaching clinical trials, it is timely to review the role of reversible protein acetylation in HIV-infected $CD4^+$ T cells.

Acetylation of host proteins is essential for cellular function

The survival and function of cells are critically dependent on their ability to rapidly integrate multiple, intersecting cell-signaling circuits. A key strategy for effectively regulating complex signals is the reversible post-translational modification (PTM) of proteins. Over 200 PTMs are known, many of which are highly conserved among a wide range of organisms (Jensen, 2006). Despite their ubiquitous presence, only a few PTMs have been comprehensively studied including acetylation of lysines. The global role of protein acetylation was initially underappreciated (Verdin

and Ott, 2015). Originally found to reversibly modify lysines in the tails of histones, acetylation was thought to regulate gene expression primarily by altering the structural properties of the chromatin environment (**Figure 2-1**). However, with the identification of acetylation-modifying enzymes and improvements in high-resolution mass spectrometry, it became clear that the regulation of cellular function by protein acetylation extends beyond the nucleus. Over 3600 novel acetyl-lysine sites have been identified in a broad range of human proteins in different subcellular compartments (Choudhary et al., 2009). Acetylation of these proteins has been linked to the regulation of diverse cellular pathways, including cell-cycle control, DNA damage response, cytoskeletal organization, and immune signaling(Shakespear et al., 2011; Spange et al., 2009).

Histone acetyltransferases (HATs) are considered the “writers” of acetylation because they transfer an acetyl group from the cofactor, acetyl-coenzyme A, to the target lysine (**Figure 2-1**). At least 26 human HATs are known, nine of which are grouped into three major families based on similarities in their structure and sequence: (1) GNAT (Gcn5-related N-acetyltransferases), including PCAF and GCN5; (2) MYST (MOZ, Ybf2/Sas3, Sas2, TIP60), including HBO1; and (3) p300/CBP proteins (E1A-associated protein of 300kDa/CREB-binding protein) (Berndsen and Denu, 2008; Li et al., 2012).

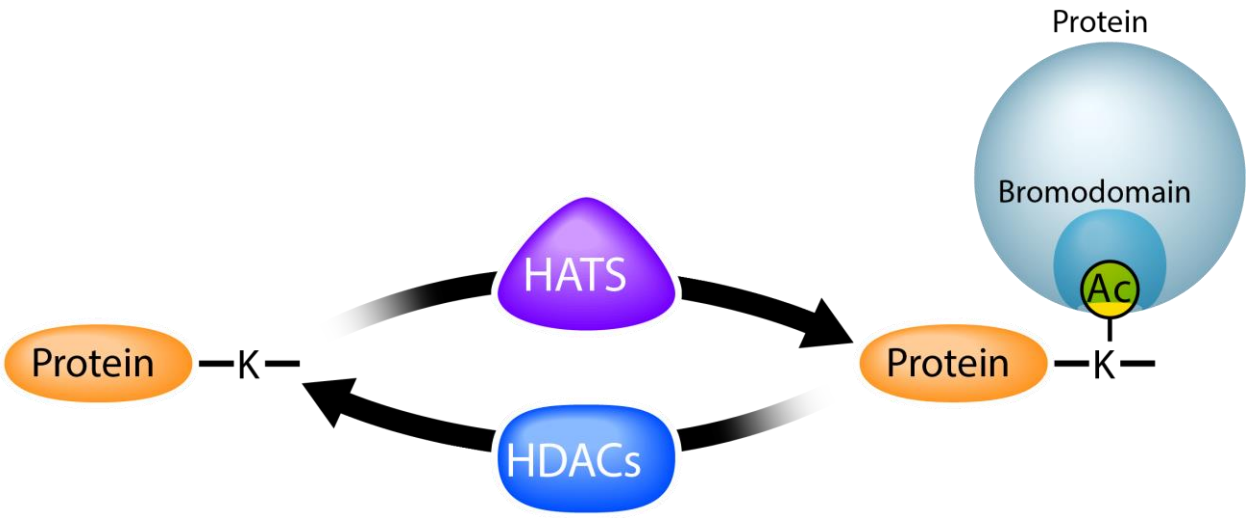

Figure 2-1: The different players in the host acetylation network. Histone acetyltransferases (HATs) transfer acetyl groups to target lysines in proteins while histone deacetylases (HDACs) remove them. Proteins containing bromodomains bind acetyl-lyisnes via distinct structural binding pocket and recruit complexes relevant for the function of the acetylated protein.

The activity of HATs is counterbalanced by HDACs, which remove the acetyl groups and are therefore considered “erasers” (**Figure 2-1**). Thus far, 18 mammalian HDACs are known, which are categorized into three classes based on distinct catalytic characteristics. Class I and II HDACs (HDACs 1–11) use a Zn^{2+}-dependent deacetylation mechanism and are inhibited by hydroxamic acids such as trichostatin A, vorinostat (SAHA), givinostat (ITF2357), and panobinostat (LBH589) (Shirakawa et al., 2013). Notably, class II HDACs shuttle between the nucleus and cytoplasm; a subset of these HDACs (class IIa) have little *in vitro* HDAC activity unless associated with the class 1 HDAC3/N-CoR complex (Jones et al., 2008). Class III HDACs are NAD^{+}-dependent sirtuin deacetylases (SIRTs 1–7), which are found in the nucleus, cytoplasm, and mitochondria, and are not responsive to classical HDAC inhibitors (Houtkooper et al., 2012).

Besides HATs and HDACs, so-called reader proteins have been identified that contain protein domains which bind specifically to acetylated lysines (**Figure 2-1**). Best known and characterized are proteins containing bromodomains—conserved ~110–amino acid protein modules that form a deep hydrophobic cavity that specifically accommodates acetyl-lysine residues (Filippakopoulos and Knapp, 2014). The human genome is predicted to encode 46 bromodomain-containing proteins, which are usually epigenetic regulators; some of these proteins contain more than one bromodomain (Filippakopoulos et al., 2012).

The three groups of acetylation-associated proteins engage in regulatory crosstalk. Many HATs, including p300/CBP and GNAT enzymes, contain bromodomains. Since they can both write and recognize acetylation marks, HATs can be recruited to acetylated sites and promote spreading of the mark (Josling et al., 2012). Because class IIa HDACs have negligible intrinsic deacetylase activity, they might function as acetyl-lysine readers rather than erasers and recruit other chromatin-modifying enzymes to sites of transcription (Bradner et al., 2010). Moreover, HAT and HDAC activities are regulated by acetylation of the enzymes themselves. p300/CBP proteins bind and regulate the activity of several HDACs (e.g. HDAC1, HDAC6, and SIRT2) by directly

acetylating lysines (Han et al., 2009; Han et al., 2008; Qiu et al., 2006). Conversely, SIRT2 can regulate the autoacetylation of p300 and thereby modulate its ability to bind to transcription pre-initiation complexes (Black et al., 2006; Black et al., 2008). HATs and HDACs not only regulate each other, but they are also intimately tied to the metabolism of cells through their cofactors acetyl-coenzyme A (HATs) and NAD^+ (Class III HDACs). This regulatory crosstalk serves to maintain a dynamic equilibrium between the acetylation and deacetylation of specific substrates within cells and to rapidly translate environmental cues into shifts in complex cellular processes.

Viruses have evolved intricate strategies to usurp complex cellular processes in support of their own propagation. HIV-1 is a complex lentivirus that reverse transcribes its RNA genome into cDNA and integrates into the host chromatin of $CD4^+$ T lymphocytes and macrophages. Through recruitment of the host transcriptional machinery, HIV promotes high-level transcription of its viral genome or becomes transcriptionally silenced in a subset of latently infected memory T cells (Ott et al., 2011).

Acetylation of the chromatin environment near the viral integration site can affect HIV transcription (Shirakawa et al., 2013). However, acetylation of non-histone proteins is also important in the viral life cycle. Multiple interactions exist between HIV and HATs, HDACs, and bromodomain-containing proteins encoded by the host. These interactions can alter the function of these epigenetic regulators, thereby disrupting the host acetylation network. Viral proteins, including the virally encoded integrase enzyme and transactivator of transcription (Tat), serve as substrates for cellular HATs and HDACs and require timely acetylation and deacetylation events for their proper function. In this review, we discuss the known mechanisms by which HIV taps into the host acetylation network as a basis for our understanding of how acetylation-targeting strategies interfere with the HIV life cycle.

HIV and the host acetylation machinery

Recent system-wide mass spectrometry approaches identified a large number of interactions of acetylation-related proteins with HIV (Fahey et al., 2011; Gautier et al., 2009; Jager

et al., 2011) , many of which have yet to be experimentally confirmed. Importantly, because these proteomic strategies rely on affinity purifications, it is unclear whether the interactions are direct or indirect via a larger protein complex. The majority of human HATs (19 of 26) interact with at least one HIV protein; some, such as p300 and p160, interact with up to five (**Figure 2-2).** As much as one third of human HDACs (6 of 18) have been formally identified as HIV interaction partners. Notable HDACs that can interact with up to three HIV proteins include HDAC1 (Tat, Vpr, and integrase) and HDAC6 (Tat, gp41, and gp120). Similarly, about one third of bromodomain-containing proteins (16 of 46) display HIV-binding potential. Interestingly, the majority of these interactions are made with HIV Tat (Tat binds to 11 HATs, 4 HDACs, and 14 bromodomain-containing proteins), underscoring the importance of reversible acetylation in the function and regulation of this accessory HIV protein. Below, we focus on confirmed interactions and modifications of critical regulators of the HIV life cycle.

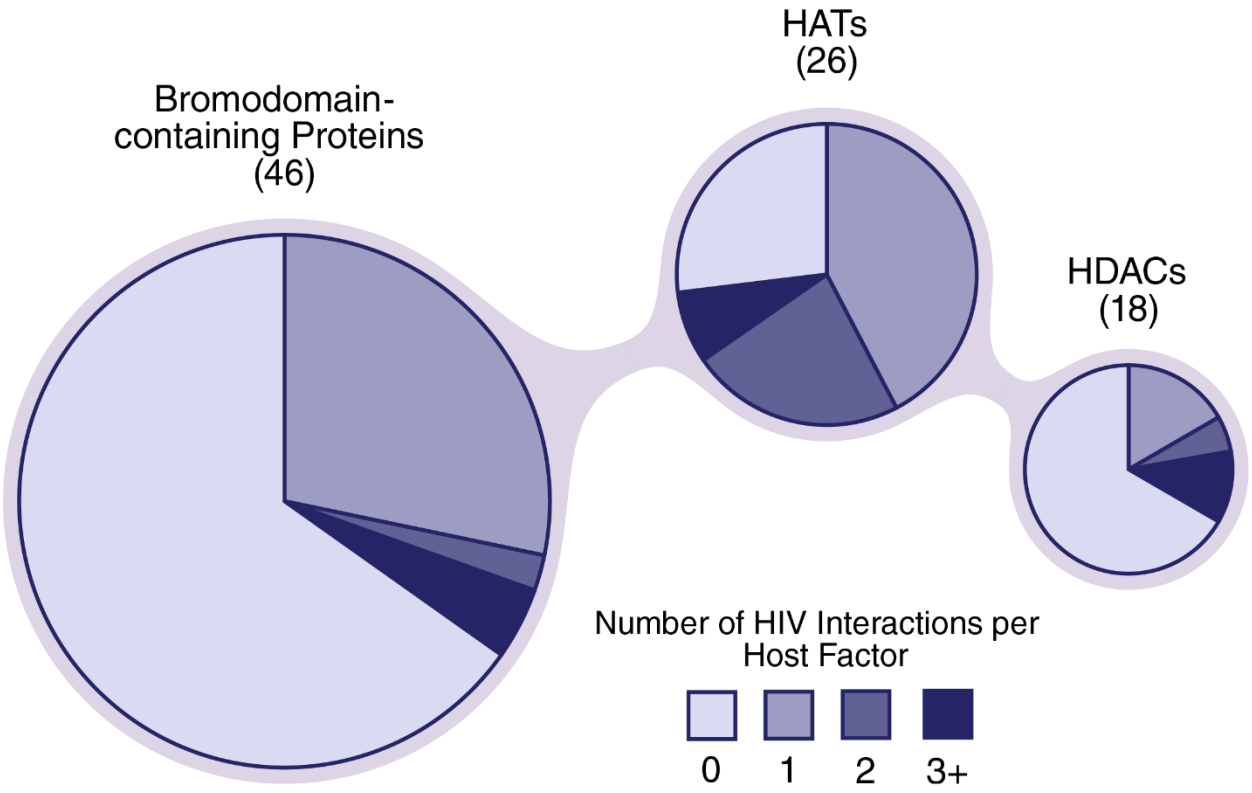

Figure 2-2: Global interactions of HIV with the host acetylation network. Recent unbiased interaction studies between HIV and host proteins have identified a high degree of interplay between HIV proteins and host acetylation factors: 19 of 26 HATs, 6 of 18 HDACs, and 16 of 46 bromodomain-containing proteins bind diverse HIV proteins. These data were determined with GPS-Prot (http://www.gpsprot.org), a web based software tool that integrates HIV-Host interaction datasets.

Acetylation of host and viral factors during HIV entry and integration

Although studies of the effect of acetylation on HIV replication have focused traditionally on transcriptional regulation, mounting evidence suggests that acetylation is critical in early steps of the viral life cycle. After HIV-1 attaches to the plasma membrane of the host cell, the viral envelope (Env) proteins gp120 and gp41 interact with the host receptor CD4 and one of two coreceptors—CXC chemokine receptor type 4 (CXCR4) and CC chemokine receptor type 5 (CCR5)—to facilitate viral fusion and entry (**Figure 2-3**) (Loetscher et al., 2000). Once within the cytoplasm, the viral nucleocapsid uses the host microtubule network to move toward the nuclear pore complex by manipulating cytoplasmic factors such as dynein (McDonald et al., 2002). During this time, the core of the HIV-1 particle progressively disassembles (viral uncoating), and the viral RNA genome is reverse transcribed by the viral reverse transcriptase enzyme to form a pre-integration complex with the proviral double-stranded cDNA at its center. The pre-integration complex, composed of viral and host factors, mediates the transport of double-stranded cDNA into the nucleus, where it integrates into the host chromatin with the assistance of host factors such as the lens epithelium-derived growth factor (LEDGF/p75) and the virally encoded integrase enzyme. During these early steps of HIV infection, several host and viral factors undergo reversible acetylation. Here, we will discuss how these early steps are regulated by the acetylation of α-tubulin, HIV-1 integrase, and cyclophilin A (**Figure 2-3**).

Microtubules are composed of α/β tubulin heteropolymers, which form key structures in cell division, vesicular trafficking, and multiple signaling pathways. These dynamic filaments are stabilized by acetylation of the α-tubulin polymer—a highly conserved mechanism that is increasingly recognized as a key factor in human health and disease (Perdiz et al., 2011; Piperno et al., 1987). As shown by Valenzuela-Fernández *et al.*, the interaction between the HIV gp120 protein and the CD4 T-cell surface receptor induces α-tubulin acetylation, leading to microtubule stabilization necessary for fusion of the virus to the host cell (Valenzuela-Fernández *et al.*, 2005). By altering the activity of HDAC6, one of two reported tubulin deacetylases (North *et al.*, 2003)–

either through overexpression of the wildtype protein or a dominant-negative mutant—the authors linked α-tubulin acetylation to early viral fusion (**Figure 2-3**).

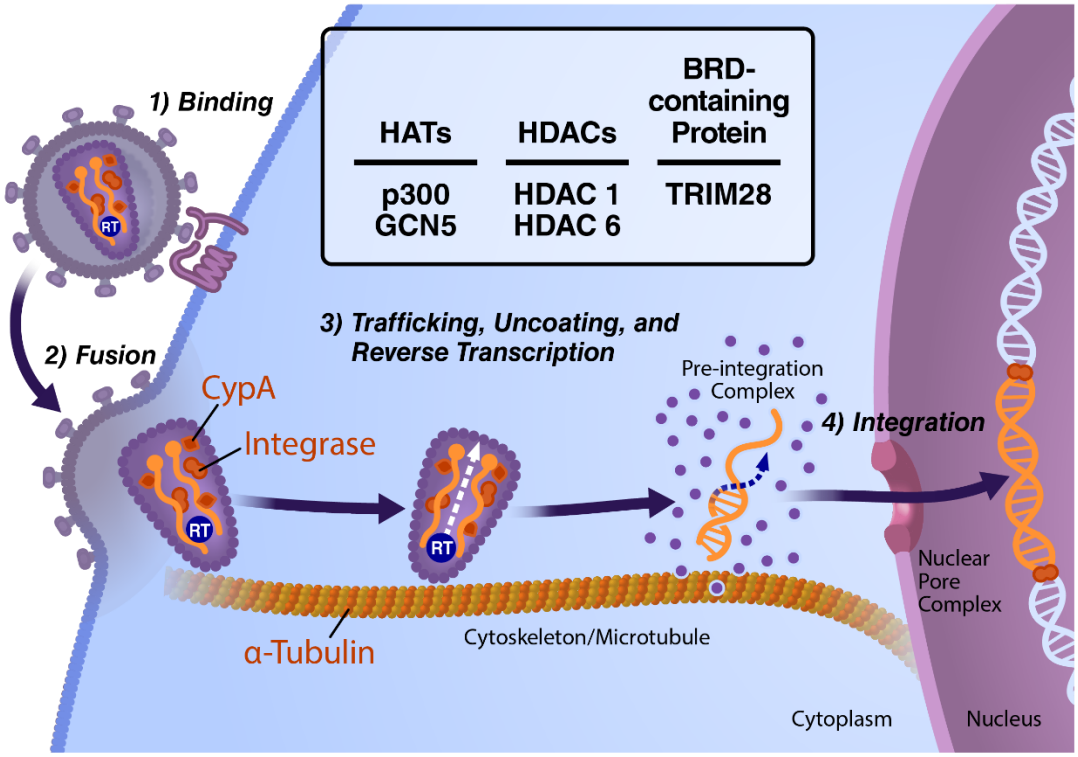

Figure 2-3: The role of reversible protein acetylation in the early HIV infection. The role of reversible protein acetylation in early HIV infection. The different steps of the early stages of the HIV life cycle are depicted with viral (integrase) and host [cyclophilin A (CypA) and α-tubulin] factors regulated by acetylation depicted in red. Engagement of HIV envelope protein gp120 with its host receptor CD4 induces α-tubulin acetylation and microtubule stabilization, a process required for successful fusion of the virus to the cell. Overexpression of HDAC6, which decreases a-tubulin acetylation, impairs virus–cell fusion and subsequent infection. Cellular CypA, a peptidylprolyl isomerase, is packaged into budding virions and regulates early steps of infection. Acetylation of CypA impairs its catalytic activity and disrupts its interaction with the HIV gag protein. The viral integrase enzyme is critical for the integration of proviral double-stranded cDNA into the host chromatin. Acetylation of the integrase enzyme by p300 enhances its affinity to genomic DNA and its strand transfer activity. It also recruits bromodomain-containing protein TRIM28 and its associated HDAC1 activity.

Recently, the matrix region of HIV Gag was shown to recruit the EB1-binding protein Kif4 to the ends of microtubules, thereby regulating the formation of acetylated α-tubulin necessary for early stages of HIV-1 infection (Sabo et al., 2013). Occurring specifically at the postentry stage of infection, this recruitment affects nuclear import, initiation of reverse transcription, and viral cDNA synthesis. Human herpes virus 8 also induces microtubule acetylation during early stages of viral infection, pointing to a common phenomenon conserved among different viruses (Naranatt et al., 2005). However, the molecular mechanism by which HIV uses the host acetylation machinery to promote α-tubulin acetylation remains unclear. Although tubulin was one of the first non-histone proteins shown to undergo acetylation, the α-tubulin acetyltransferase αTAT-1 (or MEC17 in worms) was not identified until recently (Akella et al., 2010; Shida et al., 2010). Further studies are required to determine whether HIV regulates microtubule acetylation by directly recruiting αTAT-1, by inhibiting the α-tubulin deacetylases HDAC6 and SIRT2, or by alternative mechanisms.

Integration occurs within the large pre-integration nucleoprotein complex, which consists of the viral cDNA, viral proteins (integrase, matrix, Vpr, nucleocapsid, and reverse transcriptase), and several host factors. p300 can directly acetylate the viral integrase at three carboxy-terminal lysines: K264, K266, and K273 (Cereseto et al., 2005). Acetylation increases the affinity of integrase for genomic DNA and enhances strand transfer activity (**Figure 2-3**). Conversely, point mutations in acetylation sites or inhibition of p300 inhibited viral integration and replication (Cereseto et al., 2005). An additional report confirmed the acetylation of integrase by p300, but could not find a replication defect of point mutants when an untagged viral construct was used (Topper et al., 2007). A second integrase acetyltransferase, human GCN5, has partially overlapping specificity for integrase lysine residues, suggesting that integrase acetylation is more complex than originally assumed (Terreni et al., 2010).

The acetylated residues in HIV integrase are interaction sites for host bromodomain-containing proteins. Data from a tethered catalysis yeast two-hybrid screen identified host TRIM28 (also known as KAP1 and Tif-1B) as a bromodomain factor that binds preferentially to acetylated integrase (Allouch and Cereseto, 2011). TRIM28 recruits HDAC1, thus triggering deacetylation of HIV integrase and restricting viral integration (Allouch et al., 2011). This highlights the evolution of cellular mechanisms to counter infection by exploiting viral dependence on protein acetylation. Interestingly, HDAC1 was previously identified as a component of the integrase complex (Sorin et al., 2009). Here, the authors showed, using the yeast two-hybrid system, that integrase interacts with SAP18, a component of the cellular Sin3a/HDAC complex. They further showed that this complex, packaged into HIV-1 virions, is critical for postentry viral infection. In sum, these studies suggest that HDAC1 can regulate HIV infection either positively or negatively, depending on the context of viral interaction.

Cyclophilin A (CypA), a highly conserved host peptidyl-prolyl *cis-trans* isomerase, has complex functions in diverse cellular processes such as protein folding, signal transduction, and cell-cycle regulation. During HIV infection, CypA is recruited by the group-specific antigen (Gag) precursor polyprotein, which consists of important components of the HIV virion such as matrix and capsid proteins, and is packaged into budding virions (**Figure 2-3**) (Franke et al., 1994; Thali et al., 1994). After entering target cells, CypA is associated with multiple steps of early infection, including uncoating, reverse transcription, and nuclear trafficking (Fassati, 2012). These functions are regulated by acetylation (Lammers et al., 2010). The authors used a new *in vitro* system to generate large amounts of acetylated CypA protein using synthetically evolved acetyl-lysyl-tRNA synthetase/$tRNA_{CUA}$ pair system in *E. coli.*, which co-translationally directs the incorporation of acetyllysine into a target protein in response to specifically encoded amber codons (Neumann et al., 2008). They showed that acetylation of lysine 125 (K125) inhibits the catalytic activity of the CypA enzyme and disrupts its interactions with the HIV Gag protein. It is possible, although not shown, that the positive function of HDAC1 in early viral infection is associated with deacetylation

of K125 in CypA by the co-packaged integrase/Sin3a/HDAC complex, thereby promoting optimal CypA activity (Sorin et al., 2009).

Reversible protein acetylation and HIV transcription

Once integrated into the host chromatin, the HIV genome, like a human protein coding gene, is subject to transcriptional regulation by the host RNA polymerase II (Pol II) enzyme. During the first phase of HIV-1 transcription, short incomplete viral transcripts accumulate that cannot support full viral replication (Kao et al., 1987). These incomplete transcripts results from pausing of the Pol II complex shortly after transcription starts. This elongation block is not unique to HIV and is found in many human genes (Core et al., 2008). To overcome it, HIV encodes the transcriptional transactivator Tat, an RNA-binding protein required for elongation of HIV transcription. Tat recruits a critical multicomponent host factor, the positive transcription elongation factor b (P-TEFb), to the 5' extremities of elongating HIV transcripts, specifically to a conserved RNA stem-loop structure called TAR (**Figure 2-4**). The recruitment of P-TEFb to TAR promotes transcriptional elongation through its intrinsic serine/threonine kinase activity, enhancing the processivity of Pol II and dissociating negative elongation factors that physically obstruct transcription. Subsequent splicing of these elongated HIV RNA transcripts fuel the viral life cycle and give rise to novel Tat molecules which, in an autoregulatory loop, activate HIV transcription (Weinberger et al., 2005).

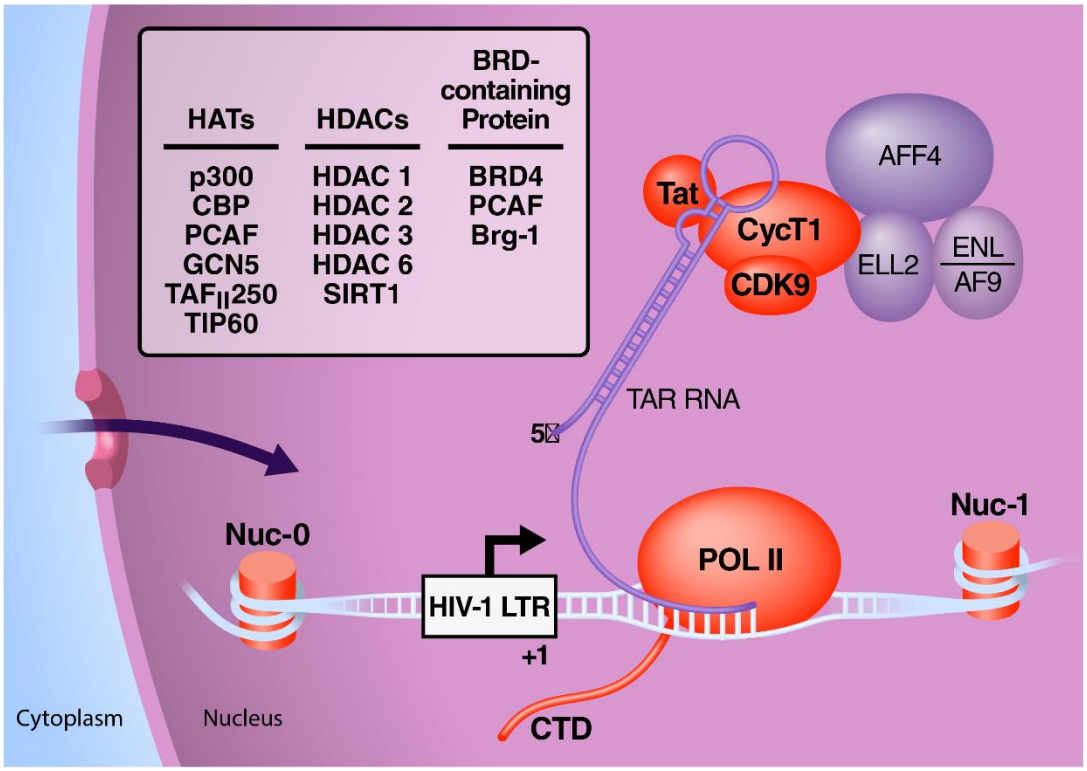

Figure 2-4: Regulation of HIV transcription by protein acetylation. HIV transcription is closely associated with the host acetylation machinery and is currently a target for acetyllysine-targeting drug regimens. Viral (Tat) and host factors [histones in nucleosomes (nuc), the Tat cofactor P-TEFb, RNA Polymerase II (Pol II)] that are targets of acetylation are depicted in orange. HATs, HDACs and bromodomains (BRD)-containing factors associated with HIV transcription are listed in the box. Proteins in purple depict factors of the super eloncation complex (SEC) recently identified as interacting with P-TEFb and Tat.

Throughout this process, viral transcription is regulated at multiple levels by reversible acetylation (**Figure 2-4**). The integrated HIV provirus is fully chromatinized, controlling access of host transcription factors to the HIV promoter in the 5' long-terminal repeat (LTR) (Verdin et al., 1993). This chromatin structure is under the control of HATs and HDACs, as first shown in studies in which the HDAC inhibitor trichostatin A potently remodeled the chromatin structure at the HIV LTR in cells (Van Lint et al., 1996) and in *in vitro* reactions (Sheridan et al., 1997). Since then, interest has grown to identify which HATs and HDACs play distinct roles in regulating HIV transcription. These efforts were recently comprehensively reviewed (Hakre et al., 2011; Shirakawa et al., 2013) Briefly, HDACs 1–3 are known to reside at the HIV LTR in cells with transcriptionally inactive (latent) HIV through interactions with various transcription factors, including YY1, LSF, CTIP2, CBP-1, NF-κB p50, c-myc and Sp1 (Shirakawa et al., 2013). In contrast, cellular HATs such as p300, CBP, PCAF, and GCN5 are recruited to the LTR by Tat and activating transcription factors such as NF-κB p65, AP-1, Myb, GR C/EBP, NFAT, Ets-1, LEF-1 and IRF (Hakre et al., 2011). Interestingly, increased histone acetylation during HIV activation appears to be associated with chromatin modifications during G_2 arrest of the cell cycle—demonstrating the ability of HIV to manipulate critical cellular processes through the host acetylation network (Thierry et al., 2004).

Many transcription factors involved in recruiting HATs or HDACs to the HIV promoter are themselves targets of acetylation. For example, the p65 subunit of NF-κB is acetylated at multiple sites. One of which is K310, a target site of SIRT1 and SIRT2; this acetylation event is critical for full transcriptional activity of p65 (Rothgiesser et al., 2010; Yeung et al., 2004). The p50 subunit of NF-κB is also acetylated at multiple sites, including K431, K440, and K441; these acetylation events enhance the DNA-binding activity and transcriptional activity of the heterodimeric NF-κB complex (Deng et al., 2003; Furia et al., 2002). Similarly, acetylation of Sp1 at K703 increases affinity of the transcription factor for DNA (Ryu et al., 2003). Interestingly, both components of the

Tat-associated P-TEFb complex, cyclin T1 and CDK9, also undergo reversible acetylation—a modification that alters their association with inhibitory ribonucleoprotein complexes and the kinase activity of CDK9 directly (Cho et al., 2009; Cho et al., 2010; Fu et al., 2007; Sabo et al., 2008).

. Three acetylation sites in the cyclin T1 subunit also serve to bind the second bromodomain of the double bromodomain and extraterminal domain (BET) protein BRD4, a process associated with activation of the P-TEFb complex (Schroder et al., 2012). Interestingly, eight lysines in the C-terminal domain of Pol II undergo reversible acetylation by p300 (Schroder et al., 2013). Acetylation of the Pol II C-terminal domain is specifically enriched downstream of polymerase-occupied gene promoters and is required for optimal activation of genes carrying paused Pol II. However, a direct connection with HIV has not yet been established (**Figure 2-4**).

Tat itself interacts with HAT enzymes including TIP60, PCAF, CBP, p300, $TAF_{II}250$, and human GCN5 (Benkirane et al., 1998; Col et al., 2001; Hottiger and Nabel, 1998; Kamine et al., 1996; Marzio et al., 1998; Weissman et al., 1998; Yamamoto and Horikoshi, 1997). These interactions can target the individual enzymes to specific transcription factor complexes (e.g. TBP, TF_{II}, NF-κB), recruit them to the HIV LTR, or modulate their catalytic activities (Caron et al., 2003). In certain cases, interactions between Tat and HATs promote extra-transcriptional effects, such as neuronal cell death (by disrupting neurotrophin signaling) or increased neoplasia (by impairing p53 tumor suppressor function) (Harrod et al., 2003; Wong et al., 2005). However, understanding the relationship between these individual interactions (i.e. temporal and spatial kinetics) within the context of the viral life cycle still remains a major challenge in the field.

Tat has at least two acetylation sites. Acetylation of lysine 28 (K28) by PCAF supports the cooperative interaction of Tat with its target RNA structure TAR and the P-TEFb cofactor, thereby promoting Pol II phosphorylation and efficient transcript elongation (D'Orso and Frankel, 2009; Kiernan et al., 1999). Acetylation of K50/51 by p300/CBP and human GCN5 terminates the P-TEFb-dependent step in Tat transactivation, mediates dissociation of Tat from TAR/P-TEFb, and

recruits instead the PCAF HAT via the PCAF bromodomain (Deng et al., 2000; Dorr et al., 2002; Kaehlcke et al., 2003; Kiernan et al., 1999; Mujtaba et al., 2002; Ott et al., 1999). Other interactions of acetylated Tat with host bromodomain-containing proteins include its recruitment of Brg-1—a component of the SWI/SNF nucleosome remodeling complex—and the HAT and transcription initiation factor $TAF_{II}250$ (Mahmoudi et al., 2006; Weissman et al., 1998). Bromodomain-containing protein BRD2 has also recently emerged as a potential regulator of the HIV LTR; this mechanism, however, appears to occur independently of Tat involvement (Boehm et al., 2013a).

Tat also interacts with host HDACs, such as SIRT1 and HDAC6, to undergo lysine deacetylation (**Figure 2-4**) (Huo et al., 2011; Pagans et al., 2005). Deacetylation of Tat by SIRT1 is necessary for optimal transactivator function—supporting a model in which timely and balanced acetylation/deacetylation events are important to fully support Tat function during HIV transcription. Tat serves as a super-substrate for SIRT1, associating avidly with the SIRT1 HDAC domain and thereby preventing other substrates (e.g. p65 K310) from accessing the enzyme (Kwon et al., 2008). By effectively inhibiting SIRT1 activity on other substrates, Tat induces hyperacetylation of p65, rendering it more active and activating infected $CD4^+$ T lymphocytes. Thus, Tat is not only a *bona fide* substrate and recruitment module for HATs, HDACs, and bromodomain-containing proteins, it also directly manipulates the activity of HATs and HDACs, resulting in reprogramming of infected T cells and manipulation of the infection rates of neighboring lymphocytes. Besides Tat, the accessory HIV protein Vpr also binds to p300/CBP HAT proteins and supports HIV transcription (Kino et al., 2002).

Acetylation during late stages of HIV infection

It remains to be determined whether acetylation also regulates the late stages of viral replication. However, it is clear that the changes in HIV entry, integration, and transcription described above will also indirectly alter the rates of virion assembly and budding. Furthermore, because stable microtubules are important for virion assembly and budding, it is likely that altering

the acetylation of α-tubulin will also directly affect these later stages of the viral lifecycle (Jolly et al., 2007). Similarly, the co-packaging of integrase with the Sin3/HDAC complex into virions is likely associated with more widespread acetylation/deacetylation processes during assembly, budding, and maturation of HIV virions (Sorin et al., 2009). However, the exact nature of the host or viral factors critical for these late steps of the viral life cycle remains unclear at this stage.

Therapeutic manipulation of the acetylation network

These intricate interactions between HIV and host acetylation-associated processes make acetylation-targeting drugs ideal candidates to support current antiretroviral therapy (ART). ART potently inhibits actively replicating HIV, but cannot eradicate the virus from patients (Chun et al., 1997; Siliciano et al., 2003; Wong et al., 1997). The major barrier to curing HIV-1 remains the persistence of long-lived, resting $CD4^+$ memory T cells harboring replication-competent but transcriptionally silenced proviruses (Chomont et al., 2009). These latent reservoirs are established early after infection, are resistant to ART, and trigger viral rebound after ART is stopped (Fischer et al., 2004; Kaufmann et al., 2004; Lewin et al., 2008; Zhang et al., 1999). One current approach is to “shock and kill” latently infected T cells with latency-reversing agents, forcing latent proviruses into active transcription under the protection of ART to eliminate them through the immune system or additional intervention.

HDAC inhibitors and reversal of HIV latency

Since early studies demonstrated that HDAC inhibitors modify the chromatin environment of the integrated provirus and potently activate HIV, considerable effort has focused on identifying HDACs that are important for maintaining the latent state (Hakre et al., 2011; Shirakawa et al., 2013). A growing library of small molecules that inhibit class I and II HDACs reactivate HIV within *in vitro* models of latent HIV infection; some of these compounds, previously approved for the treatment of cancer, have advanced into clinical trials (Campbell et al., 2015; Cillo et al., 2014; Falkenberg and Johnstone, 2014; Sgarbanti and Battistini, 2013). Compounds such as valporic acid, vorinostat, and givinostat showed success in increasing viral RNA levels in latently infected

resting T cells from treated patients; however, the results were either not reproducible (Archin et al., 2009; Blazkova et al., 2012; Routy et al., 2012; Wei et al., 2014) or pointed to the finding that repeated intake of HDAC inhibitors desensitizes cells to their latency-reversing activities (Archin et al., 2010).

The findings from studies of select latency-reactivating agents are summarized in **Table 2-1**, which also gives the working concentrations for each of the compounds used *in vitro* or *ex vivo* and outlines their mechanism of action. A more extensive list of HDAC inhibitors used *in vitro* can be found in a recent review (Wightman et al., 2012). Various comparative studies indicate that panobinostat and romidepsin are most efficient at targeting class I HDACs (Rasmussen et al., 2013; Wei et al., 2014). Notably, panobinostat, decreased the size of the latent pool in patients in a phase I clinical trial (Rasmussen et al., 2014). Furthermore, prolonged treatment with romidepsin had robust latency-reversing activity in patient-derived cells and induced virion release in a clinical study in patients (Wei et al., 2014). It remains to be shown whether panobinostat or romidepsin treatment will effectively delay viral rebound in patients. A single-drug will likely prove insufficient to overcome latency in all cells, and thus a combination treatment targeting multiple stages of the life cycle may be required (Bullen et al., 2014).

Compound Concentration	**Target**	Effects on HIV-1$^+$ Latent Cells	**Status in Trial** Trial ID	References
Romidepsin 0.1 nM – 45 nM	**Class I HDAC Inhibition**	• Increase in intracellular and extracellular HIV-1 RNA in addition to increase in viral outgrowth using *ex vivo* assays • Transient increase in plasma viremia levels in patients	**On-going Phase I Trial** NCT01933594	Bullen *et al*, 2014; Campbell *et al*, 2014; Wei *et al*, 2014; Bertino and Otterson, 2011
Panobinostat 5 nM – 30 nM	**Pan-specific HDAC Inhibition**	• Increase in intracellular HIV-1 RNA of HIV-1 *ex vivo* • Decrease in size of detectable latent reservoir	**Completed Phase I Trial** NCT01680094	Bullen *et al*, 2014; Rasmussen *et al*, 2013; Rasmussen *et al*, 2014; Wei *et al*, 2014
Vorinostat 100 nM – 5 µM	**Pan-specific HDAC Inhibition**	• Increase in HIV-1 RNA observed in patients • No detectable reduction of viral reservoir • Reactivation of HIV-1 transcription using *in vitro* and some *ex vivo* models	**Completed Phase I Trial** NCT1365065 **On-going Phase II Trials** NCT01933594 NCT01319383	Archin *et al*, 2014; Archin *et al*, 2012; Bullen *et al*, 2014; Cillo *et al*, 2014; Del Prete *et al*, 2014; Lucera *et al*, 2014; Elliott J, 2013
Valproic Acid 100 µM – 5 mM	**Pan-specific HDAC Inhibition**	• Induced transient increase in viral production in patients • High levels of toxicity in patients • Weak reactivation of latent HIV-1 transcription using *in vitro* J-Lat models	**Terminated Phase I Trial** NCT00289952	Archin *et al*, 2010; Bullen *et al*, 2014; Routy *et al*, 2012
Givinostat 30 nM – 250 nM	**Class I and Class II HDAC Inhibition**	• Increase in extracellular HIV-1 RNA with corresponding increase in supernatant p24 *in vitro*	**Completed Phase I Non-HIV Trials** NCT00792467 NCT00570661	Rasmussen *et al*, 2013; Furlan *et al*, 2011; Matalon *et al*, 2010
JQ-1 100 nM – 10 µM	**BET Protein Inhibition**	• Reactivates HIV-1 transcription using *in vitro* J-Lat models, but not primary cells using *in vitro* or *ex vivo* models	**Tested *in vitro* and *ex vivo***	Bullen *et al*, 2014; Boehm *et al*, 2013; Bisgrove *et al*, 2007; Li *et al*, 2013; Filippakopoulos *et al*, 2010
I-BET, I-BET151 100nM – 10µM	**BET Protein Inhibition**	• Reactivates HIV-1 transcription using *in vitro* J-Lat models, but not primary cells	**Tested *in vitro***	Nicodeme *et al*, 2010; Boehm *et al*, 2013; Seal *et al*, 2012
MS417 50 nM – 5 µM	**BET Protein Inhibition**	• Reactivates HIV-1 transcription using *in vitro* J-Lat models, but not primary cells	**Tested *in vitro***	Boehm *et al*, 2013; Zhang *et al*, 2012
Compound 8 1 µM – 140 µM	**Distruption of PCAF/Tat Interaction**	• Inhibits transcription from LTR-luciferase reporter, but not tested with models that express full-length virus	**Tested *in vitro***	Pan *et al*, 2007
LTK14 10 µM – 50 µM	**p300 Inhibition**	• Suppresses viral transcription *in vitro* with decreased cytotoxicity compared to parent compound isogarcinol	**Tested *in vitro***	Balasubramanyam *et al*, 2004; Mantelingu *et al*, 2007
BPRHIV001 1 µM – 140 µM	**p300 Down-Regulation**	• Inhibits transcription from LTR-luciferase reporter, but not tested with models that express full-length virus	**Tested *in vitro***	Lin *et al*, 2011

Table 2-1: Summary of emerging or clinically relevant acetylation-based therapeutic strategies to combat HIV-1 latency by reactivation or sustained suppression of the HIV-1 LTR. Clinical trial information was obtained through https://clinicaltrials.gov/.

In addition, “shock” therapies like HDAC inhibitors may exert unwanted effects on the “kill” arm of the approach. Notably, HDAC inhibitor treatment caused defects in T-cell development and distorted $CD8^+$ T cell activity, potentially diminishing the potential of these cells to effectively eliminate reactivated cells in patients (Shan et al., 2012; Tschismarov et al., 2014). Furthermore, treatment with vorinostat and panabinostat decreased interferon-γ production in primary activated $CD8^+$ T cells, resulting in impaired elimination of HIV-Gag-positive $CD4^+$ T cells in an *in vitro* model of HIV latency (Jones et al., 2014). Studies outside HIV also point to important roles of HDACs in the effector function of T cells and macrophages (Bagley et al., 2014; Cheng et al., 2014; Halili et al., 2010; Yan et al., 2014). For example, in conditional HDAC1 knockout mice, cytokine production in $CD8^+$ T cells was enhanced, but the ability to fend off a viral challenge was decreased (Tschismarov et al., 2014). It remains to be tested whether the targeted inhibition of individual HDACs is effective in reactivating latent HIV while reducing unwanted effects on T-cell function (Archin et al., 2009; Barton et al., 2014; Klase et al., 2014).

Bromodomain inhibitors and HIV transcription

Recently, novel inhibitors of bromodomain-containing proteins, especially those targeting so-called BET (bromodomain and ET domain) proteins BRD2–4 and BRDT, have shown impressive effects in cancer, immunity, and contraception (Filippakopoulos and Knapp, 2014; French, 2012; Matzuk et al., 2012). These drugs occupy the binding pockets for acetyl-peptides in bromodomains and thereby displace BET proteins from chromatin or other binding partners (Shi and Vakoc, 2014). Much of the work characterizing these compounds, particularly the freely available compound JQ1, has focused on inhibiting BRD4, owing to its relevance in some malignant midline carcinomas (Filippakopoulos et al., 2010; French, 2012). Because BRD4 is a cofactor of the P-TEFb complex and competes with the HIV Tat protein for P-TEFb binding, BET inhibitors were also tested for the ability to reactivate latent HIV. Several BET bromodomain inhibitors, including JQ1, iBET151 and MS417, activate HIV transcription in cell culture models of latency (**Table 2-1**) (Banerjee et al., 2012; Boehm et al., 2013b; Li et al., 2013), but their effect in

patient-derived cells varies (Shi and Vakoc, 2014; Zhu et al., 2012). BET inhibitors do not synergize with HDAC inhibitors to activate HIV transcription, supporting the notion that both drugs target similar molecular pathways (Bartholomeeusen et al., 2012; Boehm et al., 2013a; Loosveld et al., 2014). However, strong synergies exists with activators of the cellular protein kinase C pathway in cell culture (Boehm et al., 2013a; Jiang et al., 2014; Li et al., 2013; Wang et al., 2012) and with monoclonal antibodies against HIV in humanized mouse models of HIV (Halper-Stromberg et al., 2014). Further studies are still required to better understand the molecular mechanisms of how BET inhibitors activate HIV transcription, which BET protein is targeted, and how best to combine these inhibitors in clinical trials.

HAT inhibitors and the permanent silencing of HIV transcription

Alternatives to the "shock and kill" approach, which include durable transcriptional silencing of latently infected cells, are so far less developed. Since HIV–HAT interactions have a central role in the activation of viral transcription, a structure-guided approach could be used to develop specific inhibitors against Tat–HAT interactions (Pan et al., 2007; Vendel and Lumb, 2004; Zeng et al., 2005). While compounds such as curcumin and isogarcinol can inhibit p300/PCAF HAT activity, they also demonstrate low specificity or high levels of toxicities in cell lines (Balasubramanyam et al., 2004a; Balasubramanyam et al., 2004b). Mantelingu and colleagues demonstrated that chemical manipulation of these naturally derived compounds could increase the specificity of binding to p300, and in the case of one of the derived compounds, LTK14, suppress viral reactivation *in vitro* (Mantelingu et al., 2007). Another, more recent study, has shown that coumarin derivitaves, specifically, BPRHIV001, also inhibits p300 activity and is able to suppress Tat mediated transcription *in vitro* (Lin et al., 2011). While these studies collectively highlight the importance of p300/CBP as a potential target in HIV treatment, it is unclear whether HAT inhibitors can effectively inhibit viral rebound from latency *in vivo*. Further investigation of the molecular role of HATs, HDACs, and bromodomain-containing proteins in the

establishment and maintenance of latency are required to develop better and more targeted therapeutic interventions.

Conclusion and outlook

Protein acetylation, a highly conserved regulatory system in a broad range of organisms, can rapidly translate environment signals into critical cellular functions. Viruses such as HIV have evolved intricate strategies to manipulate this system to facilitate viral propagation at multiple steps of the viral life cycle. As a result, there is growing interest in using inhibitors of acetylation-associated proteins to disrupt these interactions. Despite an impressive body of work, much remains to be learned about the complex role of reversible acetylation during the HIV life cycle and within the immune system. The ability to continuously monitor acetyl-stoichiometric changes (i.e. frequency of each acetylated site within a cell)—on both a local and systemic scale—will be critical in assessing the biological significance of the effects of these modifications on different cellular processes. Two studies have begun to address this topic in *Saccharomyces cerevisiae* and *Escherichia coli* (Baeza et al., 2014; Weinert et al., 2014). These studies revealed that significant acetylation alterations occur in distinct subcellular compartments during specific cell-cycle phases or upon deletion of a particular HDAC. Since viruses operate in distinct host compartments at different time points, it will be interesting to use this technology to map acetylation dynamics in an infected cell during different phases of the viral life cycle. This knowledge will promote a more comprehensive understanding of the dynamics of host–virus interactions and highlight critical areas of interest for therapeutic intervention.

In addition, as new players are still continually being added to the acetylation network, novel hypotheses and opportunities for treating HIV will arise. Besides bromodomains, some tandem plant homeodomain zinc-finger proteins may also bind histones in an acetylation-specific manner (Ali et al., 2012; Qiu et al., 2012; Zeng et al., 2010). Furthermore, the tandem pleckstrin-homology domain of Rtt106, a yeast chaperone protein, binds acetylated histone H3 at lysine 56 (Su et al., 2012). Most relevant to HIV, the highly conserved YEATS domain, named for its five founding

proteins (Yaf9, ENL, AF9, Taf14, and Sas5), binds acetyl-lysine residues, with a preference for acetylated histone H3 lysine 9 (Li et al., 2014). ENL and AF9 are both members of the so-called super elongation complex (SEC), which is associated with HIV Tat and P-TEFb and critically involved in their function during HIV transcription elongation (He et al., 2010; Sobhian et al., 2010). It remains to be determined whether these interactions are dependent on the acetylation status of these factors and can be affected by acetylation-targeting drugs.

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Declarations of interest

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Chapter III: The HIV-1 Tat protein is monomethylated at lysine-71 by KMT7

Ibraheem Ali[†‡1], Holly Ramage[†1], Daniela Boehm[†1], Lynnette M.A. Dirk[§], Naoki Sakane[† ¶], Kazuki Hanada[¶], Sara Pagans[†], Katrin Kaehlcke[†], Kay Aul[†‖], Leor Weinberger[†‖], Raymond Trievel[††], Martina Schnoelzer[‡‡], Masafumi Kamada[¶], Robert Houtz[§], Melanie Ott[†‡2*]

From the [†] Gladstone Institute of Virology and Immunology, San Francisco, CA,

[‡] University of California, San Francisco, Department of Medicine, San Francisco, CA,

[§] University of Kentucky, Department of Horticulture, Lexington, KY,

[¶] Pharmaceutical Frontier Research Laboratory, Yokohama, Japan,

[‖] University of California, San Francisco, Department of Biochemistry and Biophysics, San Francisco, CA,

[††] University of Michigan, Department of Biological Chemistry, Ann Arbor, MI, and the
[‡‡] German Cancer Research Center (DKFZ), Heidelberg, Germany.

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[1] These authors contributed equally to the study.
[2] To whom correspondence should be addressed. Tel. (415) 734-4807; mott@gladstone.ucsf.edu

Running Title: KMT7 monomethylates Tat at K71
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Abstract

The HIV-1 transactivator protein Tat is a critical regulator of HIV transcription primarily enabling efficient elongation of viral transcripts. Its interactions with RNA and various host factors are regulated by ordered, transient posttranslational modifications (PTMs). Here, we report a novel Tat modification, monomethylation at lysine-71 (K71). We found that K71 monomethylation (K71me) is catalyzed by KMT7, a methyltransferase that also targets lysine-51 (K51) in Tat. Using mass spectrometry, *in vitro* enzymology, and modification-specific antibodies, we found that KMT7 monomethylates both K71 and K51 in Tat. K71me is important

for full Tat transactivation, as KMT7 knockdown impaired the transcriptional activity of wild type (WT) Tat but not a Tat K71R mutant. These findings underscore the role of KMT7 as an important monomethyltransferase regulating HIV transcription through Tat.

HIV-1 Tat Controls Viral Transcription

The HIV-1 epidemic remains a global health problem despite the growing availability of potent antiretroviral therapies. These therapies are not curative, as latent, transcriptionally silent virus can spontaneously reactivate from sanctuaries and rapidly rekindle viral infection after withdrawal of therapy (Bruner et al., 2015; Dahabieh et al., 2015; Mbonye and Karn, 2014). Therefore, the molecular mechanisms of the activation and suppression of HIV transcription are of great interest.

HIV encodes its own viral transactivator, Tat, which activates HIV transcription and facilitates its own production in a positive feedback loop. Tat is a small protein, typically found in a full-length form of ~101 amino acids (aa) or as a splice variant (72 aa) encoded only by the first exon of the *tat* open reading frame. Both isoforms efficiently transactivate the HIV promoter in the 5′ long terminal repeat (LTR). Tat interacts with the positive transcriptional elongation factor b (P-TEFb), and viral RNA through several well-characterized domains that can be found in its one-exon form: a cysteine-rich domain (aa 22–37) and a highly conserved core domain (aa 41–48), both of which participate in binding of P-TEFb (Garber et al., 1998; Yang et al., 1997; Zhu et al., 1997). The neighboring arginine-rich motif (ARM; aa 49–57) interacts with a specific stem-loop RNA structure called transactivation response element (TAR) located in the 5′ extremities of all viral transcripts (Garcia et al., 1989; Ivanov et al., 1999; Selby et al., 1989; Taube et al., 2000). Specific binding of the Tat ARM to TAR requires the coordinated binding of P-TEFb to Tat, as the cyclin T1 subunit of P-TEFb binds both the Tat cysteine-rich and core domains and loop sequences of TAR (Wei et al., 1998). C-terminal to these well-characterized domains is a glutamine-rich motif (aa 59–72). When expressed as a peptide, this region adopts a conserved

α-helical structure that is stabilized upon binding to TAR RNA and is implicated in T-cell apoptosis (Campbell et al., 2004; Loret et al., 1992).

Tat is regulated by a number of PTMs including phosphorylation, acetylation, methylation, and polyubiquitylation (Ott et al., 2011). The Tat ARM is highly modified at lysine and arginine residues. These modifications, including acetylation of K50/51 and methylation of K51 and R52/53, regulate TAR and P-TEFb binding positively (K51me) or negatively (K50ac/K51ac; R52me2/R53me2) (Mujtaba et al., 2002; Ott et al., 1999; Pagans et al., 2010; Sakane et al., 2011; Sivakumaran et al., 2009; Xie et al., 2007). The role of PTMs in the glutamine-rich motif is still unclear. Several phosphorylation sites in this domain (S62, T64, S68) enhance transcriptional activity, but are not well conserved among viral isolates (Endo-Munoz et al., 2005; Foley B, 2015). In contrast, lysine-71 is a highly conserved residue found in 74% of HIV-1 isolates across all clades reported in the HIV-1 sequence compendium (Foley B, 2015). At this residue, polyubiquitylation at K71 is required for full transactivation, but does not affect Tat stability (Bres et al., 2003).

Experimental Procedures

Materials

HEK293T, Jurkat, and TZMBL cells were obtained from American Type Culture Collection. Anti-FLAG M2 affinity gel (A2220) and anti-FLAG monoclonal rabbit antibodies (F7425) were from Sigma-Aldrich (St. Louis, MO). Tubulin antibodies (ab15246) were from Abcam and KMT7 antibodies (Clone 5F2.3, #04-805) were from Millipore. KMT7 siRNAs (#4392420) were from ThermoFisher. Tat72 proteins (unmodified, Tat K71me, Tat K51me, Tat K50Ac and Tat K51/K71me) were synthesized by PSL Peptide Specialty Laboratories (Heidelberg, Germany). Tumor necrosis factor α (TNFα), purchased from ThermoFisher (PHC3011) was resuspended in water at 100 ng/μl. Cycloheximide (MP Biomedicals, 02100183) was resuspended in water at 10 mg/ml.

In Vitro Methylation

Reactions were carried out with 3 µg of synthesized Tat peptides and 0, 1, or 2 µg of purified KMT7 in a solution of 0.1 M bicine, pH 8.2, 60 µM ^{3}H-labeled S-adenosyl methionine (^{3}H-SAM). After incubating the mixtures for 18h at room temperature, the methylation reactions were run on 15% SDS-PAGE gels, which were stained with Coomassie Blue, and destained overnight. Methylation was detected by autoradiography (22-h exposure).

Mass Spectrometry

In vitro modified and *in vivo* purified Tat peptides were analyzed by MALDI-TOF tandem mass spectrometry (MS) as described (Sakane et al., 2011).

Enzymology

Methyltransferase activity was measured with a modified radiometric assay (Houtz et al., 1991). Kinetic assays were performed with various concentrations of synthetic Tat72 peptides (unmodified, Tat K71me, Tat K51me and Tat K51/K71me). The 20 µl reactions contained Tat peptides, 0.1 M bicine, pH 8.2, 60 µM ^{3}H-SAM (3.4Ci/mmol), and 1 µg of purified full-length KMT7 (Couture et al., 2006). The reactions were incubated for 1 min at 37°C. To terminate the reaction and ensure full precipitation of the substrates, 0.5 ml of 10% TCA and 5 µl of a bovine serum albumin (BSA) solution (10 g/100 ml of water) were added. After this addition the mixture was then vortexed, incubated on ice for 3 mins, and centrifuged at 14,000 RPM for 3 minutes. To process the reaction, the protein pellets were washed with 150 µl of 0.1 M NaOH, re-precipitated in TCA, vortexed and centrifuged at 14,000 RPM for 3 minutes. The pelleted proteins were dissolved in 50 µl of formic acid, diluted by half with water, mixed with 1.25 ml of Bio-Safe II scintillant (Research Products International), and subjected to liquid scintillation. Activity was calculated after correcting for methylation in control reactions lacking either the enzyme or the substrate. The assays were performed in triplicate, and the data were plotted and fitted with SigmaPlot 11 (Systat Software) to the Michaelis-Menten equation.

Tat K71me Antibody Purification

Peptides for antibody synthesis (K71me1 Type 1, K71me1 Type 2; Fig. 4A) were synthesized by PSL Peptide Specialty Laboratories and injected into rabbits and antibodies were purified from serum with antigenic peptides as described (Pagans et al., 2011). Purified antibodies were eluted under acidic conditions, resuspended in 1% BSA and 0.1% sodium azide, and stored at –80 °C.

Dot Blot Analysis of K71me Antibodies

Tat peptides were serially diluted in water and spotted on a 0.2 μM Hybond ECL membrane. Membranes were air-dried, and nonspecific binding was blocked with nonfat dry milk (5 g/100 ml in TBST consisting of 50 mM Tris-HCl, ph 7.4, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature. Membranes were incubated with K71me antibodies diluted in blocking buffer for 1 h, washed 5 times with TBST, then incubated with HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch) at a concentration of 16 ng/ml in blocking buffer, washed 3–5 times with TBST, and analyzed using standard ECL substrate.

Detection of K71me in vivo

Tat mutants (K71A, K71R, K51/71A, K51/71R) were generated by site-directed mutagenesis of previously described Tat constructs (Pagans et al., 2010). KMT7 expression vectors (WT and H297A) were also previously described (Pagans et al., 2010). For experiments conducted in HEK293T cells, Tat constructs (2 μg) or Tat and KMT7 (2.5 μg and 6 μg, respectively) were transfected into cells using Lipofectamine 2000 and incubated for 24h prior to lysis. For experiments conducted in Jurkat cells, WT and K71R Tat72 expressing cells were produced as described (Jordan et al., 2001). After sorting, cells were transduced with lentiviruses containing control short hairpin RNAs (shRNAs) or KMT7 shRNAs (KMT7a: GCACTTTATGGGA- AATTTA; KMT7b: GTAGCTGTGGGACCTA- ATA) and a puromycin resistance cassette. After 1 week of puromycin selection at 2μg/mL, cells were lysed using IP-lysis buffer (150mM NaCl, 50mM Tris pH 7.4, 1mM EDTA, 0.5% v/v NP-40 substitute) and Tat-FLAG was immuno-precipitated from

500 µg of protein using Anti-FLAG M2 affinity gel. Immunoprecipiated Tat was analyzed using either anti-FLAG antibodies (0.16 µg/ml) or K71me antibodies (1.75 ng/ml) diluted in blocking buffer. After washing, membranes were incubated with a HRP-conjugated anti-rabbit antibody (160 ng/ml). All K71me western blots were performed at least twice.

Measuring the Half Life of Tat

WT LTR-Tat101-Dendra (LTD) constructs were subjected to site-directed mutagenesis to generate the K71R mutation in Tat. WT or K71R LTD constructs, a lentiviral construct (pCMV-ΔR8.91) and VSV-G pseudotyped envelope plasmid were co-transfected into HEK293T cells to produce lentivirus, as described (Dull et al., 1998). Jurkat T cells were infected with either WT or K71R LTD lentiviral vectors to generate polyclonal J-Lat populations as described (Jordan et al., 2003). After resilencing of the LTR, LTD jurkats were infected with KMT7b shRNAs and selected with puromycin for at least 1 week. For protein stability experiments, Tat expression was induced with TNFα (10 ng/ml) for 16 h followed by treatment with cycloheximide (10 µg/ml). Cells were fixed at various times in 2% paraformaldehyde (Alfa Aesar), incubated at 4 ^{o}C for at least 1 h, and analyzed on a FACSCalibur DxP8 (Cytek). Flow cytometry data were analyzed by FlowJo X. Data were normalized to 100% at time 0 and natural log transformed to produce a linear half-life curve, from which the slope was calculated. Two-tailed Z-tests were performed on averaged slope values from at least 4 independent experiments. The equation $t_{(1/2)}$ = ln(2)/-slope was used to generate half-life values. Time-lapse microscopy experiments were performed as described (Razooky et al., 2015).

Luciferase Assays

TZMBL cells ($1x10^5$) were transfected with a total of 100 ng of DNA containing 1, 2, 5, or 10 ng of Tat-expressing plasmids (WT, K51R, K71R, K51/71R) or empty vector using X-tremeGENE 9 (Roche Diagnostics) as recommended by the manufacturer. The cells were incubated for 48 h and lysed in 1x Promega Passive Lysis Buffer. Luciferase assays were processed with the

Promega Dual-Luciferase Reporter Assay System and measured on a Monolight 2010 luminometer. Experiments were conducted with 4 independent biological replicates with technical duplicates and the statistical significance of differences was determined with one-sided *t* tests.

Results

To determine if K51 is the only KMT7 methylation site in Tat, we performed *in vitro* methylation reactions with synthetic Tat proteins spanning aa 1–72 (Tat72). We observed substantial incorporation of ^{3}H-S Adenosyl-Methionine (^{3}H-SAM) in Tat72 proteins carrying monomethylated K51, indicating additional KMT7 methylation sites in Tat (**Figure 3-1A and 3-1B**). In Tat72 peptides with an acetyl group at K50, ^{3}H-SAM incorporation was also slightly decreased. This is consistent with previous observations that this modification reduces access of KMT7 to K51 (Sakane et al., 2011).

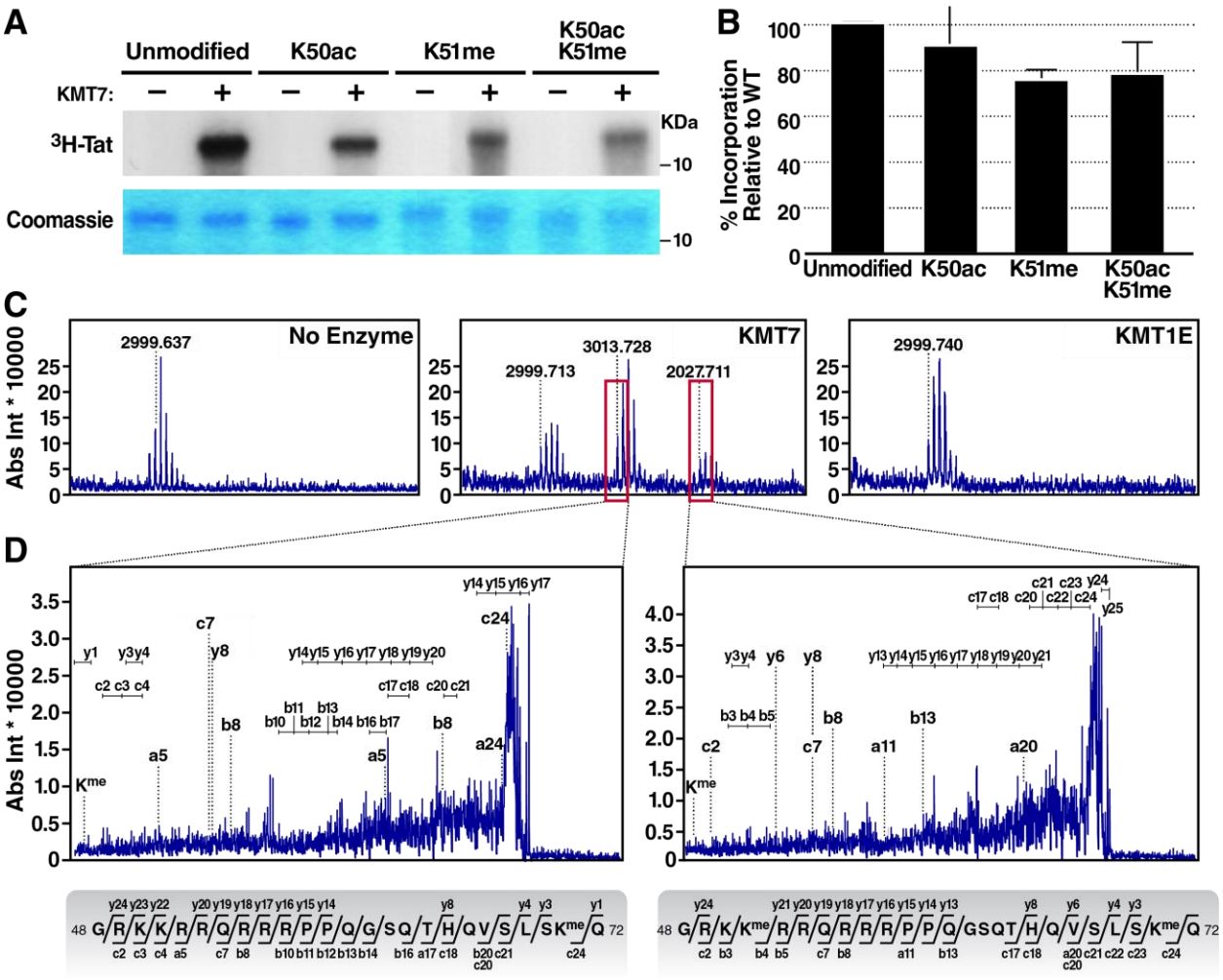


Figure 3-1: HIV-1 Tat is monomethylated at Lys-71 by KMT7. In vitro methylation assays were done with 3 µg of synthesized Tat72 proteins that were unmodified, acetylated (K50ac), methylated (K51me), or both acetylated and methylated, with or without 1 µg of recombinant KMT7 in the presence of [3H]SAM. A, top, representative autoradiogram. This image is uncropped and from one gel; the difference in background is an imperfection of the film. Bottom, Coomassie Blue stain for Tat. Molecular weight markers are noted in kilodaltons (kDa) B, quantification of three autoradiograms with ImageJ (mean±S.E.). C, in vitro methylation assays on unmodified Tat proteins with indicated enzymes were subjected to MS, shown are the zoomed regions containing Tat ions corresponding to aa 48–72. Boxed in red are peaks indicating modified Tat ions. D, MS/MS spectra of the ions boxed in 1C, and their corresponding peptide sequences. Ion annotations are found in **Table 3-S1**.

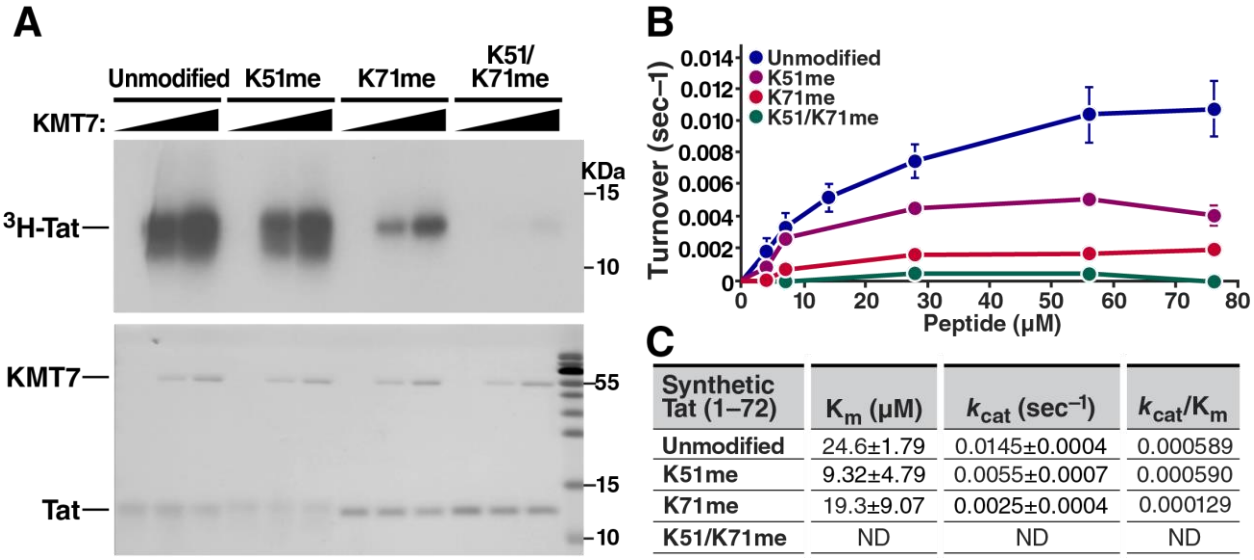
		Mass Spectrometry Ion Annotations				
	Figure 1D Left			Figure 1D Right		
	Annotation	Ion Mass	K71me	Annotation	Ion Mass	K71me
	a5	598.006		a11	1462.734	
	a17	2045.872		a20	2424.704	
	b8	1066.25		b3	341.807	
	b10	1378.103		b4	483.942	
	b11	1475.142		b5	639.89	
	b12	1573.218		b8	1080.918	
	b13	1700.565		b13	1714.677	
	b14	1757.853		c2	230.763	
	b16	1973.135		c7	941.733	
	b17	2074.58		c17	2105.045	
	b20	2438.38		c18	2242.017	
	c2	230.989		c20	2469.389	
	c3	358.925		c21	2557.104	
	c4	487.056		c22	2668.924	
	c7	2091.397		c23	2755.972	
	c17	2091.397		c24	2899.376	*
	c18	2228.051		y3	376.818	
	c20	2456.066		y4	489.843	
	c21	2543.063		y6	674.883	
	c24	2884.518	*	y8	940.85	
	y1	146.336		y13	1441.42	
	y3	375.866		y14	1538.768	
	y4	489.001		y15	1635.712	
	y8	940.094		y16	1791.815	
	y14	1538.294		y17	1947.602	
	y15	1635.481		y18	2103.61	
	y16	1791.304		y19	2232.26	
	y17	1948.125		y20	2387.675	
	y18	2104.376		y21	2545.118	
	y19	2232.49		y24	2970.69	
	y20	2388.953		$M+H^+$	3027.712	*
	y22	2672.778				
	y23	2799.973				
	y24	2956.1				
	$M+H^+$	3013.764	*			
	* ions that contain K71me					

Table 3-S1: Mass annotations for ions produced by MS/MS for Figure 3-1.

To specifically identify additional KMT7 methylation sites in Tat, we performed *in vitro* methylation reactions using non-radiolabeled SAM and subjected modified Tat proteins to MALDI-TOF MS/MS analysis developed to analyze Tat (Sakane et al., 2011). This analysis revealed monomethylation at a single additional site, K71 (**Figure 3-1C, 3-1D, Table 3-S1**) in two distinct peptides in reactions with KMT7, but not in reactions with a control enzyme KMT1E (also called SETDB1) or no enzyme (**Figure 3-1C**). In one peptide, only K71 was monomethylated (**Figure 3-1D, left**); in the other, both K51 and K71 were monomethylated (**Figure 3-1D, right**). Neither K51 nor K71 was dimethylated, underscoring the function of KMT7 as a monomethyltransferase in Tat.

KMT7 Modifies Tat at K71 in Vitro

Next, we tested whether pre-modification of K71 in Tat72 peptides affects *in vitro* methylation by KMT7. Premethylation of K71 markedly decreased ^{3}H-SAM incorporation while K51 pre-methylation had a lesser effect. Finally, premethylation of both K51 and K71 abolished methylation of Tat, demonstrating that there are no additional targets for KMT7 in Tat (**Figure 3-2A**). These findings support K51 and K71 as the sole sites for KMT7 monomethylation in Tat72.



C

Synthetic Tat (1–72)	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m
Unmodified	24.6±1.79	0.0145±0.0004	0.000589
K51me	9.32±4.79	0.0055±0.0007	0.000590
K71me	19.3±9.07	0.0025±0.0004	0.000129
K51/K71me	ND	ND	ND

Figure 3-2: KMT7 preferentially monomethylates Tat Lys-71 in vitro. A, reactions were done as in Figure 3-1A with 2 μg of synthesized Tat72 proteins that were unmodified, monomethylated at Lys-51 or Lys-71 or both, and incubated with 0, 1, or 2 μg of purified KMT7 in the presence of [3H]SAM. Top, representative autoradiogram. Bottom, Coomassie Blue stain for Tat and KMT7. B, kinetic assays were done with indicated concentrations of Tat peptides and 1μg of recombinant KMT7. Assays were conducted in triplicate; error bars indicate S.E. C, activity calculated by fitting data to Michaelis-Menten equation with SigmaPlot 11 after correction for methylation in the control reactions with either the enzyme or the substrate removed.ND, not determined in doubly modified peptides.

To better quantify dynamics of KMT7-mediated methylation of Tat72 proteins, we used pre-modified Tat proteins in a modified kinetic radiometric assay (Houtz et al., 1991). After testing for linearity with time and KMT7 enzyme concentrations, kinetics assays were performed with various concentrations of Tat72 proteins (**Figure 3-2B**). Methyltransferase activity was hyperbolic, and all Tat proteins except the Tat K51/K71me doubly modified form followed Michaelis-Menten kinetics. The doubly modified form had very little methylation, consistent with our observations using autoradiography (**Figure 3-2A**).

The catalytic turnover (k_{cat}) and Michaelis constant (K_m) of the Tat K51me proteins were 62% lower relative to the unmodified proteins. Interestingly, the k_{cat} of the K71me proteins decreased by 83% but the K_m decreased only 21%. Thus, the methylation efficiency (k_{cat}/K_m) on K51me protein was similar to that of the unmodified protein, but the K71me was 78% less than that on control proteins. Furthermore, almost no methylation was detected in the K51/K71me protein. Together, these data support the model that K71 is preferred over K51 as a target for KMT7 in Tat (**Figure 3-2B, 3-2C**) and there are no additional sites in Tat72 for KMT7 monomethylation.

MS of Tat Purified from HEK293T and Jurkat A2 Cells Reveals Monomethylation at K71 exists in vivo

To examine K71 methylation *in vivo*, we coexpressed Tat101 bearing a C-terminal FLAG tag together with either a KMT7 or empty vector in HEK293T cells for 24h. We first purified Tat from lysates by FLAG immunoprecipitation, followed by SDS-PAGE separation. The separated Tat protein was subjected to MALDI-TOF MS/MS which showed that K71 was monomethylated only when KMT7 was overexpressed (**Figure 3-3A, 3-3B; Table 3-S2**). This suggests that the fraction of Tat that is naturally monomethylated at K71 in HEK293T cells may be small compared to the unmodified form.

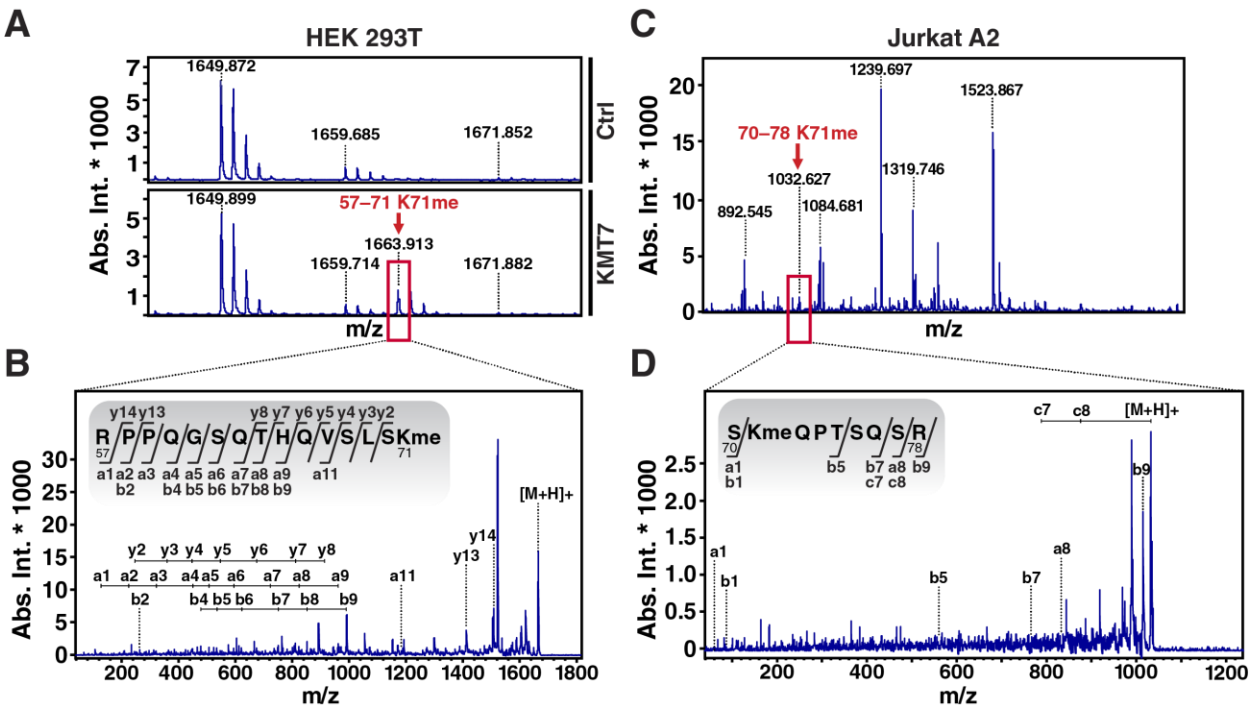


Figure 3-3: In vivo detection of Tat K71me in HEK293T and Jurkat A2 cell lines. A, WT Tat101-FLAG and KMT7 were overexpressed in HEK293T cells and analyzed by MS after FLAG affinity purification. A zoomed region of the initial MS spectrum shows the K71me containing ion found in the presence of KMT7 overexpression, but not the control. The spectrum is representative of two independent experiments. B, MS/MS of the ion containing K71me indicated by a red box in 3A and sequence of the fragment. C, Tat101-FLAG was FLAG-affinity-purified from Jurkat A2 cells after induction with TNFα and analyzed by MS. Depicted is the full spectrum with a Tat K71me-carrying ion (aa 70–78). D, MS/MS of the ion containing K71me indicated by a red box in 3C and sequence of the fragment. Ion annotations are found in **Table 3-S2**.

		Mass Spectrometry Ion Annotations					
	Figure 3B				Figure 3D		
	Annotation	Ion Mass	K71me		Annotation	Ion Mass	K71me
	a1	129.022			a1	59.492	
	a2	226.059			a8	830.203	
	a3	323.011			b1	87.782	
	a4	451.103			b5	556.341	*
	a5	508.115			b7	770.89	*
	a6	595.14			b9	1014.543	*
	a7	723.253			c7	788.592	*
	a8	824.378			c8	875.437	*
	a9	961.359			$M+H^+$	1032.623	*
	a11	1188.68					
	b2	254.011					
	b4	479.077					
	b5	536.121					
	b6	623.169					
	b7	751.173					
	b8	852.191					
	b9	989.36					
	c4	496.104					
	c8	869.144					
	y2	248.04	*				
	y3	361.104	*				
	y4	448.081	*				
	y5	547.128	*				
	y6	675.185	*				
	y7	812.298	*				
	y8	913.326	*				
	y13	1410.747	*				
	y14	1507.844	*				
	* ions that contain K71me						

Table 3-S2: Mass annotations for ions produced by MS/MS for **Figure 3-3**.

Next, we analyzed Tat methylation in J-Lat A2 T cells, in which expression of FLAG-tagged Tat101 is controlled by its natural promoter and induced by stimuli such as TNFα (Jordan et al., 2003). Tat expression was induced with TNFα, FLAG-affinity purified, and analyzed by MS. We identified a peptide fragment of 1032.627 Da, corresponding to Tat aa 70–78 with a monomethyl group at K71 (Figure 3C). MS/MS confirmed the monomethylation at K71 (**Figure 3-3D, Table 3-S2**). Together these findings show that Tat K71 is monomethylated in Jurkat T cells under conditions mimicking natural HIV infection and that this modification can be induced by KMT7 overexpression in 293T cells.

Generating Antibodies Specific for K71me

We confirmed these results using newly generated modification-specific polyclonal antibodies. We previously published methods to produce and characterize mono-, di- and tri-methyl Tat-specific antibodies at K51 (Pagans et al., 2011). Following these methods, we focused on two chemically synthesized K71me 11-mer peptides (type 1 and type 2) (**Figure 3-4A**). Peptide 1 (aa 62–72) ends with the first exon, and peptide 2 (aa 67–77) spans both Tat exons. After antigen-purification of the modification-specific antibodies, we performed dot-blot analysis of various chemically modified Tat proteins (**Figure 3-4B**). Type 1 antibodies reacted with all synthetic Tat72 proteins (WT, K51me, K71me, K50Ac and K51/K71me), but type 2 antibodies detected only the K71me Tat72 proteins. Similar results were obtained by SDS-PAGE and Western blotting (**Figure 3-4C**). The type 2 antibodies were highly specific for Tat72 proteins carrying a monomethyl group at K71, and the type 1 antibodies recognized all Tat proteins. Therefore for further analysis, we used type 2 TatK71me antibodies.

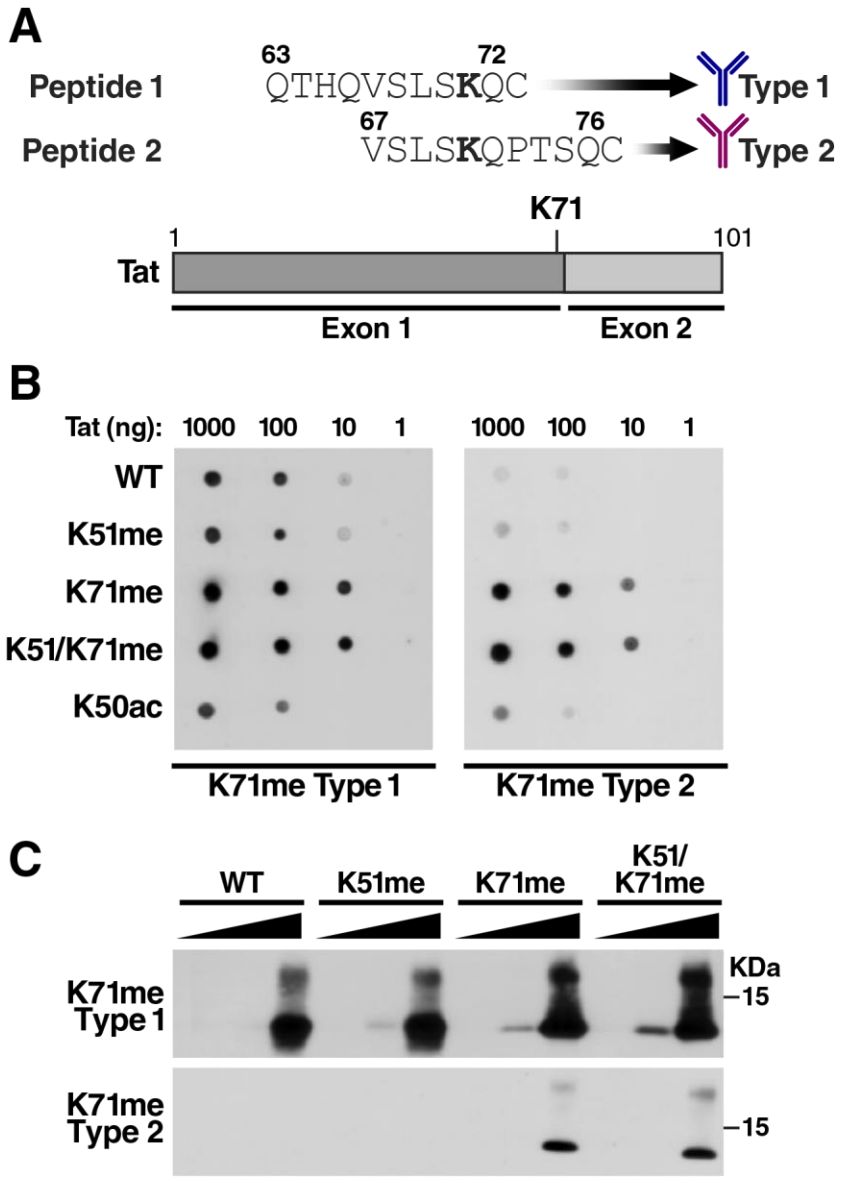


Figure 3-4: Generation of K71me-specific antibodies. A, schematic of thesynthetic methylated peptides used as antigens to generate K71me Tat antibodies. B, dot blots with synthetically modified Tat72 proteins (K51me, K71me, K51/K71me, and K50ac) incubated with antigen-purified type 1 or type 2 K71me antibodies. C, Western blot analysis of indicated Tat72 proteins (1, 10, 100 ng each) with type1 antibodies (top) and type 2 antibodies (bottom).

Detecting Tat K71me by Western Blot in vivo

To confirm the specificity of the type 2 antibodies *in vivo*, we overexpressed WT or FLAG-tagged Tat101 proteins carrying mutations at K71, K51, or K50 in 293T cells. After FLAG immunoprecipitation, we detected K71me Tat only in WT, K50 and K51mutants, but not in K71 mutants (**Figure 3-5A**). This finding indicates that endogenous KMT7 is sufficient to methylate K71 in cells. Furthermore, we were able to increase TatK71me by coexpressing Tat101 with WT KMT7 relative to coexpression with a catalytically inactive KMT7 mutant (H297A). Importantly, no change in methylation was observed on the K71R mutant (**Figure 3-5B**).

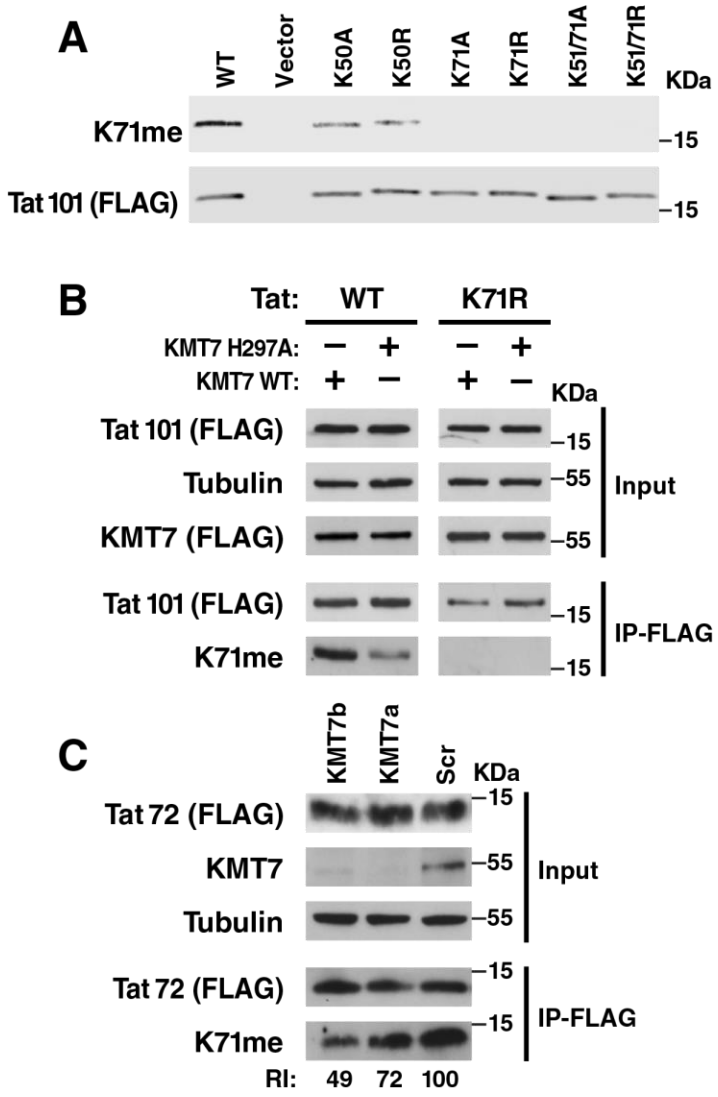


Figure 3-5: Detection of K71me Tat methylation in vivo. Lysates from 293T or Jurkat cells were FLAG-immunopurified and blotted with indicated antibodies including type 2 Tat K71me antibodies. A, Western blot analysis of indicated Tat101-FLAG mutants expressed in HEK293T cells. B, Western blot analysis of Wt or K71R Tat101-FLAG co-expressed with Wt or catalytically inactive KMT7 mutant in HEK293T cells. Tat Wt and K71R blots were cropped from the same gel and had the same exposure times. C, Western blot analysis of FLAG-purified Tat72-FLAG isolated from stable cells lines transduced with shRNAs against KMT7 (KMT7a or KMT7b) or a scrambled control. Western blots were performed with indicated antibodies. ImageJ was used for quantification.

Next, we determined whether the K71me antibodies are able to detect Tat72 in T cells. For this, we used an HIV-1 mini-genome to generate Jurkat cell lines that stably express Tat72, as described for the Jurkat A2 cells (Jordan et al., 2001). We sorted for cells with high-level GFP expression, and transduced them with lentiviruses containing one of two shRNAs targeting KMT7 (a and b) or a scrambled shRNA (Scr). The lentiviral constructs also contained a puromycin resistance gene, which allowed us to select for shRNA-expressing cells. After selection, shRNA-expressing cells were lysed and subjected to FLAG immunoprecipitation, and Tat K71me levels were determined by Western blotting (**Figure 3-5C**). Knockdown of KMT7 was robust; in accordance, Tat K71me levels were reduced by 51% in cells treated with the KMT7b shRNA and by 28% in cells treated with KMT7a shRNA (**Figure 3-5B, bottom**). These results underscore the role of KMT7 as an important K71 methyltransferase for both forms of Tat in T cells. Since a substantial amount of Tat remained methylated at K71 despite the knockdown, we suspect that a small residual pool of KMT7 may be sufficient to modify a proportion of Tat proteins, or perhaps another enzyme is capable of monomethylating Tat in cells.

K71me Does Not Affect Tat Half-life

A number of substrates modified by KMT7 are altered in their stability by downstream changes in their polyubiquitylation (Yang et al., 2009b). Since K71 has previously been reported as a site for polyubiqitination (Bres et al., 2003), we examined whether K71 methylation by KMT7 affects the stability of Tat in Jurkat cell lines expressing fluorescent Tat101-Dendra fusion proteins generated for this purpose (Jordan et al., 2003). The Tat-Dendra system has been used to determine the Tat half-life in flow cytometry and microscopy studies (Razooky et al., 2015; Singh et al., 2012). To knock down KMT7, we transduced Tat-Dendra cells with the lentiviral vectors expressing scrambled or KMT7b shRNAs used for **Figure 3-5B**. After puromycin selection, we monitored effects of KMT7 knockdown on Tat half-life only in successfully transduced cells. Knockdown of KMT7 was confirmed by western blotting (**Figure 3-6B**). Expression of WT or K71R Tat-Dendra proteins was induced with TNFα. After 16-20 h of TNFα treatment, cycloheximide was

added to inhibit *de novo* production of Tat. Cells were fixed at various times, and Tat expression was determined by flow cytometry of Dendra (**Figure 3-6A**). Tat stability did not differ in cells transduced with control and KMT7 shRNAs, excluding any prominent effect of K71 methylation on Tat stability (**Figure 3-6C**). In cells expressing scrambled shRNA, the K71R Tat mutant had a slightly longer half-life than WT Tat (10.9 vs 9.0 h, $P = 0.043$). However, time-lapse single-cell microscopy of Tat-Dendra cells revealed no statistical difference in the half-lives of WT and K71R Tat in the absence of shRNAs (**Figure 3-6D**). Together, these data affirm previous findings that K71 modifications are not involved in Tat protein stability (Razooky et al., 2015).

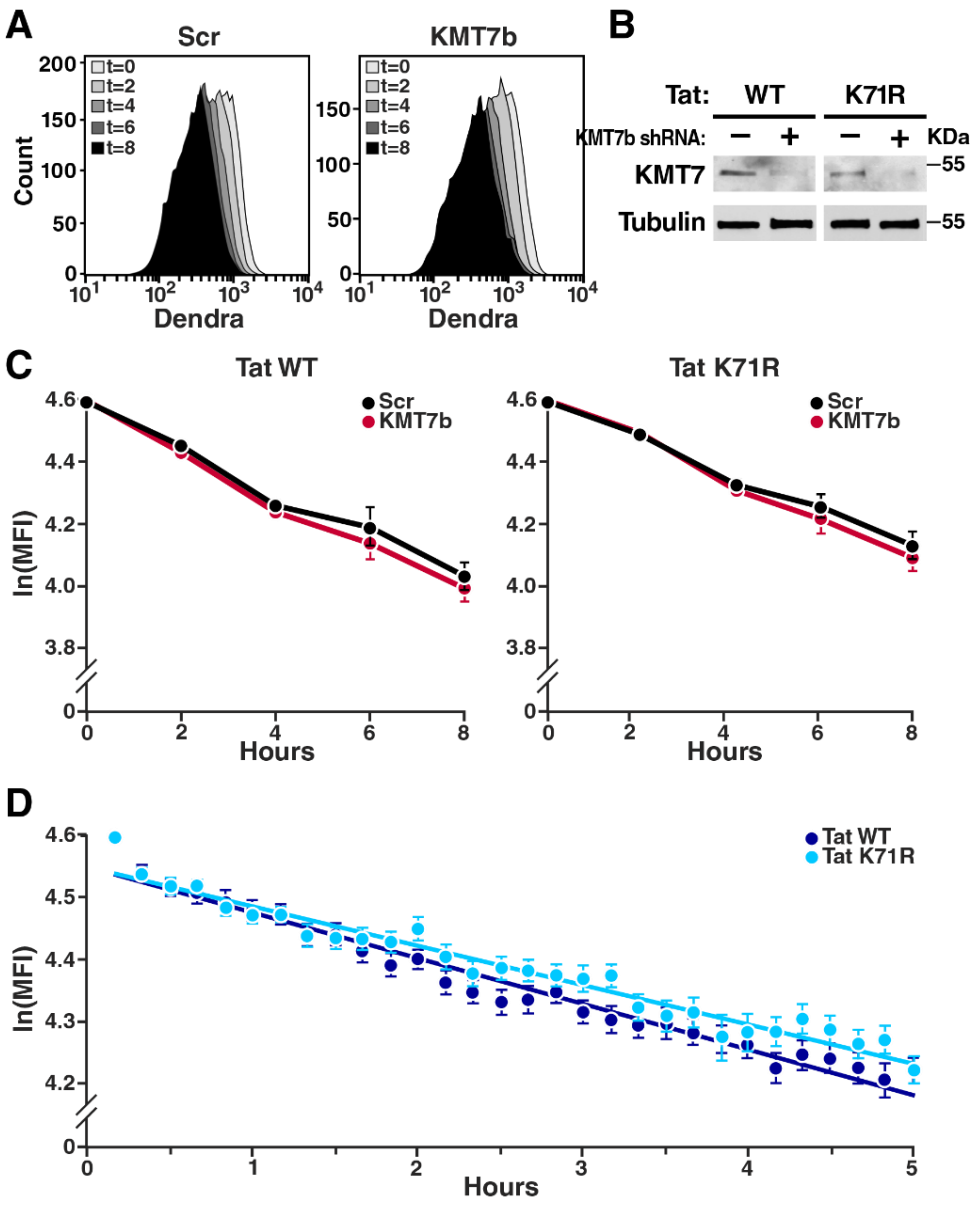


Figure 3-6: Lys-71 mutation or KMT7 knockdown does not affect Tat stability. A, flow cytometry histograms showing mean Tat-Dendra fluorescence (MFI) at indicated times (0–8 h) in WT cells stably transduced with KMT7b or scrambled shRNAs and treated with cycloheximide. B, Western blot analysis confirms knockdown of KMT7 in Tat-Dendra J-Lat lysates after puromycin selection. C, measurements of Tat half-life by flow cytometry. MFIs were normalized to t=0 after removing background, natural log transformed, and plotted over time as mean ln(MFI)±S.E. of four independent experiments in cells transduced with lentiviruses containing KMT7b or scrambled shRNAs and expressing WT or K71R Tat. D, time-lapse single cell microscopy of WT or K71R Tat-Dendra J-Lats each point represents the MFI of 50 independent cells that were tracked for at least 2 h.

K71 Methylation Enhances Tat Transactivation

Previously, we found that KMT7-mediated monomethylation of Tat K51 enhances interactions between Tat and TAR RNA and P-TEFb and activates HIV gene expression (Pagans et al., 2010). To assess the effect of K71 monomethylation on Tat transactivation, we transfected plasmids expressing WT or mutant Tat101 (K51R, K71R, or K51/71R) into TZMBL cells, which express firefly luciferase from the integrated HIV LTR when functional Tat is expressed (Montefiori, 2009). Transactivation was ~50% lower in the Tat K51R and Tat K71R mutants than in WT Tat at three different plasmid concentrations, confirming that these residues are important for Tat transactivation (Bres et al., 2003; Pagans et al., 2010). Transactivation was almost completely lost when both K51 and K71 were mutated, underscoring their combined importance in Tat transactivation. All Tat proteins were expressed at similar levels as confirmed by western blot (**Figure 3-7A**).

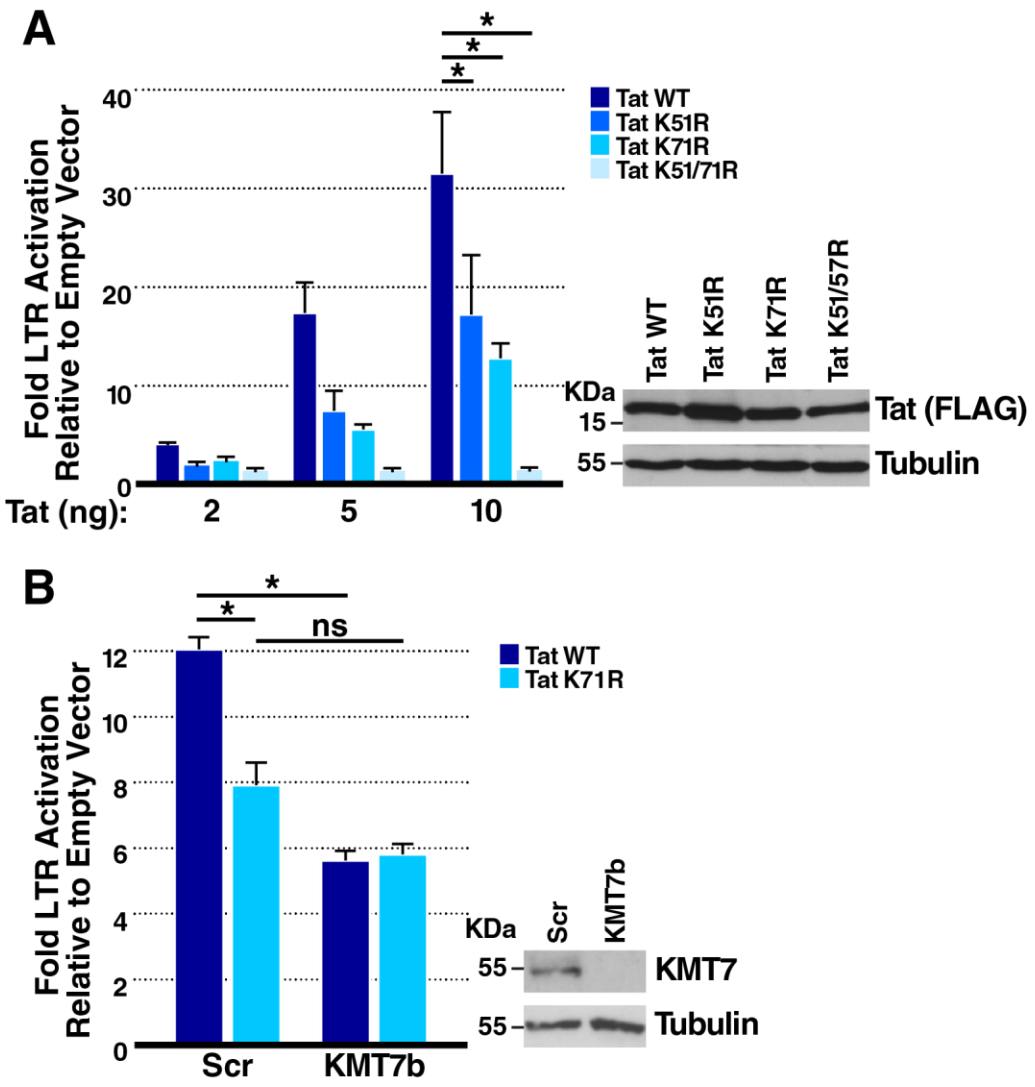


Figure 3-7: Tat K71me regulates Tat transactivation. A, TZMBL cells were transfected with increasing concentrations of plasmids (2, 5, and 10 ng) expressing WT or mutant Tat101. Luciferase values are shown from four independent experiments measured as fold expression over empty vector (mean ± S.E.). Western blot analysis was performed in cells transfected with 100 ng of Tat-FLAG plasmid and blotted with anti-FLAG antibodies. B, luciferase values in TZMBL cells transduced with KMT7b or scrambled shRNAs and then transfected with WT or TatK71R Tat-expressing plasmids (20 ng). KMT7 knockdown is visualized by Western blotting. Values are mean ± S.E. from four independent experiments. Significance was calculated with a one-sided t test. *, $p < 0.05$.

To test whether monomethylation at K71 by KMT7 contributes to Tat transactivation, we knocked down KMT7 expression in TZMBL cells before transfection with WT Tat or K71R mutant Tat101. Knockdown of KMT7 decreased the transcriptional activity of Tat WT but had no effect on K71R mutant Tat. Evidently, KMT7 activates Tat transactivation, at least in part, through K71 monomethylation (**Figure 3-7B**). Knockdown of KMT7 was confirmed by western blotting (**Figure 3-7B**). These findings indicate that K71 monomethylation, rather than regulating Tat stability, plays a positive role in the transcriptional activity of Tat.

Discussion

HIV-1 Tat is a potent viral transactivator that undergoes extensive post-translational modifications. Here, we expand the role of KMT7 in Tat function by identifying K71 as a second monomethylation site. We found that K71 is monomethylated in both functional HIV-1 Tat101 and Tat72 splice variants, underscoring its importance throughout the HIV-1 life cycle. We identified KMT7 as a robust Tat K71 monomethyltransferase in both *in vitro* and *in vivo* assays. Although KMT7 substrates often have perturbed stability, mutation of K71 or knock down of KMT7 did not affect the stability of Tat. Instead, K71 monomethylation is important for the transcriptional activity of Tat through a yet unresolved mechanism.

Notably, *in vitro* methylation and enzymology experiments indicate that the preferred methylation site of KMT7 is K71 rather than K51, previously identified as a target of KMT7. Although KMT7 has no known stringent site specificity, two consensus sequences in KMT7 targets have been described: $(K,R)^{-2}$-$(S,T,A)^{-1}$-Kme^{0}-X^{+1} (where X is a polar residue) and a newer sequence $(G,R,H,K,P,S,T)^{-3}$-$(K>R)^{-2}$-$(S>K,Y,A,R,T,P,N)^{-1}$-Kme^{0}-$(Q,N)^{+1}$-$(A,Q,G,M,S,P,T,Y,V)^{+2}$ (Del Rizzo and Trievel, 2011; Dhayalan et al., 2011). Comparing the sequences of K71me ($S^{-3}L^{-2}S^{-1}Kme^{0}Q^{+1}P^{+2}$) and K51me ($G^{-3}R^{-2}K^{-1}Kme^{0}R^{+1}R^{+2}$), K71 is more closely aligned with the newer consensus sequence than K51, possibly explaining why K71 was the preferred target in our *in vitro* studies (Del Rizzo and Trievel, 2011).

Our finding that KMT7 methylates Tat at two sites is consistent with reports that KMT7 often has multiple targets within individual substrates. For example, the RelA subunit of the NF-κB transcription factor has three KMT7 monomethylation sites (K37me, K314me, K315me) and the PCAF acetyltransferase has two (K78me, K89me) (Ea and Baltimore, 2009; Masatsugu and Yamamoto, 2009; Yang et al., 2009a). Since the addition of a monomethyl group is a rather subtle modification (14 Da), it is likely that multiple monomethylation sites act in concert to mediate appropriate regulation of the substrate protein by KMT7.

Two main consequences of KMT7-mediated methylation have emerged: alteration of protein stability and the regulation of interactions between nucleic acids and proteins (Del Rizzo and Trievel, 2011). The oncogene p53 and estrogen receptor α are both stabilized upon monomethylation by KMT7 (Chuikov et al., 2004; Subramanian et al., 2008). In contrast, RelA of NF-κB and DNMT1 are both destabilized upon monomethlyation of one or more sites by KMT7 (Esteve et al., 2009; Yang et al., 2009a). One review regarded KMT7 as a bona fide protein stability modifier, proposing the presence of a methyl/ubiquityl switch that can regulate the stability of substrates (Yang et al., 2009b). However, polyubiquitination of Tat at K71 has been linked to enhanced transcriptional activity, not degradation (Bres et al., 2003). Indeed, we found that KMT7 knockdown did not significantly affect the stability of WT or K71R Tat.

Early structural studies of Tat isolates suggested that the glutamine-rich motif (aa 59 – 72) has a degenerate α-helical structure, consistent across different Tat isolates and adopted in the presence of TAR RNA (Campbell et al., 2004). This structure is adopted partially through conserved glutamine-RNA hydrogen bonds (Q60, Q63, Q66, Q72) and an electrostatic interaction between K71 and the TAR phosphodiester backbone at nucleotides 31–35 (Campbell et al., 2004; Loret et al., 1992). K71 monomethylation could thus enhance these interactions by stabilizing the electrostatic interaction between K71 and the TAR loop (Loret et al., 1992; Pagans et al., 2010). Previously, we showed that methylation of K51 by KMT7 activates appropriate Tat/TAR/P-TEFb binding (Pagans et al., 2010). K51 lies in the ARM of Tat, which binds TAR RNA in the bulge

region. Therefore, methylation of K51 and K71 by KMT7 might coordinately enhance the binding of TAR RNA to multiple residues in Tat, potentially positioning the RNA properly with respect to Tat and P-TEFb. Future experiments are necessary to explore this model and elucidate the mechanism by which KMT7 activates Tat transactivation by monomethylating K71.

The Dynamics of Tat PTMs Influences Stochasticity of HIV-1 Viral Reactivation

The molecular events required for proper HIV-1 transcription activation are numerous and complex. However, these modifications are probably regulated based on the metabolites available in the cellular microenvironment and naturally occurring intermolecular interactions. HIV-1 Tat has access to nearly every cellular compartment, can traverse cell membranes and is a notoriously sticky protein - so naturally this makes things extra complex. Therefore, Tat could have one set of modifications in the cytoplasm, fostering certain intermolecular interactions; a different set of modifications regulated by proximity to mitochondria; and a different set of modifications that occur in the nucleus. Together, the various modifications lead to the diverse phenotypic outcomes of having Tat in cells. It would be interesting if someone was able to do time-lapse compartment-specific proteomics screens surveying HIV-1 Tat post-translational modifications.

In terms of the activities in the nucleus, we can consider the bifurcating model of HIV-1 activation proposed by Weinberger and colleagues (Weinberger et al., 2005). Here, we can assume that PTMs can push Tat towards an activated or suppressed state. Through sexual transmission, HIV-1 expansion primarily begins in the gut and so I imagine that a proportion of memory T cells that harbor latent virus are probably hanging out there or in nearby lymph nodes. These T cells are constantly surveilling their environment because they are interacting with the various things that wander through human intestines. While these cells may be in a “resting” state, they still have to constantly suppress aberrant activatory and anti-suppressive signals that they get in this microenvironment.

Latency can be reversed through a variety of mechanisms, including CD3/CD28 receptor stimulation, PKC activation, NF-κB activation, hyperacetylation, perturbations in methylation, and fluctuations in metabolic pathways such as mTOR (Ali et al., 2016; Besnard et al., 2016; Boehm et al., 2017; Burnett et al., 2010; Jordan et al., 2003). The probability that these pathways are stimulated in different ways in CD4 memory T cells in this compartment is high and could push latent cells with a small amount of Tat towards reactivation. This could be constantly occurring throughout the life-long treatment of a patient and could explain why cessation of antiretroviral therapy so quickly leads to rebound of infection. It is particularly strange that a protein that needs to overcome so many barriers for its activity, is so damn good at its job.

From a therapeutic standpoint, some have turned to a “shock and kill” methodology where they try to reactivate latent HIV-1 so that ART can then kill the virus. However, it is immensely difficult to reactivate latent HIV in patients. The pathways that robustly activate latent HIV such as CD3/CD28, PKC or NFκB stimulation make the immune system go haywire and induce massive T cell cytotoxicity or unnecessary proliferation. For more subtle approaches like KDAC inhibition, while it works well in certain model systems, clinical trials testing these compounds have been consistently disappointing (Darcis et al., 2017). Furthermore, stimulating or suppressing pathways like acetylation and methylation in an untargeted way will cause unregulated changes in chromatin structure, a surefire way of developing genome instability and cancer.

A more recent approach is to “block and lock” HIV-1 activation. The theory behind this is that the latent virus already has to pass through so many barriers, perhaps using drugs or nutritional supplements one could possibly push the probability of reactivation so low, that patients can stop ART. However, to my knowledge there have been no clinical trials to test this, and HIV-1 cure attempts are focusing more on other mechanisms for viral suppression. I suppose only time will tell if we will be able to successfully cure HIV-1. To be honest, I’m

skeptical that it will happen in my lifetime, but at least we tried. For now, perhaps the United States should focus its efforts on prevention by providing inexpensive condoms and modes of educating the public on how the disease is transmitted and how to avoid transmission.

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Chapter IV: Lysine Acetylation Goes Global: Epigenetics, Metabolism and Therapeutics.

Ibraheem Ali‡†, Ryan Conrad‡†, Eric Verdin§, Melanie Ott*†

Affiliations:

†Gladstone Institute of Virology and Immunology, San Francisco, CA; University of California, San Francisco, Department of Medicine, San Francisco, CA 94158

§Buck Institute for Research on Aging, Novato, CA, 94945

‡Authors contributed equally to this work

Correspondence:

* mott@gladstone.ucsf.edu

Abstract

Post-translational acetylation of lysine residues has emerged as a key regulatory mechanism in all eukaryotic organisms. Originally discovered in 1963 as a unique modification of histones, acetylation marks are now found on thousands of non-histone proteins located in virtually every cellular compartment. Here we summarize key findings in the field of protein acetylation over the past 20 years with a focus on recent discoveries in nuclear, cytoplasmic and mitochondrial compartments. Collectively, these findings have elevated protein acetylation as a major posttranslational modification, underscoring its physiological relevance in gene regulation, cell signaling, metabolism and disease.

Introduction

During the lifetime of a protein there are many points at which an acetyl group may be added to influence function. As early as during its translation, a protein may be N-terminally acetylated to preserve its stability, interactions or subcellular localization.(Aksnes et al., 2016) N-terminal acetylation is a major covalent modification occurring on eukaryotic proteins, with >80% of human proteins bearing an acetyl group at the α-amino position of its first amino acid. Once a protein is properly localized, acetylation of key lysine residues can occur enzymatically or

spontaneously to influence its intermolecular interactions, enzymatic functions, localization, and eventual degradation. Post-translational acetylation of lysine residues will be the primary focus of the current review.

Lysine acetylation describes the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the primary amine in the ε-position of the lysine side chain within a protein, a process that leads to neutralization of the position's positive electrostatic charge. Acetylation can occur non-enzymatically; however, in most known cases, the level of acetylation results from the balance of opposing enzymatic activities. Marks are "written" by lysine acetyltransferases (KATs) and "erased" by lysine deacetylases (KDACs). Acetylated lysine residues, amidst their many functions, can be functionally interpreted by a third group of proteins, the so called "readers", which harbor specific acetyl-lysine binding domains, most prominently bromodomains. The dynamic interplay between the writers, erasers, and readers of acetylation regulates critical epigenomic and metabolic processes, in addition to other major cellular functions.

Historically, investigators have focused on acetylation in the nucleus, where this mark regulates histone biology and transcription (Grunstein, 1997; Strahl and Allis, 2000; Struhl, 1998; Turner, 2000). Advances in mass spectrometric technologies have since revealed relevant targets of acetylation in nearly all intracellular compartments.(Choudhary et al., 2014; Duan and Walther, 2015) Compartmentalization of cellular proteins and nutrients is essential for cell specialization and function. As such, cellular acetylation is driven by the localization of enzymes, metabolites, and cofactors required to balance acetylation and deacetylation levels. Importantly, mitochondria have emerged as organelles in which acetylation is more prominent than phosphorylation(Gnad et al., 2010) and plays a key role in integrating metabolic cues with the bioenergetic equilibrium of the cell.

In this review, we provide an overview of the chemistry and biology underlying protein lysine acetylation in mammals, review recent developments in the understanding of lysine acetylation and provide examples of its function and regulation in distinct cellular compartments.

The chemistry of reversible lysine acetylation

The transfer of the acetyl group from acetyl-CoA to the ε-primary amine of a lysine residue can occur spontaneously or enzymatically. In mitochondria, acetylation is regulated in part by chemical, non-enzymatic mechanisms due to the high pH and high local acetyl-CoA concentrations within this compartment.(Santo-Domingo and Demaurex, 2012) The mechanism of non-enzymatic acetylation proceeds first via deprotonation of the lysine primary amine by naturally occurring hydroxide ions, followed by attack of the acetyl-CoA terminal carbonyl by the nucleophilic amine. A putative tetrahedral intermediate is transiently formed and decomposes into reaction products acetyl-lysine, coenzyme A, and hydroxide (**Figure 4-1**).(Wagner and Payne, 2013)

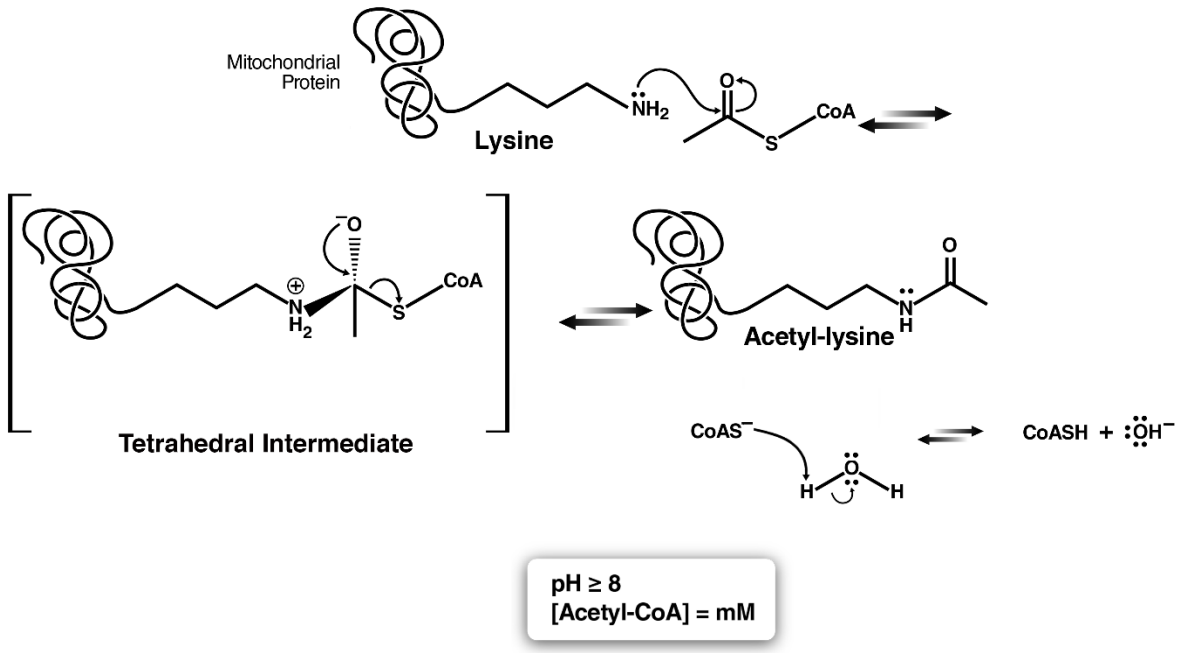


Figure 4-1: Proposed reaction mechanism of spontaneous acetylation in the mitochondria.(Wagner and Payne, 2013)

Lysine acetyltransferases

The human proteome contains 21 putative KATs that catalyze lysine acetylation (**Table 4-1**). The best characterized have been catalogued into three major families based on homology to yeast proteins, but also on structural and biochemical features of catalysis – (1) GCN5-related *N*-acetyltransferases (GNAT), (2) the p300/CREB-binding protein (p300/CBP), (3) and the MOZ, Ybf2, Sas2, and Tip60 (MYST) family. A number of other proteins have acetyltransferase activity such as TBP-associated factor 250kd (TAFII250 (KAT4)), αTubulin acetyltransferase (αTAT1), circadian locomoter output cycles protein kaput CLOCK (KAT13D) and nuclear receptor coactivator-1 (NCoA-1) but do not belong to any of the major acetyltransferase families.

The first cloned mammalian acetyltransferase was the GCN5 homolog PCAF (KAT2B). In this study, Nakatani and colleagues reported conserved sequence homology between *PCAF* and the *GCN5* genes in yeast and human. The authors performed *in vitro* acetylation assays using recombinant protein to demonstrate that PCAF (KAT2B) can acetylate whole nucleosomes while the function of human GCN5 (KAT2A) was limited to free histones.(Yang et al., 1996) Using similar assays, the enzymatic activity was demonstrated for CBP/p300 (KAT3A/B),(Ogryzko et al., 1996) TAFII250 (KAT4),(Mizzen et al., 1996) TIP60 (KAT5),(Yamamoto and Horikoshi, 1997) and NCoA-1 (KAT13A).(Chen et al., 1997; Spencer et al., 1997)

Lysine Acetyltransferases

PROTEIN NAME	ALIASES	SUBFAMILY	LOCALIZATION	STRUCTURES AVAILABLE	UNIPROT ID	REFER-ENCES
αTAT1	MEC17	N/A	Cytoplasmic	KAT Domain	Q5SQ10	17-20
KAT1	HAT1	GNAT	Nuclear, Cytoplasmic	KAT Domain	O14929	21-24
KAT2A	GCN5		Nuclear	KAT Domain, Bromodomain	Q92830	25-29
KAT2B	PCAF		Nuclear	KAT Domain, Bromodomain	Q92831	27, 30-33
ATF2	CREB2		Nuclear, Mitochondrial	DNA Binding Domain	P15336	34-37
KAT3A	CBP	P300/CBP	Nuclear, Cytoplasmic	Bromodomain	Q92793	27, 38-41
KAT3B	p300		Nuclear, Cytoplasmic	KAT Domain, Bromodomain	Q09472	27, 42-45
KAT4	TAF1, TAFII250	TAFII250	Nuclear	Complete Protein	P21675	27, 46-49
KAT5	TIP60	MYST	Nuclear, Cytoplasmic	KAT Domain (PDB: 2OU2)	Q92993	50-52
KAT6A	MYST3, MOZ		Nuclear	KAT Domain, PhD Finger	Q92794	53-55
KAT6B	MYST4, MORF		Nuclear	N/A	Q8WYB5	56, 57
KAT7	MYST2, HBO1		Nuclear	N/A	O95251	58-61
KAT8	MYST1, MOF		Nuclear, Mitochondrial	KAT Domain	Q9H7Z6	62-66
KAT9	ELP3	ELP3	Nuclear, Cytoplasmic	N/A	Q9H9T3	67, 68
GCN5L1	BLOS1	N/A	Cytoplasmic, Mitochondrial	N/A	P78537	69, 70
KAT12	GTF3C4	N/A	Nuclear	N/A	Q9UKN8	71
KAT13A	NCoA-1, SRC1	SRCs	Nuclear, Cytoplasmic	NR Binding Domain	Q15788	72, 73
KAT13B	NCoA-3, TRAM1		Nuclear, Cytoplasmic, Exosome	NR Binding Domain	Q9Y6Q9	74, 75
KAT13C	NCoA-2, TIF2, SRC3		Nuclear, Cytoplasmic	NR Binding Domain	Q15596	73, 76, 77
KAT13D	CLOCK		Nuclear, Cytoplasmic	DNA Binding Domain	O15516	78-82
KAT14	CSR2B	N/A	Nuclear, Cytoplasmic	N/A	Q9H8E8	83

Table 4-1: Putative Lysine Acetyltransferases (KATs) and their common aliases. Proteins are listed with the subcellular localization, crystal structures (if available), and UniProt ID. Reference information can be found in (Ali et al., 2018).

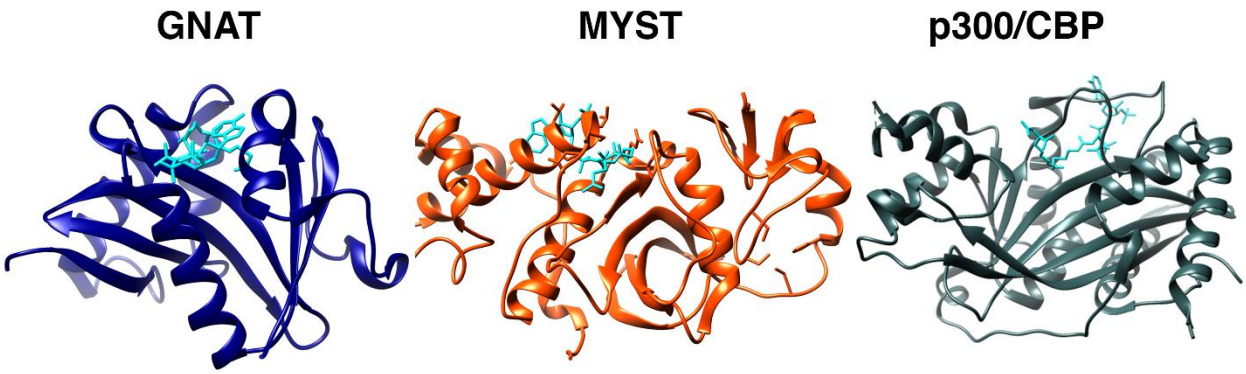


Figure 4-2: Structures of catalytic KAT domains from GNAT (human GCN5, blue, PDB: 1Z4R), MYST (human MOZ, orange, PDB:2RC4), and KAT3A/B(CBP/p300) (human KAT3B(p300), grey, PDB:3BIY) families. Acetyl-CoA is shown in cyan. Images rendered in Chimera (UCSF).

Despite considerable divergence in primary sequence, KATs from distinct families exhibit structurally homologous acetyl-CoA binding regions, which generally adopt a globular α/β fold (**Figure 4-2**). Regions flanking the central acetyl-CoA-binding cleft are not generally conserved, and may serve to guide substrate specific activities.(Berndsen and Denu, 2008) Among the KAT subfamilies, three prevailing mechanisms have been identified. GNAT family members use an active site glutamate to deprotonate the lysine ε-amine, enabling nucleophilic attack of the acetyl-CoA carbonyl, followed by formation of a transient tetrahedral intermediate and its subsequent collapse into acetyl-lysine and coenzyme A (**Figure 4-3**).(Jiang et al., 2012) The same mechanism has been proposed for KATs of the MYST family.(Berndsen et al., 2007) A two-step mechanism involving an active site acetyl-cysteine intermediate was originally proposed for MYST enzymes.(Yan et al., 2002) However, mutagenizing this cysteine residue does not affect enzymatic activity within the context of a pre-assembled ternary complex.(Berndsen et al., 2007) Mutagenesis of an active site glutamate, however, ablates activity without reducing levels of autoacetylation.(Yan et al., 2000; Yuan et al., 2012) Collectively, these data suggest that the active site glutamate plays a particularly significant role for MYST family catalysis. However, acetyl-cysteine intermediates may still be relevant depending on cellular context for MYST family members with still undefined mechanisms.

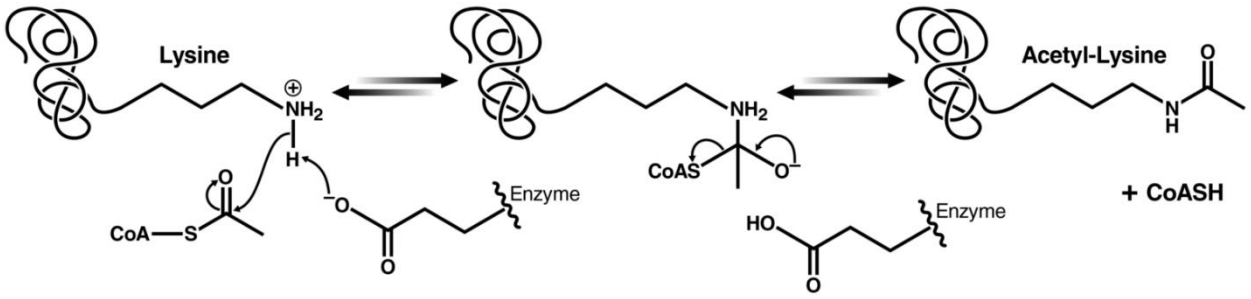


Figure 4-3: Proposed reaction mechanism for GNAT family KATs (Jiang et al., 2012).

The mechanism utilized by p300/CBP family members is categorized as a “hit and run” (Theorell-Chance) mechanism. It is ordered and rapid, and the ternary complex formed is kinetically irrelevant for catalysis.(Liu et al., 2008) Instead of an active site basic residue, aromatic residues lining a shallow catalytic pocket steer the lysine substrate and allow for nucleophilic attack of acetyl-CoA by lowering its pK_a.(Zhang et al., 2014) A tyrosine residue then acts as an acid to protonate the sulfhydryl of CoA, leaving as reaction products acetyl-lysine and CoA (**Figure 4-4**). This may partially explain the relative substrate promiscuity observed for p300.(Dancy and Cole, 2015) The mechanisms used by several KATs [i.e., KAT13D (CLOCK), KAT13A (SRC1), KAT13B (SRC3), KAT4 (TAF1), KAT9 (ELP3), and KAT12 (GTF3C4), among others] have not been formally investigated.

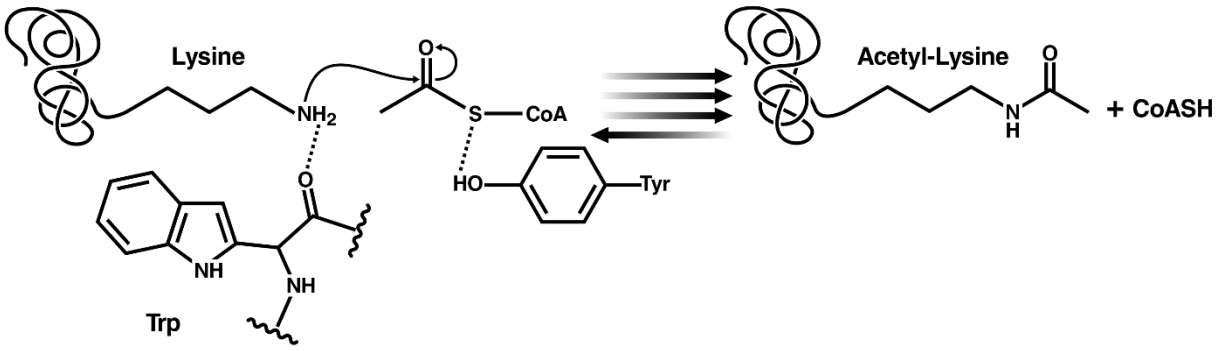


Figure 4-4: Proposed reaction mechanism for p300 family KATs.(Liu et al., 2008)

The enzymatic activity of HAT proteins may vary depending on the cellular microenvironment. For example, the substrate specificity and therefore the catalytic activity of KAT2A/B (GCN5/PCAF) may be influenced by accessory proteins within this complex that help target the acetyltransferase to its substrates, thus enhancing activity. For example, using immunoprecipitation followed by gel filtration chromatography KAT2A/B (GCN5/PCAF) can be separated from a large macromolecular structure consisting of the TBP-free-TAF complex (TFTC) and the SPT3-TAF9-GCN5-acetyltransferase (STAGA). (Demeny et al., 2007; Martinez et al., 1998; Ogryzko et al., 1998) (Demeny et al., 2007; Martinez et al., 1998; Ogryzko et al., 1998) (Demeny et al., 2007; Martinez et al., 1998; Ogryzko et al., 1998) (Demeny et al., 2007; Martinez et al., 1998; Ogryzko et al., 1998). These complexes are large, up to 2 megadaltons, and likely vary in composition across the genome to transduce highly specific stimuli.(Nagy and Tora, 2007)

Recent studies have identified two putative mitochondrial KAT enzymes, suggesting that acetylation in the mitochondria can be enzymatically triggered and raising interesting questions about the catalytic mechanisms of these proteins. GCN5-like protein 1 (GCN5L1) was proposed to act as a mitochondrial KAT and a counter-regulator to SIRT3, a mitochondrial lysine deacetylase. Notably, robust *in vitro* acetylation required the presence of additional mitochondrial factors suggesting that GCN5L1 activity may not be direct.(Scott et al., 2012) In addition, KAT8 (MOF) localizes specifically to mitochondria in HeLa cells and its catalytic activity is required for appropriate mitochondrial gene expression.(Chatterjee et al., 2016) However, it remains unclear whether KAT8 (MOF) enzymatic activity regulates mitochondrial protein acetylation.

Autoacetylation is an important mechanism of HAT enzymatic regulation. In 2004, Cole and colleagues identified a cluster of key lysine residues within an activation loop motif of KAT3B (p300) that must be acetylated in order for the enzyme to have robust catalytic activity.(Karanam et al., 2006; Thompson et al., 2004) In this model, the activation loop regulates KAT3B (p300) activity by competing with substrates for the active site. Upon hyperacetylation, the activation loop

is displaced allowing for substrates to interact with the active site.(Karanam et al., 2007; Karukurichi et al., 2010) Active site autoacetylation appears to be a conserved process as RTT109, a yeast acetyltransferase, autoacetylates its active site at K290 to increase its affinity for acetyl-CoA.(Albaugh et al., 2011) Similar to KAT3B (p300), KAT8 (MOF) also requires autoacetylation for its activity, shifting the structure of the protein to allow for better substrate binding and catalytic activity *in vitro* and *in vivo.*(Yuan et al., 2012) In contrast, KAT13D (CLOCK) gene acetylates its dimerization partner BMAL1, a modification that facilitates the assembly of a CRY1-CLOCK-BMAL1 complex and suppresses its activity in a negative feedback loop essential for circadian rhythmicity.(Hirayama et al., 2007)

Lysine deacetylases and sirtuins

The reversible nature of lysine acetylation is essential to its function in the regulation of critical cellular processes. The possible existence of enzymatic deacetylation was first suggested in 1978 when it was observed that n-butyrate treatment induced the differentiation of Friend erythroleukaemic cells into hemoglobin-synthesizing normoblast-like cells, a phenotype that correlated with strong histone hyperacetylation.(Riggs et al., 1977) This early work characterizing n-butyrate and Trapoxin(Kijima et al., 1993; Yoshida et al., 1995) as KDAC inhibitors paved the way for Schreiber and colleagues to purify the first KDAC from bovine calf thymus lysates using a Trapoxin based affinity matrix.(Taunton et al., 1996) Following this, and in rapid succession, KDACs 2–11 were discovered through sequence homology analyses to yeast deacetylases.(Emiliani et al., 1998; Fischle et al., 1999; Gao et al., 2002; Guardiola and Yao, 2002; Hubbert et al., 2002; Yang et al., 1997; Zeng et al., 1998)

At the same time, the silent information regulator (Sir) protein family, known to suppress gene expression at telomeres and ribosomal DNA,(Fritze et al., 1997; Gotta et al., 1997) gained attention as potential deacetylase enzymes. Mutation of Sir proteins in yeast induced hyperacetylation of histones.(Braunstein et al., 1993) In 1999, Frye and colleagues identified five

human cDNAs with sequence homology to the yeast Sir2 gene and shortly after, Sir2 was identified as an NAD^+ dependent histone deacetylase.(Frye, 1999; Imai et al., 2000) The family known as Sirtuins was completed using a phylogenetic classification scheme identifying the last two members, SIRT6 and SIRT7.(Frye, 2000)

KDACs and sirtuin proteins are mechanistically and structurally distinct (**Figure 4-5**). They are formally categorized into four distinct enzyme classes based on structural homology with yeast transcriptional repressors and unique catalytic mechanisms.(Delcuve et al., 2012; Seto and Yoshida, 2014) (**Table 4-2**) Class I, II, and IV enzymes are Zn^{2+}-dependent and form KDACs 1–11. Class I enzymes (KDAC1, 2, 3, 8) localize mainly to the nucleus, while class II (KDAC4–7, 9, 10) and Class IV (KDAC11) enzymes generally shuttle between the nucleus and cytoplasm. The Sirtuin proteins 1–7 encompass the class III enzymes and are described in the text below. Similar to KATs, KDACs are also often found in large, macromolecular complexes that function primarily in gene repression. For example CoREST, NuRD, and Sin3 complexes harbor a catalytic core composed of a KDAC1:KDAC2 dimer, and the NCoR complex contains KDAC3.(Kelly and Cowley, 2013; Watson et al., 2012)

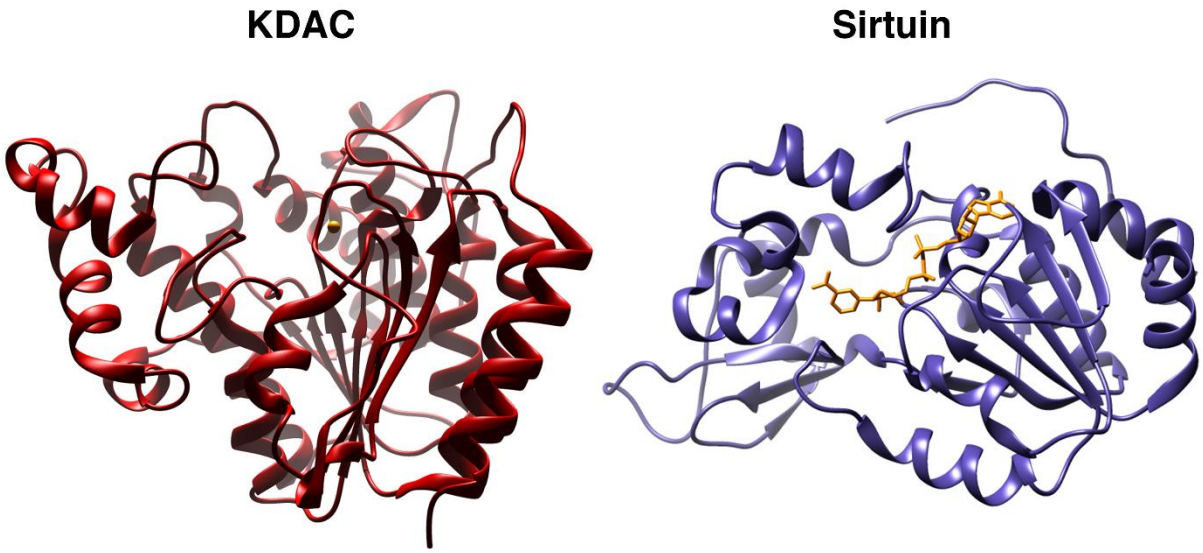


Figure 4-5: Structures of catalytic KDAC domains from KDAC (human KDAC2, red, PDB:4LXZ) and Sirtuin (human SIRT1, purple, PDB:4I5I families). KDAC zinc and Sirtuin NAD are shown in yellow. Images rendered in Chimera (UCSF).

Zn^{2+}-dependent Lysine Deacetylases (KDACs)

PROTEIN NAME	SUBFAMILY	LOCALIZATION	STRUCTURES AVAILABLE	INHIBITORS	UNIPROT ID	REFER-ENCES
KDAC1	I	Nuclear, Cytoplasmic	Complete Protein	Panobinostat, Vorinostat, Romidepsin	Q13547	123-125
KDAC2		Nuclear, Cytoplasmic	Deacetylase Domain	Panobinostat, Vorinostat, Romidepsin	Q92769	126-128
KDAC3		Nuclear, Cytoplasmic	Deacetylase Domain	Panobinostat, Vorinostat	O15379	128-130
KDAC8		Nuclear, Cytoplasmic	Complete Protein	Panobinostat, Vorinostat, PCI-34051	Q9BY41	131-136
KDAC4	IIA	Nuclear, Cytoplasmic	Deacetylase Domain	Panobinostat, Romidepsin	P56524	137-141
KDAC5		Nuclear, Cytoplasmic	N/A	Panobinostat	Q9UQL6	142-144
KDAC7		Nuclear, Cytoplasmic, Mitochondrial	Deacetylase Domain	Panobinostat	Q8WUI4	145-150
KDAC9		Nuclear, Cytoplasmic	N/A	Panobinostat, Vorinostat	Q9UKV0	151, 152
KDAC6	IIB	Nuclear, Cytoplasmic	Deacetylase Domain	Panobinostat, Vorinostat, Romidepsin, Tubastatin A	Q9UBN7	112, 153
KDAC10		Nuclear, Cytoplasmic	N/A	Panobinostat	Q969S8	111, 154
KDAC11	IV	Nuclear, Cytoplasmic	N/A	N/A	Q96DB2	110, 155

Table 4-2: Zn^{2+} dependent lysine deacetylases (KDACs). Proteins are listed with subcellular localization, relevant crystal structures (if available), common inhibitors, and UniProt ID. Reference information can be found in (Ali et al., 2018).

Mechanistic insight into KDAC catalysis derives from studies of HDLP, a deacetylase homolog from the bacterium *Aquifex aeolicus*.(Finnin et al., 1999) Like HDLP, KDACs utilize an active site histidine to deprotonate a critical water molecule, enabling nucleophilic attack of the acetyl group carbonyl (**Figure 4-6**). Decomposition of the oxyanionic tetrahedral intermediate releases acetate and the deacetylated lysine as reaction products. The divalent cation (Zn^{2+}) is important for positioning and polarizing a catalytic water molecule, and is positioned itself by aspartic acid and histidine residues of a classical catalytic triad (charge-relay network). This Zn^{2+} is a critical target of inhibitors of the class I, II and IV KDACs, which mainly function via chelation.

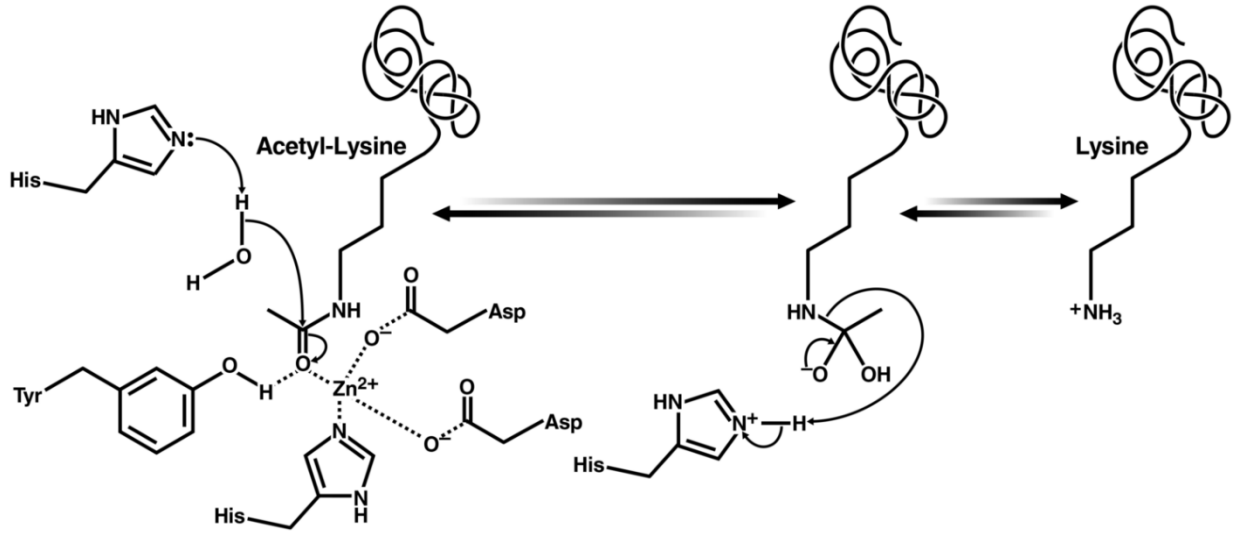


Figure 4-6: Proposed reaction mechanism for class I, II, and IV KDACs (Newkirk et al., 2009).

Class III KDACs function independently of an active site metal and, instead, rely on nicotinamide adenine dinucleotide (NAD^+) as a cofactor for catalytic activity.(Gao et al., 2013) Of the seven sirtuins in mammals, only SIRT1, 2, 3 have robust lysine deacetylase activity. More limited deacetylase activity has been reported for SIRT5, SIRT6 and SIRT7; while SIRT4 has no reported deacetylase activity (**Table 3**).(Ford et al., 2006; Laurent et al., 2013; Liszt et al., 2005; Nakagawa et al., 2009; North et al., 2003) SIRT6 and SIRT7 localize primarily to the nucleus, SIRT1 and SIRT2 shuttle between the nucleus and cytoplasm, and SIRT3 is a bona fide mitochondrial matrix protein.(Michishita et al., 2005) Unlike class I, II, and IV KDACs, sirtuins are not found in large repressive macromolecular complexes. However, certain binding partners regulate their enzymatic activity. For example, the active regulator of sirtuin (AROS) has been shown to stimulate SIRT1-mediated deacetylation of p53,(Kim et al., 2007) while deleted in breast cancer 1 (DBC1) negatively impacts SIRT1 activity.(Kim et al., 2008; Zhao et al., 2008)

NAD$^+$ -dependent Sirtuin Deacetylases

PROTEIN NAME	LOCALIZATION	STRUCTURES AVAILABLE	INHIBITORS	UNIPROT ID	REFERENCES
SIRT1	Nuclear, Cytoplasmic	Deacetylase Domain	EX-527, Nicotinamide	Q96EB6	168-175
SIRT2	Nuclear, Cytoplasmic	Complete Protein	EX-527, Nicotinamide	Q8IXJ6	175-181
SIRT3	Mitochondrial	Deacetylase Domain	EX-527, Nicotinamide	Q9NTG7	175, 180, 182–186
SIRT5	Mitochondrial, Nuclear, Cytoplasmic	Deacetylase Domain	Nicotinamide, Suramin	Q9NXA8	186–191
SIRT6	Nuclear	Complete Protein	N/A	Q8N6T7	164, 192–195
SIRT7	Nuclear	N/A	N/A	Q9NRC8	196, 197

Table 4-3: NAD$^+$ dependent sirtuin deacetylases. Proteins are listed with subcellular localization, relevant crystal structures (if available), common inhibitors, and UniProt ID. Reference information can be found in (Ali et al., 2018).

The sirtuin reaction mechanism proceeds by nucleophilic addition of acetyl oxygen to the anomeric (C1') carbon of the nicotinamide ribose via S_N1, concerted S_N2, or dissociative S_N2-like mechanisms, resulting in the formation of a C1′-O-alkylamidate intermediate (**Figure 4-7**). Next, a histidine residue abstracts a proton from the 2′-hydroxyl group of the NAD^+ ribose, which then attacks the C1′-O-alkylamidate carbon, generating a bicyclic intermediate. A base deprotonates a water molecule, enabling its attack of the bicyclic intermediate. Collapse of the bicyclic intermediate generates the deacetylated lysine and O-acetyl-ADP-ribose.(Sauve and Schramm, 2003) Sirtuins likely also have weak ADP ribosyltransferase activity via incomplete catalysis through this described mechanism. ADP ribosyltransferase activity has been formally reported for SIRT4 and SIRT6.(Haigis et al., 2006) The mitochondrial SIRT5 enzyme exhibits broad deacylase activity, accepting malonyl- and succinyl-lysine substrates.(Du et al., 2011; Park et al., 2013; Rardin et al., 2013a) The biological function of this distinct activity is not yet clear.

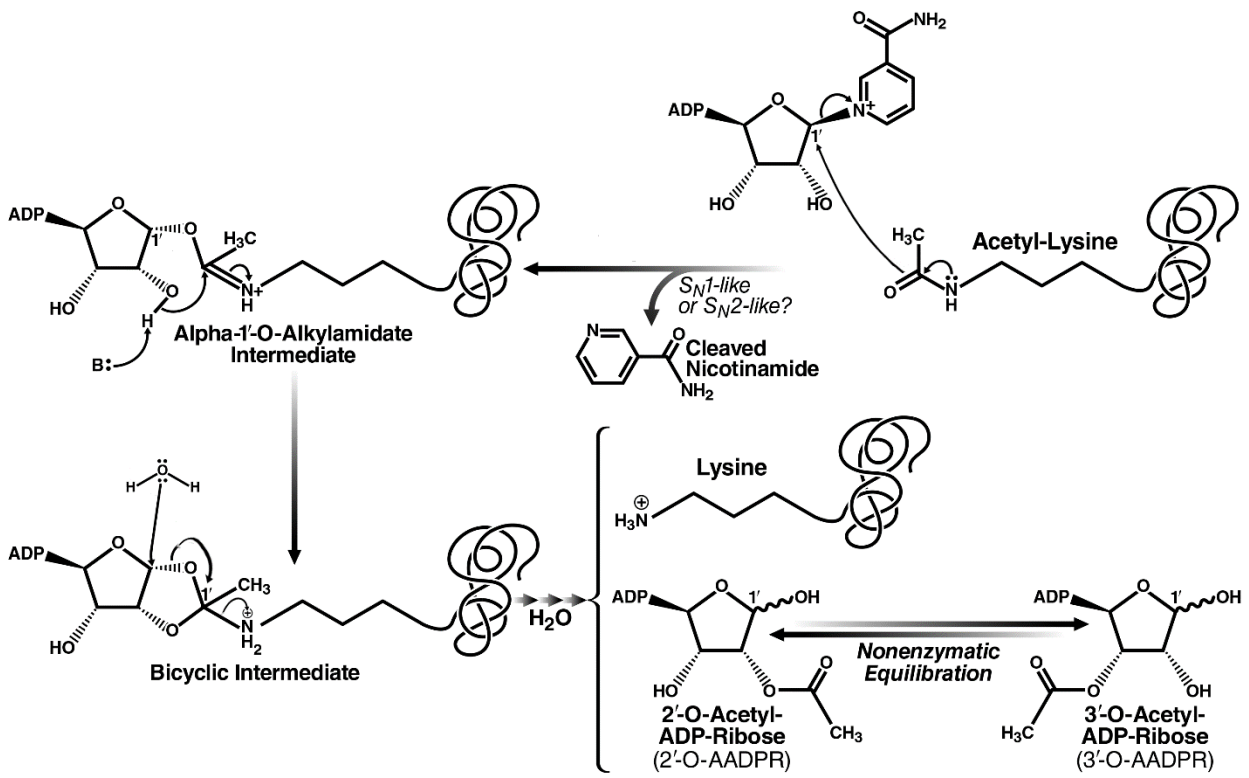


Figure 4-7: Proposed reaction mechanism for class III KDACs/sirtuins(Hirsch and Zheng, 2011).

Acetyl-lysine binding modules

An important function of lysine acetylation is the generation of novel recognition surfaces for the binding of proteins harboring “reader” domains specific for the posttranslationally modified residue. The best-characterized reader module of acetyl-lysines is a structurally conserved protein domain called the bromodomain. The first reference to the bromodomain is traced to the characterization of the *Drosophila* gene brahma (*brm*), a regulator of homeotic genes now known to be a core catalytic component of SWI/SNF chromatin remodelers.(Tamkun et al., 1992) The conserved structural motif discovered in the *brm* gene was termed a bromodomain, yet it is etymologically distinct from elemental bromine. Apart from the observation of its frequent occurrence in transcriptional regulators, the bromodomain was relatively uncharacterized from the time of its discovery in 1992(Tamkun et al., 1992) to the determination of its structure by Zhou and colleagues in 1999.(Dhalluin et al., 1999) NMR studies of the KAT2B (PCAF) bromodomain revealed that this domain binds acetyl-lysine residues on histones and described the structural details of this interaction.

The bromodomain is approximately 110 amino acids in length, and there are 61 distinct bromodomains encoded by 46 proteins (**Table 4-4**). The bromodomains are conserved from yeast to humans and are encoded in an increasing number of factors during eukaryotic evolution.(Haynes et al., 1992) In mammals, bromodomains can be divided into several distinct subfamilies based mostly on structural homology.(Fujisawa and Filippakopoulos, 2017; Sanchez and Zhou, 2009) While most bromodomain-containing proteins encode one bromodomain, up to six bromodomains have been documented in a single protein (Polybromo-1). The so-called bromo- and extraterminal (ET) domain-containing (BET) proteins encode a characteristic double bromodomain motif and are implicated in recruiting the positive transcription elongation factor b (P-TEFb) and other factors to signal inducible genes, including those regulated by the transcription factor *c-myc* in several cancers.(Delmore et al., 2011; Mertz et al., 2011; Ott et al.,

2012) Nearly all bromodomain-containing proteins are nuclear factors that bind chromatin to regulate its structure and function. They function mostly as transcriptional coactivators (i.e., KAT3B (p300), BRD4), but repressive functions of certain bromodomain-containing proteins are also known (i.e., BAZ2A, ZYMND11). Remarkably, many nuclear KATs harbor bromodomains. The KAT2A (GCN5) bromodomain is important for chromatin remodeling(Syntichaki et al., 2000) and regulation of sequential histone acetylation events.(Cieniewicz et al., 2014) A recent structural analysis of the core catalytic domain of KAT3B (p300) showed an assembled configuration of the bromodomain, PHD, RING and KAT domains with the RING domain positioned over the KAT domain substrate-binding pocket, providing insight into how chromatin-substrate targeting and KAT regulation might be linked.(Delvecchio et al., 2013)

PROTEIN NAME	BROMO-DOMAINS	LOCALIZATION	BROMO-DOMAIN STRUCTURE	UNIPROT ID	REFERENCES
BPTF	1	Nuclear, Cytoplasmic	Yes	Q12830	27, 211, 212
KAT3A (CBP)	1	Nuclear, Cytoplasmic	Yes	Q92793	27
KAT3B (p300)	1	Nuclear, Cytoplasmic	Yes	Q09472	27
BRWD1	2	Nuclear, Cytoplasmic	1 of 2	Q9NSI6	27
PHIP	2	Nuclear, Cytoplasmic	1 of 2	Q8WWQ0	27, 213, 214
BRPF1	1	Nuclear, Cytoplasmic	Yes	P55201	56, 215-219
TRIM24	1	Nuclear, Cytoplasmic	Yes	O15164	220, 221
SP100	1	Nuclear, Cytoplasmic	Yes (4PTB)	P23497	222, 223
KAP1	1	Nuclear, Cytoplasmic	Yes	Q13263	224-228
ZMYND11	1	Nuclear, Cytoplasmic	Yes	Q15326	229, 230
KAT2A (GCN5)	1	Nuclear	Yes	Q92830	27
KAT2B (PCAF)	1	Nuclear	Yes	Q92831	27
CECR2	1	Nuclear	Yes	Q9BXF3	27
BRDT	2	Nuclear	2 of 2	Q58F21	27, 231-235
BRD4	2	Nuclear	2 of 2	O60885	27, 46, 236-239
BRD3	2	Nuclear	2 of 2	Q15059	27, 240
BRD2	2	Nuclear	2 of 2	P25440	241-244
BAZ1A	1	Nuclear	No	Q9NRL2	245, 246
BRD8B	2	Nuclear	No	Q9H039	247
BAZ1B	1	Nuclear	No	Q9UIG0	248, 249
BRD9	1	Nuclear	Yes	Q9H8M2	27, 46, 250-252
BRD7	1	Nuclear	Yes	Q9NPI1	250, 253
BRPF3	1	Nuclear	No	Q9ULD4	59, 254, 255
BRD1	1	Nuclear	Yes	O95696	27, 256
ATAD2B	1	Nuclear	Yes	Q9ULI0	27, 257, 258
TRIM33	1	Nuclear	Yes	Q9UPN9	259-262
SP110	1	Nuclear	No	Q9HB58	263, 264
SP140	1	Nuclear	No	Q13342	265, 266
SP140L	1	Nuclear	No	Q9H930	267
BAZ2B	1	Nuclear	Yes	Q9UIF8	268, 269
BAZ2A	1	Nuclear	Yes	Q9UIF9	270, 271
KMT2A	1	Nuclear	Yes	Q03164	272-275
TAF1L	1	Nuclear	Yes	Q8IZX4	27, 237
TAF1	2	Nuclear	2 of 2	P21675	27, 46, 48, 49, 276, 277
ZMYND8	1	Nuclear	Yes	Q9ULU4	278-280
PBRM1	6	Nuclear	6 of 6	Q86U86	27, 281, 282
BRG1	1	Nuclear	Yes	P51532	27, 283-285
SMARCA2	1	Nuclear	Yes (5DKC)	P51531	286
BRWD3	2	Nuclear, Extracellular	No	Q6RI45	287
ATAD2	1	Nuclear, Exosome	Yes	Q6PL18	27, 288-291
ASH1L	1	Nuclear, Tight Junctions	Yes	Q9NR48	27, 292, 293

Table 4-4: Bromodomain containing proteins. Proteins are organized according to their observed subcellular localization. UniProt IDs refer to human proteins. References correspond to protein localization and relevant crystal structures drawn from mouse and human data. Reference information can be found in (Ali et al., 2018).

The bromodomain structure is well characterized, with >400 high-resolution X-ray crystal structures available and near complete structural coverage across the protein family. The domain is composed of four left-handed α-helices (αZ, αA, αB, and αC) connected by two loops (ZA and BC loops, **Figure 4-8**). (Dhalluin et al., 1999) This structure forms a hydrophobic cavity that serves as the acetyl-lysine recognition site. A hydrogen bond mediated by a conserved bromodomain asparagine residue and the acetyl-lysine carbonyl serves as the ligand recognition mechanism. Tyrosine residues lining the bromodomain cleft also play a significant role in ligand positioning via pi-pi stacking and hydrogen bond formation with critical water molecules. Helical regions of bromodomains are moderately conserved, but the length and sequence of the loop regions vary considerably. Some bromodomains cooperatively bind multiply acetylated peptides, such as the testis-specific BET protein BRDT.(Moriniere et al., 2009) Others are controlled by posttranslational modifications on nearby proteins. *In vitro*, the bromodomain:acetyl-lysine interaction is relatively weak (K_d = low micromolar). *In vivo*, the combined affinities of adjacent or proximal protein domains (i.e., helicase, SAND, distinct bromodomain) may modulate specificities and/or strength of binding.

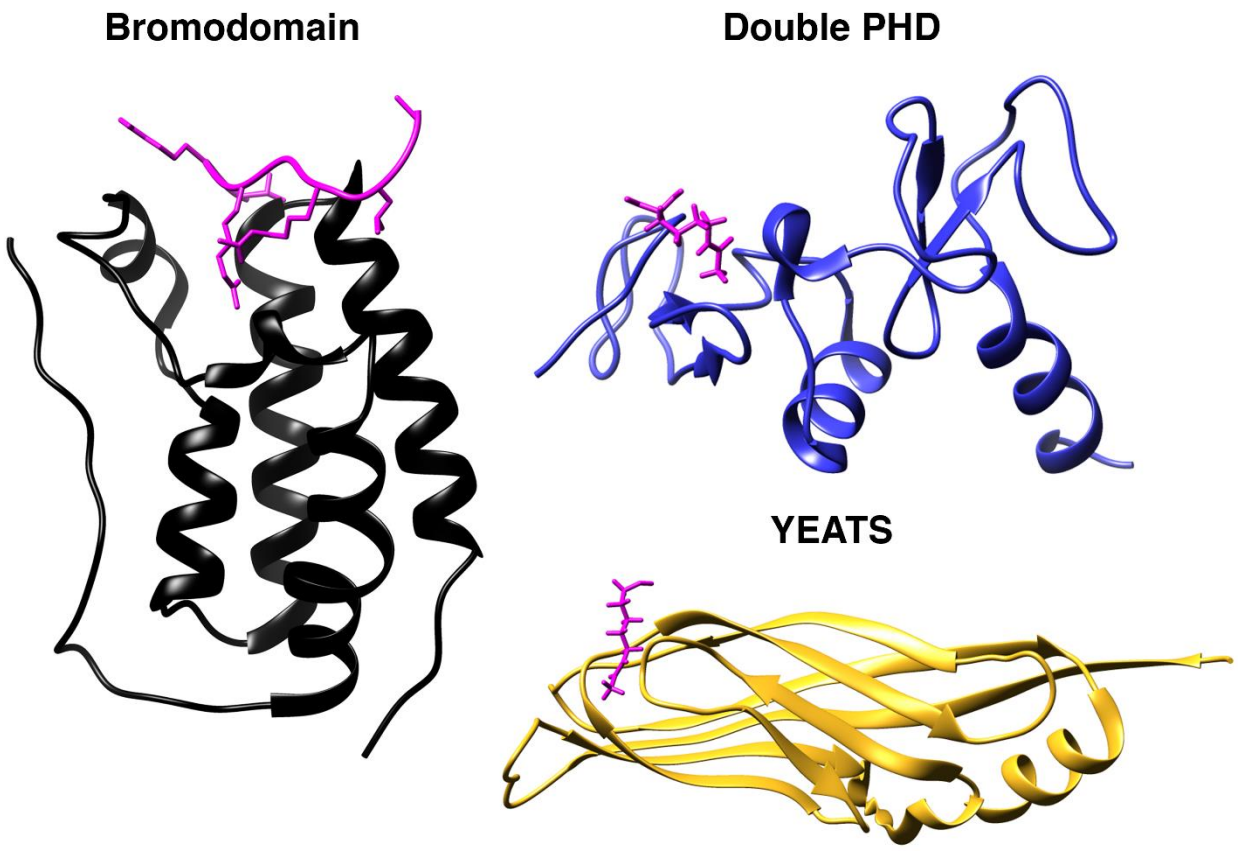


Figure 4-8: Structures of acetylation reader domains: Bromodomain (human BRD4, black, PDB:3UVW), double PHD (human DPF3, blue, PDB:2KWJ), and YEATS (human AF9, yellow, PDB:4TMP). Acetyl-lysine ligands shown in pink. Images rendered in Chimera (UCSF).

Several other protein domains have been reported to accept acetyl-lysine residues as ligands. The plant homeodomain (PHD) finger domain is generally recognized as a methyl-lysine reader domain, but when present in tandem in the protein DPF3b, it binds acetylated lysine residues on histone H3 and H4 molecules (**Figure 4-8**).(Zeng et al., 2010) The tandem PHD:acetyl-lysine binding mode is mechanistically distinct from that of the bromodomain, utilizing aspartic acid within the first PHD domain to form a hydrogen bond with the acetyl amide of the ligand. Interestingly, this aspartic acid also serves to recognize N-terminally acetylated peptides in addition to acetyl-lysine residues. Notably, proteins other than DPF3b encode tandem PHD domains, such as the CHD4 chromatin remodeler and KAT6A (MOZ), both of which have been shown to bind acetylated histones.(Musselman et al., 2012; Qiu et al., 2012)

The YEATS domain also recognizes acetyl-lysine residues.(Li et al., 2014) YEATS domains are present in five human proteins (YEATS2, ENL, AF9, TFIIF, and GAS41). AF9 and ENL are both components of the so-called superelongation complex (SEC), a multimeric complex containing P-TEFb, AFF1/AFF4 scaffolds, and the ELL1/ELL4 elongation factors.(Luo et al., 2012) Structurally, the YEATS domain adopts an Immunoglobulin fold (**Figure 4-8**), and its interaction with acetyl-lysine is mediated by several hydrogen bonds in addition to aromatic residues important for ligand positioning.(Luo et al., 2012) Acetylated H3K9 is a ligand for the AF9 YEATS domain, and the ENL YEATS domain exhibits a preference for acetylated H3K27, although ENL correlates genome-wide with both acetylated H3K9 and H3K27 in acute myeloid leukemia (AML) cells.(Wan et al., 2017) The two other YEATS domain-containing proteins, GAS41 and YEATS2, belong to chromatin-remodeling complexes. The AF9 YEATS domain has an expanded binding repertoire of acyl-lysine marks, and can also accommodate modifications, such as crotonylation.(Li et al., 2016) Importantly, translocations between genes encoding ENL/AF9 and MLL methyltransferase occur frequently, and the resultant fusion proteins are oncogenic drivers.(Krivtsov and Armstrong, 2007) Specifically, the ENL YEATS domain is

required for tethering the SEC to enforce oncogenic gene expression programs in AML.(Wan et al., 2017)

While the bromodomain, YEATS and tandem PHD domains specifically recognize acetyl-lysine residues, readers have recently been found that specifically bind unmodified lysine residues. The SET protein functions through its acidic-domain to bind the C-terminus of the transcription factor p53 only when p53 is not acetylated. The function appears to be conserved, as proteins with similar domains, such as VPRBP, DAXX and PELP1, also bound preferentially to non-acetylated p53. In addition, the SET acidic-domain recognizes non-acetylated lysine-rich domains of histone H3, KU70 and FOXO1, suggesting broad implications for this mechanism of recognition.(Wang et al., 2016a)

The widening scope of protein acetylation

In 1997, over three decades after the discovery of acetylation on histones and tubulin, the transcription factor p53 was identified as a non-histone KAT substrate.(Gu and Roeder, 1997) By 2000, 10 more nuclear proteins and transcription factors were found to be substrates of acetylation, leading to speculation that acetylation may rival phosphorylation as a post-translational modification(Kouzarides, 2000). Six years later, the first acetylome screen identified 388 acetylation sites in 195 proteins, more acetylation sites than were identified in the previous 40 years.(Kim et al., 2006) Since then, more than 155 systems-wide acetylome studies have revealed the existence of thousands of acetylation sites on many cellular proteins, connecting lysine acetylation to virtually every cellular function and most biological outcomes (**Figure 4-9**). Mass spectrometry analyses of acetylation have been conducted in a wide-variety of species ranging from gram-positive(Kosono et al., 2015; Liu et al., 2016) and -negative bacteria(Weinert et al., 2017), budding yeast(Downey et al., 2015), plants(Konig et al., 2014; Smith-Hammond et al., 2014a; Smith-Hammond et al., 2014b), to eukaryotic human pathogens(Miao et al., 2013; Xue

et al., 2013), rodents and humans. These have provided valuable insight into the stoichiometry and dynamics of lysine acetylation, as well as interactions with other PTMs.

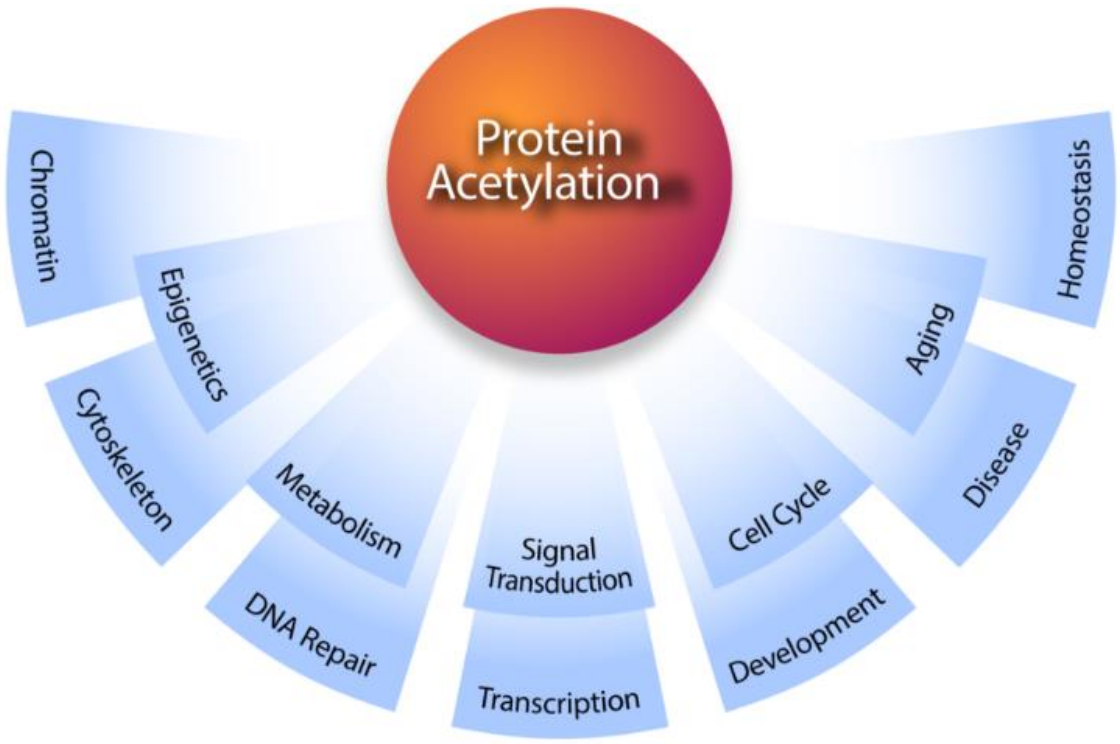


Figure 4-9: Acetylome studies reveal the scope of biological functions regulated by acetylation in mammalian cells.

Proteomic-based studies generally rely on an enrichment step in which pan-acetyl lysine antibodies are used to purify acetylated proteins from trypsin-digested lysates.(Choudhary et al., 2009; Kim et al., 2006) Notably, the use of an antibody raised against a single antigen can conceivably bias which proteins are purified from lysates, suggesting that most current studies only capture a subset of cellular acetylation sites.(Kim et al., 2016; Svinkina et al., 2015) Stable isotope labeling of amino acids in culture (SILAC),(Zhu et al., 2016b) and a label-free approach,(Rardin et al., 2013b) have been used to assess the dynamics of acetylated protein stoichiometry. These studies have revealed that in mammalian cells individual acetylation sites appear conserved across species, but not across tissue types.[4, 5] A high degree of overlap is observed in human, rat and mouse liver tissues, yet little overlap exists between rat liver and rat heart.(Bing and Zhaobao, 2010; Lundby et al., 2012) Acetylation occurs in regions with defined secondary structure, such as α-helices and β-sheets, unlike phosphorylation.(Choudhary et al., 2009; Kim et al., 2006)

Nuclear protein acetylation levels are high in tissues with actively dividing cells and in tumors. Many acetylation sites are found on proteins related to DNA damage, cell-cycle control, and transcription **(Figure 4-10)**.(Beli et al., 2012; Kim et al., 2006), (Choudhary et al., 2009) Mitochondrial acetylation is primarily found on proteins related to cellular metabolic processes, and is enriched in highly metabolically active tissues such as brown fat, heart, and liver, and likely plays a role in other tissue types depending on their metabolic activity and capacity to respond to insulin.(Bing and Zhaobao, 2010; Lundby et al., 2012; Rardin et al., 2013b) Cytoplasmic acetylation has been relatively understudied despite the fact that tubulin was the second protein discovered to be acetylated.(L'Hernault and Rosenbaum, 1983; Piperno and Fuller, 1985; Piperno et al., 1987) Notably, it is difficult to exclusively study cytoplasmic acetylation because cellular fractionation methods are imperfect, and many proteins tend to shuttle between the cytoplasm and other subcellular compartments. With these caveats in mind,

cytoplasmic acetylation is observed predominantly in liver, peri-renal and testis fat, tissues with high cellular concentrations of acetyl-CoA. [73]

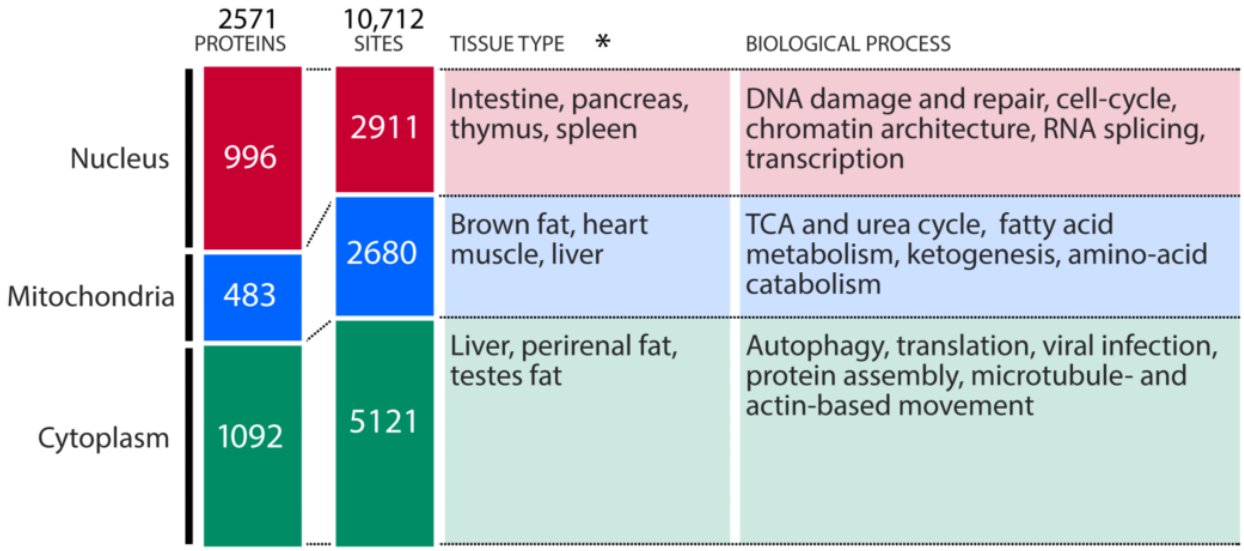


Figure 4-10: Acetylated proteins identified in proteomic studies: compartmentalization, tissue enrichment (*in rodent) and related biological processes as described in ref: (Choudhary et al., 2009), (Rardin et al., 2013b), (Lundby et al., 2012).

Several groups have taken genetic approaches to probe KAT- or sirtuin- specific acetylation sites. Examples include KAT2A/KAT2B knockdown studied in HeLa cells(Fournier et al., 2016), KAT13D(CLOCK) knockout studied in mouse liver tissues(Masri et al., 2013), SIRT1 knockout studied in mouse embryonic fibroblasts(Simon et al., 2012) or liver tissues(Kim et al., 2015) and SIRT3 knockout studies in mouse liver tissues(Rardin et al., 2013b; Sol et al., 2012) (**Figure 4-11**).

ENZYME	Δ ACETYLATED PROTEINS | SITES	TISSUE OR CELL TYPE	BIOLOGICAL PROCESS
KAT2A KAT2B	398 | 1569	HeLa cells	Chromatin organization, Pol II transcription elongation, histone lysine methylation, cell cycle, actin-mediated cell contraction
KAT13D	179 | 306	Mouse liver	Glycolysis, TCA cycle, amino acid metabolism, fatty acid metabolism
SIRT1	1800 | 4623	MEFs	DNA repair, signaling, transcription factor assembly, transcription elongation, spliceosome assembly, fatty acid metabolism, TCA cycle, small molecule metabolism
	114 | 807	Mouse liver	
SIRT3	179 | 306	Mouse liver Mitochondria	Fatty acid metabolism, TCA cycle, amino acid catabolism, ketone body metabolism, electron transport chain

Figure 4-11: Genetic approaches to identify acetylation sites, tissue type, and biological process as described in refs (Rardin et al., 2013b), (Fournier et al., 2016), (Masri et al., 2013), (Simon et al., 2012), (Kim et al., 2015) and (Sol et al., 2012).

The first integrative studies provided evidence for coordinated regulation of PTMs.(Duan and Walther, 2015) For example, one study noted coordination between acetylation and phosphorylation in the nucleus upon DNA damage, but most changes in phosphorylation occurred in the cytoplasm.(Beli et al., 2012) Surveys of acetylation and succinylation sites found substantial overlap between both acylation sites in mitochondria, suggesting potential competition between these modifications.(Gu et al., 2016; Weinert et al., 2013) Environmental cues, such as caloric restriction, microbiome components, and viral infection, and drugs, such as KDAC inhibitors and aspirin, also affect global acetylation levels.(Liu et al., 2015; Scholz et al., 2015; Tatham et al., 2017; Zhong et al., 2016; Zhu et al., 2016b)

Nuclear acetylation regulates gene expression

Histone acetylation

When nucleosomal histones are assembled with DNA, each subunit displays an N-terminal tail that can be post-translationally modified. Core nucleosomes are composed of pairs of histones H2A, H2B, H3 and H4; variants of these histones are important in chromatin regulation and gene expression. Histone H1 and its accompanying variants are regarded as 'linker' histones as they connect core nucleosomes into denser 30-nm fibers.(Robinson and Rhodes, 2006) Acetylation sites have been observed on all histone subunits, including linker histones, and occur in both the tail and globular domains (**Summarized in Figure 4-12**). Acetylation occurs abundantly in the tail domains while acetylation in the globular domain appears less abundant.(Tweedie-Cullen et al., 2012) Tail acetylation sites are evenly spaced among the nucleosomal histone subunits and possess some functional redundancy.(Ma et al., 1998) Acetylation sites are well conserved, in contrast to methylation, where species-specific differences exist.(Garcia et al., 2007a) Together, the specific array of histone modifications, known as the "histone code," may serve as a highly dynamic regulatory system for gene expression control in mammalian cells.

HISTONE	LYSINE MODIFICATION	
	Tail Domain	Globular Domain
H2A	K5, K9, K13, K15	K36, K118
H2B	K5, K11, K12, K15, K16, K23, K24	K46, K57, K120
H3	K4, K9, K14, K18, K23, K27	K36, K37, K56, K64, K79, K112, K115, K112
H4	K5, K8, K12, K16, K20, K31	K77, K79, K91
H1	*K16, K33	K45, K63, K74, K89, K96, K105, K167

Figure 4-12: Histone lysine acetylation sites and their domain location.(Huang et al., 2015) Each histone has additional isoforms not listed in this table. *Histone H1 N-terminal domain is structurally distinct from tail domains found in histone H2-4.

Acetylation of key lysine residues generally thought to disrupt the electrostatic interactions between the phosphodiester backbones of DNA and lysine-rich nucleosomes to expose DNA to transcription machinery.(Luger et al., 1997) Key advances in the analysis of histone acetylation comes from the use of electron transfer dissociation and electron capture dissociation mass spectrometry.(Syka et al., 2004; Udeshi et al., 2008) These methods allow for the analysis of long histone peptides (>20 a.a.) and therefore the identification of multiple modifications on individual histone proteins. Using this and other methodologies, several comprehensive studies have observed the combinatorial patterns of histone modifications on each subunit.(Garcia et al., 2007b; Garcia et al., 2008; Phanstiel et al., 2008; Tan et al., 2011; Tweedie-Cullen et al., 2012) These analyses are essential to determine whether specific modifications are compatible on the same histone at the same time, potentially identifying important rules for the histone code.

The best-studied acetylation sites are found on histone H3 and H4, but acetylation of H2A and H2B tails has also been correlated with increased transcriptional activity.(Gatta and Mantovani, 2011; Puerta et al., 1995) Histone 3 lysine acetylation has been observed on 14 residues, six of which are located on the tail region and eight in the globular domain.(Huang et al., 2015) H4 is acetylated at 9 lysines, six in the tail region and three in the globular domain.(Huang et al., 2015) In addition, lysine acetylation has been observed on H2A tails at four sites,(Poplawski et al., 2014; Tweedie-Cullen et al., 2012; Zheng et al., 2013) and the globular domain at two sites **(Figure 4-12)**.(Chen et al., 2012) Turnover of histone acetylation is unequal at different sites.(Zheng et al., 2013) While acetylation of histone tails generally has a fast turnover (<30mins), with the exception of H3K4, H2AK13 and K2AK15, nearly all globular domain modifications were more stable with a half-life greater than 2 hours.(Zheng et al., 2013)

Histone H1 is highly modified and was first identified to be acetylated *in vivo* in 2004.(Garcia et al., 2004; Vaquero et al., 2004) Multiple proteomic approaches have identified H1 acetylation sites at 11 lysine residues (K16, K33, K45, K63, K74, K89, K96, K105, K167, K168,

K190), albeit at low frequency.(Kamieniarz et al., 2012; Park et al., 2013; Singh et al., 2013; Tan et al., 2011; Tweedie-Cullen et al., 2012; Wisniewski et al., 2007) Given its role in DNA condensation, histone H1 was originally thought to act primarily as a suppressor of gene expression, but its function is now understood to be more nuanced.(Happel and Doenecke, 2009) For example, H1.4K34ac is detected in distal and proximal promoter regions of highly transcribed genes in induced pluripotent stem cells and cancer cancer cell lines, induced pluripotent stem cells (iPSCs) and testicular germ cell tumors.(Kamieniarz et al., 2012) In addition, an inverse relationship between the presence of H1 on chromatin and acetylation marks on H3 and H4 has been described.(Bernier et al., 2015; Sun et al., 2015)

Transcription factor acetylation

Notably, nuclear lysine acetylation is not restricted to histones, but is also found on numerous transcription factors including p53, NF-κB, and STAT3. Mechanistically, acetylation modulates transcription factor activity at multiple steps by inducing nuclear translocation or protein stabilization by sterically preventing ubiquitination, modifying molecular complex composition, and facilitating chromatin binding specificities. Proteomics studies have identified many known acetylation sites on transcription factors, of which we only list those with additional functional studies (**Table 4-5)**. We illustrate these phenomena using well-characterized case studies below (**Figure 4-13**).

PROTEIN NAME	ACETYL-LYSINE	WRITER	ERASER	UNIPROT ID	REFS
AR	K630, K632, K633	KAT2B, KAT3A, KAT3B, KAT5	KDAC1, KDAC7	P10275	362-365
ATM	K3016	KAT5	Unknown	Q13315	52, 366
BCL6	K379	KAT3B	Unknown	P41182	367
BMAL1	K537	KAT13D	SIRT1	O00327	101, 368
CDK9	K44, K48	KAT2A, KAT2B, KAT3B	KDAC3, SIRT2	P50750	369-372
ChREBP	K672	KAT3B	SIRT1	Q9NP71	373, 374
CREB	K91, K94, K136	KAT3A, KAT3B	SIRT1, KDAC8	P16220	375, 376
CRTC2	K628	KAT3A, KAT3B	SIRT1	Q53ET0	377
CycT1	K380, K386, K390, K404	KAT3B	KDAC1, KDAC3	O60563	315, 378, 379
E2F1	K117, K120, K125	KAT2A, KAT2B, KAT3B	HDAC1	Q01094	380-382
EKLF	K270, K284	KAT3A, KAT3B	Unknown	Q13351	383, 384
ERα	K226, K268, K299, K302, K303	KAT3A	SIRT1	P03372	385-387
Foxo1	K242, K245, K248, K262, K265, K274	KAT3B	SIRT1, SIRT2, SIRT3	Q12278	379-393
Foxp3	K31, K327, K263, K268	KAT3B, KAT5	KDAC7, KDAC9, SIRT1	Q9BPZS1	50, 394-396
FXR	K217	KAT3B	SIRT1	Q96RI1	397, 398
GABPB1	K69, K340, K369	KAT3B	SIRT7	Q06547	399
GATA1	K158, K246, K252, K312	KAT3A, KAT3B	KDAC5	P15976	400-403
HIF1α	K10, K11, K12, K19, K21, K709	KAT2B, KAT3B	KDAC1, KDAC4, SIRT1, SIRT2	Q16665	404-408
HIF2α	K385, K685, K741	KAT3A	SIRT1	Q99814	409, 410
HMG17	K2	KAT2B	Unknown	P05204	411
IFNαR2	K399	KAT3A	Unknown	P48551	412
Myc	K143, K148, K157, K275, K317, K323, K371	KAT2B, KAT3B, KAT5	SIRT1, SIRT2	P01106	315, 413-416
Notch1	K1764, K1770, K1771, K1772, K1785, K1935, K2050, K2068, K2146, K2147, K2150, K2154, K2161, K2164	KAT2B, KAT3B	SIRT1	P46531	417
p53	K120, K321, K373, K381, K382	KAT6A, KAT3B	KDAC1, SIRT1, SIRT2	P04637	315, 418-422
PAF53	K373	KAT2B	SIRT7	Q9GZS1	423
PRLR	K277, K339, K412, K456, K466, K472, K505, K514, K517, K526, K533, K536, K590, K601	KAT3A	KDAC6, SIRT2	P15471	424
Pygo2	K11, K43, K44, K47	KAT3A, KAT3B	Unknown	Q9BRQ0	425
RelA	K122, K123, K218, K221, K310, K314, K315	KAT2B, KAT3A, KAT3B	KDAC3, SIRT1, SIRT2	Q04206	426-430
Rb	K873, K874	PCAF	SIRT1	P06400	431
RORγ	K69, K81, K99, K112	KAT3B	KDAC1, SIRT1	P51449	432, 433
RPB1	K1888, K1909, K1916, K1923, K1937, K1958, K1972, K1986	KAT3B	Unknown	P24928	434, 435
SMAD7	K64, K70	KAT3B	KDAC1,KDAC3,KDAC5,KDAC6	O15105	436, 437
Sp1	K703	KAT3B	Unknown	P08047	315, 438
Sp3	K551	KAT3B	Unknown	Q02447	439-441
SREBP1c	K289, K309	KAT3B	SIRT1	P36956	442
STAT2	K390	KAT3A	Unknown	P52630	412, 443
STAT3	K49, K87, K685	KAT3A, KAT3B	KDAC1, KDAC2, KDAC3	P40763	444-446
STAT5b	K359, K694, K696, K701	KAT3A	SIRT2, KDAC6	P51692	424, 447
HIV-1 Tat	K28, K50, K51	KAT2A, KAT2B, KAT3B	SIRT1	P04608, P04610	448-451
UBF1	K352	KAT3A	Unknown	P17480	452
YY1	K173, K174, K178, K179, K180, K181	KAT2B, KAT3B	KDAC1, KDAC2	P25490	453

Table 4-5: Selection of Acetylated Transcription Factors, their Writers and Erasers. Reference information can be found in (Ali et al., 2018).

Several excellent reviews have documented the functions of transcription factor acetylation.(Choudhary et al., 2014; Park et al., 2015; Thiagarajan et al., 2016; Wang et al., 2016b) Here we exemplify a few key principals (**Figure 4-13**). In the case of transcription factors such as NF-κB and STAT3 that are cytoplasmic when inactive, signaling begins with an extracellular stimulus that leads to a cascade of PTMs resulting in changes in dimer structures and translocation from the cytoplasm to the nucleus.(Greene and Chen, 2004; Zhuang, 2013) STAT3 activation is marked by specific phosphorylation and acetylation events that allow for dimerization and subsequent nuclear localization.(Avalle et al., 2017) While phosphorylation is thought to be dominant for dimerization and DNA binding, several studies suggest a phosphorylation-independent mechanism of dimerization.(Braunstein et al., 2003; Kumar et al., 1997). Chin and colleagues demonstrate that acetylation at K685 by KAT3A/B (CBP/p300) induces homodimerization and nuclear translocation of STAT proteins.(Xu et al., 2016) RelA is a subunit of NF-κB generally sequestered in the cytoplasm through its interaction with its negative regulator IκBα. Upon cell stimulation, RelA is acetylated by KAT3A/B (CBP/p300) at several residues. Acetylation at K221 disrupts the RelA-IκBα interaction, allowing for nuclear translocation and increased DNA binding (**Figure 4-13A)**.(Chen et al., 2002; Pejanovic et al., 2012)

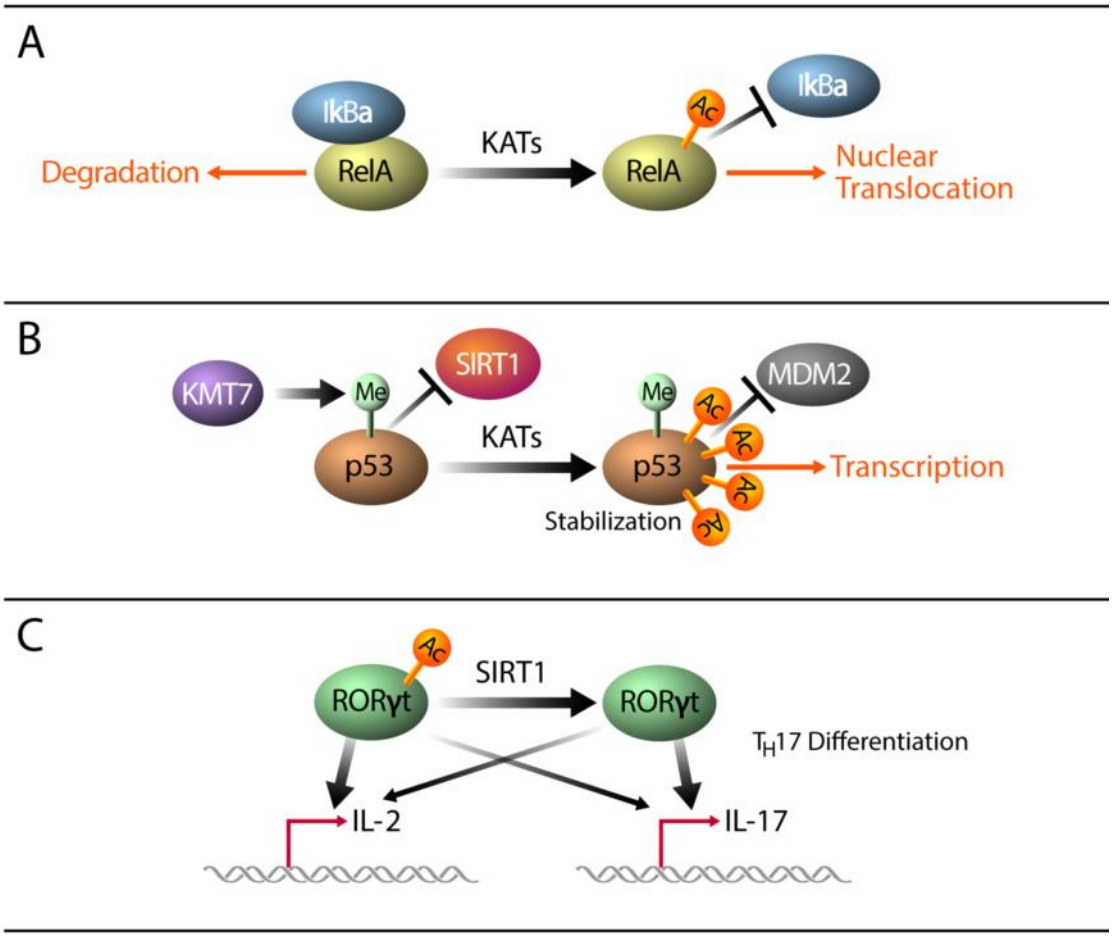


Figure 4-13: Mechanisms driving acetylation dependent regulation of transcription factors.

Changes in acetylation can also induce changes in protein stability as is the case for the p53 transcription factor. Acetylation can directly compete with ubiquitination at distinct lysine residues.(Caron et al., 2005) It can also mediate structural changes to prevent ubiquitination by sterically hindering interaction with ubiquitin ligases.(Caron et al., 2005; Goel and Janknecht, 2003) At homeostasis, p53 is maintained at low levels in the nucleus primarily through ubiquitin-mediated proteolysis.(Brooks and Gu, 2011) Upon DNA damage, p53 becomes highly acetylated at its carboxy-terminal domain (CTD), preventing MDM2-mediated ubiquitination and degradation.(Luo et al., 2000; Rodriguez et al., 2000) Acetylation of the p53 CTD is mediated primarily by KAT3B (p300), but KAT6A (MOZ) also acetylates p53 at K120 and K382.(Rodriguez et al., 2000; Rokudai et al., 2013) Crosstalk exists between factor acetylation and other PTMs, notably lysine methylation. For example, lysine methyltransferase 7 (KMT7, SET7/9)-mediated monomethylation at K372 promotes acetylation and stabilization of p53 by disrupting its interaction with the deacetylase SIRT1 (**Figure 4-13B)**.(Huang et al., 2006; Ivanov et al., 2007; Liu et al., 2011; Son et al., 2016) A similar phenomenon has been reported for RelA acetylation at K310 which prevents KMT7-mediated monomethylation at K314 and K315, thus stabilizing the protein.(Yang et al., 2010) Furthermore, acetylation of p53 is recognized by KAT3A/B (CPB/p300) and KAT4 bromodomains to facilitate acetylation of histone H3 and histone H4 at p53 response genes, which induces cell-cycle arrest or apoptosis.(Barlev et al., 2001; Li et al., 2007a; Wu et al., 2014)

Acetylation can further influence the DNA binding affinity and promoter specificity of transcription factors such as the T cell lineage master regulators RAR-related orphan receptor gamma (RORγ) and Forkhead box O proteins (FoxO). RORγ is acetylated by KAT3B(p300) and deacetylated by KDAC1 and SIRT1 at K69, K81, K99, and K112, the latter activating DNA binding of this lineage-determining transcription factor.(Lim et al., 2015; Wu et al., 2015) Deacetylation of RORγ increases transcription of the interleukin-17 (IL-17) gene but decreases activation of IL-2

(**Figure 4-13C**).(Lim et al., 2015) Similarly, acetylation of FoxO proteins by KAT3B (p300) facilitates dissociation from promoters of genes such as *p27* and *MnSOD*, a process that is reversible upon SIRT1, SIRT2 or SIRT3 overexpression.(Daitoku et al., 2011) Interestingly, acetylation of the Forkhead box P3 protein (FOXP3) enhances stability and function of the transcription factor, a master regulator of regulatory T cell identity.(Sakaguchi et al., 2010) FOXP3 acetylation is regulated by a balance between KAT3B (p300) and KAT5 (TIP60) acetylation and KDAC7, KDAC9 and SIRT1 deacetylation at K31, K263, and K274.(Kwon et al., 2012; Li et al., 2007b; Liu et al., 2013; van Loosdregt et al., 2011) Of note, acetylation of KAT5 (TIP60) by KAT3B (p300) increases its ability to acetylate FOXP3, highlighting the multiple layers of KAT cooperation required for appropriate signaling and regulatory T cell function.(Liu et al., 2013; Samanta et al., 2008; Xiao et al., 2014)

Acetylation of the basal transcription machinery

In addition to transcription factors, a growing number of factors associated with the RNA polymerase II complex are acetylated. Basal transcription factor acetylation has been studied in less detail and these data we summarize here. Choudhary et al lists eight TBP-associated proteins (TAFs) that are acetylated in the nucleus.(Choudhary et al., 2009) These proteins compose the basal transcription factor TFIID and contribute to transcription initiation of the RNA polymerase II complex.(Bieniossek et al., 2013) The function of TAF acetylation in TFIID function remains largely unknown, but acetylation of TAF(I)68, the second largest subunit of the TATA box-binding protein-containing factor TIF-IB/SL1, enhances binding to rDNA and was linked to enhanced RNA polymerase I transcription.(Muth et al., 2001) A more recent study highlighted the importance of CBP in RNA Pol II regulation at promoters in Drosophila. In this system, CBP was present at the promoters of nearly all expressed genes was found to play a role in promoter-proximal pausing, especially at genes with CBP and GAF co-occupancy.(Boija et al., 2017)

Acetylation of the positive transcription factor b (P-TEFb) is studied in detail.(Cho et al., 2009; Fu et al., 2007; Sabo et al., 2008) P-TEFb is composed of a cyclin T subunit and the cyclin-dependent kinase CDK9, which when assembled in an active elongation complex, is critical to phosphorylate negative elongation factors and the C-terminal domain (CTD) of the largest RNA polymerase II subunit at serine 2 activating transcription elongation by the polymerase complex.(Hsin and Manley, 2012; Meinhart et al., 2005) P-TEFb is stored predominantly in the nucleoplasm in a ribonucleoprotein complex (7SK RNP) but is released and activated upon increased transcriptional demand.(McNamara et al., 2016; Zhou et al., 2012) This release is caused in part by acetylation of four sites in cyclin T1 (K380, K386, K390, K404) that dissociates acetylated cyclin T1/CDK9 from the 7SK RNP and activates CDK9 activity on negative elongation factors and the polymerase CTD.(Cho et al., 2009) CDK9 also is subject to acetylation at two lysine residues (K44, K48).(Fu et al., 2007; Sabo et al., 2008) K48 acetylation disrupts ATP binding and inhibits CDK9 kinase activity directly(Cho et al., 2010; Sabo et al., 2008), while K44 acetylation activates P-TEFb activity. Additional cellular elongation factors found to be acetylated by mass spectrometry include FACT members SUP16H and SSRP1, but also the RTF1 subunit of the PAF1 complex as well as SUB1 and the CTD phosphatase FCP1(Choudhary et al., 2009), but these marks have not yet been studied functionally.

The CTD of RNA polymerase II is acetylated. The CTD is a long and flexible domain structure composed of heptapeptide repeats with the consensus sequence YSPTSPS, which is conserved across eukaryotes. Interestingly, the CTD has expanded in metazoans to include a C-terminal region of heptad repeats that are less strictly aligned with the consensus sequence. In mammals, this region contains eight heptad repeats where the serine in position 7 is replaced with a lysine.(Simonti et al., 2015) Acetylation of these lysine residues is mediated by KAT3B(p300) and is enriched downstream of transcription start sites in actively transcribed genes, linking this modification to polymerase pausing.(Schroder et al., 2013) Accordingly,

activation of signal-induced genes is inhibited when lysines are mutated to arginines in the CTD. However, the acetylated RNA polymerase II is not only found on signal-induced genes, but on many actively transcribed genes, implicating additional functions for CTD acetylation in transcription.(Schroder et al., 2013) Notably, both RNA polymerase I and III subunits are also acetylated(Choudhary et al., 2009). PAF53, a regulatory subunit of RNA polymerase I, is acetylated by KAT3A(CBP) at K373.(Chen et al., 2013) PAF53 acetylation is maintained at low levels by SIRT7, which facilitates robust rRNA transcription. Induction of stress by glucose deprivation suppresses SIRT7 activity leading to hyperacetylation of PAF53 and suppression of rRNA transcription.(Chen et al., 2013)

Protein stability and aggregation in the cytoplasm

Tubulin and HSP90 are regulated by KDAC6

Lysine acetylation in the cytoplasm is historically a very “old” concept, as tubulin was the first non-histone acetylation substrate identified.(L'Hernault and Rosenbaum, 1983, 1985; Piperno and Fuller, 1985; Piperno et al., 1987) Tubulin forms microtubules, a major structural element in the cytoplasm, composed of α/β tubulin dimers.(Burton et al., 1975; Howes et al., 2014; Tilney et al., 1973) Acetylation of α-tubulin occurs on the luminal side of microtubules at K40 and is catalyzed predominantly by α-tubulin acetyltransferase αTAT1, a non-canonical KAT homologous to zebrafish or *C. elegans* MEC17.(Akella et al., 2010; Kalebic et al., 2013; Shida et al., 2010) It is unclear whether acetylation is a cause or a consequence of tubulin stability, although this mark is generally considered a marker of protein stability. αTAT1 overexpression destabilizes microtubules; however, this is mainly attributed to enhanced αTAT1-tubulin interactions, and not considered a consequence of increased acetyltransferase activity.(Kalebic et al., 2013) Tubulin is deacetylated by KDAC6(Hubbert et al., 2002) and SIRT2.(North et al., 2003) KDAC6 is the major tubulin deacetylase and KDAC6 overexpression increases the chemotactic motility of murine fibroblasts, possibly due to tubulin destabilization.(Hubbert et al., 2002) SIRT2 plays an

important role in tubulin deacetylation in response to macrophage and NLRP3 inflammasome activation(Misawa et al., 2013; North et al., 2003) and also regulates tubulin acetylation on mitotic spindles.(Nagai et al., 2013)

HSP90 gained considerable attention due to its potential as a therapeutic target in hematologic malignancies.(Kramer et al., 2014) HSP90 exists in two major isoforms: HSP90α which is stress-inducible and tightly regulated; and HSP90β which is constitutively expressed(Kramer et al., 2014). HSP90 acetylation is detected on up to 22 distinct residues on HSP90α, and 5 distinct residues HSP90β.(Choudhary et al., 2009; Kramer et al., 2014; Scroggins et al., 2007) KDAC6 deacetylates HSP90 influencing glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) signaling.(Jimenez-Canino et al., 2016; Kovacs et al., 2005) KDAC1 may also influence HSP90 acetylation,(Nishioka et al., 2008) though KATs responsible remain elusive. Acetylation predominantly occurs on the middle domain of HSP90 where it regulates intermolecular interactions and chaperone activity.(Jimenez-Canino et al., 2016)

Tau and Alzheimer's disease

Acetylation also regulates microtubule-associated proteins (MAPs) with Tau as a prominent example.(Weingarten et al., 1975; Witman et al., 1976) Tau is highly expressed in neurons, and mutations in Tau serve as important markers for dementia and Alzheimer's disease.(Wang and Mandelkow, 2016) These mutations are linked to microtubule-binding repeats causing neurological defects associated with the disruption of Tau-microtubule interactions. Tau aggregation produces paired helical filaments seen in neurofibrillary tangles present in the brains of individuals afflicted with neurodegeneration.(Goedert and Jakes, 2005)

Acetylation was identified on more than a dozen lysine residues in Tau using *in vitro*, cell-based and mass spectrometric assays.(Cohen et al., 2011; Min et al., 2010; Morris et al., 2015) Acetylation is a common feature across MAP family members as the microtubule-binding domains of MAP2 and MAP4 are also acetylated.(Hwang et al., 2016) Tau, like MAP2 and 4 proteins,

possesses intrinsic acetyltransferase activity.(Cohen et al., 2016; Cohen et al., 2013; Hwang et al., 2016) In addition, several KATs have been identified to modify Tau including KAT3A/B(CBP/p300) and KAT2B(PCAF).(Cohen et al., 2011; Min et al., 2010) Deacetylases that target Tau include SIRT1, SIRT2 and KDAC6 with robust activities by SIRT1 and KDAC6.(Min et al., 2010; Noack et al., 2014; Xiong et al., 2013)

Several acetylation sites on Tau are well characterized.(Tracy and Gan, 2017) These include K274, K280 and K281. Acetylation of these sites reduces Tau interaction with microtubules by interfering with functions of the microtubule-interacting domain.(Cohen et al., 2011; Sohn et al., 2016) Acetylation of K274 and K281 leads to mislocalization of Tau, while K280 acetylation promotes Tau aggregation.(Cohen et al., 2011; Tracy et al., 2016) Acetylation of a distinct site, K174, slows cellular turnover of Tau and contributes to cognitive defects in mouse models of Alzheimer's disease. Notably, acetylation of specific RXGS motifs in Tau inhibit phosphorylation and aggregation of the protein, indicating opposing effects of different acetylation sites in Tau on neurogenerative pathogenesis.(Cook et al., 2014) These sites are also targeted by distinct KDACs: RXGS motifs are preferentially deacetylated by KDAC6, while SIRT1 targets K174, K274, K280 and K281.(Cook et al., 2014; Min et al., 2010) As Tau is decorated with many posttranslational modifications including lysine methylation and ubiquitination, these modifications can competitively inhibit Tau acetylation *in vitro* and *in vivo*.(Funk et al., 2014; Morris et al., 2015; Thomas et al., 2012)

Mitochondrial proteins are heavily acetylated

Mitochondrial acetylation regulates cell metabolism

Acetylation is widespread in mitochondrial proteins: 1/3 of mitochondrial proteins are acetylated,(Anderson and Hirschey, 2012) and many proteins carry multiple acetylated lysines.(Choudhary et al., 2009; Kim et al., 2006) Mitochondrial acetylation is strongly conserved from *Drosophila* to humans.(Weinert et al., 2011) Not surprisingly, acetylated proteins are

involved in major functions of mitochondria (e.g., TCA cycle, oxidative phosphorylation, β-oxidation of lipids, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and the urea cycle).(Wang et al., 2010; Zhao et al., 2010) Mitochondrial metabolism results from high concentrations of acetyl-CoA from aerobic catabolism of pyruvate, β-oxidation of long-chain fatty acids, and decarboxylation of malonyl-coA.(Berg et al., 2012)

Three of the seven class III deacetylases (SIRT3, 4 and 5) are mitochondrial.(He et al., 2012a) SIRT3 has robust NAD^+-dependent protein deacetylase activity and mice lacking SIRT3 show significant hyperacetylation of mitochondrial proteins,(Lombard et al., 2007) while mice lacking SIRT4 or SIRT5 do not. Proteins that become hyperacetylated in the absence of SIRT3 control the shift to a fasting metabolism when the source of energy switches from glucose to lipids and amino acids. Thus, SIRT3 is linked to the energy status of the cell,(Anderson et al., 2003; Bitterman et al., 2002; Lin et al., 2000; Lin et al., 2004; Lin et al., 2002) and it is expressed at the highest levels in metabolically active tissues (e.g., liver, kidney, and heart).(Ahn et al., 2008; Palacios et al., 2009) SIRT3 expression is also increased in glucose-poor, fasting states, including calorie restriction in liver and kidney.(Alhazzazi et al., 2011; Caton et al., 2011; Hallows et al., 2011; Hirschey et al., 2010; Tauriainen et al., 2011)

An important unresolved question regarding mitochondrial protein acetylation is the mechanism of acetylation itself. Is a mitochondrial KAT required? Mitochondria contain high concentrations of acetyl-CoA in millimolar amounts(Garland et al., 1965), and therefore a non-enzymatic mechanism could account for the high level of mitochondrial protein acetylation.(Paik et al., 1970) Indeed, increased mitochondrial protein acetylation is associated with physiological conditions that result in higher levels of acetyl-CoA (e.g., fasting, calorie restriction, high-fat diet, and ethanol intoxication).(Fritz et al., 2012; Hirschey et al., 2011; Hirschey et al., 2010; Picklo, 2008; Schwer et al., 2009)

Three mitochondrial KATs have been reported. One is GCN5L1, which is homologous to a prokaryotic acetyltransferase.(Scott et al., 2012) Mitochondrial protein acetylation is lower when the enzyme is lacking, and increased when it is overexpressed.(Scott et al., 2014) The second is the nuclear MYST family acetyltransferase KAT8 (MOF). It controls nuclear and mitochondrial respiratory genes by regulating oxidative phosphorylation.(Chatterjee et al., 2016) KAT8 (MOF) is important in tissues that are energetically demanding. For example, conditional knockouts of this gene result in hypertrophic cardiomyopathy and cardiac failure in mouse. However, the function of KAT8 (MOF) mediated mitochondrial acetylation in these cell types is not yet clear. Third, is acetyl-CoA acetyltransferase 1 (ACAT1), a regulator of the pyruvate dehydrogenase complex in mitochondria. ACAT1 was reported to influence acetylation of two mitochondrial proteins: PDHA1 and PDP1.(Fan et al., 2014) ACAT1 knockdown led to a decrease in acetylation of PDHA1 and PDP1, inhibiting their function and leading to changes in glucose homeostasis that could contribute to the Warburg effect. It is critical to note that none of the studies of mitochondrial KATs use *in vitro* methodologies to show that acetylation of mitochondrial substrates is direct. This leaves a possibility that GCN5L1, KAT8 (MOF) or ACAT1 may modulate mitochondrial Acetyl-CoA levels or pH, influencing the efficiency of spontaneous acetylation in this cellular compartment.

SIRT3 is also important to the respiratory chain. Mice without SIRT3 use 10% less O_2 and make 50% less ATP than wild-type mice.(Ahn et al., 2008; Jing et al., 2011) SIRT3 deacetylates and activates mitochondrial respiratory chain complexes (e.g., NDUFA9 (complex I)(Ahn et al., 2008) and SDHA (complex II))(Cimen et al., 2010; Finley et al., 2011) and regulates ATP synthase.(Bao et al., 2010)

Metabolic targets of SIRT3

SIRT3 is a key enzyme in metabolism, necessary for efficient fatty acids utilization in the liver and of lipid-derived acetate and ketone bodies in peripheral tissues during fasting. The first

identified target of SIRT3 is acetyl-CoA synthetase 2, which generates acetyl-CoA from acetate in extrahepatic tissues during fasting.(Hallows et al., 2006; Schwer et al., 2006) During fasting, acetate is made by the liver from acetyl-CoA and can be used as energy by other tissues.(Shimazu et al., 2010) SIRT3 regulates fatty acid oxidation by deacetylation and activation of long chain acyl-CoA dehydrogenase during fasting.(Hirschey et al., 2010) β-Oxidation intermediates (e.g., long chain fatty acids) accumulate in mice that lack SIRT3.(Hirschey et al., 2010) SIRT3 also regulates ketone body production by deacetylating and activation 3-hydroxy-3-methylglutaryl-CoA synthase 2, a key step in the synthesis of ketone bodies.

In amino acid metabolism, SIRT3 regulates the aminotransferase that forms glutamine by transferring an α-amino to α-ketoglutarate. Another enzyme, glutamate dehydrogenase (GLUD1), regenerates α-ketoglutarate from glutamate and releases nitrogen as ammonia in the urea cycle.(Berg et al., 2012) SIRT3 accelerates the urea cycle by activating ornithine transcarbamoylase (OTC) Humans with urea cycle disorders and mice without SIRT3 have similar metabolic profiles including increased levels of serum ornithine and reduced levels of citrulline.(Hallows et al., 2011)

Other pathological conditions exhibit lower levels of SIRT3. Tumors often have reduced levels of SIRT3. As a result, glucose use is enhanced because of increased levels of reactive oxygen species (ROS) that activate hypoxia-inducible factor 1 alpha (HIF1α), that, in turn, activates glycolytic genes.(Finley et al., 2011; Kim et al., 2010; Shulga et al., 2010) SIRT3 also deacetylates and activates isocitrate dehydrogenase 2 and increases ROS levels as a byproduct of oxidative phosphorylation.(Someya et al., 2010) SIRT3 deacetylates and activates the ROS-scavenging enzyme manganese superoxide dismutase to reduce oxidative damage in the liver.(Chen et al., 2011; Qiu et al., 2010; Tao et al., 2010) Mice without SIRT3 show greater oxidative stress,(Qiu et al., 2010) particularly on a high-fat diet,(Hirschey et al., 2011) and have higher ROS levels than normal under calorie restriction.(Someya et al., 2010)

Therapeutic Targeting of Lysine Acetylation

KDAC inhibitors

The manipulation of lysine acetylation using small molecules now known to be KDAC inhibitors was instrumental in the discovery of this modification. N-butyrate was known to control gene expression and to induce differentiation of acute erythroid leukemia cells.(Candido et al., 1978; Riggs et al., 1977; Vidali et al., 1978) Trichostatin A and tetrapeptide trapoxin are potent KDAC inhibitors.(Kijima et al., 1993; Yoshida et al., 1990a; Yoshida et al., 1990b) Suberoylanilide hydroxamic acid (SAHA) induces terminal differentiation and apoptosis in transformed cells and inhibits KDAC1 and KDAC3.(Richon et al., 1998) SAHA (also known as Vorinostat) was approved by the Food and Drug Administration (FDA) to treat cutaneous T cell lymphoma. The anti-epileptic drug valproic acid also inhibits KDACs(Gottlicher et al., 2001) and is in clinical trials for various indications. Other KDAC inhibitors are approved by the FDA (some of which are displayed in **Figure 4-14**), while others remain in clinical development.(Mottamal et al., 2015; Subramanian et al., 2010)

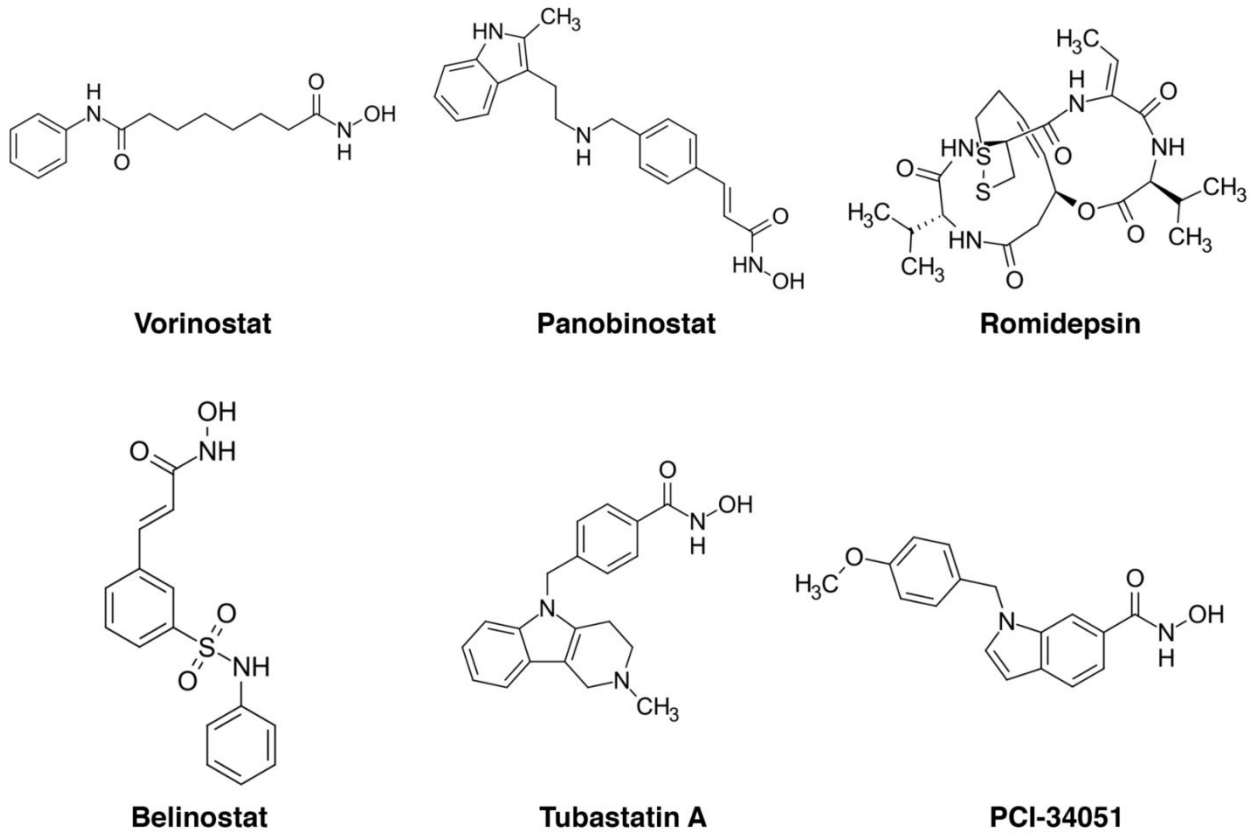


Figure 4-14: Selected chemical structures of KDAC inhibitors.

Several hypotheses may explain the mechanisms of action of KDAC inhibitors. These small molecules might induce DNA damage and cell cycle interruption, cause ROS to accumulate, or activate apoptotic pathways.(Xu et al., 2007) Most likely, in some way, these small molecules encourage apoptosis or hinder proliferation.(Takai et al., 2004) Hyper-acetylation from small-molecule KDAC inhibitors has been observed at the tumor suppressor gene *CDKN1A*(Gui et al., 2004) and in reactivation of latent HIV.(Lusic et al., 2003) Thus, acetylation-mediated transcriptional disruptions might explain the effects of KDAC inhibition on cellular proliferation and other phenotypes.

KDAC inhibitors targeting class I/II/IV enzymes generally chelate the divalent metal ion required for catalysis, although not all inhibitors exploit this mechanism.(Lobera et al., 2013) Available small molecules mostly target class I and II KDACs with limited selectivity for individual KDACs.(Falkenberg and Johnstone, 2014) However, emergent small molecules are active against a more restricted range of KDACs. Preclinical examples include specific inhibition of the cytoplasmic KDAC6 by Tubastatin A and of KDAC8 by PCI-34051.(Balasubramanian et al., 2008; Butler et al., 2010) Importantly, the subset of differentially acetylated proteins differs depending on the KDAC inhibitor used. (Scholz et al., 2015)

Sirtuin modulators

SIRT1 is an attractive target for modulation given early connections between Sir2 and replicative lifespan in yeast.(Kaeberlein et al., 1999; Lin et al., 2000). Indeed, as discussed, sirtuin activity closely ties key metabolic and epigenomic processes. However, specific targeting of sirtuins, while exciting, has proven difficult. Adding to this challenge, initial clinical studies with sirtuin activators have been inconsistent. While it is clear that sirtuin genetic deletion results in large changes in acetylation substrates and gross chromosomal abnormalities that lead to DNA damage,[351, 569] more extensive work is required to understand this family of genes and their therapeutic potential.

Polyphenolic compounds, namely the phytochemical resveratrol, were originally shown to activate sirtuin activity by enhancing cofactor and substrate binding via engagement of the SIRT1 N-terminus.(Howitz et al., 2003; Wood et al., 2004) These polyphenols lack potency in sirtuin binding, have low retention times in humans, and likely have considerable off-target effects.(Erdogan et al., 2017; Walle et al., 2004) More recent high-throughput screening methodologies uncovered other SIRT1 activators, such as SRT1720, with interesting biological effects that lead to extended lifespan and improved health in mice and some efficacy against xenografted tumor growth models.(Chauhan et al., 2011; Mercken et al., 2014; Mitchell et al., 2014)

Controversy has erupted about the action of resveratrol and SRT1720 **(Figure 4-15)**. Two studies demonstrated *in vitro* that resveratrol-mediated SIRT1 activation required the presence of a fluorophore conjugated to substrate peptides,(Beher et al., 2009; Pacholec et al., 2010) an observation that was supported by structural data.(Cao et al., 2015) *In vivo*, resveratrol induces hypoacetylation for a subset of non-fluorophore labeled peptides, but also induces hypteracetylation of other substrates while leaving a large proportion of genes unchanged.(Hubbard et al., 2013; Lakshminarasimhan et al., 2013) These contradictory effects in global protein acetylation could be due to off target effects, such as inhibition SIRT3 or activation of SIRT5.(Gertz et al., 2012) Importantly, they also may be explained by significant sequence specificities of resveratrol-mediated SIRT1 activation due to allosteric mechanisms.

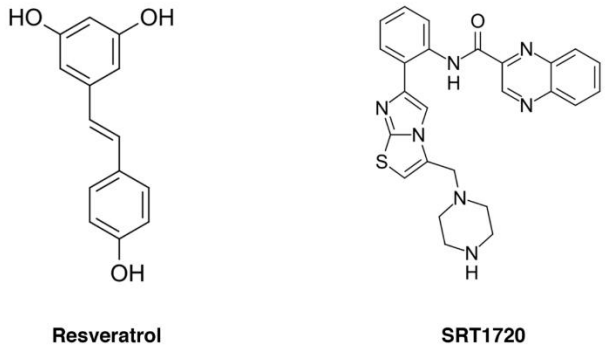


Figure 4-15: Selected chemical structures of sirtuin activators.

A number of specific inhibitors of sirtuin activity have been identified **(Figure 4-16)**. Examples include indols, such as EX-527 targeting SIRT1, along with compounds such as sirtinol and tenovin derivatives.(Gertz et al., 2013; Mai et al., 2005; McCarthy et al., 2012; Napper et al., 2005) A growing number of SIRT2-specific inhibitors have been tested, including as AGK2,(He et al., 2012b; Luthi-Carter et al., 2010; Riepsamen et al., 2015) SirReal inhibitors,(Rumpf et al., 2015) and most recently 33i.(Erburu et al., 2017) Due to the high degree of conservation among sirtuin active sites, not surprisingly, several inhibitors bind to two or more sirtuins: cambinol with an IC_{50} ~60 μM for SIRT1 and SIRT2,(Heltweg et al., 2006) salermide targeting SIRT1 and SIRT2,(Lancelot et al., 2013; Lara et al., 2009) and suramin that inhibits SIRT5 but also strongly inhibits SIRT2 and SIRT1.(Cui et al., 2014; Schuetz et al., 2007) Inhibitors that target SIRT3 are also being tested.(Alhazzazi et al., 2016; Salo et al., 2013) Not much is known about the mechanism behind sirtuin inhibitors; however, in some cases, they likely function by interfering with NAD^+ engagement.(Gertz et al., 2013)

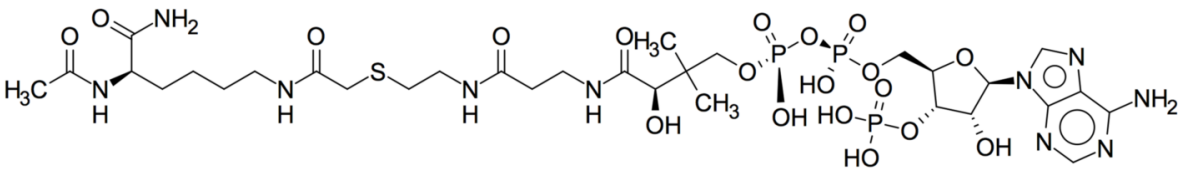
Figure 4-16: Selected chemical structures of sirtuin inhibitors

KAT inhibitors

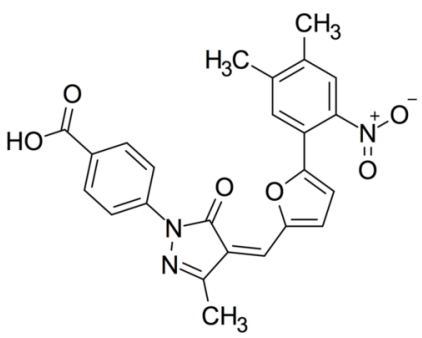
KAT3A/B(CBP/p300) has emerged as a potential therapeutic target for respiratory diseases, HIV infection, metabolic diseases, and cancer.(Dekker and Haisma, 2009) However, the relatively shallow substrate-binding site in p300 is a challenging drug target, and most compounds to date target the acetyl-CoA binding site in the enzyme.(Marmorstein, 2001) Early KAT inhibitors include several phytochemicals, such as curcumin,(Marcu et al., 2006) garcinol,(Arif et al., 2009) and anacardic acid.(Sun et al., 2006) Chemical inhibitors were originally developed as bi-substrate acetyl-CoA mimics,(Lau et al., 2000) pioneered by the Cole laboratory, and later replaced by smaller, more selective synthetic compounds, such as C646.(Gao et al., 2013) C646 is a pyrazolone-furan (**Figure 4-17**) that was discovered via virtual ligand screening. It efficiently reduces histone acetylation levels within cells and displays cytotoxic properties toward certain cancer cells.(Gao et al., 2013) Notably, a recent study characterized A-485, the most potent and specific p300 inhibitor identified to date.(Lasko et al., 2017) A-485 was found to be 1000-fold more potent than other cell permeable HAT inhibitors including C646 and highly specific to the KA3A/B(CBP/p300) BHC (bromodomain HAT-C/H3) domains. A-485 was also found to suppress proliferation in 61 cancer cell lines with an EC50 <2 μM indicating the compound may have some therapeutic potential, especially against hematological malignancies and prostate cancer.(Lasko et al., 2017) Importantly, further characterization of potential off target effect and studies in pre-clinical animal models are likely necessary prior to moving forward in any clinical setting.

Another study used naturally occurring acyl-CoA derivatives conjugated to biotin to affinity-purify KATs. Palmitoyl-CoA was recovered and found to inhibit GCN5 (KAT2A). This metabolite among other acyl-CoA derivatives, were also able to bind PCAF (KAT2B) and MOF (KAT8) and modestly reduce levels of histone acetylation, underscoring that Acyl-CoA cofactors may act as endogenous regulators of lysine acetyltransferase activities.(Montgomery et al., 2015)

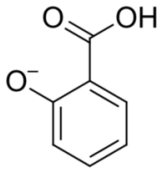
Interestingly, some long chain fatty acid metabolites such as myristic acid (required to produce myristoyl-CoA) have also been reported to activate deacylation activity in sirtuins, especially SIRT6.(Feldman et al., 2013)



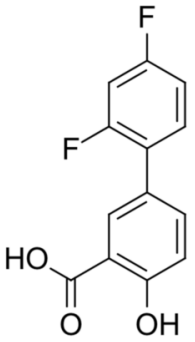
Lys-CoA



C646



Salicylate



Diflunisal

Figure 4-17: Selected chemical structures of KAT inhibitors

Salicylate inhibits KAT3A/B(CBP/p300) acetyltransferase activity by directly competing with acetyl-CoA and down-regulates the specific acetylation of histones and non-histone proteins in cells.(Shirakawa et al., 2016) Furthermore, diflunisal, an FDA-approved drug containing a salicylic acid substructure, inhibited KAT3A/B(CBP/p300) more potently than salicylate. Both drugs are orally bioavailable and inhibited p300-dependent myelogenous leukemic cell growth *in vitro* and *in vivo*, pointing to a potential new clinical application. In addition, p300-induced tau acetylation was inhibited by salicylate or its derivative salsalate, which enhanced tau turnover and reduced tau level.(Min et al., 2015) In a mouse model of Alzheimer's disease, administration of salsalate after disease onset rescued tau-induced memory deficits and prevented hippocampal atrophy, underscoring the clinical potential of KAT inhibitors in Alzheimer's disease.

Bromodomain inhibitors

Small-molecule inhibition of bromodomains is the most recent advancement in efforts to pharmacologically target the protein acetylation network. Rather than disrupting enzymatic catalysis, these compounds target protein:protein interactions by inhibiting bromodomain recognition of its acetyl-lysine residue-containing ligand. The first bromodomain drug discovery attempts were described in the HIV field targeting the interaction of the acetylated form of the viral transactivator Tat (acK50) with the bromodomain of KAT2B/PCAF, a critical step in transcription from the integrated HIV provirus.(Dorr et al., 2002; Mujtaba et al., 2002; Zeng et al., 2005) The structure-based approach led to the discovery of a class of N1-aryl-propane-1,3-diamine compounds that selectively inhibited the acTat:PCAF interaction, albeit with relatively low potency. Also, the intracellular introduction of acetylated histone H4 peptides induced dissociation of BRD4 from chromatin and reduced cell growth.(Nishiyama et al., 2008) A year later, a patent from Mitsubishi Pharmaceuticals indicated that thienodiazepines bind BRD4 bromodomains.(Miyoshi, 2009) This patent report spurred the discovery of a lead compound, JQ1, with therapeutic activity against a rare squamous epithelial cancer called the NUT midline carcinoma.(Filippakopoulos et al., 2010) The NUT midline carcinoma is cytogenetically defined

by a translocation of the BRD4 gene that results in an in-frame fusion with the nuclear protein in testis (NUT), a tissue-specific acetyltransferase.(Grayson et al., 2014)

At the same time as the initial report of JQ1, the laboratory of Alexander Tarakhovsky in collaboration with GlaxoSmithKline reported the discovery of I-BET, a synthetic compound mimicking acetylated histones and disrupting containing chromatin complexes responsible for expression of inflammatory genes in activated macrophages, thus conferring protection against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis.(Nicodeme et al., 2010) Interestingly, BET inhibitors also support immunotherapeutic applications by suppressing expression of Programmed Cell Death Protein Ligand 1 (PDL1),(Zhu et al., 2016a) which increases cytotoxic T-cell activity and limits tumor progression in mice. Since the characterization of BET inhibitors and their preclinical application in cancer and immunology disease models, their potential utility in modulating male fertility,(Matzuk et al., 2012) neurocognitive function,(Korb et al., 2015) cardiovascular disease,(Anand et al., 2013) and viral infections(Conrad and Ott, 2016) has been described.

JQ1, I-BET, and related compounds are powerful inhibitors of both bromodomains of the BET protein BRD4, with similar activity also against bromodomains of BRD2, BRD3 and the testis-specific BET protein BRDT.(Filippakopoulos et al., 2010) They function primarily by competing with acetyl-lysine binding by forming a hydrogen bond with a critical asparagine residue that otherwise engages the acetyl-lysine. The pharmacophore is a methyltriazole that is common to most available BET inhibitors (**Figure 4-18**). Recently, second generation BET inhibitors have been described, including bivalent compounds that target both BET bromodomains and achieve potency orders of magnitude above that of JQ1.(Tanaka et al., 2016), Phthalimide-conjugated BET inhibitors that function as hetero-bifunctional small molecules have also been reported, which direct BET proteins to E3 ligase activity of cereblon, allowing for rapid and exquisitely specific destruction of BET proteins within the cell.(Winter et al., 2015)

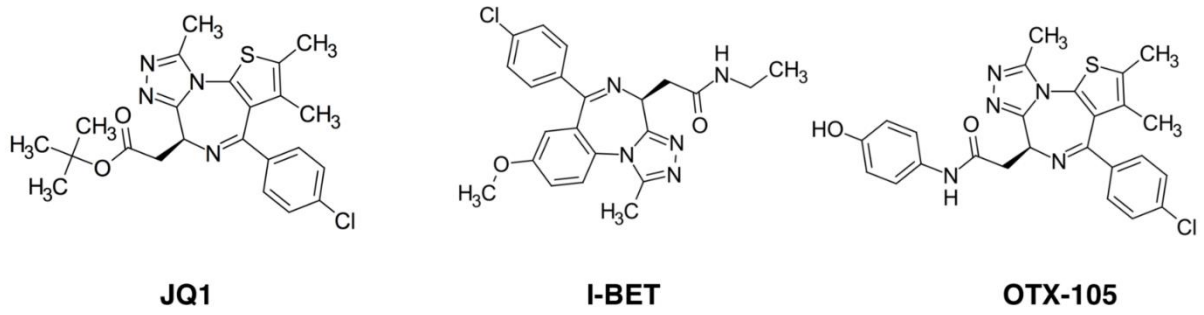


Figure 4-18: Selected chemical structures of BET inhibitors.

More than 20 early clinical trials are in progress with BET inhibitors.(Filippakopoulos et al., 2010; Liu et al., 2017) Their focus is primarily on the treatment of various hematological malignancies, as BET proteins are co-activators of several critical oncogenes, including *MYC*,(Delmore et al., 2011) in addition to important regulators of cell proliferation and fate, such as *MYB*,(Roe et al., 2015) *BCL2*, and *FOSL1*.(Lockwood et al., 2012) Transcriptional disruption of these genes is linked to anti-neoplastic phenotypes observed under BET inhibition, likely operating via local removal of BRD4 and associated transcription factors (i.e., P-TEFb) from acetylated chromatin or acetylated transcription factors (i.e., TWIST,(Shi et al., 2014) GATA-1,(Lamonica et al., 2011; Zhu et al., 2016a) and ERG(Roe et al., 2015)) in addition to indirect effects on transcription and the cell cycle. Several BET inhibitor trials have completed Phase I or reported tolerability and partial clinical outcomes.(Berthon et al., 2016; Odore et al., 2016) Thus far, BET inhibitors appear well-tolerated with dose-limiting side effects as diarrhea, fatigue, and reversible thrombocytopenia.(Theodoulou et al., 2016a)

As BET inhibitors are rapidly advancing into clinical trials, inhibitors of non-BET bromodomains are also being developed.(Chen et al., 2016; Drouin et al., 2015) Current non-BET bromodomain inhibitors have been described mainly for bromodomains of acetyltransferases (i.e., p300/CBP) and chromatin remodeling components (i.e., BRD7, BRG1). Most non-BET targeting small molecules are at the stage of being chemical probes,(Theodoulou et al., 2016b) and it has emerged that druggability varies among individual bromodomains.

Future and perspectives for protein lysine acetylation

Lysine acetylation has moved from being a specialized mark on histones to a critical modification controlling cell fate, proliferation, and metabolism. The modification causes a change in the electrostatic charge of its cognate lysine residue, recruits reader proteins, and is tightly linked to fluctuations in key cellular metabolites, such as NAD^+ and acetyl-CoA. In respective cellular compartments, lysine acetylation regulates diverse molecular outcomes, such as gene-

specific chromatin processes, enzymatic regulation, protein multimerization, localization and stability. Reader protein domains, including the bromodomain, tandem PHD, YEATS, and acidic domains have evolved to specifically bind to acetylated or non-acetylated lysine residues, thus coordinating the acetylation response. Our understanding of other acylation marks is rapidly evolving; examples include lysine crotonylaton, succinylation and malonylation, with shared enzymes that place and remove the marks such as KAT3A/B and sirtuins, respectively.(Hirschey and Zhao, 2015; Sabari et al., 2015) Pharmacological targeting of lysine acetylation is an established and briskly advancing field, starting from KDAC inhibitors, moving to sirtuin activators, and now including KAT and bromodomain inhibitors. The effects and mechanisms underlying these compounds are still being uncovered, and future studies must consider the role of newer acylation marks in drug action.

Other open questions concern the issue how partitioning of critical metabolites contributes to the function of lysine acetylation in distinct cellular milieus. A considerable degree of diversity of non-histone acetylation has emerged in metazoans, especially in mammals. This could be due to a more discreet compartmentalization of acetyl-CoA in lower eukaryotes. In yeast, acetyl-CoA is 20–30 fold enriched in mitochondria as compared to other cellular compartments.(Weinert et al., 2014) In this context, acetyl-CoA does not permeate past the mitochondrial membrane and allows for distinction between acetyl-CoA as a metabolic intermediate and a cofactor for lysine acetylation. In mammals, this distinction is not as clear, and the question how other acyl group donors such as succinyl-CoA or malonyl-CoA compartmentalize remains yet unexplored. The opening of the lysine acetylation field to nutrition, exercise and aging as well as its growing influence on disease pathogenesis and treatment of cancer, neurodegeneration, and HIV is exciting and signals far-reaching significance. Lysine acetylation may be key to the understanding of how such processes are molecularly defined. In the future, lysine acetylation and its directed intervention hold promise and is aimed at significantly improving health- and lifespan in humans.

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Chapter V: Crosstalk Between RNA Polymerase II CTD Acetylation and Phosphorylation

Ibraheem Ali[1,2], Diego Garrido Ruiz[3], Zuyao Ni[4], Jeffrey R. Johnson[1], Heng Zhang[6], Pao-Chen Li[1], Ryan J. Conrad[1,2], Xinghua Guo[4], Jinrong Min[6], Jack Greenblatt[4], Matthew Jacobson[3, 5], Nevan J. Krogan[1, 5], Melanie Ott[1,2]

Affiliations:

1 J. David Gladstone Institutes, San Francisco, California, 94158, USA

2 Department of Medicine, University of California San Francisco, San Francisco, California, 94143, USA

3 Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, California, 94143, USA

4 Donnelly Centre, University of Toronto, Toronto, Ontario, Canada

5 California Institute for Quantitative Biosciences (QBC) at UCSF, San Francisco, California, 94143, USA

6 Structural Genomics Consortium, University of Toronto, Ontario, Canada

Correspondence: melanie.ott@gladstone.ucsf.edu

Abstract

Multiple posttranslational modifications of the RNA polymerase II C-terminal domain (CTD) coordinate passage through the transcription cycle. The crosstalk between different modifications is poorly understood. Here, we show how acetylation of lysine residues at position 7 of characteristic heptad repeats (K7ac), a modification only found in higher eukaryotes, regulates phosphorylation of serines at position 5 (S5p), a conserved mark of polymerases initiating transcription. Using mass spectrometry, we identified Regulator of Pre-mRNA Domain-containing (RPRD) proteins as reader proteins of K7ac. K7ac enhanced *in vitro* binding of CTD peptides to the CTD-interacting domain (CID) of RPRD1A and RPRD1B proteins and inversely regulated S5p levels genome-wide. Treatment with deacetylase inhibitors globally enhanced levels of K7ac- and

decreased levels of S5-phosphorylated polymerases ≥500 base pairs downstream of transcription start sites of expressed genes, consistent with acetylation-dependent S5 dephosphorylation via a previously identified RPRD-associated S5 phosphatase. Consistent with this model, RPRD1B knockdown increased S5p, but also enhanced K7ac levels, indicating that RPRD proteins recruit a K7 deacetylase. Collectively, our data identify RPRD CIDs as K7ac reader domains and reveal auto-regulatory crosstalk between K7ac and S5p via RPRD proteins at the transition from transcription initiation to elongation in higher eukaryotes.

RNA Polymerase II CTD modifications regulate its function.

The RNA Polymerase II (Pol II) complex is highly conserved in all eukaryotic cells and responsible for the production of most gene expression products (Buratowski, 2003; Eick and Geyer, 2013). RPB1, the largest subunit of the complex, contains the catalytic core of the complex and a unique regulatory region called the C-terminal domain (CTD). In eukaryotes, the CTD is composed of twenty or more repeats with a heptad consensus sequence, $Y_1S_2P_3T_4S_5P_6S_7$, which is highly conserved from yeast to human. In multicellular eukaryotes, the CTD is expanded and contains a varying number of non-consensus repeats depending on the organism (Chapman et al., 2008). The 52 repeats of the mammalian CTD can be divided into 21 consensus repeats proximal to the enzymatic core, and 31 non-consensus repeats distal from the core with less fidelity to the consensus. Divergence from the consensus sequence most commonly occurs at position 7, which can be replaced with an asparagine (N), threonine (T), or a lysine (K) instead of the consensus serine (Eick and Geyer, 2013). The CTD is intrinsically disordered and functions as an interaction platform for accessory proteins required for transcription and transcription-associated RNA processing events (Buratowski, 2009; Jasnovidova and Stefl, 2013).

The heptad repeats within the CTD are extensively and dynamically post-translationally modified at different times during the transcription cycle. Of the seven consensus CTD residues, 5 can be phosphorylated (Y1, S2, T4, S5, and S7). The two remaining proline residues can

undergo isomerization into *cis* or *trans* conformations (Heidemann et al., 2013). Serine-5 phosphorylation (S5p) and serine-2 phosphorylation (S2p) are the most thoroughly studied CTD modifications (Buratowski, 2009; Jasnovidova and Stefl, 2013). Serine-5 is phosphorylated by the cyclin-dependent kinase 7 (CDK7) subunit of general transcription factor TFIIH, is enriched at promoters, and decreases successively towards the 3' end of genes (Brookes et al., 2012; Ebmeier et al., 2017). The phosphorylated serine-2 mark, placed by several kinases (CDK9, CDK12, CDK13 and BRD4), starts to accumulate downstream of transcription start sites and steadily increases towards the 3' ends of genes, reflective of its critical role in productive polymerase elongation (Bartkowiak et al., 2010; Devaiah et al., 2012; Nechaev and Adelman, 2011). The distribution of consensus Pol II modifications is best studied in yeast, revealing a fixed transition point from S5p to S2p enriched, on average, 450 base pairs (bp) downstream of transcription start sites (TSS) (Bataille et al., 2012; Kim et al., 2010; Mayer et al., 2010; Tietjen et al., 2010). Similar to S5p, Serine-7 phosphorylation (S7p) is catalyzed by CDK7, is enriched near promoters and in gene bodies, and regulates the expression snRNA genes (Brookes et al., 2012; Egloff et al., 2012). Tyrosine-1 phosphorylation is enriched near promoters, and has been linked to enhancer and antisense transcription (Descostes et al., 2014). Threonine-4 phosphorylation is enriched in coding regions and is required for cell viability and transcription termination (Hintermair et al., 2012).

Posttranslational modifications (PTMs) specifically found in non-consensus repeats include asymmetric dimethylation of a single arginine (R1810me2), conserved among some metazoa, that regulates transcription of small nuclear and nucleolar RNAs (Sims et al., 2011). In addition, lysine residues at position 7 of eight heptad repeats are acetylated by the acetyltransferase p300/CBP (KAT3A/B) (K7ac), and were recently also found to be mono- and di-methylated by a yet unknown methyltransferase (Dias et al., 2015; Schroder et al., 2013; Voss et al., 2015; Weinert et al., 2018). These lysine residues evolved in higher eukaryotes in the common ancestor of the metazoan lineage, and are highly conserved among vertebrates (Simonti et al.,

2015). While lysine-7 mono- and di-methlyation marks are found near promoters, K7ac is enriched in gene bodies (Dias et al., 2015). K7 residues are required for productive transcription elongation of immediate early genes in response to epidermal growth factor stimulation (Schroder et al., 2013). Importantly, K7ac marks are found at ~80% of actively transcribed genes, with a peak in signal +500 bp downstream of the TSS, indicating that the modification could more broadly regulate the transition from transcription initiation to productive elongation (Schroder et al., 2013). In a genetic model where all eight K7 residues were mutated to arginines (8KR), cells expressing 8KR RPB1 exhibited altered expression of genes relating to development, multicellularity and cell adhesion, underscoring a critical role of K7ac in the development of higher eukaryotes (Simonti et al., 2015).

Effector proteins interacting with differentially modified CTDs often contain a so-called CTD-interacting domain (CID), which is one of the best-studied CTD-binding modules and is conserved from yeast to humans (Ni et al., 2011). The mammalian Regulator of Pre-mRNA Domain-containing (RPRD) proteins 1A, 1B and RPRD2 proteins are homologues of the yeast transcription termination factor *Rtt103*, and each contains a CID (Ni et al., 2011). *Rtt103* and RPRD CIDs can bind CTD peptides carrying S2p, but not S5p; S7p and unmodified K7 residues reside at the edge of the CID binding cleft, and can be substituted without altering the binding affinity (Jasnovidova et al., 2017a; Meinhart and Cramer, 2004; Ni et al., 2014). RPRD1A and RPRD1B are found in macromolecular complexes that associate with Pol II and transcription regulatory factors, including the S5-phosphatase RPAP2 (Liu et al., 2015; Morales et al., 2014; Ni et al., 2011; Ni et al., 2014; Patidar et al., 2016). RPRD1A, also called P15RS, regulates G1/S cell cycle progression and suppresses Wnt and β-catenin signaling via interactions with the class I lysine deacetylase HDAC2 and transcription factor 4 (TCF4) (Jin et al., 2018; Liu et al., 2015; Liu et al., 2002; Wu et al., 2010). RPRD1B, also called CREPT, was identified in a mass spectrometry-based screen for mammalian Pol II-interacting proteins; it is upregulated in various cancers, and regulates genome stability and transcription termination (Lu et al., 2012; Morales et

al., 2014; Patidar et al., 2016; Zhang et al., 2018). Although the homology with *Rtt103* implies a conserved role in transcription termination and explains why the proteins are enriched in 3' ends of eukaryotic genes, an additional less well-defined role of RPRD proteins has emerged at 5' ends of genes in higher eukaryotes. This involves a mechanism to regulate genome stability through the resolution of R-Loops, which are DNA-RNA hybrids (Lu et al., 2012) as well as the recruitment of RPAP2 to initiating RNA Pol II (Ni et al., 2014).

In this study, we provide molecular insight into the role of RPRD proteins at the 5' ends of genes and newly connect RPRD proteins with K7ac. We find that RPRD proteins via their CIDs specifically interact with K7ac, and that this interaction promotes S5-dephosphorylation at and beyond +500 bp downstream of the TSS. This data supports a model where vertebrates evolved specific crosstalk between S5p and K7ac to ensure precise transcription initiation dynamics and a timely transition to a productive elongation phase at a defined distance to the TSS.

Preferential Binding of RPRD Proteins to Acetylated RPB1

To identify proteins that interact with Pol II K7ac, we performed stable isotope labeling with amino acids in cell culture (SILAC). We overexpressed HA-tagged RPB1 proteins, either wild type or 8KR mutant, in HEK293T cells. The proteins also contained a known α-amanitin resistance mutation enabling propagation of successfully transfected cells in the presence of α-amanitin, which induces the degradation of endogenous Pol II (Bartolomei and Corden, 1987). After culture of cells in differential metabolic labeling media, RPB1-containing complexes were purified via their HA tag and subjected to mass spectrometric analysis (**Figure 5-1A**). We found all members of the RPRD family preferentially bound to wildtype RBP1, including RPRD1A, RPRD1B, RPRD2 along with several of their interacting partners such as RPAP2, RPAP3, and RUVB1, which were previously identified by mass spectrometry (Ni et al., 2011; Patidar et al., 2016) (**Figure 5-1B**).

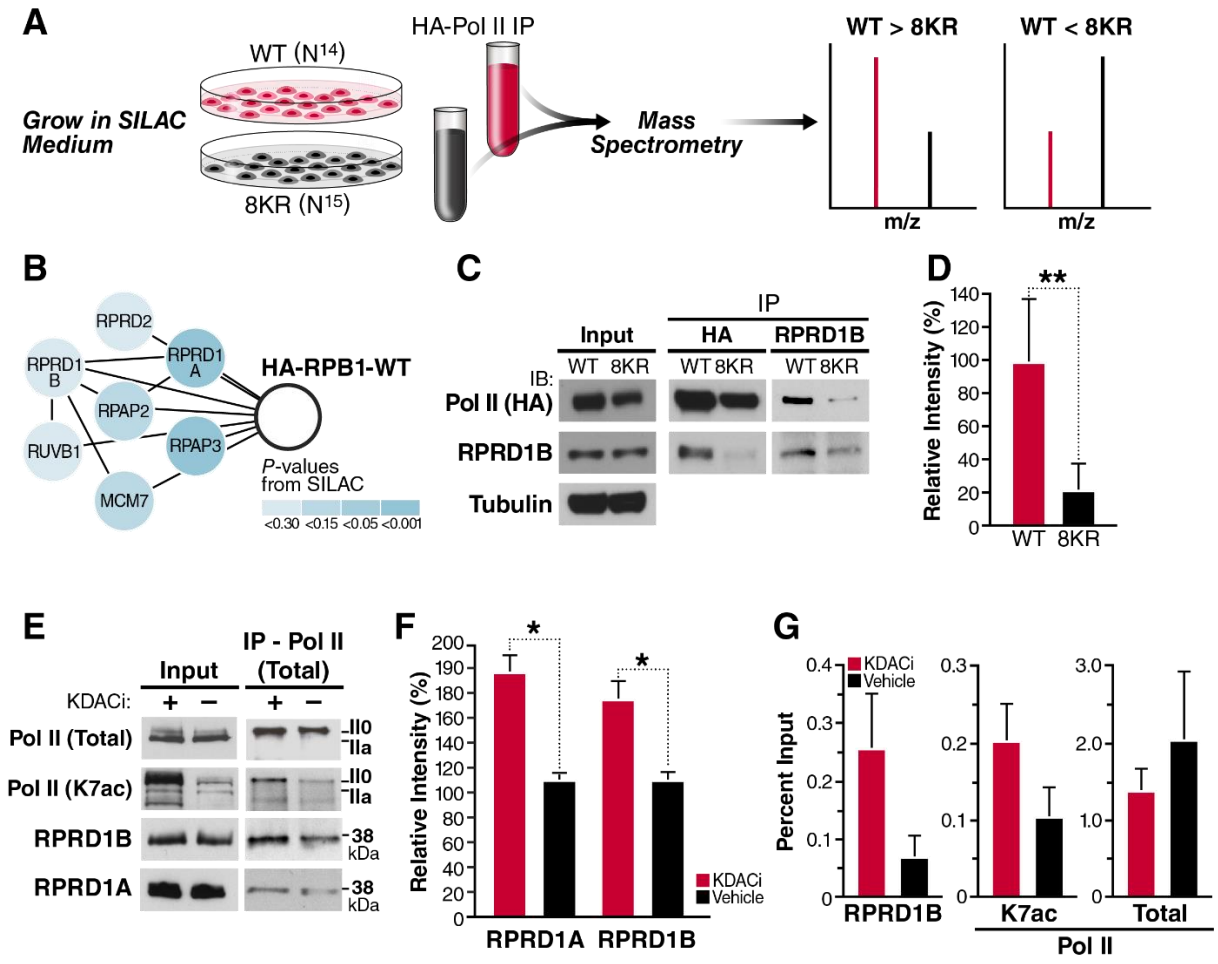

Figure 5-1: RPRD proteins interact with RPB1 in an acetylation-dependent manner. A. SILAC based mass spectrometry screen was used to identify factors that bind preferentially to WT Pol II compared to the 8KR mutant. B. Components of the RPRD1B/RPRD1A complex that were identified as WT interactors. C. Reciprocal IPs using antibodies against RPRD1B or hemagglutinin (HA) from WT and 8KR cells. D. Densitometry using ImageJ of n=4 independent RPRD1B western blots after HA- immunoprecipitation. E. NIH3T3 cells were treated with KDAC inhibitors (30nM Panobinostat and 5uM Nicotinamide) for 2 hours. IP from 500ug of nucleoplasm pre-cleared with IgG followed by western blotting with the indicated antibodies. Pol II (Total) blots were performed with an N-terminal H224 antibody F. Densitometry of RPRD1A and RPRD1B western blots produced from three independent Pol II (Total-H224) IP elutions. Error bars are SEM, * $p < 0.05$ for a one-tailed T test. G. ChIP-qPCR of Leo1 at +33 nt downstream of TSS using the indicated antibodies from 4 independent chromatin preps, total Pol II ChIP experiments were done with the 8WG16 antibody. Values are represented as percent of input with IgG subtracted, error bars are SEM.

As RPRD1B is the most thoroughly studied of the RPRD family, we confirmed preferential binding of RPRD1B to wild type relative to 8KR mutated RPB1 in repeated co-immunoprecipitation experiments by performing either pull down of endogenous RPRD1B and blotting for HA-RPB1 protein or the reverse (**Figure 5-1C**). The enrichment of endogenous RPRD1B proteins after wild type, and not mutant, HA-RPB1 immunoprecipitation was consistent among four independent experiments and statistically significant ($p = 0.0098$) (**Figure 5-1D**). We also tested interaction between endogenous RPB1, RPRD1A and RPRD1B proteins in NIH3T3 cells treated with lysine deacetylase (KDAC) inhibitors. KDAC inhibitor treatment induced robust hyperacetylation of endogenous RPB1 in input material as tested with an antibody specific for K7ac (Schroder et al., 2013), but did not change total overall RPB1 protein levels (**Figure 5-1E**). Following pulldown of endogenous Pol II, more RPRD1A and RPRD1B proteins were recovered when cells were treated with KDAC inhibitors as compared to vehicle-treated cells, confirming positive regulation of the RPB1:RPRD interaction by acetylation **(Figure 5-1E and 5-1F)**.

Next, we tested *in vivo* recruitment of RPRD1B to a known target gene, *Leo1* (Ni et al., 2011). Using chromatin immunoprecipitation (ChIP) followed by quantitative PCR, we found RPRD1B recruitment to the *Leo1* promoter (+33 bp) consistently enhanced in NIH3T3 cells treated with KDAC inhibitors as compared to vehicle-treated cells (**Figure 5-1G).** Similar to what we observed by western blotting, K7 residues were hyperacetylated at the Leo1 promoter in response to KDAC inhibition in ChIP analysis with the K7ac-specific antibody. Importantly, total Pol II occupancy did not increase under KDAC inhibition, confirming specific K7 hyperacetylation and enhanced RPRD1B recruitment in response to KDAC inhibition (**Figure 5-1G**).

Direct Interaction of K7ac with RPRD CTD-Interacting Domains (CIDs)

To test whether K7ac modulates CTD binding to the RPRD CID domains, we performed isothermal titration calorimetry (ITC) to test the interaction between synthetic CTD peptides and purified CID domains from both RPRD1A and RPRD1B proteins. We generated CTD peptides spanning roughly 3 heptad repeats (20 amino acids) with repeat 39 at the center. This region was

chosen as it is acetylated and phosphorylated *in vivo* (Voss et al., 2015; Weinert et al., 2018), and contains two consecutive K7 residues (**Figure 5-2A**). Peptides were synthesized in an unmodified (UnM), acetylated (K7ac), or phosphorylated (S2p and S5p) state. S2p was included as a positive control as it enhances CTD:CID interactions, while S5p served as a negative control (Ni et al., 2014; Pineda et al., 2015). In addition, we combined S2p and S5p with K7ac to investigate potential combined effects. Compared to the unmodified CTD, binding of the RPRD CIDs to CTD peptides carrying K7ac had a significantly lower K_d (2.3-fold reduction for RPRD1A and 3.8-fold for RPRD1B), indicating enhanced binding. (**Figures 5-2B–D**). S2p itself had a robust effect in enhancing CID binding, as previously observed, but combining K7ac with S2p further decreased the K_d by 2.8-fold and 4.2-fold, respectively. S5p-carrying peptides did not interact with CID proteins as expected (data not shown). Together these data indicate that K7ac enhances the interaction of RPRD proteins with the Pol II CTD with and without additional S2p marks.

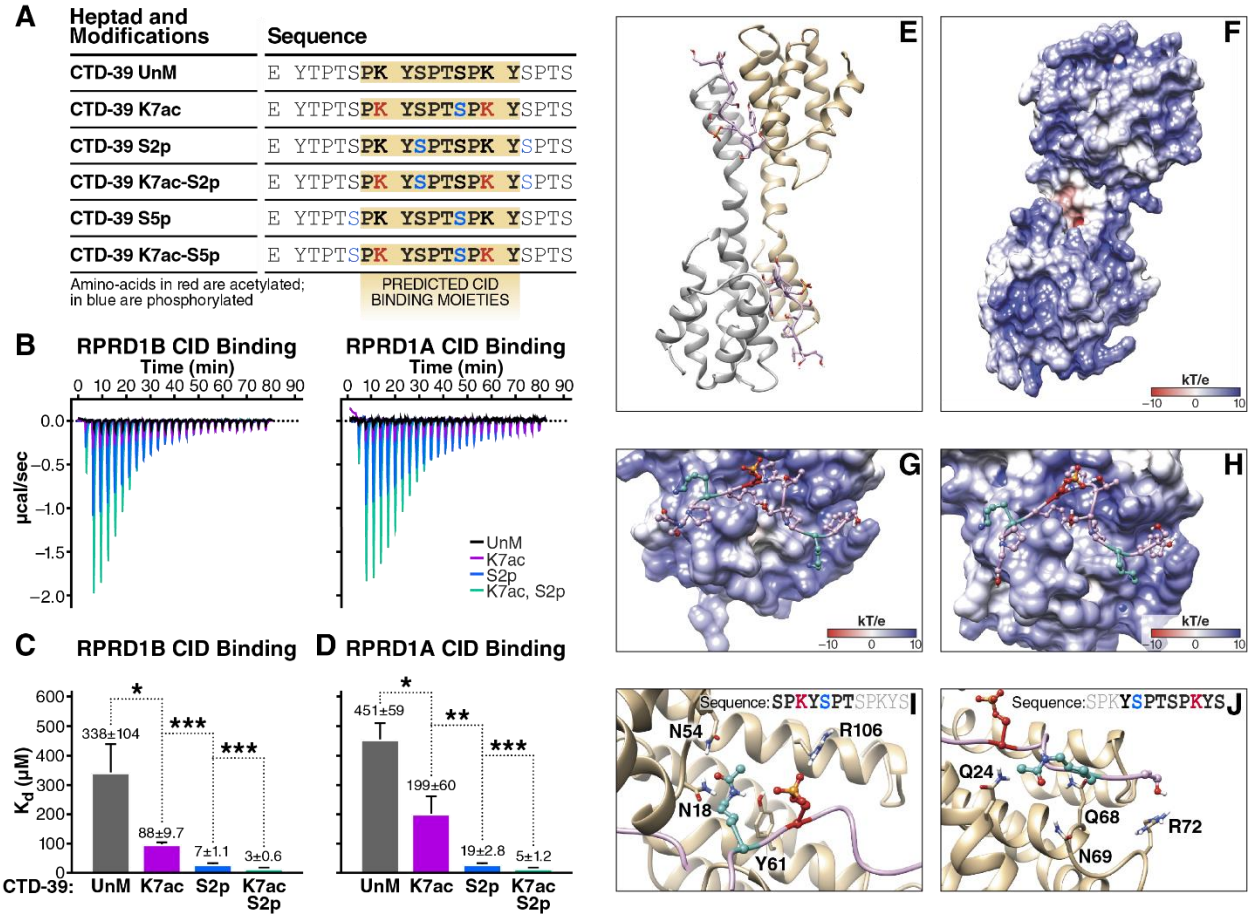

Figure 5-2: RPRD CID domains recognize acetylated and phosphorylated CTD peptides. Isothermal titration calorimetry (ITC) experiments measuring in-vitro binding affinity between RPRD1A and RPRD1B CID domains and modified CTD peptides. A. Table of CTD-39 peptides with indicated post-translational modifications and predicted RPRD CID binding moieties. B. Representative ITC plots of CTD-CID interactions. C Kd values measured by ITC for RPRD1B CID and modified CTD-39 binding. D. Kd values measured by ITC for RPRD1A CID and modified CTD-39 binding. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.005$ using a one tailed T test. E. Dimer model from RPRD1B crystal structure (pdb:4Q94) containing two recognition modules and two peptides fragments of the CTD. F. Electrostatic potential surface for RPRD1B CID. G. First CTD peptide fragment model from crystal structure superimposed with the electrostatic potential surface around the corresponding binding site. H. Second CTD peptide fragment model from crystal structure superimposed with the electrostatic potential surface around the corresponding binding site. I. Recognition elements around first K7ac and S2p in the CTD peptide fragment. J. Recognition elements around second K7ac in the CTD peptide fragment.

To better understand the mechanism behind K7ac-dependent stabilization of binding between the CID and CTD peptides, we performed molecular modeling using previously published RPRD1B CID structures bound to CTD peptides [pdb: 4Q94 (dimer) and 4Q96 (tetramer)]. RPRD protein dimerization is believed to occur through coiled coil domain interactions that are not present in these structures (Mei et al., 2014; Ni et al., 2014), which nonetheless dimerize and tetramerize by domain swapping. We proceeded with *in silico* analyses using both structures and searched for consistencies between both. Because phosphorylation and acetylation change the net charge of the peptide fragment, we first calculated the electrostatic potential of the CID structure to investigate the charge distribution along the binding cleft (Dolinsky et al., 2004). We found that the recognition module within the CID, both in the dimeric and tetrameric structures, has a positively charged binding pocket (**Figures 5-2E–H, Figure 5-S1A–D**), which will enhance the binding of peptides with S2p. Similarly, acetylation, and thus neutralization, of the positively charged lysine residues favored interaction with this binding pocket by enhancing electrostatic stability.

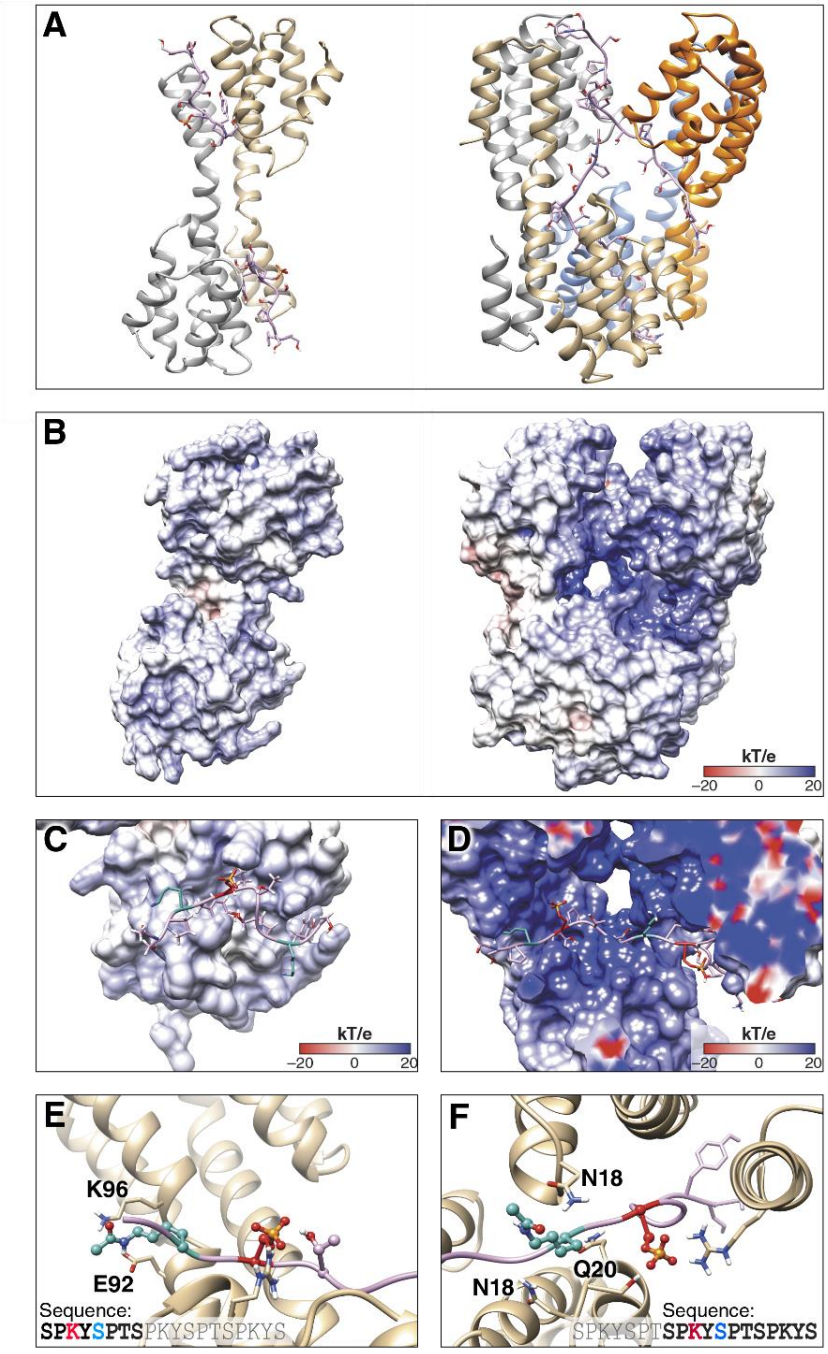

Figure 5-S1: Dimer and tetramer models of RPRD1B:CTD complex share similar features. Comparison between dimeric and tetrameric models of RPRD1B:CTD complex. A. Dimer (left) and tetramer (right) models from crystal structures. B. Electrostatic potential surface for dimer (left) and tetramer (right) models. C. Dimer model CTD peptide fragment superimposed with the electrostatic potential surface around the corresponding binding site. D. Tetramer model CTD peptide fragment superimposed with the electrostatic potential surface around the corresponding binding site. E. Recognition elements around first K7ac and first S2p in the CTD peptide fragment in the tetramer model. F. Recognition elements around second K7ac and second S2p in the CTD peptide fragment in the tetramer model.

We also obtained 20 ns molecular dynamics trajectories to refine the models and identify residues within the CID that directly bind to S2p and K7ac, and thus contribute to the recognition of these post-translational modifications. Through these simulations, we reproduced the previously reported coordination between S2p and arginine-106 (R106), and observed that the two K7 residues in the acetylated state formed transient interactions with nearby CID residues (**Figure 5-2I and 5-2J**). In particular, the first acetylated K7 residue in the CTD peptide formed transient hydrogen bonds with three CID residues (N18, N54 and Y61) in the same vicinity. The second acetylated K7 residue interacted with CID residues at the other end of the binding cleft (Q24, Q68, N69 and R72) (**Figure 5-2I and 5-2J)**. Similar results were obtained in simulations of the tetrameric structure, which also showed coordination between S2p and R106 along with similar transient hydrogen-bonding between K7ac and several residues in the CID (N18, Q20, E92 and K96) (**Figure 5-S1E and 5-S1F**). Together, the transient interactions coupled to the overall positive electrostatic potential around the recognition module explain the stabilization of K7ac-modified CTD peptides over the non-acetylated or unmodified peptides.

Increased K7ac Correlates with Reduced S5p Downstream of Transcription Start Sites

RPDR1 proteins are known to interact with RPAP2, the mammalian homolog of yeast *Rtr1* and a known S5 phosphatase (Ni et al., 2011). We tested the influence of K7ac on S5p levels by performing ChIP-seq with antibodies specific for K7ac, S5p and total unmodified (8WG16) Pol II in chromatin isolated from NIH3T3 cells treated with KDAC inhibitors. Average TSS–anchored occupancy profiles were generated for all expressed genes (Ramirez et al., 2016) and normalized to genomic background signal (**Figure 5-S2A-C**). KDAC inhibition increased genome wide Pol II-K7ac occupancy as expected, but interestingly only from ≥500 bp downstream of the TSS onwards. It decreased K7ac levels proximal to this in an area, where total Pol II occupancy was strongly enhanced (**Figure 5-3A and 5-3B**). In this region, S5p levels were also increased as compared to vehicle-treated cells, while beyond the ≥500 bp mark S5p levels decreased below

the level of control cells, mirroring enhanced K7ac (**Figure 5-3C**). While total Pol II occupancy increased immediately downstream of the TSS in response to KDAC inhibition, potentially explaining the TSS-proximal S5p enhancement, beyond +500–1000 bp, total Pol II occupancy on average remained unchanged, underscoring that the inverse relationship between K7ac and S5p at this point was not confounded by changes in total Pol II levels.

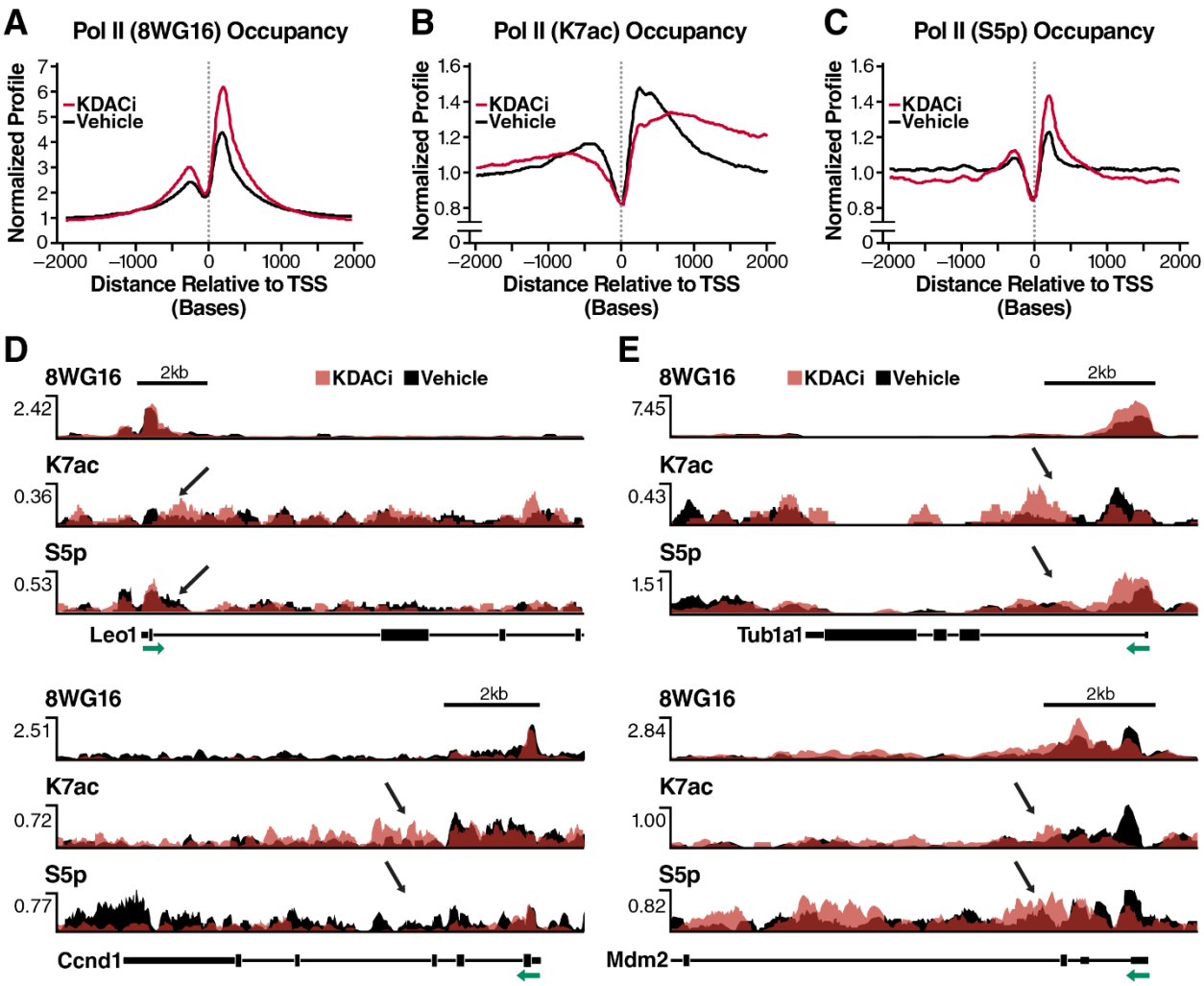

Figure 5-3: An inverse relationship between K7ac and phosphorylation is induced upon KDAC inhibition. A-C. TSS profiles generated from ChIP-seq data of expressed genes in NIH3T3 cells. Normalized profiles are measured in reads per million and expressed as fold change relative to background signal. D. Occupancy profiles of RNA Pol II PTMs measured as reads per million on selected RPRD1B occupied genes. S5p is measured using the RNA Pol II 4H8 antibody. E. Occupancy profiles of RNA Pol II PTMs on control genes. Green arrows indicate the direction of transcription relative to the TSS of the depicted gene. Black arrows indicate the site of affected PTMs in response to HDACi.

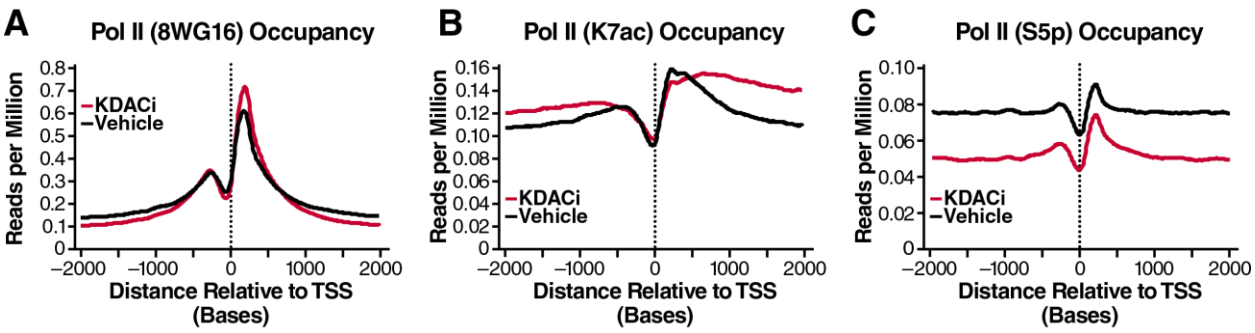

Figure 5-S2: An inverse correlation between K7ac and phosphorylation on chromatin is induced upon KDAC inhibition. A-C. TSS profiles generated from ChIP-seq data of expressed genes in NIH3T3 cells measured in reads-per-million. S5p is measured using the RNA Pol II 4H8 antibody.

A focused analysis of known target genes of RPRD1B such as *Leo1* and *Cyclin D1* (Lu et al., 2012; Ni et al., 2011) showed corresponding profiles with lowered S5p and enhanced K7ac levels downstream of the TSS in response to KDAC inhibition (**Figure 5-3D**). But ~10% of actively expressed genes, including *Tub1a1* and *Mdm2*, did not show a downregulation of S5p in response to KDAC inhibition despite strong upregulation of K7ac, indicating that these genes are not controlled by RPRD proteins but possibly alternative mechanisms (**Figure 5-3E**). Unfortunately, we could not examine the occupancy of RPRD proteins genome-wide as the available antibodies did not show a sufficient signal-to-noise ratio in ChIP-seq experiments (data not shown).

RPRD1B Controls Genes Involved in Multicellularity, Development and Cell Adhesion

Overexpression of RPRD proteins has been shown to decrease S5p levels at the *Leo1* gene consistent with the model that RPRD proteins recruit the S5 phosphatase RPAP2 (Ni et al., 2011). We now performed the inverse experiment and knocked down RPRD1B in NIH3T3 cells using lentiviral shRNAs. A 50% knockdown efficiency was sufficient to induce global S5 hyperphosphorylation as observed by western blotting, indicating a critical role of RPRD1B in overall S5 dephosphorylation. Surprisingly, we also observed a consistent upregulation of K7ac levels in RPRD1B knockdown cells, pointing to the recruitment of a K7 deacetylase by RPRD proteins in addition to the S5 phosphatase **(Figure 5-4A)**. No change in total Pol II levels was observed.

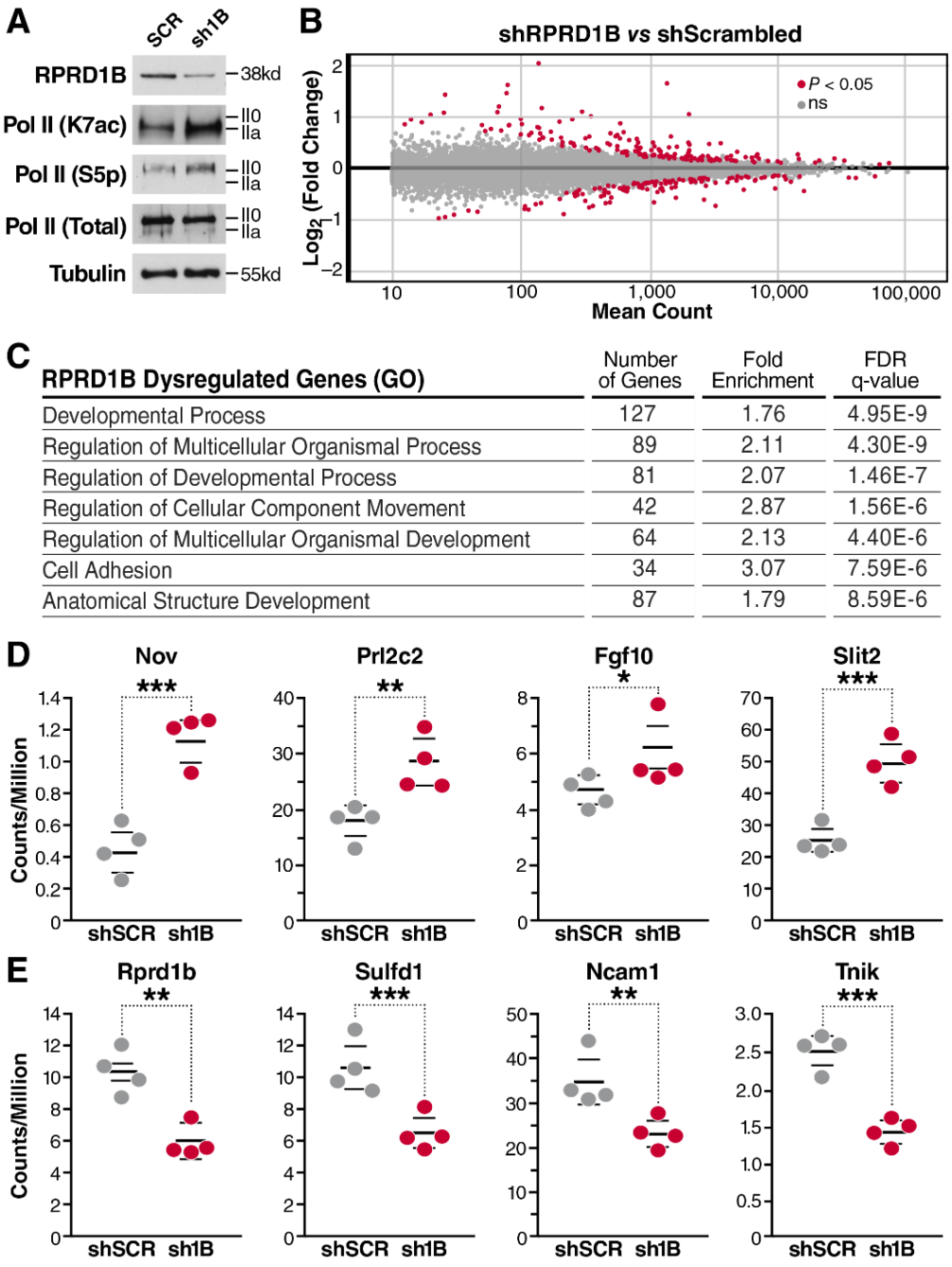

Figure 5-4: RPRD1B knockdown dysregulates genes relating to development and multicellularity and disturbs Pol II PTM homeostasis. NIH3T3 cells were treated with shRNAs against RPRD1B (sh1B) or a scrambled sequence (shScr) and selected with puromycin for 1 week. A. Western blotting from whole cell lysates to monitor RPRD1B protein expression upon knockdown and the effect on Pol II K7ac and S5p. B. RNA-seq highlighting 271 significantly dysregulated genes in red. C. Gene ontology analysis of genes significantly dysregulated in response to RPRD1B knockdown. D. DESeq counts per million for selected upregulated genes associated with regulation of multicellular organismal process. E DESeq counts per million for selected downregulated genes in response to RPRD1B knockdown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ using a one tailed T test.

RNA-seq on RPRD1B knockdown cells identified 271 differentially expressed genes as compared to control shRNA-treated cells (**Figure 5-4B**). RPRD1B was among the most significantly downregulated genes with an mRNA knockdown efficiency of 41% ($p = 0.0019$; **Figure 5-4E**), similar to what was observed for protein expression. Gene Ontology analysis on dysregulated genes indicated that RPRD1B knockdown induced changes in genes related to developmental processes, multicellular organismal development and cell adhesion, consistent with previous findings that complete knockout of the factor causes embryonic lethality (Morales et al., 2014) **(Figure 5-4C).** Furthermore, this is consistent with studies indicating K7ac specifically evolved in higher eukaryotes, and regulates developmental genes with significant enrichment for evolutionary origins in the early history of eukaryotes through early vertebrates (Schroder et al., 2013; Simonti et al., 2015).

The majority of dysregulated genes associated with multicellular organismal processes was upregulated in response to RPRD1B knockdown (69.7%). Examples include *Nov*, an immediate-early gene important for regulating proliferation and development, the Prolactin 2c2 gene *Prl2c2* involved in embryonal development, the fibroblast growth factor 10 gene *Fgf10* necessary for organogenesis, and the SLIT homolog 2 gene *Slit2* involved in neural ECM-mediated signaling (**Figure 5-4D**). Examples of downregulated genes include the ECM-associated sulfatase 1 gene *Sulf1* and neural cell adhesion molecule *Ncam1* **(Figure 5-4E)**. *Tnik,* an essential activator of the Wnt signaling pathway, was also down-regulated (**Figure 5-4E**), but most other Wnt-regulated genes remained unchanged. Together, these data identify RPRD1B as a regulator of genes involved in multicellular organismal development and further support the model that RPRD proteins are relevant reader proteins of the K7ac mark in higher eukaryotes.

RPRD1B Knockdown Perturbs both K7ac and S5p Marks Genome-Wide

Next, we performed ChIP-seq in RPRD1B knockdown cells using antibodies against K7ac, S5p and total Pol II. The most striking finding was the induction of a distinct TSS-proximal increase in K7 acetylation with minimal changes to total unmodified (8WG16) Pol II occupancy (**Figure 5-**

5A and 5-5B, Figure 5-S3A and 5-S3B). This is consistent with the observation that K7ac was induced upon RPRD1B knockdown in western blot experiments and underscores a model where RPRD1B recruits a K7 deacetylase that counterbalances K7 acetylation within the first 500–1000 bp of mRNA production (**Figure 5-5A**). This peak in K7ac levels was mirrored by a TSS-proximal decrease in S5p, possibly through residual RPRD (and RPAP2) proteins remaining in the RPRD1B knockdown cells inducing S5 dephosphorylation in response to K7 hyperacetylation (**Figure 5-5C, Figure 5-S3C).** Beyond this TSS-proximal region, S5p levels were elevated relative to cells treated with control shRNAs, consistent with the observation that reduced RPRD1B levels globally induce S5 hyperphosphorylation due to the lack of S5 phosphatase recruitment.

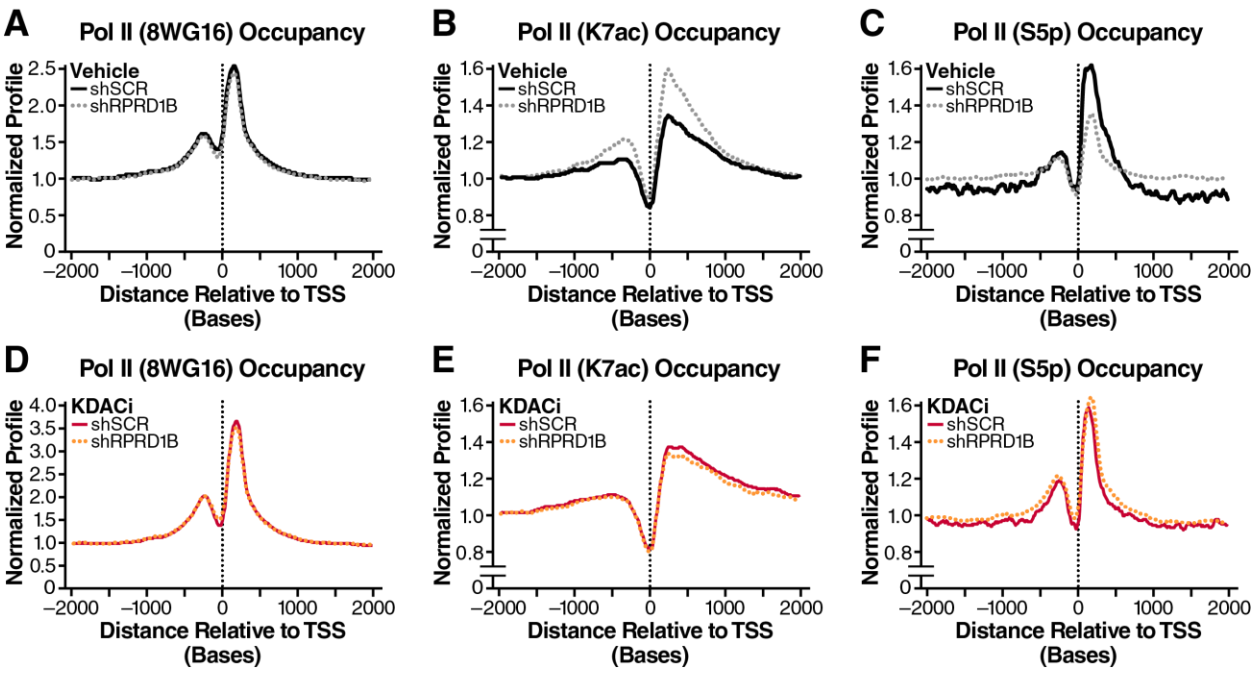

Figure 5-5: Increased S5p and K7ac levels in response to RPRD1B knockdown. TSS profiles of expressed genes in NIH3T3 cells. Normalized profiles are measured in reads per million normalized to background signal. A-C TSS profiles generated from ChIP-seq data from NIH3T3 cells expressing either scrambled or RPRD1B targeted shRNAs treated with Vehicle control. D-F TSS profiles generated from ChIP-seq data from NIH3T3 cells expressing either scrambled or RPRD1B targeted shRNAs treated with KDACi. S5p is measured using the RNA Pol II 4H8 antibody. Profiles are representative of two independent experiments.

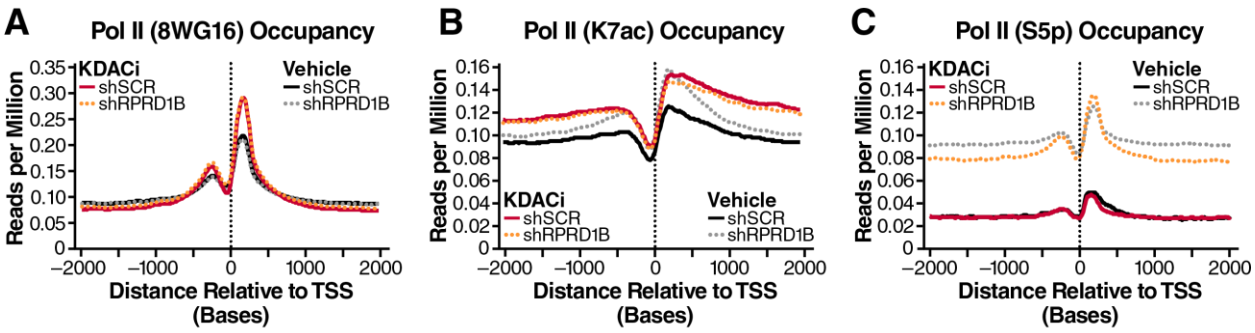

Figure 5-S3: RPRD1B knockdown induces hyperacetylation and hyperphosphorylation on chromatin near TSSs. NIH3T3 cells were treated with shRNAs targeting a scrambled sequence (shSCR) or RPRD1B (shRPRD1B), then with KDACi or a Vehicle control for 2 hours followed by ChIP-seq. A-C. TSS profiles generated from ChIP-seq data shown as reads-per-million.

When RPRD1B knockdown cells were treated with KDAC inhibitors, the inverse relationship between K7ac and S5p observed after either KDAC inhibitor treatment or RPRD1B knockdown were no longer detected (**Figure 5-5D-F).** This indicates that RPRD1B plays a critical role in mediating the changes in S5p levels observed after KDAC inhibition. It further supports the model that RPRD proteins recruit a KDAC in addition to a phosphatase, considering that knockdown of RPRD1B and KDAC inhibition each increased K7 acetylation to the same level, and no further increase was observed when both were applied together. Collectively, these results underscore the close link between acetylation of K7 residues and dephosphorylation of S5 via RPRD proteins at a unique distance from the TSS (+500-1000 bp) and point to the importance of an RPRD-associated K7 deacetylase.

Discussion

In this study, we report a new molecular function for K7ac in terminating S5p in the transition from transcription initiation to elongation. We show that K7ac enhances the recruitment of RPRD proteins to the initiated Pol II complex in order to facilitate S5 dephosphorylation; this occurs presumably via RPAP2, their interacting S5 phosphatase **(Figure 5-6A)**. Surprisingly, we found that lack of RPRD1B protein expression also increased K7ac levels, indicating that in addition to binding an S5 phosphatase, RPRD proteins may also recruit a K7 deacetylase. This provides a unique autoregulatory mechanism as binding to RPRD proteins to K7ac ultimately leads to the removal of the mark. As previous studies have highlighted the importance of S2p in enhancing the interaction between the CTD and RPRD CID domains (Ni et al., 2014; Pineda et al., 2015), the emergence of S2p downstream of K7ac may serve to maintain RPRD recruitment to complete S5 dephosphorylation during the early phase of transcription elongation (Ni et al., 2014). When levels of K7ac were perturbed –by KDAC inhibitor treatment (**Figure 5-6B)** or RPRD1B knockdown (**Figure 5-6C**)– recruitment of the S5 phosphatase was either enhanced, resulting in increased S5 dephosphorylation and lower S5p levels genome-wide, or recruitment was diminished, enhancing S5p levels, respectively. Therefore, these data support a model in

which dynamics of K7 acetylation evolved to blunt the peak of S5 phosphorylation at a precise distance from the TSS in higher eukaryotes, likely facilitating the transition between transcription initiation and productive elongation.

We previously observed a transient enrichment in the occupancy of K7-acetylated Pol II located approximately +500 bp downstream of the TSS when we normalized K7ac peaks to total Pol II occupancy on expressed genes (Schroder et al., 2013). This fits well with the self-limiting nature of K7ac wherein the modification recruits its own terminating enzyme via the RPRD reader proteins. Here we observed characteristic changes in S5p levels at and beyond the +500 bp mark that support the model that K7ac and S5p are inversely correlated. The +500 bp mark further corresponds well with the proposed hand-off site between S5p and S2p previously determined in yeast, underscoring that higher eukaryotes may have evolved K7ac to maintain this transition at the same genomic position (Mayer et al., 2010). In our current study, peaks proximal to the +500 bp mark behaved on average differently than peaks at or beyond the mark. Possible explanations are: A) RPRD proteins are recruited specifically to the +500 bp location (at the peak of K7ac) and exert their effect on S5p in this region and beyond; B) the balance between S5 phosphorylation and K7 acetylation immediately downstream of the TSS is shifted towards S5p and efficient dephosphorylation can only occur after CDK7 levels are additionally lowered beyond the TSS (Ebmeier et al., 2017). C) It is the transition of Pol II into elongation and the occurrence of S2p at the transition point that allows for efficient RPRD association and efficient S5 dephosphorylation; D) the RPRD complex changes before and after the +500 bp mark with the K7 KDAC highly enriched closer to the TSS, and less enriched beyond +500 bp. We envision that multiple of the mechanisms might be in place to explain the observed changes. Interestingly, similar peak enrichments of CTD PTMs as we describe for the sense strand of transcription were observed along divergent transcription of the negative strand of DNA, the significance of which remains unknown.

An important finding of our study is that K7ac enhances binding of the RPRD CIDs to CTD peptides. We show that the affinity of the CID:K7ac interaction is ~90 μM, which lies in the range of acetyl-lysine interactions with bromodomains, the latter being the "classical" Kac recognition domain (Muller et al., 2011). Interestingly, the K7ac:CID interaction accommodates additional phosphorylation marks such as S2p. This supports previous findings that the CID domain creates a positively charged "channel" in which the CTD peptide is dynamically situated depending on its PTM status (Jasnovidova et al., 2017a; Ni et al., 2014). K7ac recognition occurs by electrostatic and hydrogen bonding with various residues of the CID. Principles of specific recognition of phosphorylated amino acids have been well-studied and are consistent with the features we have highlighted for S2p. Lysine acetylation has received less study; our results suggest that side chains containing amide groups (N and Q) play an important role, forming transient hydrogen bonds with the amide group of acetylated K7 residues. It is especially interesting that N18, N69 and Q24, which form hydrogen bonds with the lysine amide in the dimer or tetramer models are conserved across RPRD proteins in mammals (Ni et al., 2014). This flexible mechanism of recognition could allow for the RPRD complex to be first recruited to the distal region of the CTD via K7ac alone. Furthermore, this could allow for the interaction between CID domains to accommodate for serine-to-asparagine substitutions at position 7, also in the distal region of the CTD. The complex can then migrate along the consensus repeats within the CTD using S2p and possibly also S7p to remove S5p along the full length of the vertebrate CTD (**Figure 5-6A**) (Egloff et al., 2012; Schuller et al., 2016).

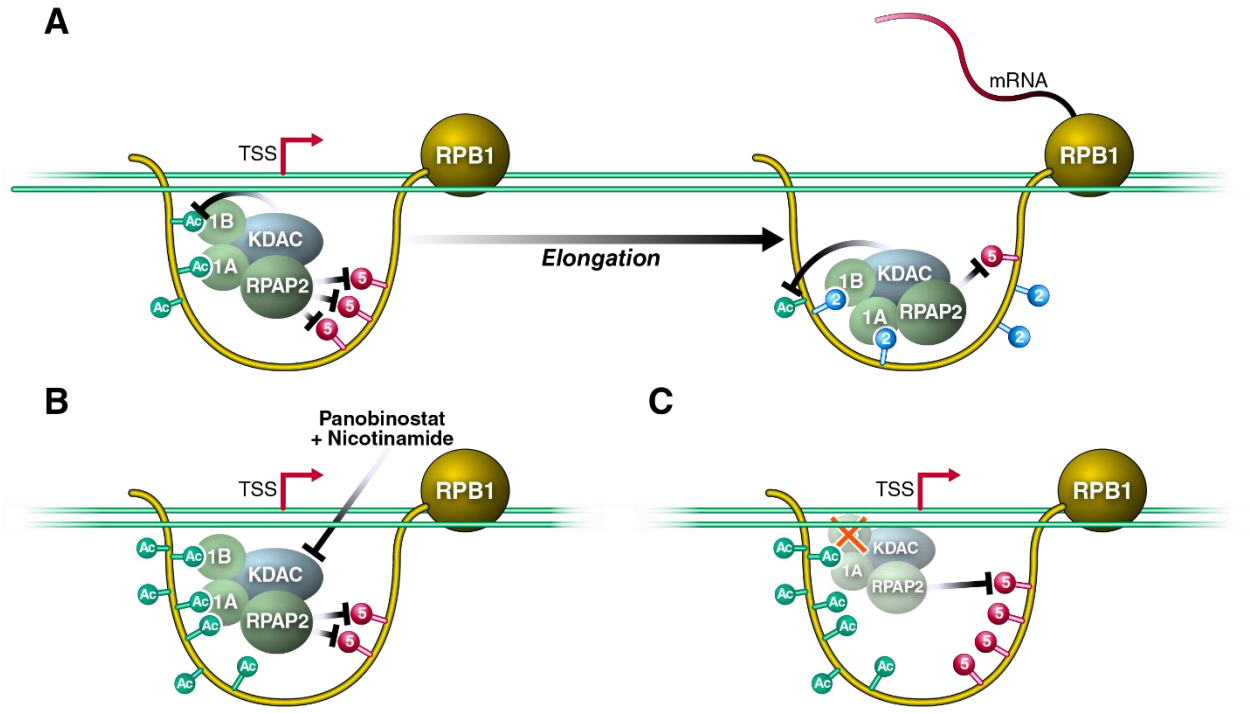

Figure 5-6: RPRD proteins are recruited to the RPB1 CTD via acetylation and phosphorylation to antagonize S5-phosphorylation. A. Model of RPRD complex reader and effector functions along modified residues within the CTD. 1B- RPRD1B 1A- RPRD1A, KDAC is a yet to be identified K7 deacetylase. B. KDAC inhibition induces K7 hyperacetylation and downregulation of S5-phosphorylation. C. Knockdown of RPRD1B perturbs the recruitment of the complex to result in both S5 hyperphosphorylation and K7 hyperacetylation. See text for details.

Serine-7 phosphorylation is the third-best studied PTM of the CTD. In mammals, S7p is enriched with S5p near promoters, but is uniquely stable in gene bodies (Descostes et al., 2014). Similar to K7ac, S7-phosphorylated residues are considered docking stations for RPAP2 regulating S5-dephosphorylation and expression of snRNA genes (Egloff et al., 2012). Interestingly, S7p also enhances CTD:CID interaction consistent with the enhancement of electrostatic stability we describe here for K7ac (Egloff et al., 2012; Ni et al., 2014). Recent studies have succeeded in examining individual repeat PTMs *in vivo* and showed that CTD heptads are generally phosphorylated at one position per repeat (Schuller et al., 2016; Suh et al., 2016). This supports a model of dynamic movement along the 52 mammalian CTD repeats proposed here where the RPRD complex may start at distal non-consensus regions and work its way up to more proximal consensus regions to reach all S5p marks and allow maximal placement of S2p. An interesting, yet unexplored connection may also exist between other minor phosphorylation sites (Y1 and T4) and K7 acetylation. Y1p in yeast is known to dissociate *Rtt103* during the initiation and elongation phases of transcription to prevent premature termination of transcription, and could interfere with K7ac recognition (Mayer et al., 2012). In contrast, T4p was shown to enhance the interaction with the *Rtt103* CID domain, and could be conserved in mammals (Jasnovidova et al., 2017b). As *Rtt103* and RPRD proteins are closely related, Y1p and T4p may also regulate the recruitment of RPRD proteins in addition to K7ac, S2p and S7p.

While RPAP2 is a known S5 phosphatase, the nature of the RPRD-associated deacetylase remains unknown. Previous studies have identified HDAC2 as a RPRD1A-associated deacetylase, thus being a possible candidate for a K7 deacetylase (Liu et al., 2015). This is consistent with our previous observations that class I/II KDACs are involved in deacetylation of the hypophosphorylated form of Pol II during or after transcription initiation (Schroder et al., 2013). Notably, HDAC2, unlike HDAC1 and 3, is found at promoter-proximal regions in addition to gene bodies, underscoring its potential as the RPRD-associated K7ac deacetylase (Wang et al., 2009). Interestingly, RPRD1B has previously been shown to interact

with p300/CBP to regulate gene expression in cancer (Zhang et al., 2018). This points to a fine-tuned balance between RPRD proteins in the recruitment of K7 acetyltransferases and deacetylases. Future studies will investigate whether differences exist between RPRD1A and RPRD1B proteins with respect to controlling K7 acetylation.

Gene expression changes as a consequence of RPRD1B knockdown were moderate, but the cellular pathways found to be altered in response to RPRD1B knockdown showed a relevant list of genes. These were mainly involved in development of multicellular organisms and were strikingly similar to differentially regulated pathways found in wildtype and 8KR- Pol II expressing cells (Simonti et al., 2015). We have previously shown that K7ac evolution in higher eukaryotes presented a unique mode by which transcription elongation is regulated in mammals. We propose that this regulation of K7ac is linked to the now reported recruitment of RPRD proteins and the corresponding S5 dephosphorylation, a step tightly controlled in its dynamics in yeast. The question of why the need arose to control S5p with K7ac in multicellular organisms at a defined distance from the TSS remains unanswered but will be further examined. At this point, our data underscore a key role of controlled CTD PTM regulation at the transition from initiation to elongation important for the expression of developmentally relevant genes; they further demonstrate that this control depends on precise interactions with the RPRD complex, which performs reader and effector functions at a well-defined time during the transcription cycle.

Experimental Procedures

Antibodies and Reagents

Dynabeads Protein G (ThermoFisher, 10003D), Dynabeads Protein A (ThermoFisher, 10001D), Bovine Calf Serum (Gemini, 100-506), 293T and NIH3T3 cells are from ATCC. Panobinostat (CAS 404950-80-7) and α-amanitin (CAS 23109-05-9) were purchased from Santa Cruz Biotechnology. All other chemicals and reagents were purchased from Sigma.

Cell Fractionation and Immunoprecipitation

Cell fractionation was performed using the Dignam & Roeder method with minor modifications. 293T or NIH3T3 cells were pelleted and washed in cold DPBS. Pellets were resuspended in 5 volumes of DR Buffer A (10mM HEPES-KOH pH 7.9, 10mM KCl, 1.5 mM $MgCl_2$, 0.5mM DTT, 1x HALT, 30nM Panobinostat and 5μM Nicotinamide). Cells were Dounce homogenized with 10 strokes using a tight pestle (Wheaton) and cytoplasmic lysates were set aside or decanted. Nuclear pellets were resuspended in DR Buffer C (20mM HEPES, 0.42M NaCl, 1.5mM MgCl2, 0.2mM EDTA, 25% glycerol, 0.5mM DTT, 1x HALT, 30nM Panobinostat and 5μM Nicotinamide), sonicated using the Sonic Dismembrator 500 (ThermoFisher Scientific). 500μg of nucleoplasm was precleared with normal IgG (Santa Cruz) conjugated to the appropriate beads and immunoprecipitation was performed using anti HA- agarose beads (Sigma, A2095) or antibodies bound to Dynabeads. Immunoprecipitates were eluted either by boiling in 2x Laemmli buffer (agarose) or incubating in Elution Buffer (50mM $NaHCO_3$, 1% SDS) and adding 2x Laemmli buffer (Dynabeads).

Lentiviral transduction of RPRD1B shRNAs

VSV-G pseudotyped lentiviruses were produced to contain a Puromycin resistance gene and a shRNA against RPRD1B (NM_027434.2-1003s21c1) or a scrambled control. Cells were transduced with 0.5mL unconcentrated virus and selected using 2μg/mL Puromycin for 1 week prior to experimentation.

Chromatin Immunoprecipitation in NIH3T3 cells

NIH3T3 cells were grown under normal conditions (10% BCS, 1x Penicillin and Streptomycin, 2mM L-Glutamine). We treated $6x10^7$ cells with a lysine deacetylase inhibitor cocktail (30nM panobinostat, 5uM Nicotinamide) or a vehicle control (DMSO, water) for 2h. Cells were fixed with 1% formaldehyde for 15 minutes, thoroughly washed with DPBS, and resuspended in ChIP lysis buffer #1 (10mM Tris pH 7.4, 10mM NaCl, 0.5% NP-40, 1x HALT, 30nM Panobinostat and 5μM Nicotinamide). After sitting on ice for 10 minutes, cells were briefly vortexed and nuclei were pelleted. Nuclei were treated with MNase (NEB, M0247S) for 25 minutes at RT, pelleted and

resuspended on ice in ChIP lysis buffer #2 (50mM Tris HCl pH 8.0, 10mM EDTA, 0.5% SDS, 1x HALT, 30nM Panobinostat and 5μM Nicotinamide). Chromatin was further sheared by sonication using the Sonic Dismembrator 500 (ThermoFisher Scientific) and preserved at -80ºC until immunoprecipitation. 20-40ug chromatin was used for each IP with the antibody concentrations listed in **Table 5-S2**. IPs were diluted into a final volume of 800uL with ChIP Dilution buffer (167mM NaCl, 16.7 mM Tris HCl pH 8.0, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS) and left at 4ºC overnight. IPs were washed then eluted in ChIP elution buffer (50mM $NaHCO_3$, 1% SDS) and decrosslinked at 65ºC for 16hrs. Samples were treated with RNAse A (Thermofisher, EN0531) for 20 minutes and DNA were purified using the QIAquick PCR purification kit (Qiagen, 28106). Primer sequences are available upon request. For samples that were deep-sequenced, 2ng of immunoprecipitated DNA from each reaction was used to create libraries using the Ovation Ultra-Low Library prep kit (Nugen, 0344-32) following manufacturer recommendations and libraries were deep sequenced on the HIseq 4000 or NextSeq 500 using single-end 50bp or single-end 75bp sequencing, respectively.

RNA sequencing

RNA was prepared from $1x10^6$ NIH3T3 cells using the QIAgen RNeasy Plus Kit. Libraries were prepared with the Ovation Ultralow System V2 kit pn: 7102-32 / 0344-32 and libraries were deep sequenced on NextSeq 500 using paired-end 75pb sequencing. RNA seq analysis was done using the Illumina RNAexpress application v 1.1.0.

ChIP-seq data analysis

Barcodes were removed and sequences were trimmed using Skewer (Jiang et al., 2014). For each ChIP 50-60 million reads were aligned to the *Mus musculus* mm10 genome assembly using Bowtie with the –a -l 55 -n 2 -m 1 parameter (Langmead et al., 2009). Peaks were called and sequence pileups normalized to reads per million using MACS2 -B -SPMR -g mm -no-model -slocal 1000 (Zhang et al., 2008). TSS profiling was done using -plotProfile on matrices generated with 10bp bins using the computeMatrix function found in the Deeptools 2.2.3 build (Ramirez et

al., 2016). Normalized profiles are calculated as fold change in signal relative to the observed background signal which we define as the signal from the 10bp bin at -3000 relative to TSS. Reproducibility of data was assessed by principal component analysis (Ramirez et al., 2016).

Stable Isotope Labeling of Amino Acids in Culture (SILAC) of WT and 8KR Polymerases

SILAC (Stable isotope-labeled amino acid) labeling was performed according to the manual of SILAC Protein Quantitation Kit (LysC) –DMEM (Thermo Scientific cat. no. A33969). In brief, 293T cells stably expressing Pol-II-WT-HA and Pol-II-8KR-HA were grown in the light medium (L-Lysine-2HCl) or heavy medium ($^{13}C_6$L-Lysine-2HCl), respectively. After growing 7 doubling time in the respective medium, incorporation efficiency of heavy L-lysine in 293T-Pol-II-8KR-HA cells was determined and the efficiency was more than 99%. To immunoprecipitate the HA proteins, 5 mg of total cell lysate from Pol-II-WT-HA and 5mg of total lysate from Pol-II-8KR-HA cells in p300 lysis buffer were mixed together (total 500 uL), and 100 uL of HA agarose (Roche) were added. After overnight immunoprecipitation at 4C, the HA-agarose was washed 4 times with 1 mL of cold p300 lysis buffer to remove non-specific binding proteins. The bound proteins were eluted twice by 100 ul of 0.1 M Glycine, pH 2.5 after 30 min incubation. Each elution was stored in separate tube. 10 uL of 1 M Tris-HCl pH 8.0 was added into each elution to neutralize the pH. The quality of the elution was monitored by Protein Silver Staining (Pierce). Two elusions were combined and 50 uL out of the 200 uL combined elusions were sent to Mass Spectrometry. Two independent biological repeats were performed.

Mass Spectrometry Analysis

Sample were analyzed on a Thermo Scientific LTQ Orbitrap Elite mass spectrometry system equipped with an Easy-nLC 1000 HPLC and autosampler. Samples were injected onto a pre-column (2cm x 100 um I.D. packed with 5 um C18 particles) in 100% buffer A (0.1% formic acid in water) and separated by a 120 minute reverse phase gradient from 5% to 30% buffer B (0.1% formic acid in 100% ACN) at a flow rate of 400 nl/min. The mass spectrometer continuously collected spectra in a data-dependent manner, acquiring a full scan in the Orbitrap (at 120,000

resolution with an automatic gain control target of 1,000,000 and a maximum injection time of 100 ms) followed by collision-induced dissociation spectra for the 20 most abundant ions in the ion trap (with an automatic gain control target of 10,000, a maximum injection time of 10 ms, a normalized collision energy of 35.0, activation Q of 0.250, isolation width of 2.0 m/z, and an activation time of 10.0). Singly and unassigned charge states were rejected for data-dependent selection. Dynamic exclusion was enabled to data-dependent selection of ions with a repeat count of 1, a repeat duration of 20.0 s, an exclusion duration of 20.0 s, an exclusion list size of 500, and exclusion mass width of + or - 10.00 ppm.

Raw mass spectrometry data were analyzed using the MaxQuant software package (version 1.2.5.8) (Cox and Mann, 2008). Data were matched to the SwissProt human proteins (downloaded from UniProt on 2/15/13, 20,259 protein sequence entries). MaxQuant was configured to generate and search against a reverse sequence database for false discovery rate calculations. Variable modifications were allowed for methionine oxidation and protein N-terminus acetylation. A fixed modification was indicated for cysteine carbamidomethylation. Full trypsin specificity was required. The first search was performed with a mass accuracy of +/- 20 parts per million and the main search was performed with a mass accuracy of +/- 6 parts per million. A maximum of 5 modifications were allowed per peptide. A maximum of 2 missed cleavages were allowed. The maximum charge allowed was 7+. Individual peptide mass tolerances were allowed. For MS/MS matching, a mass tolerance of 0.5 Da was allowed and the top 6 peaks per 100 Da were analyzed. MS/MS matching was allowed for higher charge states, water and ammonia loss events. The data were filtered to obtain a peptide, protein, and site-level false discovery rate of 0.01. The minimum peptide length was 7 amino acids. Results were matched between runs with a time window of 2 minutes for technical duplicates.

Isothermal Titration Calorimetry

RNA Pol II CTD peptides were purchased from Peptide 2.0 (Chantilly, VA). ITC experiments were performed as previously described (Ni et al., 2014)

Molecular modeling

Crystallographic structures were used for the dimer (pdb:4Q94) and tetramer (pdb:4Q96) models. Electrostatic potential surfaces were calculated using an adaptive Poisson-Boltzmann solver (APBS) from the PDB2PQR server using the Amber force field and PROPKA to assign protonation states (Dolinsky et al., 2004). Amber's LEaP program was used with the Amber ff14SB force field and the following force field modifications: phosaa10 (phospates), ffptm (phosphorylated serines) and ALY.frcmod (acetyllysines). The TIP3P water model was used to solvate the system in a cubic periodic box, such that the closest distance between any atom in the system and the periodic boundary is 10 Å. Net positive charge in the box was neutralized by adding counterions (Cl-) until neutrality. Energy minimization was performed in two steps: using harmonic restraints on the protein (10.0 kcal mol^{-1} $Å^{-2}$) and an unrestrained minimization. For each minimization we ran 1000 steps of steepest descent and 1000 steps of conjugate gradient minimization at a constant volume with a non-bonded cutoff of 9 Å. The equilibration was done in three steps. First, the system was heated from 0 to 300 K with a restrained equilibration (10.0 kcal mol^{-1} $Å^{-2}$) for 20 ps at constant volume with a non-bonded cutoff of 9 Å, using the SHAKE algorithm to constrain bonds involving hydrogens, and the Andersen thermostat. The second round of equilibration was performed lowering the harmonic restraints (1.0 kcal mol^{-1} $Å^{-2}$) on the system for 20 ps (other parameters identical). The third round was performed for 1 ns at constant pressure of 1.0 bar with non-bonded cutoff of 9 Å at 300 K with the Andersen thermostat. Simulations were performed without restraints using new velocities with random seeds at constant pressure of 1 bar with non-bonded cutoff distance of 9 Å. 20ns simulations were run with 2 fs timestep per construct. Coordinates and energy were saved every picosecond (500 steps) (Case et al., 2005). Molecular graphics and analyses were performed with the UCSF Chimera package (Pettersen et al., 2004).

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Author Contributions

I.A. designed the study, validated SILAC hits, conducted HDACi experiments, western blotting and quantification, performed ChIP-qPCR and ChIP-seq experiments and analyses, performed knockdown experiments, and statistical analyses. P.C.L. and J.J. performed SILAC experiments and mass-spectrometric data analysis. Z.N., H.Z., and J.M. performed ITC experiments. D.G.R. performed molecular modeling experiments. R.J.C. supported ChIP-seq analysis. X.G., J.G., M.J., and N.K. supervised experiments. M.O. supervised the study design and data collection. I.A. and M.O. wrote the manuscript.

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Chapter VI: Outlooks and Conclusions

The role of post-translational modifications is widely appreciated in cellular biology and pharmacology. As technological advances progress, researchers will continue to develop a better understanding of the roles protein acetylation and methylation play in mammalian systems. This body of work demonstrates that protein acetylation can be found all over mammalian cells. Acetylation plays an important role in a number of biological processes by altering the molecular function of proteins, signaling pathways and more. Disruption of acetylation can lead to the development of cancer, abnormalities in organismal development and genome instability (Ali et al., 2018). To me, it seems that the future of this research is going to look at the spaciotemporal dynamics of acetylation in response to various stimuli. This will probably be achieved through time-lapse mass spectrometry experiments looking at the dynamics and stoichiometry of the modification simultaneously on thousands of proteins. In addition, I think it will be really important for future investigators to study how the consumption of metabolites influences development and signaling outcomes in the context of various protein modifications. This could be achieved through large-scale epidemiological and nutritional studies that look at epigenetic changes in response to chronic dietary preferences or deficiencies. It might help us understand the onset of chronic diseases that plague long-lived and aging populations.

Viruses such as HIV-1 harness cellular pathways such as that of acetylation or metheylation and manipulates them to enable infection. (Boehm and Ott, 2017; Jeng et al., 2015). Unfortunately, these pathways also seem to be highly interconnected with signaling from other modifications and sometimes work antagonistically in the same signaling cascade. As a consequence, most studies or diseases targeted for epigenetic regulation will probably require a multi-drug treatment strategy with a specific time-course. From the perspective of HIV-1 Tat regulation, it would be interesting to see how Tat PTMs change depending on the cellular compartment in which it is found or stage of infection. This could shed light on the efficacy of

strategies for “Shock and Kill” or “Block and Lock” by producing a better sense of how modifications change with respect to space and time during infection. However, I think it might be a bit unrealistic to think that this will be a sustainable means of curing the disease. Between the cost of drug-development and issues around access and pricing of drugs, there will probably need to be a fundamental shift in the American healthcare system or most people won’t even be able to afford the treatments even if they work.

Probably the most effective strategy for minimizing the harm that is caused by HIV-1 infection will be through targeted public health approaches, especially in rural parts of Africa and South-East Asia where the virus spread remains rampant. It is also notable that in order to address HIV-1 cure, ensuring spread prevention and therapeutics equitably reach those that are infected (not just those who have the money for treatment) is paramount. Communities of color have lower rates of trust in medicine and science because of historic mistreatment of these communities, therefore more can be done in these populations to minimize spread, morbidity and mortality caused by this disease. Before we start pumping patients full of more drugs with minimal efficacy and significant off-target effects prioritizing alternative options may be important.

RNA Polymerase II CTD acetylation, if it turns out to be a relevant modification, will change the paradigm for understanding mammalian transcription. It is clear that both placement and the removal of K7ac is probably necessary for appropriate transcription elongation (Ali, 2018; Schroder et al., 2013). There is a great deal to study in the context of this particular modification. Studies investigating the regulation of CTD acetylation in response to stimulation in primary cells will help us get a better understanding of the importance of these modifications beyond the work of cell lines. Investigating the proteins that recognize the modification similar to RPRD proteins will be important for understanding its role in transcription. It will also be important to define the KDAC that removes the modifications. Our preliminary evidence suggests that Class I and Class III KDACs may play roles in influencing CTD acetylation. It may also be important to investigate

the role of each individual lysine residue for the CTD, especially those that have been identified using mass spectrometric approaches (Voss et al., 2015). This can be achieved with point mutational studies of individual CTD lysine residues or with monoclonal antibodies trained to specific residue clusters.

Considering that CTD acetylation is implicated in promoter-proximal pausing and early elongation. It is important to study this modification in the context of specific mammalian cellular stimuli. Some preliminary evidence has shown that CTD acetylation is enhanced in primary bone-marrow derived macrophages in a time-dependent manner after activation using LPS or INFγ. Promoter-proximal pausing is a critical step in regulating gene expression. Many genes during development are paused and activated through specific stimuli. CTD acetylation should be assessed in primary cell models, as it is likely that acetylation levels are artificially dysregulated in cell-line systems and probably has a more dynamic range in cells that are better transcriptionally regulated. As this K7ac antibody is quite robust for both ChIP and western blotting. It may be useful to make the antibody commercially available and optimized so that it can be used in other important studies. With many developmental avenues to pursue this type of work needs more than just one person.

Overall, it goes without question that protein post-translational modifications play an extremely important role in regulating transcription activation and cellular signaling. However there is a lot to learn about how metabolism and metabolites influence these processes. Many in the field of aging are beginning to see how important specific nutrients and metabolites are in regulating acetylation and methylation. However, how the dysregulation of these systems leads to cancer, auto-immunity, and immune disruption needs to be better understood. I have always been one to advocate for preventative medicine over reactive medicine. I was taught as a kid that you are what you eat. Epidemiologists are telling us that if you eat and smoke crap, you get sick. There is an epigenetic basis to these facts, and more needs to be done to study this.

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