

The Activity and Kinetic Mechanisms of Histone Lysine Methyltransferases Involved in Cancer

by

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Abstract

Epigenetic regulation of gene expression is mediated in part by chromatin-modifying enzymes that catalyze post-translational modifications on histones. Among these, histone lysine methyltransferases (KMTs) play a central role in gene regulation and are frequently implicated in disease aetiology. Despite their recognized importance, the catalytic mechanisms, substrate specificity, and kinetic activities of most histone methyltransferases remain poorly understood.

In this thesis, a novel method was developed to rapidly purify recombinant histones in less than one day providing substrates for biochemical investigation of KMTs. Using these substrates, as well as nucleosomes and modified nucleosomes, the histone methyltransferases SET7/9 (KMT7 or SETD7) and DOT1L (KMT4) were systematically characterized through substrate profiling and bi-substrate kinetic analyses. Substrate profiling was performed to define enzyme-specific residue selectivity and methylation patterns, followed by initial-rate kinetics, substrate-variation experiments, and product inhibition studies to resolve catalytic mechanisms. The methods used include mass spectrometry, proteomics and other biochemical techniques.

The kinetic analyses demonstrate that SET7/9 and DOT1L exhibit distinct substrate preferences and sequential reaction mechanisms. These results establish that histone lysine methylation is controlled by intrinsic kinetic and substrate-recognition systems. This thesis provides an insight into KMT activity and mechanism and offers insights for drug discovery programs.

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Dedication

This thesis is dedicated to my wonderful parents, Tunde and Grace Ige, and my sweet daughter Grace Ige who have been unyielding in their support.

Publications and Contribution of Authors

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Chapter 1: Introduction

1.1 Posttranslational Modifications to Histone H3 Lysine and Arginines

In eukaryotes, histones package DNA, organizing it into nucleosomes consisting of 147 bp of DNA and an octamer of histones(1). Nucleosomes are the basic units of chromatin which can be condensed or packed into denser higher orders of organization called heterochromatin or conversely, more loosely packed into euchromatin. When densely packed into heterochromatin, this level of organization can restrict access of the transcriptional machinery to DNA and reduce gene expression. Conversely nucleosomes can be less condensed resulting in euchromatin, allowing access of transcriptional machinery to DNA and resulting in increased gene expression. Post-translational modifications (PTMs) on histones can modulate gene expression by regulating chromatin condensation and recruiting transcription factors. Histone H3 in particular is known to have at least 17 kinds of PTMs, including acetylation (ac), ADP-ribosylation (ar), biotinylation (bio), citrullination (ci), crotonylation (cr), formylation (fo), O-GlcNAcylation (glc), glutathionylation (glu), methylation (me), phosphorylation (ph), propionylation (pr), succinylation (succ), and ubiquitylation (Ub) (Figure 1.1). The exact roles of each PTM are not yet completely understood and some are more common than others. The majority of PTMs are found on histone lysines and arginines therefore this introduction will focus on 5 PTMs to histone H3 lysines and arginines. The enzymes that catalyze the transfer of these PTM are called writers, those that remove the PTM are called erasers and protein domains that recognize (bind to) the PTM are called readers.

1.1.1 Acetylation

Histone acetylation (Kac) involves the addition of an acetyl group to the Nε of histone lysine residues. It is catalyzed by lysine acetyltransferases (KATs) such as KAT3B (p300) and KAT3A [CREB-binding protein (CBP)], with acetyl-CoA as the acetyl group donor. It has been shown that acetylation of lysine residues on histone H3 occurs at histone K4, K9, K14, K18, K23, K27, and K36, K56 and K79. In particular, histone H3K4ac, H3K9ac and H3K27ac are associated with active transcription. Histone acetylation marks are removed by “histone deacetylases” (HDACs) also called lysine deacetylases (KDAC) (2,3). The acetylation and deacetylation of histones regulate the expression of many genes, some of which are involved in apoptosis and cell proliferation (4). Examples of KATs include KAT3B (also known as p300/CBP-associated factor (PCAF)) and KAT2A (Also known as General Control Non-repressed 5 protein (GCN5) (5). Mechanistically, Kac can sterically block interactions and neutralizes the positive charge of the histones, thereby reducing electrostatic interactions with the negatively charged DNA backbone, leading to a more open euchromatin structure and higher transcription levels (6). Beyond these steric and electrostatic, changes acetyl-lysine binds to proteins that possess bromodomains (BRDs) which are an example of a reader domain. The bromodomain is a conserved structural motif characterized by a left-handed 4-helix bundle that forms a deep hydrophobic pocket. This pocket is precisely shaped to accommodate the acetyl-lysine side chain, where an asparagine residue often forms a critical hydrogen bond with the carbonyl oxygen of the acetyl group, thereby anchoring the reader protein to the chromatin (7).

The recruitment of these bromodomain-containing readers is essential for the assembly of the transcriptional machinery. Major coactivator complexes utilize this interaction to target specific genomic loci; for example, the SWI/SNF (BAF) chromatin remodeling complex uses

bromodomains in its catalytic subunits, such as BRG1, to dock onto acetylated histones and physically displace nucleosomes to clear a path for RNA polymerase. Similarly, the Bromodomain and Extra-Terminal (BET) family of proteins, most notably BRD4 (bromodomain-containing protein 4), acts as a molecular scaffold that binds to acetylated enhancers and promoters to recruit the P-TEFb complex, which is necessary for RNA Polymerase II elongation. Some KATs, such as KAT3A (CBP), KAT3B (p300) and KAT2 (GCN5), also contain their own bromodomains, allowing them to bind to existing marks and spread acetylation across a genetic region in a self-reinforcing feedback loop (8).

While bromodomains are the primary readers of the Kac mark, other specialized domains have evolved to recognize this modification with high specificity. The YEATS domain, found in proteins like MLLT1 (Myeloid/Lymphoid or Mixed-Lineage Leukemia Translocated to 1), also known as ENL (Eleven-Nineteen Leukemia protein) and MLLT3 (Mixed-Lineage Leukemia; Translocated to, 3), also known as AF9 (ALL1 Fused 9), represents a distinct class of readers that frequently target acetylated histone H3K9 or H3K27 and are often involved in the misregulation of gene expression in leukemias (9). Readers known as plant homeodomain (PHD) fingers and Tandem Tudor domains can work in conjunction with bromodomains to increase binding affinity and specificity for multi-acetylated histone tails. Collectively, these readers translate the chemical signal of lysine acetylation into a coordinated biological response, ensuring that the necessary enzymes and transcription factors are precisely positioned to drive gene expression (10).

Kac is associated with a plethora of epigenetic functions. For example, it was also found that K56ac is present in human embryonic stem cells (hESCs) and may be involved in the human core transcriptional network of pluripotency (11).

1.1.2 Methylation

Histone methylation can happen on lysine or arginine residues with up to 3 and 2 methyl groups being transferred, respectively. It does not affect the charge of the resultant methylated residues at physiologic pH, but could sterically block interactions that happen in its absence. More importantly and analogous to acetylation, such methylation can recruit proteins that can increase or decrease transcription (12) depending on the histone residue position and type of methylation. It is also thought that methylation increases hydrophobicity and may prevent H-bond networks from forming with target proteins or DNA (13)

Histone lysine residues can undergo monomethylation (Kme1), dimethylation (Kme2), and trimethylation (Kme3) catalyzed by lysine methyltransferases (KMTs) (14). Histone arginine residues can undergo monomethylation and dimethylation (catalyzed by protein arginine methyltransferases (PRMTs) (15,16). Dimethylation can also occur either in a symmetric or asymmetric manner. Symmetric dimethylation (Rme2s) refers to methylation on two separate terminal nitrogen atoms (*N*, *N'*) whereas asymmetric dimethylation (Rme2a) means two methylations on the same terminal nitrogen atom (*N*, or *N*). KATs utilize S-adenosyl-L-methionine (SAM) as the methyl donor in these methylation reactions.

Mono methylation of histone H3K4 (H3K4me1) is often found in the enhancer regions and histone H3K4me3 in the promoters of actively transcribed genes. Therefore, such methylation events are believed to activate transcription, directly or indirectly. For example, enzymes that catalyze promoter histone H3K4me3 recruit transcription factor TFIID which in turn recruits RNA polymerases (17). Methylated histone H3K36 usually designates regions of active chromatin and thus are believed to be involved in active transcription (18). In addition, histone H3K36me is involved in splicing regulation (19), DNA damage response (20), and DNA methylation (21).

The KMT SET7/9, which is also called SETD7 or KMT7, is a unique member of the SET domain-containing family of lysine methyltransferases. Unlike many other KMTs that form multi-protein complexes, SET7/9 functions primarily as a monomer. It is characterized by its ability to catalyze the addition of a single methyl group (monomethylation) to lysine residues. While it was originally identified as the enzyme responsible for H3K4me1, a mark typically associated with active gene enhancers, subsequent research has revealed that its primary physiological impact may stem from its extensive activity with non-histone proteins, including p53, E2F1, and various hormone receptors (22–24).

SET7/9 is widely expressed across human tissues, though its protein levels vary significantly depending on the metabolic and proliferative state of the cell. High levels of expression are consistently found in the liver, skeletal muscle, and pancreas, where it plays a fundamental role in insulin signaling and glucose homeostasis (25,26). Within the cell, SET7/9 is a nucleocytoplasmic shuttling protein. While much of its epigenetic work occurs in the nucleus, a significant fraction resides in the cytoplasm to regulate signaling scaffold proteins and transcription factors before they translocate into the nucleus. Its expression is dynamically regulated by environmental cues; for instance, high glucose conditions or oxidative stress often upregulate SET7/9, linking its activity to the cellular stress response.

Genome-wide chromatin immunoprecipitation (ChIP) and ChIP-seq studies suggest that SET7/9 localizes primarily to transcriptionally active chromatin regions. SET7/9 occupancy is enriched near promoter regions and transcription start sites (TSSs), where histone H3K4me1 may be associated with transcription initiation and enhancer activity (27,28). In addition to promoters, SET7/9 binding has been observed at enhancer elements and regulatory regions of actively

transcribed genes, consistent with the distribution of H3K4me1 as a mark of transcriptional competence and enhancer priming (29,30).

SET7/9 lacks a canonical DNA-binding domain, and it is therefore recruited to specific genomic loci through interactions with transcription factors, coactivators, and chromatin-associated complexes. SET7/9 associates with transcriptional regulators such as p53, NF- κ B, estrogen receptor α (ER α), and TAF10, enabling locus-specific recruitment and modulation of gene expression (31–33). Interaction with RNA polymerase II-associated complexes and chromatin remodelers further facilitate its localization to actively transcribed regions (25). In addition, SET7/9 recruitment is influenced by chromatin context and pre-existing histone modifications, suggesting that epigenetic crosstalk contributes to its targeting and function (34,35). Beyond histones, SET7/9 methylates numerous non-histone proteins involved in transcription, DNA repair, and signaling pathways, reinforcing its role as a regulatory hub linking chromatin modification to cellular signaling networks (25,36).

The enzyme's ability to act as a metabolic sensor positions SET7/9 as a molecular integrator linking cellular metabolic state to transcriptional regulation. By methylating p53 at Lysine 372 (K372), it stabilizes the tumor suppressor and promotes the expression of pro-apoptotic genes like BAX(31). However, as seen in various cancers, this guardian role can be subverted if the enzyme is co-opted to stabilize oncogenic receptors. Because SET7/9-deficient mice are viable and fertile, the enzyme has become an attractive therapeutic target; inhibiting it may disrupt specific disease pathways (like chronic inflammation or hormone-driven cancers) without causing the systemic toxicity associated with more fundamental epigenetic regulators.

SET7/9 has been implicated in several types of cancer due to its role in regulating gene expression and maintaining chromatin homeostasis. Notably, SET7/9 mutations and deletions have been linked to breast cancer and prostate cancer.

SET7/9 plays a complex and often contradictory role in the progression of breast cancer, functioning as either an oncogene or a tumor suppressor depending on the molecular landscape of the cell. One of its most significant roles is the epigenetic regulation of the Estrogen Receptor alpha (ER α) (33). By methylating ER α at the lysine 302 (K302) residue, SET7/9 stabilizes the receptor and enhances its transcriptional activity, thereby driving the proliferation of hormone-dependent cancer cells. This oncogenic function is further supported by its interaction with the transcription factor RUNX2, which facilitates tumor growth and invasion. Consequently, high expression levels of SET7/9 have been clinically correlated with shorter overall survival and a higher risk of relapse in specific patient cohorts, particularly those with HER2-enriched and basal-like subtypes (37).

Conversely, SET7/9 can exhibit tumor-suppressive characteristics by modulating DNA repair mechanisms and cellular metabolism. In certain contexts, SET7/9 inhibits the Gli-1 signaling pathway; a deficiency in the enzyme leads to increased tumor growth and metabolic reprogramming, such as enhanced glycolysis. Furthermore, the loss of SET7/9 expression has been linked to cisplatin resistance in aggressive breast cancers, including Triple-Negative Breast Cancer (TNBC). This resistance occurs because the absence of SET7/9 leads to the stabilization of PARP1, a protein critical for DNA damage repair, on both mRNA and protein levels. This finding has significant therapeutic implications, as combining cisplatin with FDA-approved PARP inhibitors like Niraparib (Zejula) has shown synergistic effects in overcoming resistance in SET7/9-deficient tumors (38).

The regulation of SET7/9 levels within the cell is primarily managed by the E3 ubiquitin ligase TRIM21, which targets SET7/9 for proteasomal degradation -depending on cell cycle progression. This regulatory axis suggests that the balance between TRIM21 and SET7/9 may dictate the clinical trajectory of the disease, with TRIM21 acting as a tumor suppressor by lowering SET7/9-mediated RUNX2 activation (37). Given its central role in ER α stabilization, SET7/9 is emerging as a potential pharmacological target. Notably, Cyproheptadine, an FDA-approved antihistamine, has been identified as a SET7/9 inhibitor capable of reducing ER α expression and inhibiting the growth of estrogen-responsive tumors (22,39). As research continues, the SET7/9-TRIM21 axis remains a critical focal point for developing personalized treatment strategies that account for the diverse molecular subtypes of breast cancer.

In prostate cancer (PCa), SET7/9 has been identified as a key regulator of androgen receptor (AR), the master transcription factor for prostate cell growth. SET7/9 catalyzes the monomethylation of the AR at lysine 632 (K632), a modification that is essential for the receptor's transcriptional potency. This methylation facilitates crucial inter-domain communication between the N- and C-termini of the AR, enhancing its recruitment to androgen-responsive elements (AREs) on target genes such as PSA and TMPRSS2 (23,40). Consequently, SET7/9 expression is significantly up-regulated in the nuclei of malignant prostate epithelial cells compared to benign tissue, and its presence is positively correlated with increased cell proliferation and anti-apoptotic signaling.

Beyond direct receptor modification, SET7/9 drives PCa progression through the epigenetic regulation of various non-histone proteins and chromatin modifiers. It methylates the histone demethylase KDM4A (also called JMDD2A), an interaction that stimulates KDM4A's oncogenic activity and its cooperation with ETS transcription factors like ETV1. This SET7/9- KDM4A axis promotes tumor invasion and metastasis, specifically by up-regulating downstream effectors like

NPM3(41). Additionally, SET7/9 methylates the pioneer factor FOXA1, which typically acts as a transcriptional repressor; the loss of SET7/9-mediated control over FOXA1 has been implicated in the reprogramming of the AR cistrome, further contributing to disease progression (24). Other targets include the ROR α 2 isoform, which, upon methylation by SET7/9, associates with the TIP60 coactivator complex to enhance the transcription of genes involved in tumor proliferation (42).

The therapeutic potential of targeting SET7/9 in prostate cancer is particularly promising in the context of Castration-Resistant Prostate Cancer (CRPC). Because SET7/9 is required for maximal AR function even in androgen-independent environments, its inhibition could bypass standard hormone therapy resistance. Small-molecule inhibitors, such as (R)-PFI-2, and the repurposed drug cyproheptadine have demonstrated the ability to disrupt SET7/9-mediated pathways, leading to reduced AR activity and inhibited tumor growth in preclinical models. Furthermore, because SET7/9-null mice show no major developmental defects, targeting this enzyme may offer a high therapeutic index with minimal impact on normal cells (43). As such, SET7/9 may be a valuable prognostic biomarker for aggressive prostate cancer and a viable target for next-generation epigenetic therapies.

1.1.3 Ubiquitylation

Histone lysine ubiquitylation (KUb) results in the ligation of ubiquitin, a 76 amino acid protein, to histone lysine residues via an amide bond formed with the lysine $N\epsilon$, and the carboxylate of an aspartate residue from ubiquitin. Ubiquitin is found throughout eukaryotic cells on many different proteins (hence the name) and has a variety of functions depending on the protein that is ubiquitinated. However, here we will focus on histone lysine ubiquitylation. KUb can happen singly at a single residue (mono) or multiple times at different residues (multi-mono), polymerize

at a single residue (poly) or from multiple polymers at different residues (multi-polyubiquitination) (44). Lysine residues on histone H2A and H2B are known to be monoubiquitinated. Such ubiquitination can interact or crosstalk with other histone modifications and eventually regulate gene transcription (45). For example, the association between histone H2BK120Ub and histone H3K79me has been well documented (46). KUb is triggered by the cooperation of three enzymes, namely: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. E3 ubiquitin ligase determines the substrate specificity of the reaction. It has been shown that NEDD4 is a ubiquitin E3 ligase that triggers ubiquitination on various proteins (47). Histone H3 ubiquitination has been shown to be vital for histone H3 K9, K14, K27 and K56 acetylation by recruitment of the KAT2A (also called GCN5) (47).

1.1.4 ADP Ribosylation

The transfer of one ADP-ribose moiety from NAD⁺ to specific amino acid residues of histones, releasing nicotinamide is known as mono ADP-ribosylation (48). Such mono ADP-ribosylated residues may be available for further ADP-ribosylation. This subsequent addition gives rise to poly ADP-ribosylated proteins and branching can be introduced with $\alpha(1'-2')$ ribose–ribose linkages (48). ADP-ribosylation is catalyzed by a group of enzymes named poly (ADP-ribose) polymerases (PARPs). Histone lysine, arginine and other amino acid residues can be modified with ADP Ribose (49). Histone residues that are known to be ADP-riboseylated include histones H2AK13, H2BK30, H3K27, H3K37 and H4K16, H1S7 and H1S188 (50,51). Three families of mammalian proteins that mono- and/ or poly-ADP-ribosylate arginine and lysine residues have been identified (49). The first family are called ARTDs (ADP-ribosyltransferases) because they possess sequence homology to bacterial diphtheria toxin. The second family, in their own case, show sequence homology to clostridial C2 and C3 toxin, hence the name ARTC (52). The third family are the

NAD⁺-dependent protein deacetylases, the sirtuins (53). ARTCs and sirtuins are thought to mediate mono-ADP-ribosylation, whereas among the ARTDs there are both mono- and poly-ADP-ribosyltransferases (48). The attachment of ADP-ribose reverses the positive charge on the lysine and arginine residues suggesting that ADP-ribosylation of histones may promote the euchromatin mode to facilitate the DNA repair process (54), replication and transcription (48). Poly-ADP-ribosylation of histones H3 and H4 has been reported to regulate their assembly (55).

1.1.5 Crotonylation

Histone lysine crotonylation (Kcr) is the transfer of crotonyl groups to lysine residues of histones. Like histone acetylation, histone lysine crotonylation occurs on the N ϵ - group of lysine but is distinguished from acetylation by its planar orientation and four-carbon length. It is also primarily associated with active transcription (56). Kcr could be important in the control of male germ cell differentiation (56) and might be implicated in some diseases (57,58). In addition to acetylation, p300 also possesses crotonyl transferase activity and histone crotonylation directly stimulates transcription to a greater degree than p300-catalyzed histone acetylation (59). Moreover, Sirtuin family deacetylases such as SIRT1 to SIRT3 remove Kcr in a site-specific manner (60,61). Some bromodomains like BRD9 and TAF1 can interact with Kcr, but with lower affinity than Kac (62). While AF9 YEATS domain displays selectively higher binding affinity for Kcr over Kac (63). Given these findings, the distinction between the functional consequences of histone Kac and Kcr is not very clear.

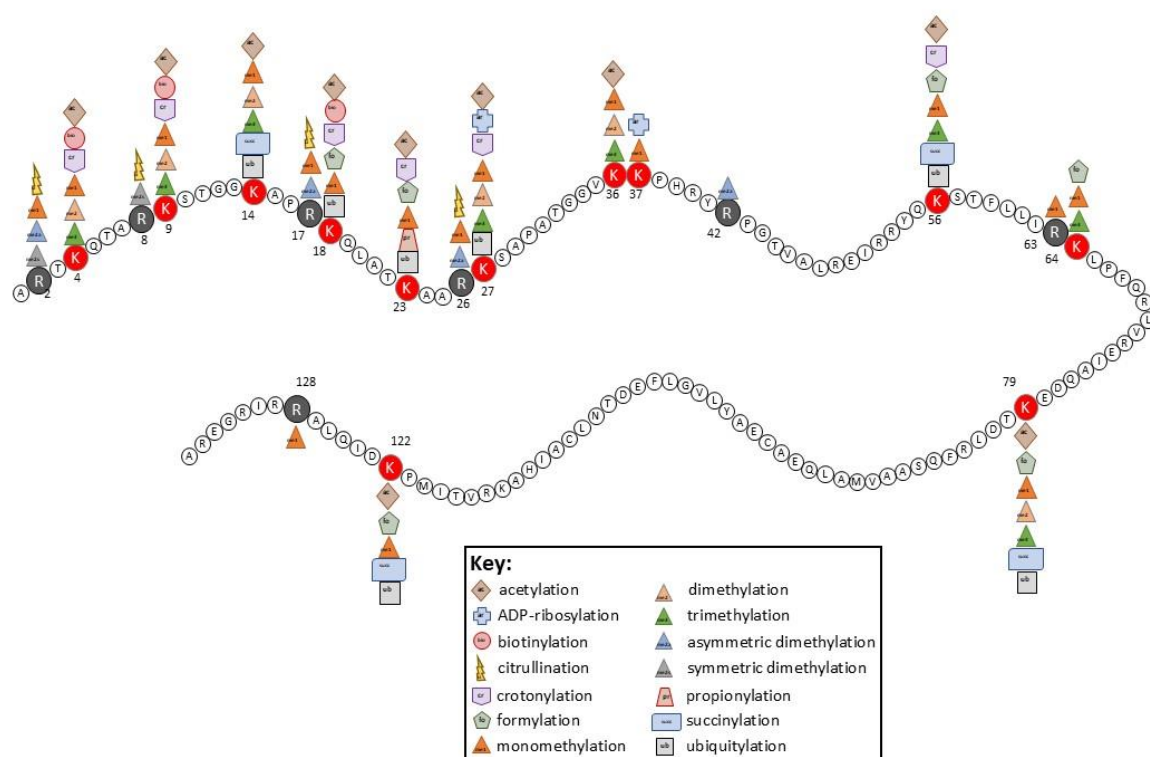


Figure 1.1. Modifications to Histone H3 Lysine and Arginine Residues.

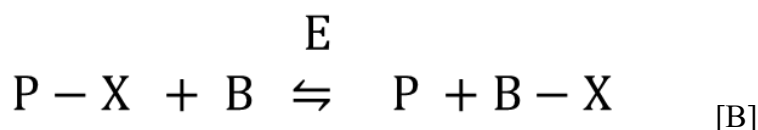
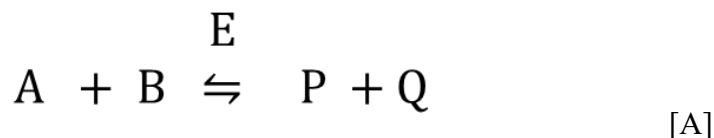
KMTs, PRMTs, and KATs govern the deposition of histone PTMs dictating transcriptional states, cell identity, and genome stability. Therefore, dysregulation of the enzymes that catalyze histone PTMs is strongly associated with cancer and other diseases, making them attractive targets for therapeutic intervention.

Importantly, histone-modifying enzymes are transferases and therefore operate through multi-substrate reaction mechanisms, typically requiring both a histone substrate and a small-molecule cofactor such as S-adenosyl-L-methionine or acetyl-CoA. As a result, their catalytic behavior cannot be fully understood using single-substrate kinetic models. Instead, defining the bi-substrate kinetic mechanism, is essential for understanding how substrates bind, how products are released, and how inhibitors interact with distinct enzyme states. This mechanistic knowledge directly informs rational drug design, as effective inhibitors must be tailored to the enzyme's kinetic

mechanism, binding order, and transition states. Consequently, an understanding of bi-substrate enzymatic mechanisms provides a necessary framework for interpreting histone PTM catalysis and for developing selective epigenetic drugs.

1.2 Kinetic Enzymatic Mechanisms of Bi-Bi substrate Reactions

Epigenetic writer enzymes require two substrates a donor molecule serving as a source of the modification and the target protein (histone) where the modification goes, and they yield two products, the remains of the donor molecule and the modified protein, t Since these writer enzymes utilize two substrates and produce two products their enzymatic mechanisms are known as Bi-Bi (substrate and product) reactions. Examples of such Bi-Bi epigenetic writers include arginine or lysine methyltransferases (PRMTs or KMTs) whose substrates include histone arginine or lysine residues and SAM, producing the products methylated histone residues and S-adenosyl-L-homocysteine (SAH) (Scheme 1). KATs whose substrates include histone lysine residues and AcCoA, produce acetylated histone residue and HS-CoA as products. Bi-Bi enzymatic mechanisms typically take the form shown in Scheme 1 where E is the enzyme, A (P-X) and B are the substrates and P and Q (B-X) the products.



Scheme 1. Bi-Bi enzymatic mechanisms. A and B are substrates and P and Q are products. P-X is another way of representing A and B-X another way of representing Q when describing transferase reactions like those catalysed by writers. In this case X represents the modification being transferred for example for KMTs and PRMTs X is the methyl-group P-X is SAM and B-X is the methylated protein. These reactions do not suggest an order to binding of substrates to, nor release of products from the enzyme.

1.2.1 Significance of Bi-substrate Kinetic Parameters

The kinetic parameters that appear in bi-substrate rate and double reciprocal equations describe distinct steps in enzyme catalysis. V_{Max} represents the maximal rate of reaction (max velocity) achieved when both substrates are present at saturating concentrations under steady-state conditions while v_o or initial reaction rate (initial velocity), is the rate at which products are formed. The Michaelis constants K_{MA} and K_{MB} correspond to the apparent substrate concentrations of A and B, respectively, required to achieve half-maximal velocity when the other substrate is saturating; these parameters reflect a combination of substrate binding and product release. In contrast, the equilibrium dissociation constants K_{SA} and K_{SB} describe the equilibrium binding affinities of substrates A and B for the free enzyme E and are defined by the ratio of dissociation to association rate constants. Unlike Michaelis constants, dissociation constants tell us exclusively about substrate–enzyme binding interactions and are independent of chemical catalysis. Turnover number (k_{cat}) refers to the maximum number of substrate molecules converted to products by an enzyme. . Enzyme-catalyzed bi-substrate reactions fall under two major categorizations (64).

1 Ping pong (Double displacement) Reactions

2 Sequential (Single displacement) Reactions

1.2.1.1 Ping pong Reactions

Ping pong reactions by one or more products being released before all substrates have been added. In these kinds of reactions, the substrates do not interact. They interact with the enzyme (E) individually. The group X is transferred from the first substrate A (B-X) to the enzyme making a covalent enzyme intermediated F ($F = E-X$) and the first product P is released (Ping). In the second

phase of the reaction, X transferred from the enzyme to the second substrate B to yield the second product Q ($Q = B-X$) which is then released by the enzyme (Pong) Figure 1.2 (65).

The rate equation for ping pong reactions is shown in Equation 1.1:

$$v_o = \frac{V_{\text{Max}}[A][B]}{K_{\text{MB}}[A] + K_{\text{MA}}[B] + [A][B]} \quad [\text{Equation 1.1}]$$

Where K_{MA} and K_{MB} are the Michaelis constants for substrates A and B respectively and V_{Max} is the maximal velocity of the enzyme obtained when both A and B are present at saturating concentrations (65).

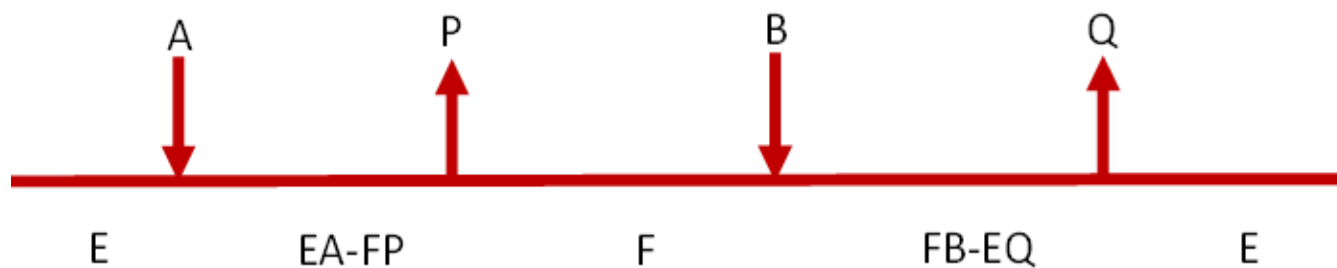


Figure 1.2. Cleland shorthand notation for a Double displacement Bi-Bi reaction.

If we rearrange the rate equation, we can get.

$$\frac{1}{v_o} = \frac{K_{\text{MA}}}{V_{\text{Max}}[A]} + \frac{K_{\text{MB}}}{V_{\text{Max}}[B]} + \frac{1}{V_{\text{Max}}}$$

Considering $\frac{1}{v_o}$ vs $\frac{1}{[A]}$ (Figure 1.3)

$$\frac{1}{v_o} = \frac{K_{\text{MA}}}{V_{\text{Max}}} \cdot \frac{1}{[A]} + \frac{K_{\text{MB}}}{V_{\text{Max}}[B]} + \frac{1}{V_{\text{Max}}}$$

With secondary plots, we can obtain the values of the constants.

The formula for the intercepts is

$$\frac{1}{V_{\text{Max}}} + \frac{K_{\text{MB}}}{V_{\text{Max}}[\text{B}]}$$

Therefore, plotting intercepts vs $\frac{1}{[\text{B}]}$ will look like

$$\text{Intercepts} = \frac{1}{V_{\text{Max}}} + \frac{K_{\text{MB}}}{V_{\text{Max}}} \cdot \frac{1}{[\text{B}]}$$

The properties of the graph will be

$$\text{intercept of intercepts} = \frac{1}{V_{\text{Max}}}$$

$$\text{slope of intercepts} = \frac{K_{\text{MB}}}{V_{\text{Max}}}$$

Therefore,

$$V_{\text{Max}} = \frac{1}{\text{intercept of intercepts}}$$

$$K_{\text{MB}} = V_{\text{Max}} \cdot \text{slope of intercepts}$$

However, the formula for slopes is

$$\frac{K_{\text{MA}}}{V_{\text{Max}}}$$

This is because the curves are parallel to each other and have the same slope. They are independent of the substrates' concentration.

$$K_{\text{MA}} = V_{\text{Max}} \cdot \text{slopes}$$

Similarly, for $\frac{1}{v_0}$ vs $\frac{1}{[\text{B}]}$ (Figure 1.3)

$$V_{\text{Max}} = \frac{1}{\text{intercept of intercepts}}$$

$$K_{\text{MA}} = V_{\text{Max}} \cdot \text{slope of intercepts}$$

The equation for slope in this case is $\frac{K_{\text{MB}}}{V_{\text{Max}}}$

$$K_{\text{MB}} = V_{\text{Max}} \cdot \text{slope}$$

Diagnostic Plots for Ping Pong Bi-Bi Reactions

The double reciprocal plots for double displacement Bi-Bi Reactions with one of the substrates kept constant, say [B] gives a straight line of slope $\frac{K_{\text{MA}}}{V_{\text{Max}}}$ with intercept $\frac{K_{\text{MB}}}{V_{\text{Max}}[B]} + \frac{1}{V_{\text{Max}}}$. Since the slope is independent of [B], plots for different values of [B] will yield plots of parallel lines. A plot of $\frac{1}{v_0}$ against $\frac{1}{[B]}$ for different values [A] likewise yields a group of parallel lines. These parallel lines are diagnostic of a Ping Pong or double displacement mechanism.

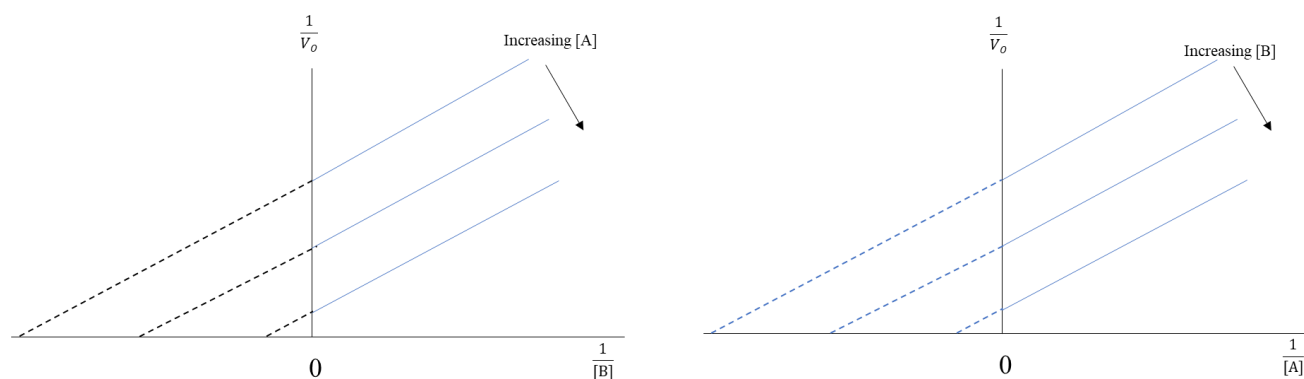


Figure 1.3. Diagnostic Plot for Ping Pong Reactions.

1.2.1.2 Sequential Reactions

Some Bi-Bi reactions involve sequential binding of the substrates to the enzyme forming a ternary complex before catalysis takes place. In this way the enzyme acts as a platform that brings the two substrates together for the reaction to take place and they are sometimes called single displacement reactions (66). With respect to transferases reactions, the group to be transferred, X is directly passed from A (P-X) to B, yielding P and Q (B-X) (Scheme 1B). In contrast to Ping Pong Bi-Bi reactions, the products of sequential Bi-Bi reactions are not released until all substrates bind to the enzyme. Sequential reactions can be further classified into (i) Ordered sequential mechanism and (ii) Random sequential mechanism.

Ordered sequential reactions are where there is an obligatory order to binding of substrates to, and dissociation of products from, the enzyme. Hence, the first substrate must bind to the enzyme before the second substrate can and the first product must dissociate before the second (Figure 1.4).

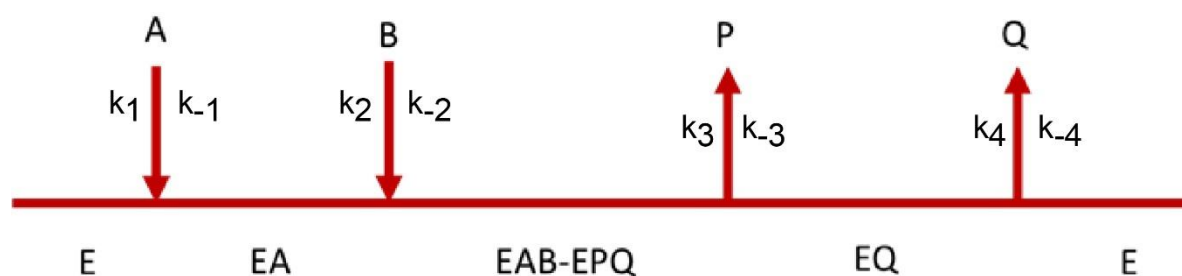


Figure 1.4. Cleland shorthand notation for an Ordered sequential Bi-Bi reaction. A and B are the first and second substrates to bind to the enzyme, respectively and P and Q are the first and second products to dissociate from the enzyme, respectively. The microscopic rate constants for the forward substrate association processes are k_1 , k_2 , the forward constants for the product dissociation processes are k_3 , k_4 the reverse constants for substrate dissociation processes are k_{-1} , k_{-2} , and the reverse constants for the product association processes are k_{-3} , k_{-4} .

In random sequential mechanisms, there is no preference for the order of substrate binding to the enzyme. There is no compulsory order of substrate addition to the enzyme such that either substrate may bind first, and the ternary complex forms through more than one pathway (Figure 1.5)

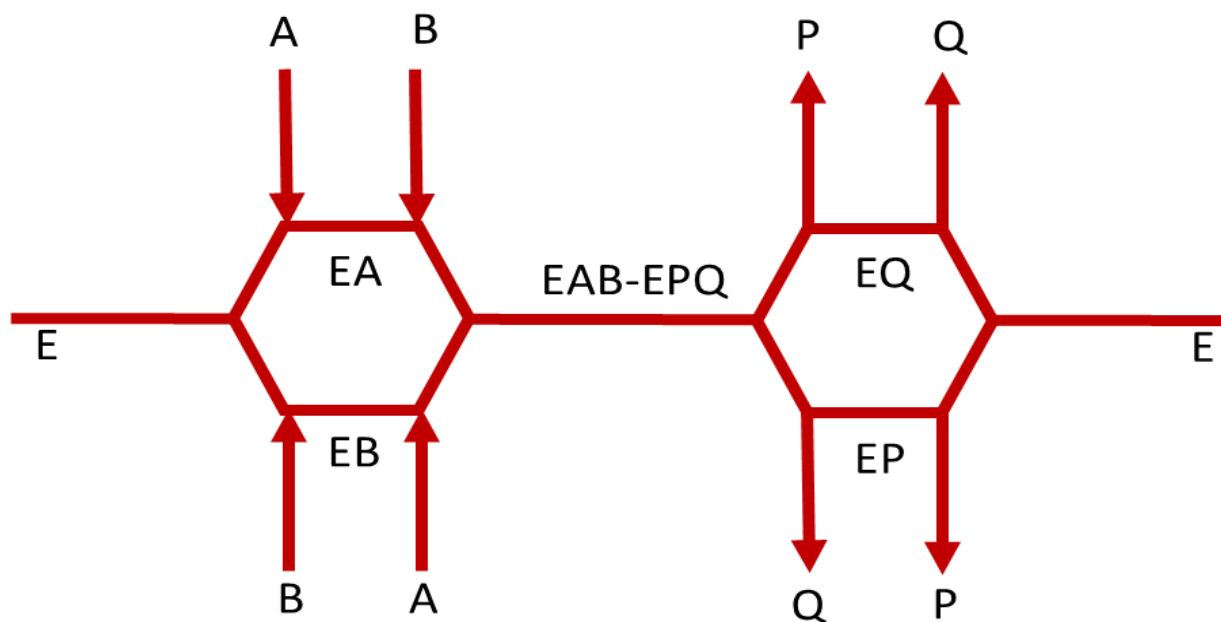


Figure 1.5. Cleland shorthand notation for a Random Single displacement Bi-Bi reaction

The initial rate equation for a sequential reaction is shown in Equation 1.2. The equation applies to the ordered sequential Bi-Bi enzymatic mechanism reaction under steady state conditions where $d[EAB]/dt = 0$. The parameter $K_{SA} = [E][A]/[EA] = k_{-1}/k_1$ is a simple equilibrium dissociation constant. The Michaelis constants are best described by the microscopic rate constants (Figure 1.4) as follows: $K_{MA} = (k_3 k_4)/(k_1(k_3 + k_4))$ and $K_{MB} = ((k_{-2} + k_3)k_4)/(k_2(k_3 + k_4))$. Therefore, for the ordered sequential mechanism K_{MA} and K_{MB} reflect dissociation of the enzyme substrate complex to both free substrates and products after catalysis. This means that these constants do not simply reflect substrate binding affinity but include catalysis and product affinity.

If the steady state assumption is made, the equivalent equation for the random sequential kinetic mechanism has been described as “far too complicated for the unambiguous experimental determination of key rate parameters” since this equation has 18 constants (67). Thankfully previous studies have shown that the steady state random sequential kinetic mechanism is almost never observed, and the rapid equilibrium assumption can usually be made (68). To invoke the rapid equilibrium assumption, the binding of substrates must be governed by equilibrium dissociation constants rather than Michaelis constants and the rate limiting step of the mechanism is the catalytic conversion of EAB to EPQ. Interestingly when this is done, the rate equation for the random sequential Bi-Bi enzymatic mechanism is the same as Equation 1.2 except that all constants are equilibrium dissociation constants. Therefore, for a rapid equilibrium random sequential kinetic mechanism $K_{SA} = [E][A]/[EA]$, $K_{MB} = [EA][B]/[EAB]$, $K_{MA} = [EB][A]/[EAB]$ and K_{SB} (see below) $= [E][B]/[EB] = K_{SA}K_{MB}/K_{MA}$.

$$v_o = \frac{v_{\text{Max}}[A][B]}{K_{SA} K_{MB} + K_{MB} [A] + K_{MA} [B] + [A][B]} \quad [\text{Equation 1.2}]$$

Diagnostic Plot for Sequential Reactions

Equation 1.2 which represents the ordered sequential mechanism and the rapid equilibrium random sequential mechanism can be rearranged to give to give a reciprocal plot where $[A]$ varies with fixed $[B]$ (Equation 1.3).

$$\frac{1}{v_o} = \frac{K_{MA}}{v_{\text{Max}}} \left(\frac{K_{SA}K_{MB}}{[B]K_{MA}} + 1 \right) \frac{1}{[A]} + \frac{1}{v_{\text{Max}}} \left(\frac{K_{MB}}{[B]} + 1 \right) \quad [\text{Equation 1.3}]$$

Equation 1.3 shows that $[B]$ affects the slope and the intercept of the plot. Therefore plotting

$\frac{1}{v_o}$ against $\frac{1}{[A]}$ with constant $[B]$ yields a linear plot with a slope equal to $\frac{K_{MA}}{v_{\text{Max}}} \left(1 + \frac{K_{SA}K_{MB}}{K_{MA}[B]} \right)$ and an

intercept on the $\frac{1}{v_0}$ axis equal to $\frac{1}{V_{\text{Max}}} \left(1 + \frac{K_{\text{MB}}}{[\text{B}]}\right)$. Equation 1.3 can be rearranged to Equation 1.4

$$\frac{1}{v_0} = \frac{K_{\text{MB}}}{V_{\text{Max}}} \left(\frac{K_{\text{SA}}}{[\text{A}]} + 1 \right) \frac{1}{[\text{B}]} + \frac{1}{V_{\text{Max}}} \left(\frac{K_{\text{MA}}}{[\text{A}]} + 1 \right) \quad [\text{Equation 1.4}]$$

Plotting $\frac{1}{v_0}$ versus $\frac{1}{[\text{A}]}$ is also linear with the slope equal to $\frac{K_{\text{MB}}}{V_{\text{Max}}} \left(1 + \frac{K_{\text{SA}}}{[\text{A}]}\right)$ and intercept,

$$\frac{1}{V_{\text{Max}}} \left(1 + \frac{K_{\text{MA}}}{[\text{A}]}\right).$$

For the particular case of the random sequential kinetic mechanism, plotting $\frac{1}{v_0}$ vs $\frac{1}{[\text{A}]}$ for constant $[\text{B}]$ yields an equation like Equation 1.3 except that $K_{\text{SB}} = [\text{E}][\text{B}]/[\text{EB}] = K_{\text{SA}}K_{\text{MB}}/K_{\text{MA}}$ and can be written as Equation 1.5.

$$\frac{1}{v_0} = \frac{K_{\text{MA}}}{V_{\text{Max}}} \left(\frac{K_{\text{SB}}}{[\text{B}]} + 1 \right) \frac{1}{[\text{A}]} + \frac{1}{V_{\text{Max}}} \left(\frac{K_{\text{MB}}}{[\text{B}]} + 1 \right) \quad [\text{Equation 1.5}]$$

Accordingly, for the random sequential kinetic mechanism, and assuming rapid equilibrium binding, it is possible to write Equation 1.2 as Equation 1.6 to represent all equilibrium dissociation constants K_{SA} , K_{MB} , K_{MA} , and K_{SB} . However, it must be noted that it is impossible to define K_{SB} , (as it is described above) for an ordered sequential mechanism since it implies that B can bind to the enzyme first or in the absence of A. Therefore, the equations that govern the rapid equilibrium random sequential kinetic mechanism and the steady state ordered sequential kinetic mechanism take the same form but the constants K_{MB} , and K_{MA} , define fundamentally different parameters. In an ordered sequential kinetic mechanism K_{MB} , and K_{MA} are Michaelis constants that include processes that result in the dissociation of EAB and EPQ into products

$$v_0 = \frac{V_{\text{Max}}[\text{A}][\text{B}]}{K_{\text{SB}} K_{\text{MA}} + K_{\text{MB}} [\text{A}] + \frac{K_{\text{SA}} K_{\text{MB}}}{K_{\text{SB}}} [\text{B}] + [\text{A}][\text{B}]} \quad [\text{Equation 1.6}]$$

Regardless, if the mechanism of substrate binding is random or ordered, a reciprocal plot of varying concentrations of one substrate at fixed concentrations of the other, produces a characteristic series of lines that intersect to the left of the $\frac{1}{v_0}$ axis. This pattern of lines is indicative of a sequential mechanism and is easily distinguished from a ping-pong mechanism but this pattern cannot determine if the mechanism is ordered sequential or random sequential (Figure 1.6).

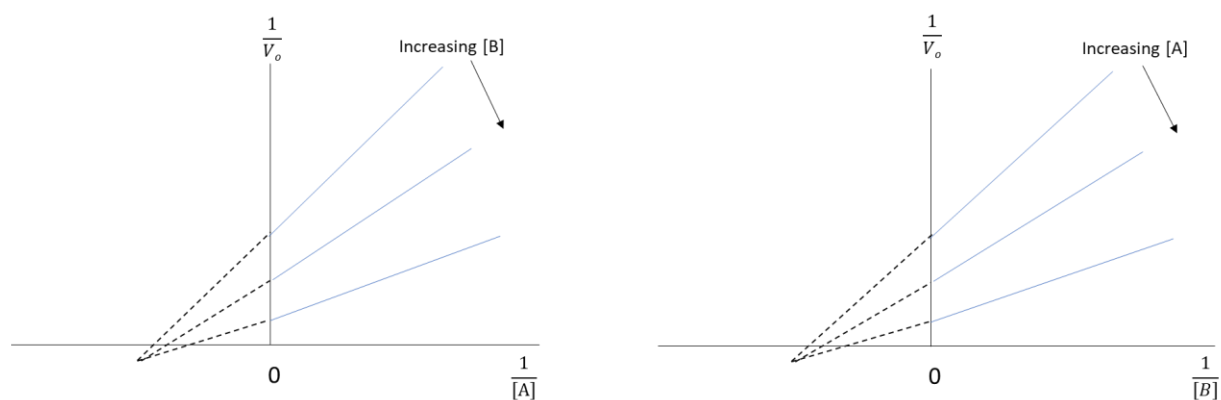


Figure 1.6. Diagnostic Plot for Sequential Reactions.

1.2.1.3 Differentiating Random and Ordered Sequential Mechanisms

In a rapid-equilibrium random sequential mechanism, both substrates (A and B) and both products (P and Q) bind to the free enzyme in a random fashion. As a result, both P and Q can act as competitive inhibitors of both substrates: P competes with A when B is held constant, and Q competes with B when A is held constant. This symmetric competitive inhibition pattern is a hallmark of a random sequential mechanism and reflects the fact that both substrates and products can access the same enzyme form.

In contrast, an ordered Bi-Bi mechanism requires substrates to bind in a defined sequence, typically with A binding first to form EA, followed by B. Product release is also ordered, with one product (usually P) leaving before the other (Q). Because of this obligatory order, the inhibition patterns become asymmetric. Product Q, which often structurally resembles the first substrate A, competes directly with A for binding to the free enzyme and therefore acts as a competitive inhibitor of A when B is held constant. However, Q does not compete with B for free enzyme, since B only binds after A has already bound. Instead, Q can bind to the EA complex, interfering with both B binding and catalysis, which makes Q a mixed inhibitor of B when A is held constant. Product P, on the other hand, binds only to later enzyme forms in the catalytic cycle rather than to free enzyme, so it behaves as a mixed inhibitor of both A and B.

This difference in inhibition patterns is summarized in the Table 1.1 for an ordered mechanism, P shows mixed inhibition with respect to both substrates, while Q is competitive with A and mixed with B; for a rapid-equilibrium random mechanism, both P and Q are competitive inhibitors of both A and B. These distinct patterns provide a practical way to distinguish random from ordered sequential mechanisms using product inhibition experiments (69).

Table 1.1. Differentiating Random and Ordered Sequential Mechanisms

Mechanism	Product Inhibitor	A Variable	B Variable
Ordered	P	Mixed	Mixed
	Q	Competitive	Mixed
Rapid Equilibrium Random	P	Competitive	Competitive
	Q	Competitive	Competitive

1.3 Objectives

Primary Objective

The primary objective of this research was to define the catalytic activity, substrate specificity, and kinetic mechanisms of the histone lysine methyltransferases SET7/9 (KMT7) and DOT1L (KMT4), and to define their mechanistic roles in epigenetic regulation and cancer biology.

Specific Objectives

1. Preparation of Histone Substrates and KMT Enzymes

To develop a rapid and efficient method for the purification of recombinant histones, and to establish optimized procedures for the production and purification of recombinant SET7/9 and DOT1L enzymes. These enzymes and histone substrates will provide the experimental foundation for biochemical and kinetic analyses of KMT activity.

2. Development of Methods for Measuring KMT Activity

To develop and optimize analytical methods for measuring KMT activity, including the detection of methylated lysine residues and the quantification of methylation products. These methods will be used to evaluate substrate preferences, residue selectivity, methylation patterns, and the influence of chromatin structure and pre-existing histone modifications on enzyme activity.

3. Characterization of Substrate Specificity and Enzymatic Kinetic Mechanisms

To characterize the substrate specificity and catalytic mechanisms of SET7/9 and DOT1L using substrate profiling, initial-rate kinetic analysis, and product inhibition studies. These experiments will define catalytic pathways, substrate binding order, product release mechanisms, and the kinetic mechanisms of these enzymes.

Understanding whether a Bi-substrate enzyme follows a Ping-Pong or Sequential mechanism is central to mechanistic enzymology. It shapes how inhibitors behave, how catalytic efficiency is interpreted, and how structural states relate to dynamic binding steps. These distinctions form the foundation of accurate kinetic modelling and mechanism-based drug design.

Because histone methyltransferases are SAM-dependent Bi substrate enzymes, understanding Bi–Bi kinetics mechanisms are essential for interpreting how enzyme inhibitors are developed and used in the management of diseases. In Chapter 2, we look at a disease in which a histone methyltransferase is implicated (Mixed Lineage Leukemia).

Chapter 2: Pleiotropic effects of drugs targeting epigenetic processes in Mixed Lineage Leukemia

2.0 Abstract

Histone lysine methylation regulates gene expression and chromatin structure, with major roles in development and disease. In mixed lineage leukemia (MLL), KMT2A fusion proteins aberrantly recruit DOT1L to activate HOXA genes, promoting leukemogenesis. DOT1L inhibitors like pinometostat show therapeutic promise but risk off-target effects due to DOT1L's broader roles in immune function, cardiac health, and thermogenesis. Similarly, EZH2 inhibition can impact fertility and immune responses. This review highlights the need for selective, context-specific epigenetic therapies to minimize systemic toxicity and maximize efficacy, underscoring the importance of balancing therapeutic impact with physiological safety.

2.1 Epigenetics and Histones

Epigenetics is the study of heritable changes in gene expression that are not directly caused by the DNA sequence itself. Epigenetics is involved in cellular processes, orchestrating development, differentiation, and preserving cellular identity. Key epigenetic mechanisms such as DNA methylation, hydroxymethylation, histone modifications, and non-coding RNAs (lncRNA) regulate chromatin structure and tune gene expression based on cell type and placement within the cell cycle, and response to environmental stimuli (70). Among the myriad epigenetic modifications, histone lysine methylation stands out for its intricate role in modulating gene expression, making it a promising frontier for groundbreaking advancements. Our focus in this review is driven by the limited research in this area, presenting an untapped opportunity for novel insights into drug development and disease management. Through this lens, we aim to shed light on its immense therapeutic potential and underscore its significance in epigenetic research.

Histones package eukaryotic DNA into a condensed form, facilitating genomic stability and regulation. The histone octamer, composed of two copies each of histones H2A, H2B, H3, and H4, forms the core around which approximately 147 base pairs of DNA are wrapped to create a nucleosome, the fundamental unit of chromatin (35,71). The positively charged histones interact with the negatively charged phosphate backbone of DNA, stabilizing the structure through electrostatic interactions (72). Chromatin generally exists in two forms: heterochromatin, which is tightly packed and transcriptionally silent, and euchromatin, which is not tightly packed and is transcriptionally active. Histone PTMs such as acetylation, phosphorylation, and methylation modulate chromatin structure. PTMs, regulating the accessibility of transcriptional machinery to DNA. Histone PTMs not only control chromatin compaction but also serve as binding sites to recruit transcription factors, chromatin remodelers, co- activators, and co- repressors (73). For instance, histone lysine acetylation neutralizes the positive charge of lysine residues, thereby weakening electrostatic interactions between histones and DNA (74). This reduction in charge promotes chromatin relaxation, increasing accessibility to transcriptional machinery and facilitating gene expression. More importantly, acetylated lysines serve as binding sites for reader domains within transcriptional complexes, promoting transcriptional activation.

Not all histone modifications alter charge. Modifications such as methylation can sterically block H-bonding or other interactions with DNA but are primarily involved in binding to reader domains (73).

2.2 Histone Methylation and Demethylation

Histone methylation is involved in regulating chromatin structure and gene expression without altering the net charge of histone residues. Both lysine (K) and arginine (R) residues in histones can be methylated. Prominent methylation sites on histone H3 include residues such as K4, K9,

K27, K36, and K79, while key methylation sites on histone H4 include K20 and R3. Lysine residues can be mono-, di-, or tri-methylated, while arginine residues can be mono- or symmetrically/asymmetrically di-methylated. Importantly, lysine methylation is reversible, in contrast to arginine methylation, where some types are thought to be irreversible, though this is debated (74).

Two eraser histone lysine demethylases primarily catalyze histone lysine demethylation: lysine-specific demethylase 1 (LSD1), a flavin-dependent monoamine oxidase, and the JmjC domain-containing demethylases, which employ a Fe (II)- and α -ketoglutarate-dependent oxidative mechanism to remove methyl groups from lysine residues (75). These enzymes mediate the dynamic regulation of chromatin states by adding and removing methyl groups (Figure 2.1).

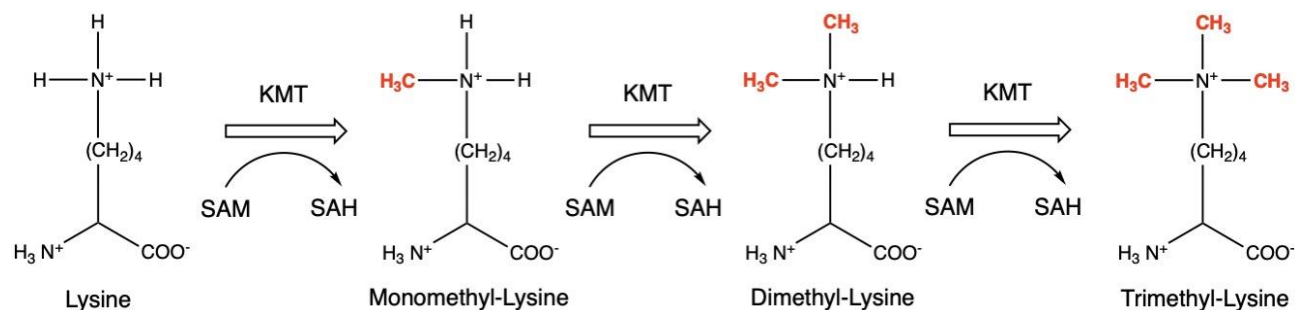


Figure 2.1. Histone Lysine Methylation. Histone Lysine methyltransferases can catalyze the addition of up to three methyl groups in the presence of S-Adenosyl Methionine.

Histone methylation is catalyzed by the writers HMTs sometimes more broadly called protein methyltransferases PMTs. These enzymes transfer methyl groups from the co-factor SAM to the $\text{N}\epsilon$ - group of lysine, or the terminal $\text{N}\omega$ group of arginine residues on histone tails, which are the flexible, N-terminal extensions of histone that collapse into the nucleosome core (76). PMTs are classified into two major groups: protein arginine methyltransferases (PRMTs), which catalyze arginine methylation, and protein or histone or lysine methyltransferases (PKMT or HMT or

KMT), which catalyze lysine methylation (77). KMTs are further divided into two subgroups: those that contain a SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain and those that do not (78).

The SET domain, composed of ~130 amino acids, forms the catalytic core of the SET domain containing KMTs. It binds to both SAM and the histone substrate, facilitating methyl group transfer through a nucleophilic attack on the methyl group of SAM by the lysine N ϵ group. A critical tyrosine residue within the SET domain deprotonates the lysine, promoting the nucleophilic attack (Figure 2.2). SET domain-containing KMTs form the largest family of histone methyltransferases. There are a few non-SET domain-containing KMT that target histones but the most relevant to this discussion is DOT1L. DOT1L methylates histone H3 at K79 in the globular domain, a process that is unique to this enzyme and is restricted to being in the context of nucleosomes, which provides additional substrate specificity (79,80). Histone methylation can lead to either transcriptional activation or repression depending on the methylated residue, its methylation state (mono-, di-, or tri-methylation), and the associated protein complexes. For example, tri-methylation of histone H3K4 is generally associated with active transcriptional promoters, whereas mono- and di-methylation of histone H3K4 is often found at enhancer regions of actively transcribed genes (81). The methylation of histone H3K4 facilitates the recruitment of the general transcription factor TFIID, which in turn promotes RNA polymerase II binding and transcription initiation (80)

Conversely, histone H3K9me₃, H3K27me₃, and H4K20me₃ are often associated with transcriptional repression, but this depends on where the mark is found (e.g. gene body vs promoter) and the context of other modifications and proteins. For instance, H3K27me₃ by Polycomb Repressive Complex 2 (PRC2) silences gene expression, but in combination with

histone H3K4me3 the gene is said to be in a poised state where it is not expressing but ready to be rapidly activated for expression. Gene body H4K20me1 is associated with active gene expression but centromeric H4K20me3 is repressive (82). Furthermore, histone H3K36 methylation is enriched in gene bodies and is involved in transcription elongation, co-transcriptional mRNA splicing, and the stabilization of nucleosomes following transcription, but its roles differ slightly depending exactly where in the gene body and the number of methyl groups (80).

The SET domain-containing KMTs exhibit diverse substrate specificity and functional roles that included histone and non-histone targets. However, in this manuscript we will focus on histone modifications. For example, SUV39 family KMTs, characterized by conserved cysteine-rich pre-SET and post-SET domains, catalyze histone H3K9 di and tri-methylation H3K9me2 and me3, typically involved in transcriptional repression and heterochromatin formation. In contrast, the SET1 and SET2 families catalyze histone H3K4me3 and histone H3K36 methylation, respectively, with the former associated with transcriptional activation and the latter with transcription elongation and mRNA processing (83). EZH1/2, members of the EZH family of KMTs, catalyze histone H3K27me2/me3 as part of the PRC2 complex. Unlike most KMTs, EZH1/2 lack pre- and post-SET domains but contain a conserved CXC domain critical for their methyltransferase activity, which is only active in the context of the PRC2 complex (83). The SUV420H family of KMTs catalyze histone H4K20me2/me3, which are associated with active gene expression (82). These enzymes contain only the SET and post-SET domains (84). The SMYD family of KMTs exhibits unique structural configurations, containing a MYND domain and a SET-I motif. SMYD family members, such as SMYD1 and SMYD2, interact with proline-rich motifs in binding partners via the MYND domain, whereas SMYD3 is involved in DNA binding (85). The PRDM family of KMTs contains a catalytic PR/SET domain. Although they differ in sequence from other

SET domains, they adopt a similar tertiary structure. SETD7, SETD8, and DOT1L represent other important KMTs, with SETD7 catalyzing histone H3K4me1 and SETD8 catalyzing histone H4K20 me1(86).

Arginine methylation also contributes to transcriptional regulation. Methylation of histone H3R2, R16, and R26 by coactivator-associated arginine methyltransferase 1 (CARM1) or protein arginine *N*-methyltransferase 4 (PRMT4) is associated with transcriptional activation, whereas arginine methylation on other residues can repress transcription, depending on the location. For example, methylation of histone H4 at R3 promotes downstream histone acetylation, further enhancing transcriptional activation (80).

Histone methylation is a highly dynamic and context-dependent epigenetic modification critical in regulating chromatin architecture and gene expression. The complex network of histone methyltransferases, associated demethylases, and effector proteins provides control over gene expression, chromatin remodeling, and, eventually, cellular function. Anomalies in these mechanisms and their far-reaching effects are involved in many diseases, including cancer and developmental disorders (87). An example is mixed lineage leukemia, whose etiology is strongly linked with anomalies in histone methylation (88).

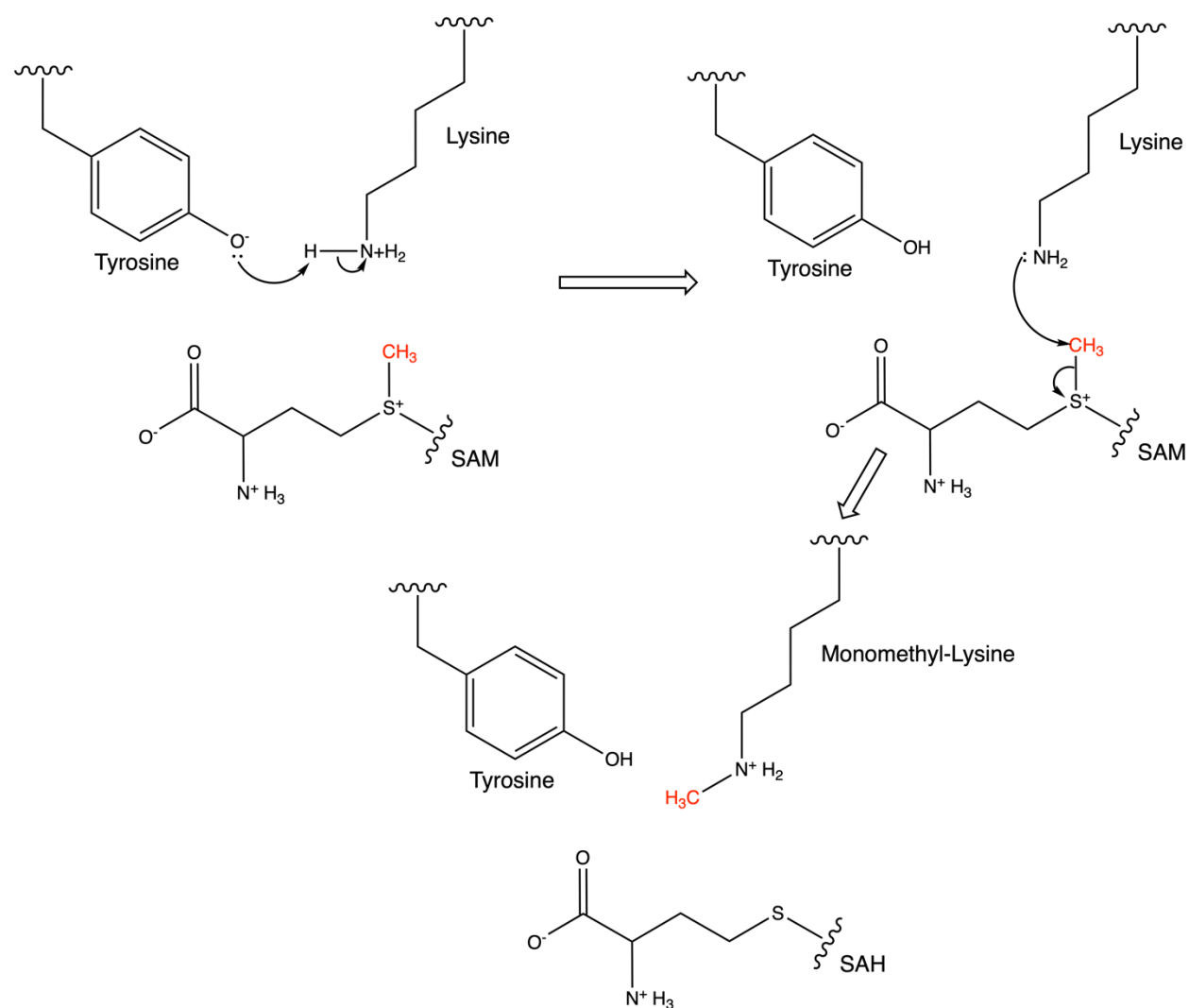


Figure 2.2. Catalytic Mechanism of a SET Domain Protein Methyltransferase.

2.3 Mixed Lineage Leukemia, History, Etiology, and the Role of Histone Methylation

A distinct subset of patients initially diagnosed with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML) exhibit significantly worse clinical outcomes compared to other leukemia cases. This group includes infants under one year of age diagnosed with ALL, who demonstrate aggressive disease progression and poor survival rates (88). Leukemic blasts, in these cases, frequently co-express markers from both the lymphoid and myeloid lineages. Sometimes, complete lineage switching is observed during treatment, with leukemias initially diagnosed as

ALL relapsing as AML (89). This observation led to the designation of these leukemias as mixed lineage leukemia (MLL) (90).

Even before this designation, cytogenetic studies identified specific chromosomal translocations in these leukemias, most notably translocations involving chromosome 11 at locus 11q23, such as t(4;11)(q21;q23) (91). These translocations are characteristic of a subset of ALL associated with poor prognosis (92,93). It has since been established that translocations of the 11q23 locus are a hallmark of MLL. Although treatment of pediatric leukemias has significantly improved, with five-year survival rates approaching 90%, MLL patients, especially infants, continue to have poor outcomes, with survival rates around 40% (94).

A second peak of MLL incidence is observed in older patients, often those previously treated with topoisomerase II inhibitors. These therapy-related leukemias, known as therapy-related AML (t-AML) or ALL (t-ALL), share common genetic alterations with MLL. Approximately 70% of infant ALL and 10% of all other ALL cases are associated with 11q23 abnormalities (95). Therapy-related leukemia manifests primarily as AML, with 11q23 translocations present in around 10% of therapy-related AML and 3% of de novo AML cases (96).

KMT2A is a large, ubiquitously expressed multi-domain protein involved in hematopoiesis, including in stem and progenitor cells. It functions as part of a nuclear complex that includes proteins with diverse roles in transcriptional regulation (97). KMT2A was cloned from the breakpoint region at 11q23 (98,99), and its involvement in leukemia led to its designation as mixed lineage leukemia (MLL) protein. The functional KMT2A protein is produced through post-translational cleavage, yielding a 180-kDa C-terminal subunit (KMT2A^C) and a 320-kDa N-terminal subunit (KMT2A^N), which reassociate to form the core KMT2A complex (100–102).

KMT2A^N contains AT hook DNA-binding domains that help to guide the complex to its genomic targets. Moreover, a CxxC domain is present which binds to unmethylated CpG sequences, this facilitates the recruitment of histone deacetylases (HDACs) and other transcriptional regulators (103,104). KMT2A^N also recruits the tumor suppressor protein Menin, which is involved in transcriptional activation through interactions with lens epithelium-derived growth factor (LEDGF) (105). The C-terminal subunit KMT2A^C contains a SET domain responsible for catalyzing H3K4me1/me2/me3, (106,107).

KMT2A^C is essential in promoting efficient transcription by recruiting additional proteins that facilitate transcriptional activation. For instance, KMT2A catalyzes H3K4me3, which enhances transcriptional activation (108,109). It also aids the recruitment of other factors, such as KAT8 [MOF (Males absent on the first)], specifically acetylating histone H4K16, which contributes to chromatin decondensation and promotes transcription (110,111).

Transcription factors such as p53 and β -catenin are known to recruit the KMT2A complex to initiate transcription (94). Additionally, the tumor suppressor protein Menin, encoded by *MEN1*, binds to KMT2A through an RXRFP motif within the first 10 amino acids of KMT2A. Menin and KMT2A colocalize at the promoter of *HOXA9*, a gene essential for hematopoietic differentiation, and in the absence of Menin, KMT2A and its fusion proteins fail to regulate *HOXA9* expression properly (112). Dysregulation of *Hox* genes, particularly *HOXA9*, is a critical factor in KMT2A-driven leukemogenesis (113).

HOXA genes, belonging to the homeobox (*HOX*) gene family, are essential regulators of development and differentiation in normal cell lines. These genes encode transcription factors that play a pivotal role in controlling the expression of downstream genes responsible for critical processes such as patterning, segmental identity, and tissue-specific development during

embryogenesis (114). *HOXA* genes play a critical role in cellular differentiation in normal cell lines by guiding stem or progenitor cells to develop into specific cell types, ensuring proper tissue formation. They are also involved in spatial organization, establishing correct positioning and structural alignment of tissues along the anterior-posterior axis (115).

HOXA genes regulate cellular proliferation, maintaining tissue homeostasis and preventing uncontrolled cell division (116). They support tissue repair and regeneration by facilitating cell renewal and repair mechanisms after injury, enabling effective recovery and restoration of tissue functionality (115). Dysregulation of *HOXA* genes can disrupt these processes, leading to developmental abnormalities or contributing to oncogenesis. Such dysregulation has been implicated in cancer and other diseases, emphasizing the importance of maintaining proper *HOXA* gene function (117).

Chromosomal translocations of 11q23, which disrupt the *KMT2A* gene, result in fusion proteins that eliminate the methyltransferase activity of *KMT2A*^C while retaining the DNA-binding capacity of *KMT2A*^N. These translocations occur in a small breakpoint cluster region and result in the in-frame fusion of *KMT2A* with various partner genes (118). These fusion events are thought to occur through mechanisms that vary with age. As mentioned above, exposure to topoisomerase inhibitors such as etoposide can lead to double-strand DNA breaks, with the 11q23 locus being particularly vulnerable (119). Alternatively, DNA breaks may occur during the early stages of apoptotic fragmentation, and subsequent aborted repair processes result in translocations, as evidenced by the prevalence of 11q23 breaks in early apoptotic cells (120).

2.4 *Hox* genes and the role of DOT1L in KMT2A

HOXA proteins, particularly HOXA9 and its dimerization partner MEIS1, are key hematopoietic oncoproteins overexpressed in various leukemias, including those with KMT2A rearrangements (121). Typically, HOXA transcription factors are master regulators of embryonic development and play an essential role in normal hematopoietic differentiation. *HOXA* expression is high in stem cells and early progenitors and must be downregulated for proper maturation. Regardless of the fusion partner, the deregulation of *Hox* genes, particularly HOXA9, is considered the key driver of leukemogenesis in KMT2A-associated leukemias. Therefore, continuous ectopic expression of *HOXA* genes blocks differentiation, leading to a rapidly proliferating pool of pre-leukemic precursor cells. This is consistent with evidence that inhibiting excessive *HOXA* expression is a promising therapeutic strategy for KMT2A-associated leukemias (121). Previous studies have shown that reversing overexpression of *HOXA9* with shRNA in cell lines harboring the *KMT2A-MLLT3* translocation is alone sufficient to force differentiation and arrest growth, eventually leading to cell death (122). This suggests that reducing *HOXA9* expression or activity is sufficient to treat the *KMT2A-MLLT3* translocation-associated leukemias (123,124).

KMT2A fusion leukemias, can form between KMT2A^N and other proteins such as AF4/FMR2 family member 1 (AFF1) and MLL Translocated To 3 (MLLT3) formerly known as ALL1-Fused gene from chromosome 4 (AF4) and ALL1-Fused gene from chromosome 9 (AF9), respectively. These fusion proteins recruit DOT1L to the *HOXA9* locus. This ultimately results in the addition of histone H3K79 methylation to histone H3K4 methylation with, leading to the abnormal overexpression of *HOXA9* and subsequent leukemogenesis (125) (Figure 2.3).

DOT1L catalyzes the mono-, di, and tri-methylation of histone H3 at lysine 79 (H3K79), (126). The di- and trimethylated form of histone H3K79 (H3K79me_{2/3}) are associated with transcription downstream of the TSS and have been implicated in various cellular processes, including DNA repair mechanisms, embryogenesis, cell cycle progression, hematopoiesis, in orchestrating immune cell formation and function and cardiac function (127). As noted, DOT1L interacts with several KMT2A fusion proteins, which are characteristic of KMT2A-rearranged leukemias (128) which yield increased H3K79me_{2/3} at KMT2A target genes which would otherwise catalyze histone H3K4me_{1/2/3} (129,130). Therefore, DOT1L has emerged as a promising therapeutic target for KMT2A-rearranged leukemias. DOT1L inhibitors, reduce histone H3K79me_{2/3} resulting in reduced *HOXA9* expression and leukemic cell death (124).

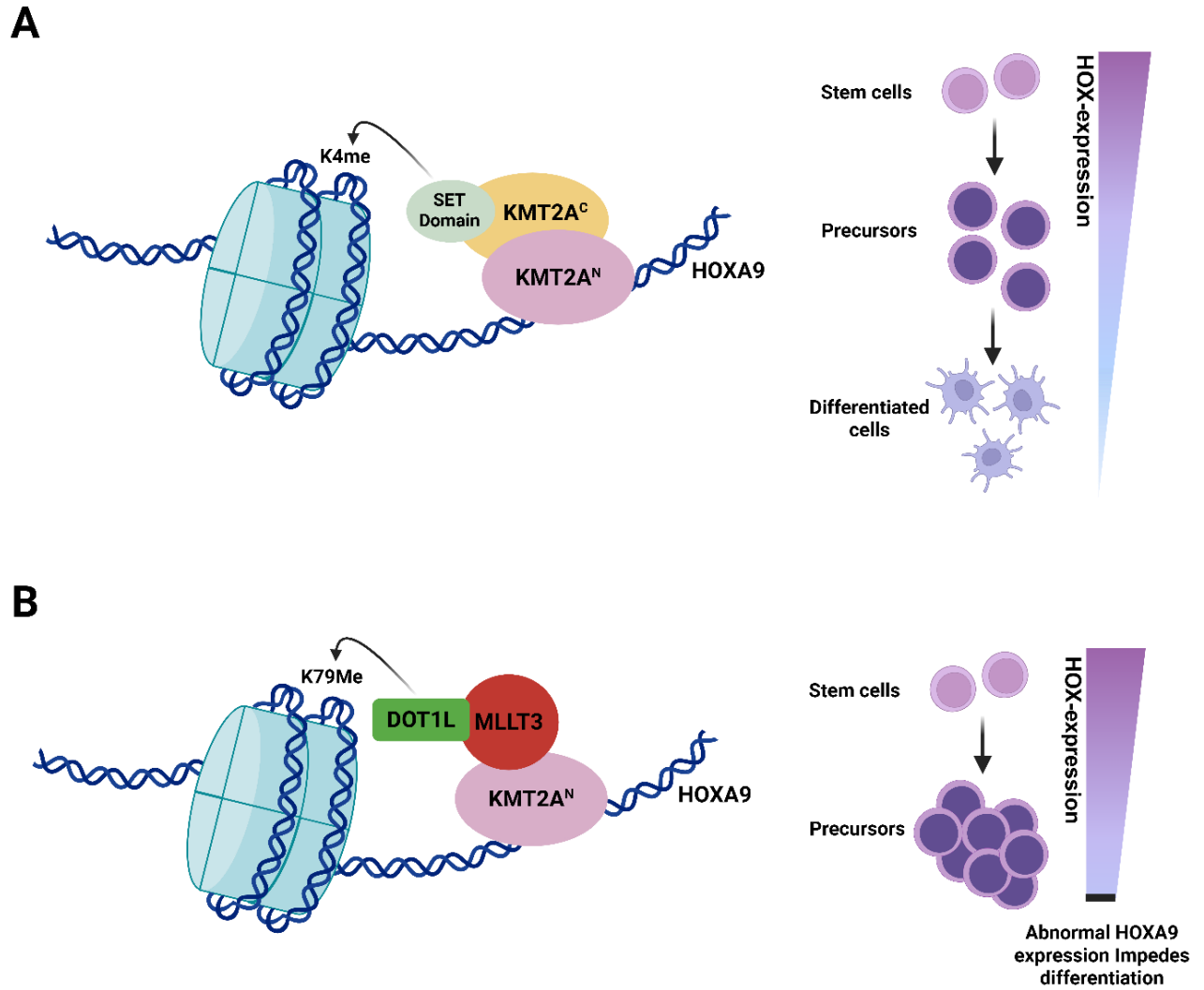


Figure 2.3. Mechanism of *HOXA9* regulation in normal and leukemic hematopoiesis. In normal hematopoietic development, the KMT2A protein binds to target DNA regions near *HOXA9* and, through its SET domain, deposits histone H3K4 methylation (K4me), allowing proper *HOX* gene expression. This regulated expression supports the progression from stem cells to differentiated cells. (A) In KMT2A-rearranged leukemia, the KMT2A fusion protein (KMT2A-MLLT3) aberrantly recruits DOT1L, which catalyzes H3K79 methylation (K79Me), leading to persistent *HOXA9* overexpression. This disrupts the normal differentiation process, resulting in the accumulation of precursor cells and contributing to leukemogenesis in Mixed Lineage Leukemia (B).

2.5 KMT inhibitors as Drug Candidates

In 2011, the first selective small-molecule inhibitor of DOT1L, EPZ004777, was developed. This compound exhibits picomolar potency *in vitro* (126). The design and synthesis of EPZ004777 were guided by the structure of the cofactor SAM and the enzyme's active site, resulting in a molecule highly selective for DOT1L with more than 1000-fold greater selectivity over nine other methyltransferases, despite the structural similarity to SAM. As expected, EPZ004777 was found to act as a competitive inhibitor concerning SAM. Structural studies further elucidated a ligand-induced conformational adaptation in DOT1L's catalytic site upon binding of EPZ004777 (131,132). Subsequent advancements led to the development of SGC0946, a DOT1L inhibitor with enhanced potency *in vitro* and cellular models. In MCF10A cells, SGC0946 was found to be approximately ten times more effective than EPZ004777 at reducing histone H3K79 methylation (132). A significant milestone in DOT1L inhibitor development occurred in 2013 with the introduction of EPZ-5676 (pinometostat), the first DOT1L inhibitor to advance to clinical trials (79). Another DOT1L inhibitor, postulated to modify histone H3K79 covalently, has also been reported, demonstrating high *in vitro* potency and selectivity for DOT1L over other methyltransferases (133,134).

In addition to DOT1L inhibitors, other histone methyltransferase inhibitors have entered the clinical landscape. In 2020, the FDA granted accelerated approval to tazemetostat (Tazverik), an EZH2 (Enhancer of zeste homolog 2) inhibitor, for the treatment of adult and pediatric patients aged 16 and older with metastatic or locally advanced epithelioid sarcoma that is not amenable to surgical resection. Tazemetostat was also approved for adults with relapsed or refractory (R/R) follicular lymphoma (FL) harbouring EZH2 mutations following at least two prior systemic therapies. It was further approved for adult patients with R/R FL, but no satisfactory treatment

alternatives were found. The Cobas EZH2 Mutation Test was concurrently approved as a companion diagnostic for tazemetostat. Unfortunately, it was voluntarily withdrawn by its manufacturer in early 2026.

Although histone methyltransferases such as DOT1L and EZH2 are primarily recognized for their roles in chromatin regulation, growing evidence suggests that they can also catalyze the methylation of non-histone proteins, including the Androgen Receptor (AR). This occurs when PRNCR1 binds to the C-terminally acetylated AR at the enhancers, recruiting the DOT1L enzyme to methylate the N-terminus of AR (135,136). This expands the scope of their biological functions and indicates that inhibiting these enzymes may impact additional non-histone substrates involved in diverse regulatory and signaling pathways (137,138) (Figure 2.4).

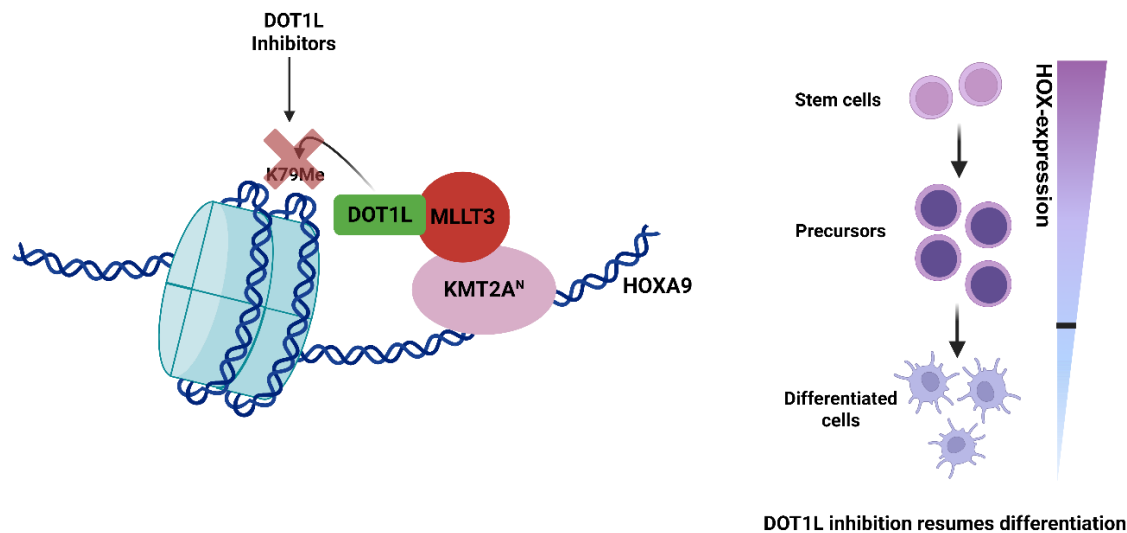


Figure 2.4. DOT1L inhibition restores differentiation in KMT2A-rearranged leukemia. DOT1L inhibitors block histone H3K79 methylation (K79Me) mediated by the KMT2A-MLLT3 fusion complex, leading to reduced *HOXA9* expression. This reactivation of normal epigenetic regulation enables precursor cells to differentiate into mature blood cells, counteracting the leukemogenic block in differentiation.

Table 2.1. Epigenetic therapies targeting histone lysine methylation and Bromodomain and extraterminal (BET) proteins: Indication and Clinical Trial Status.

Drug	Enzyme Target	Indication	Clinical Trial ID	Status
Tazemetostat (EPZ-6438)	EZH2	Soft Tissue Sarcoma	NCT02601950	Completed
Valemetostat (DS-3201)	EZH1/EZH2	Hematologic malignancies and solid tumors.	NCT02732275	Active
Epsametostat (HH2853)	EZH1/EZH2	Relapsed/Refractory Peripheral T-cell Lymphoma	JPRN-jRCT2031230059	Active
Tulmimetostat (CPI-0209)	EZH1/EZH2	Solid Tumors and Lymphomas	NCT04104776	Active
Litametostat (CPI-1205)	EZH2	Advanced Solid Tumors	NCT03525795	Completed
Mevrometostat (PF-06821497)	EZH2	Castration Resistant Prostate Cancer, and Follicular Lymphoma	NCT03460977	Active
GSK3326595	PRMT5	Solid Tumors and Non-Hodgkin's Lymphoma	NCT02783300	Completed

Advanced Solid				
JNJ-64619178	PRMT5	Tumors, NHL, and Lower Risk MDS	NCT03573310	Active
MTAP-deleted Solid				
TNG908	PRMT5	Tumors	NCT05275478	Completed
Ladademstat (ORY-1001)	KDM1 (also called LSD1)	Acute Myeloid Leukemia (AML)	EudraCT No.: 2018-000482-36	Completed
Bomedemstat (IMG-7289/ MK-3543)	KDM1 (also called LSD1)	Myelofibrosis	NCT03136185	
Seclidemstat (SP-2577)	KDM1 (also called LSD1)	Refractory Ewing or Ewing-related Sarcomas	NCT03600649	Active
Metastatic Castration-				
ZEN-3694	BET	Resistant Prostate Cancer	NCT02711956	Completed
RO6870810 (CPI-0610)	BET	Myelofibrosis	NCT02158858	Active
KMT2A-rearranged				
Pinometostat (EPZ-5676)	DOT1L	leukemias.	NCT01684150	Completed

2.6 The problem with KMT inhibitors

A thorough understanding of epigenetic mechanisms is crucial for successfully applying epigenetic-targeted drugs. Alterations in epigenetic enzymes can change chromatin structures, impacting cellular processes like transcription, replication, and DNA repair. Even minor modifications in epigenetic enzyme activity can significantly affect cellular functions.

The roles of KMTs in modifying histones are thought to take place in the nucleus where in principle, those KMTs can be recruited to many genes to alter their expression. The KMT inhibitors we discussed are not restricted to any particular part of the nucleus and can by inhibition of KMT, result in changes in expression of many genes. However, using AML as an example, DOT1L inhibitors like EPZ5676 only have one target gene: HOXA9. Therefore, inhibition of DOT1L at all other genes would be considered off-target and a potential cause of toxic effects.

2.6.1 DOT1L (KMT4)

Despite its therapeutic promise, pinometostat, for example, has been associated with adverse effects on immune response. Histone H3K79 methylation is involved in regulating immune function (139), and pinometostat impairs host defense mechanisms by deregulating key genes involved in innate immunity, such as *RUBICON*, *TRIM25*, and *BCL3*, (140) in human lung epithelial cells (141)..

Histone H3K79 methylation might have a general role in controlling the host response of pathogens that are interferon inducers (139). Its inhibition may lead to unpredictable changes in the expression of multiple genes. It has been shown that such changes in expression may include other histone modifying enzymes such as KDMs, KATs, and HDACs. This would reflect in changes in total histone and residue-specific histone modifications, and this could in turn precipitate down-stream changes in gene expression (124). In fact, Lilloco and colleagues showed

that pinometostat paradoxically increased the total histone mono- and dimethyl-lysine produced *in vitro*. This might be a result of downstream changes in the expression of genes coding for histone modifying enzymes. They showed that pinometostat reduced the expression of other KMTs like SETD7 and SMYD3 in both MOLM-13 (which express HOXA9) and K562 (which do not express HOXA9), (124). The implication of this is reflected in the fact that DOT1L is important for its involvement in orchestrating immune cell formation and function (142). The effects of DOT1L inhibitors have been shown to affect immune response against infections (140). Specifically, DOT1L plays a crucial role in B cell development and immune memory. DOT1L knockout has been found to reduce the expression of several genes essential for proper B cell activity, signaling, and migration, including *SIPR2*, *ACKR2*, and *ACKR4* (127,143). DOT1L is also important in proper T cell development and differentiation (143).

DOT1L is important in several other physiological roles that if DOT1L were to be inhibited, could lead to negative outcomes. For example, DOT1L is an important regulator of joint and cartilage health, and its activity can help prevent osteoarthritis (144). Monteagudo et al., demonstrated DOT1L's importance in joint health by injecting mice with pinometostat, which triggered osteoarthritis in the mice. *In vitro* testing was done using human chondrocytes with osteoarthritic characteristics including the low expression of *COL2A1*, which codes for type two collagen, and increased expression of *COL1A1*, which codes for type 1 collagen. DOT1L inhibition by pinometostat lead to a further reduction in *COL2A1* expression, and higher expression of *COL1A1*. DOT1L's mechanism of aiding cartilage health is thought to decrease Wnt signaling in cartilage cells by directly blocking the activity of the Wnt activator Sirtuin 1 (SIRT1). Human chondrocytes exposed to DOT1L inhibition resulted in higher expression of Wnt signaling target genes, several of which are associated with poor cartilage health (144). Furthermore, the presence of DOT1L has

been shown to interfere with the activity of SIRT1, and this in turn was shown to decrease Lymphoid enhancer-binding factor 1 (LEF1) and T cell factor 1 (TCF1) expression, transcription factors that increase expression of Wnt signaling-associated genes, while SIRT1 activation led to an increase in LEF1 and TCF1. Also, osteoclast differentiation is potentially affected since knockdown of DOT1L in mice results in increased osteoclastic reabsorption of bone and overall lower bone density and integrity (145).

DOT1L is important in murine cardiac health and its expression can potentially reduce risk of heart failure and diseased cardiac physiology (146). DOT1L inhibition can lead to changes in heart morphology and physiology, as mice that were born with no DOT1L expression in their cardiac tissues were found to have altered cardiac structure, with increased cardiac cell proliferation and dilated, enlarged, and heavier hearts, similar to dilated cardiomyopathy seen in humans with heart failure. Accordingly, these mice exhibited half the normal cardiac ejection fraction which resulted in death by 6 months. Increases in cardiomyopathy due to DOT1L inhibition/knockout are thought to be caused by the loss of DOT1L's regulation and subsequent increase in expression of dystrophin (*Dmd*). *Dmd* promotes the connection of cardiac sarcomeres to the extracellular matrix, helping generate contractile force in cardiomyocytes. Mice where DOT1L is silenced had reduced *Dmd* levels in cardiac tissue. Moreover, ChIP assays showed significant decreases in histone H3K79me at the *Dmd*, gene indicating that DOT1L directly regulates *Dmd* expression (146). It is possible that inhibiting DOT1L results in conditions in mice that closely resemble heart failure in humans, and perhaps loss of function of DOT1L in humans could theoretically result in altered cardiac physiology as well.

DOT1L increases heat generation, in brown adipose tissue (BAT) by increasing expression of genes involved in upregulating heat production. BAT can generate heat by increasing expression

of uncoupling protein 1 (*Ucp1*) which generates heat in mitochondria rather than generating ATP through respiration. BAT is higher in human newborns and animals, than adult humans and is thought to play a role in metabolism, temperature regulation, and obesity (147,148). In response to exposure to a cold environment the transcription factor *Zc3h10* increases *Ucp1* expression. Accordingly, overexpression of *Zc3h10* in mice results in higher *Ucp1* expression, increasing heat generation, and preventing food-induced obesity (148). DOT1L expression is 2-fold higher in BAT vs liver, stomach, and spleen cells. Moreover, DOT1L expression increases with exposure to cold and increases *Ucp1* expression (149). At the *Ucp1* promoter, DOT1L interacts with *Zc3h10*, resulting in an 11-fold higher *Ucp1* expression whereas DOT1L in the absence of *Zc3h10* yields no *Ucp1* expression and a 3-fold increase in expression with *Zc3h10* alone (149) Inhibiting DOT1L in mice impairs BAT thermogenesis by reducing *Ucp1* levels by over 50%, hindering the mice's abilities to cope with colder temperatures. Overexpression of *Zc3h10* could not counteract DOT1L inhibition and led to increased weight in the mice (149).

2.6.2 EZH2

Adverse pleiotropic effects can theoretically be an issue when inhibiting any epigenetic enzyme that regulates the expression of multiple genes. Another example of where adverse pleiotropic effects might arise is the inhibition of EZH2.

EZH2 is the catalytic KMT found in PRC2 and is responsible for mono-, di-, and tri-methylation of histone H3K27. Histone H3K27me3 is a repressive mark as histone H3K27me3 acts as a binding site for PRC1, a polycomb complex that silences gene expression by compacting chromatin and monoubiquitinating H2AK119 (151). EZH2 is important in development and is necessary for proper nervous system development (152), skeletal development (153), and X-chromosome inactivation. (154). EZH2 has become a therapeutic target for treating cancers, as overexpression

of EZH2 in humans has been linked to several cancers and is important for cancer survival and proliferation (155). While inhibition of EZH2 can help treat certain cancers, EZH2 is important in numerous physiological processes and inhibiting EZH2 can result in the altered expression of many different genes.

An adverse pleiotropic effect that can follow EZH2 inhibition is the effect on oocyte health (156). Females are born with a set number of oocytes that eventually develop into mature eggs, Inhibition of EZH2 by tazemetostat can lead to negative effects on female reproduction, germline cell fate, and the growing oocyte cells, potentially leading to future congenital problems in offspring, including reduced weight (156). Developing egg cells in mice have high levels of H3K27me3, more so than in somatic cells, suggesting that EZH2 plays an important role in developing egg cells. Inhibition of EZH2 by tazemetostat leads to a significant reduction of histone H3K27me3 in mice egg cells and primary oocytes; levels did not go back to normal within 10 days, indicating potential long-term or even epigenetic changes in primary oocytes. Although tazemetostat is contraindicated in pregnancy and for 30 days after pregnancy, if histone H3K27me3 depletion is a long-term effect of EZH2 inhibition, or if it is a permanent epigenetic change, it is possible that future offspring may be affected long after drug exposure. For example, mice offspring born after maternal histone H3K27me3 depletion suffered from reduced growth, which can lead to metabolic health problems later in life.

EZH2 expression can also affect male reproductivity as it regulates spermatogenesis. The expression of EZH2 is elevated in male mice germ cells during the last phases of meiosis and in undifferentiated germ cells (157), whereas EZH2 knockout mice were shown to have lower amounts of mature sperm cells compared to control mice. In EZH2 KO mice, altered expression and activity levels of proteins important for germ cell stem cell self-renewal, including lower levels

of Ret and increased activity of NEUROG3 and KIT. The increased activity of NEUROG3 and KIT in EZH2 KO mice (EZH2 directly inhibits these proteins) favours germ cell differentiation into spermatozoa rather than continuing self-renewal. These results indicate that inhibition of EZH2 could result in altered germ cell differentiation and lower sperm count due to unregulated apoptosis, potentially affecting male fertility.

EZH2 expression also affects pulmonary health. Asthma/allergy pulmonary responses are caused by allergen exposure and the subsequent release of inflammatory mediators and IgE antibodies in the lungs, in response to cytokines released by immune cells, including T cells and invariant natural killer T (iNKT) cells (158). T cell development, including iNKT and T helper cells, is affected by EZH2 expression, and loss of function of EZH2 alters the amount of T cells produced, increasing the risk of experiencing asthma/allergy-like episodes (159). Moreover, increases in IgE, helper T cells, iNKT cells, B cells, neutrophils, airway hypersensitivity, and airway inflammation were seen in EZH2 KO mice. Increases in iNKT2 cells in the lungs of EZH2 KO mice are caused by the increased expression of *Zbtb16*, which codes for the transcription factor PLZF and is important for T cell differentiation. The iNKT cells of EZH2 KO mice had increased cytokine interactions and increased levels of IL-4, and T helper cells had increased levels of IL-4 and IL-13, increasing the risk of airway hypersensitivity and the asthma/allergy response. Therefore, therapeutic inhibition of EZH2 may increase the risk of allergic responses.

2.7 Inherent difficulties in targeting ubiquitous enzymes

The inherent problems associated with targeting enzymes that are ubiquitously expressed are well known. In contrast to receptors that tend to show at least some tissue specificity, enzymes can show little tissue specificity or even if expressed in a specific organ, the enzyme can distribute throughout the body via the circulation provided it is not membrane bound.

For example, β_1 -adrenoceptors are predominantly found in cardiac and renal tissues, β_1 -adrenoceptors in the lungs and α_1 -adrenoceptors are primarily located in vascular and visceral smooth muscles. This tissue specificity provides a significant advantage in drug design, as therapeutic agents can be localized to specific tissues, thereby reducing off-target effects. For instance, β_1 -selective adrenoceptor antagonist, such as metoprolol work in the heart to slow heart rate and in the kidneys to potentially reduce renin and because of their selectivity, they do not have off target effects β_2 receptors in the lungs (160).

In contrast, the drugs targeting enzymes involved in epigenetic processes may have wide tissue distributions and therefore cannot be expected to have tissue specific effects. Further complicating this issue, these drugs do not confine themselves to particular genes or even regions of the genome in any given cell. Therefore, using DOT1L inhibitors for AML caused by translocations of KMT2A (where reduction of HOXA9 alone is sufficient to treat the disease) the activity of the drug at all other gene loci where the DOT1L works would be, by definition, an off-target effect.

2.8 The Need for Targeted Therapy

The fundamental role of altered epigenetic modification patterns in tumorigenesis establishes epigenetic regulatory enzymes as important targets for cancer therapy. It is generally assumed that the therapeutic benefit of global epigenetic modification is related to the reactivation of aberrantly silenced genes and the silencing of oncogenic ones. However, global epigenetic alteration is not selective for cancer cells, and side effects caused by off-target binding could severely curtail the specificity of this direction of therapy.

Several drugs have been developed to target epigenetic enzymes in the management of different diseases. 5-azacytidine and decitabine have shown significant clinical benefit in the therapy of myelodysplastic syndrome and myeloid leukemias (162,163), while HDAC inhibitors such as

vorinostat and romidepsin are approved for the treatment of cutaneous T-cell lymphoma (164,165). Several others are undergoing clinical testing, and pinometostat (EPZ 5676) has been in clinical trials for treatment of mixed lineage leukemia. However, EPZ 5676 inhibits DOT1L through the genome causing changes in expression of many genes that themselves are also involved in influencing gene expression. (166,167).

All in all, there is a need to look for ways to attempt drug-targeting programs for drugs that modulate HMTs and epigenetics in general. This will certainly reduce the side effects and may also lead to a significant dose reduction.

2.9 Conclusion remarks and future perspectives

Histone-modifying enzymes, such as methyltransferases and acetyltransferases, are often broadly involved in regulating gene expression across many genes in cell types and contexts. While such inhibitors elicit desired therapeutic effects, their lack of selectivity can lead to significant off-target interactions, resulting in an imbalance in the risk-benefit profile. The widespread effects of histone modification inhibitors can cause toxicity and diminish the overall therapeutic benefits, as the unintended epigenetic changes on non-target genes may introduce harmful side effects.

Chapter 3: Denaturation, rapid dilution refolding, and single-step purification of the core histones using desalting size-exclusion chromatography

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3.0 Abstract

Nucleosomes, composed of DNA and histone octamers, regulate gene expression through histone modifications such as lysine acetylation and methylation. These modifications are added by writers, removed by erasers, and interpreted by readers to control gene expression, chromatin structure, and essential cellular processes such as differentiation and development. Accurate post translational modifications analysis requires high-purity histones due to the sensitivity of epigenetic assays. Recombinant histones are expressed in *E. coli* as inclusion bodies, requiring denaturation, refolding, and purification. These traditional purification methods involve complicated and lengthy protocols taking days and potentially exposing the histones to oxidation and proteolytic degradation. We developed a rapid method for refolding histones from inclusion bodies in a one-step purification using a desalting column achieving >90% purity. This method is compared to our previous High Performance Liquid Chromatography (HPLC)-based protocol. Our single-step desalting purification reduces purification time from multiple days to one day, lowers operational cost, and eliminates the need for reverse-phase HPLC, making high-purity histone production accessible to laboratories without specialized chromatography infrastructure.

3.1 Introduction

Nucleosomes are the smallest repeating structural units of chromatin, composed of 147 bp of DNA wrapped around an octamer consisting of two copies of histones H2A, H2B, H3, and H4. This structure plays a dual role by compacting genomic DNA and regulating the accessibility of genetic information for transcription, replication, and repair. The N-terminal tails of histones serve as platforms for a wide range of PTMs, including acetylation, methylation, phosphorylation, and ubiquitination (171,172). These modifications do not alter the underlying DNA sequence but can profoundly affect chromatin structure and gene expression, constituting a major branch of epigenetic regulation (35,173,174). The addition, recognition, and removal of histone PTMs are mediated by writers, readers, and erasers, respectively. Writers are enzymes responsible for adding histone PTMs. For instance, KATs, such as KAT3A (also called p300/CBP-associated factor (PCAF)), catalyze the addition of acetyl groups to lysine residues on histones, which generally promotes transcription (174). PKMTs such as the SET domain-containing lysine methyltransferase 7/9 (SET7/9) and PRMTs like PRMT1 and PRMT6 catalyze the transfer of methyl groups to lysine or arginine residues, respectively. The functional outcome of histone methylation, whether activation or repression, depends on the specific site and degree of methylation. Erasers remove histone modifications, thereby allowing dynamic regulation of chromatin states. HDACs such as Sirtuin 1 (SIRT1) remove acetyl groups, typically resulting in chromatin condensation and transcriptional repression (175). KDMs such as KDM1 (also called LSD1) remove methyl groups and can either activate or repress transcription depending on the context. Readers are specialized protein domains that bind to histone modifications and are usually part of a larger protein. Bromodomains bind to acetylated lysines, while chromodomains, and plant homeodomain (PHD) fingers bind to methylated histone lysine residues. Reader domains are often part of chromatin

remodeling complexes, transcription factors, or even other writers or erasers. The patterns of histone PTMs govern chromatin dynamics and gene expression. The profound effects of histone PTMs underscore the need for their precise characterization. As such, efficient production of pure histones is required for studying the activity of histone writers and erasers.

Bacteria are typically used to overexpress histones. Due to the rapid expression and possibly lack of endogenous histones in bacterial systems, they form insoluble inclusion bodies. Although inclusion bodies usually provide a high yield and are relatively straightforward to isolate, they are, by nature, unfolded and are therefore insoluble and inactive. Following isolation via sequential washing steps to remove contaminants, the inclusion bodies consist mostly of histone as measured by SDS-PAGE (176). For existing protocols, the histone-containing inclusion bodies are denatured, solubilized and subsequently purified using complicated multi-step processes consisting of several rounds of gel filtration and cation exchange chromatography under denaturing conditions, typically 6–8 M urea (176–178). A final dialysis step is typically employed to refold the histones while removing urea and salts, yielding purified histones suitable for downstream applications. This multi-step process takes at least 3 days because two dialysis steps are involved with each dialysis step being done overnight (Figure 3.1A).

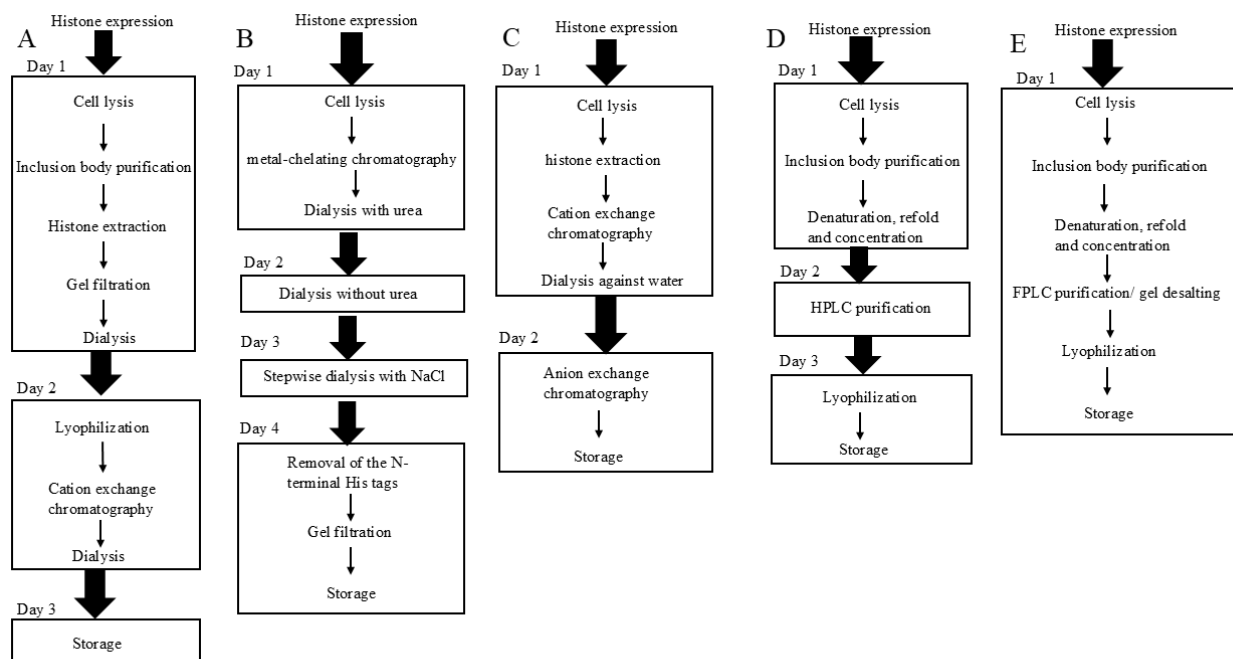


Figure 3.1. Different histone purification protocols. Shown are different schematic workflows: The conventional workflow of Luger et al.,(177) (A). Newer protocols developed by Tanaka et al.,(179) (B), and Klinker et al., (180)(C). Our initial method which utilizes rapid dilution Pak et al.,(181) (D) and our new method (E).

Although effective, the histone purification workflows in Figure 3.1A through 1D are labor-intensive and time consuming, with the dialysis steps frequently constituting the primary time bottleneck. Two major problems with such multi-day protocols are the potential for hydrolysis of histones either enzymatic (due to protease contamination) or non-enzymatic, and the potential for oxidation of sensitive amino acids such as methionine, histidine, and tyrosine. Oxidation can be particularly insidious since it could affect writer, reader, and eraser binding but will not affect the apparent histone molecular weights on SDS-PAGE or Western Blot. To address these limitations, several ostensibly streamlined approaches have been developed. One such method involves the use of affinity tags, as demonstrated by Tanaka et al. (Figure 3.1B), wherein recombinant human histones were expressed with a hexahistidine (6His) tag and purified using immobilized metal

affinity chromatography (IMAC) under denaturing conditions (6 M urea) followed by dialysis to remove the urea (179). This method was developed to make histone octamer precursors for recombinant nucleosomes. Following purification, histone H2A/H2B and histone H3/H4 dimers were formed by mixing the denatured histones together and then dialyzing overnight, followed by thrombin cleavage overnight to remove the 6His tag. The following day the resulting mixture was purified using Sephadex 70 size exclusion chromatography to purify the histone H2A/H2B and histone H3/H4 dimers from the 6His tags and the thrombin (179). Although seemingly less complicated this approach necessitates an additional time-consuming tag-removal step and subsequent purification to remove the 6His tag complicating the workflow and ultimately taking several days to get to the final purified product.

Other supposedly more efficient methods, which include alternative dialysis-based protocols, have been explored to enhance efficiency. However, these techniques fail to address the fundamental challenges of the multi-step, multi-day protocol, which are inherently labor-intensive and increase the potential for histone oxidation and degradation (176,178,180). For example, Klinker and colleagues employed cation exchange chromatography under denaturing conditions following cell lysis, inclusion body isolation and denaturation and solubilization (180). The resulting solution then underwent dialysis to remove excess salts and denaturing agents and was subsequently subjected to anion exchange chromatography for further purification.

We developed a rapid method of denaturation, and refolding of histones from inclusion bodies and a one-step desalting purification strategy that eliminates the need for ion exchange, chromatography, and dialysis. Our new method (Figure 3.1E) is capable of denaturing, refolding, and purifying histones in a single day on the 100-mg level. Our novel approach utilizes a desalting

column with a fast protein liquid chromatography (FPLC) apparatus but could in principle be performed without an expensive FPLC by using open columns and a simple UV detector. We directly compare our method to our previous histone purification method that utilizes reverse phase high-performance liquid chromatography (HPLC) in place of a desalting column (181), and we compare these generally to previous, much more complicated, multi-step protocols (Figure 3.1). The new histone purification method described herein (Figure 3.1E) is straightforward, efficient, and economical. It also has a comparable yield and purity to our previous method (Figure 3.1D). Moreover, the final product, lyophilized dry powder histone will remain stable for long periods at -80 °C and can be easily reconstituted in water or any buffer.

3.2 Methods

3.2.1 Bacterial cell culture

The plasmids encoding the genes for *Xenopus laevis* histones H2A, H2B, H3, and H4, each within the pET3a vector, were generously provided by Dr. Karolin Luger from the University of Colorado (176). These plasmids were transformed into *Escherichia coli* BL21(DE3) pLysS (Thermo Scientific) cells, which were cultured in Luria-Bertani (LB) broth with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. The bacteria were streaked onto LB agar plates containing the appropriate antibiotics for each histone construct. A single colony from a streaked LB agar plate was used to inoculate a starter culture, which was incubated until slightly turbid, pelleted and used to inoculate 2 L of LB media with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C while shaking at 250 rpm.

The histones were expressed according to the methods developed by Luger *et al.* (176). Briefly, upon reaching the desired OD_{600nm} of 0.6-0.8, protein expression was induced with 0.2 mM

isopropyl β -D-1-thiogalactopyranoside (IPTG) for 2 hours. Cells were harvested by centrifugation at $8900 \times g$ for 10 minutes at 4 °C, and the pellets were stored at –80 °C for further processing.

3.2.2 Lysis and washing of inclusion bodies

Cells were lysed and inclusion bodies washed according to our previous methods (181). Briefly, cell pellets were resuspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Thermo Scientific), 1 mM β -mercaptoethanol (BME), 2 protease inhibitor tablets (Thermo Scientific) (one per 50 mL sample volume), 20 U/mL DNase I (Thermo Scientific), and 0.33 mg/mL lysozyme (Amresco)) and sonicated on ice, applying 30 pulses at 50% amplitude, repeated three times and centrifuged at $20,000 \times g$ (Thermo Scientific Sorvall RC 6 Plus – Fiberlite F13-14x50cy) for 25 minutes at 4 °C and the supernatant discarded. The pellet containing the inclusion bodies was washed by resuspension in lysis buffer but with 1% Triton X-100 and sonicated as above. The washed inclusion bodies were pelleted by centrifugation at $12,000 \times g$ for 20 minutes at 4 °C (Thermo Scientific Sorvall RC 6 Plus – Fiberlite F13-14x50cy) and the supernatant was decanted. The wash step was repeated at least 3 times.

3.2.3 Denaturing, refolding, and concentration of recombinant histones from inclusion bodies

Inclusion body denaturation, refolding, and concentration followed our previous methods (181) with minor variation. Inclusion bodies were denatured in 20 mM Tris (pH 7.5), 8 M guanidine hydrochloride [GuHCl] and 10 mM dithiothreitol [DTT] at room temperature for 1 hour with nutation. The solution was then centrifuged at $20,000 \times g$ for 10 minutes at 4°C, and the supernatant was retained.

Denatured inclusion bodies containing histone were added to refolding buffer: 100 mM Tris (pH 7.5), 400 mM arginine, 2 mM EDTA, 2 mM DTT, 2 mM PMSF, and 4 protease inhibitor tablets in

a total volume of 200 mL at a rate of 100 μ L/min while being vigorously mixed on ice. Refolded histones were concentrated using a 10-kDa molecular weight cutoff centrifugal filter (Amicon) and then centrifuged at $4,000 \times g$ (Thermo Scientific Sorvall RC 6 Plus – Fiberlite F13-14x50cy) for 15–30 minutes at 4 °C until the volume was reduced by more than half. The final solution was then filtered through a 0.2 μ m syringe filter (if needed this can be stored at –80 °C). The concentrated solutions of histones H2A, H2B, H3 and H4 were divided into two equal fractions, one purified via HPLC and the other via desalting column using an FPLC (see details below). Washed inclusion bodies consist predominantly (>85%) of histones (177). Therefore, efficiency is presented here as purified histone product per unit processing time (mg/hour).

3.2.4 HPLC purification

HPLC purification followed the methods of (181) with minor variations. Briefly, a Shimadzu 10A VP series HPLC with an SPD-10AV VP UV-VIS detector, an LC20AB binary pump and a manual injection system with a 10 mL loop were used. Trifluoroacetic acid (TFA) was added to the histone concentrate to make the final concentration 0.1% TFA in histone concentrate. Thereafter, 5 mL of the concentrate was injected at a time, onto the HPLC system connected to a semi-preparative Zorbax C8 9.4 x 250 mm column eluted via gradient. The histones were eluted with mobile phases A: 0.1% aqueous TFA, and B 0.1% TFA in acetonitrile (181) using a linear gradient of 5% B to 95% B over 45 minutes at 2 mL/min, monitoring at 260 and 280 nm. Histones typically eluted between 38 and 42 min and fractions were collected and lyophilized.

3.2.5 Desalting column purification

The remaining portion of each concentrated histone was desalted at room temperature using a HiPrep 26/10 desalting column connected to a GE ÄKTA Purifier 10 FPLC system, equipped with a FRAC-950 fraction collector, P-900 pump, UV-900 detector, and pH/C-900 conductivity

monitor. Up to 20 mL was injected onto the column pre-equilibrated with 50 mM ammonium bicarbonate at pH 8, at 3 mL/min at room temperature (~20 °C). Ammonium bicarbonate buffer was freshly prepared immediately prior to use and kept sealed to minimize CO₂-related pH drift. Reducing agents were intentionally omitted during desalting because they can chemically react with the Primesep 200 column used in our LC-MS/MS method (see below). Our previous results have shown that the absence of such reducing agents has no effect on lysine methyltransferase reactions (182). Moreover, the short duration of the desalting step minimizes the potential for oxidation. Peaks were detected by monitoring UV absorbance at 280 nm and conductivity in mS/cm. Histones eluted just after 20 mL, and these collected fractions were lyophilized, and the salt peak eluted ~60 mL.

3.2.6 Histone purity and molecular weight determination

Samples from the steps of purification were prepared in a saturated urea solution mixed with 5X sample dilution buffer, without heating and 10 µL loaded onto an 18% Mini-PROTEAN Tetra Vertical Electrophoresis Cell run at 200 V for 50 minutes. Gels were fixed, stained with Coomassie Brilliant Blue, and destained using a solution of 10% methanol and 10% acetic acid until protein bands were visible. The gel was imaged using the Invitrogen iBright 1500 system. The purity of gel bands was determined using densitometry with the software ImageJ (183). For each lane all peaks were integrated, and the histone purity was measured by dividing the band with maximum area (the histone in question) by the area of all peaks combined and multiplying by 100 to give a percentage purity. The purity was measured as the mean of two measurements from two independent gels and the standard deviation (SD). For each of the core histones, Student's two-tailed t-tests assuming equal variance were used to determine significant differences between histone purified via HPLC or desalting.

Histone molecular weights were measured with a Nexera ultra-high performance liquid chromatography (UHPLC) system coupled to a Shimadzu 8040 triple quadrupole mass spectrometer (LC-MS/MS). A Waters Acquity UPLC BEH C18 1.7 μ M 2.1 X 100 mm column was used to separate analytes at 40 °C. Mobile phase A was 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The flow rate was 0.3 mL/min. The LC program consisted of 5% B to 95% B over 8 minutes, followed by a 2-minutes wash with 100% B, and re-equilibration at 5% B for 5 minutes. The total run time was 10 minutes. Mass spectra were acquired using a Q3 scan over the range 400–2000 m/z in positive-ion mode. Deconvolution to obtain the protein mass was achieved using the LabSolutions Multi-Charged Ion Analysis Software (Shimadzu). The H^+ “adduct” ion model was selected, peaks were detected using the slope detection algorithm, and no additional smoothing, baseline correction, or filtering was applied.

3.2.7 Methylation experiments

The histones were utilized in methylation reactions using the histone modifying enzymes protein arginine methyltransferase 1 (PRMT) 1 (181); protein arginine methyltransferase 6 (PRMT6) (184); and histone lysine methyltransferase (SET7/9). Although SET7/9 is now officially known by its HGNC designation SETD7 (Q8WTS6), the name SET7/9 is much more commonly used, has historical precedent in the literature and we will therefore use it throughout this article. The reaction mixture contained 50 nM SET7/9, 50 nM PRMT1 or 100 nM PRMT6, 100 mM ammonium bicarbonate buffer (pH 9), 200 nM stable isotope-labeled internal standard $^{13}C_6^{15}N_2$ lysine, 5 μ M histone substrates (histones H2A, H2B, H3, and H4), and 25 μ M *S*-adenosyl-L-methionine (SAM) (Cayman Chemical Co). The reaction volume was 50 μ L. The reactions were conducted at 30 °C for 30 minutes and were terminated by the addition of ice-cold methanol, transferred to 300 μ L HPLC inserts, and dried in a Thermo Scientific Savant SPD2010 SpeedVac

Concentrator at 70 °C for at least 1 h. Dried samples were acid hydrolyzed in the vapor phase using 6 M HCl at 110 °C for 24 h. The hydrolyzed samples were then dried in a vacuum centrifuge at 70 °C for at least 30 minutes and reconstituted in 50 µL of 0.05% formic acid for analysis by LC-MS/MS analysis as described below.

3.2.8 Histone methylation assay using LC-MS-MS

Chromatographic separation and mass spectrometric analysis were performed using the LC-MS/MS described above. Quantification of lysine and arginine methylation was performed using our previous method, with minor modifications(124). Modified amino acids were separated on a Primesep 200 HPLC column (SIELC Technologies) maintained at 40 °C. The mobile phases consisted of (A) 0.05% aqueous formic acid and (B) 1% formic acid in 50% aqueous acetonitrile. The chromatographic run employed an isocratic hold at 0% B for 1.5 minutes, followed by a linear gradient to 85% B over 30 seconds, and held at 85% B for 3 minutes. Detection was carried out in positive multiple reaction monitoring (MRM+) mode using dual ionization sources: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Instrument parameters were set as follows: nebulizing gas flow at 2 L/min, drying gas flow at 15 L/min, desolvation line temperature at 250 °C, and heating block temperature at 400 °C. The precursor and product ion mass-to-charge (m/z) values for lysine, arginine, their methylated derivatives, and the internal standard ($^{13}\text{C}_6$ $^{15}\text{N}_2$)-lysine are summarized in Table S3.1 (124,185).

3.2.9 Concentration measurements

Each purified histone was reconstituted in deionized water to a final concentration of 1 mg/mL. Concentration was initially estimated via UV absorbance using the extinction coefficients for histones H2A, H2B, H3 and H4 provided by Luger *et al.* (176). 100-µL aliquot of each solution was transferred to 300 µL HPLC-compatible glass inserts and subsequently dried in a vacuum

centrifuge at 70 °C for at least 1 hour. The dried histones were subjected to vapor-phase acid hydrolysis by exposure to 6 N hydrochloric acid at 110 °C for 24 hours. Following hydrolysis, the samples were again dried in a vacuum centrifuge at 70 °C for at least 30 minutes and reconstituted in 100 µL of 0.05% aqueous formic acid. LC-MS/MS was used to analyze the resulting solutions for the quantification of lysine residues. The concentration of each histone was determined by normalizing the concentration of lysine measured by the number of lysine residues in each histone.

3.3 Results

3.3.1 Desalting and HPLC purification

Core histones H2A, H2B, H3 and H4 were purified using a HiPrep 26/10 desalting column, with elution occurring after 20 mL, as indicated by a prominent absorbance peak at 280 nm (Figure S3.1), consistent with the expected elution profile for a protein. A conductivity peak was observed beyond 60 mL, likely reflecting the presence of salts from the denaturation and/or refolding buffers. The histone and salt peaks have a resolution of 5.6, well beyond the generally accepted baseline of 1.5 and suggesting that there is very little potential for contamination of the purified histone by any of the salts used for denaturation and refolding. When purified by HPLC, histones eluted between 38 and 42 minutes, with UV absorbance monitored at both 260 nm and 280 nm (Figure S3.2). Earlier eluting peaks in the void volume were likely attributable to salts from the buffer systems used during protein denaturation and refolding. Typical recombinant histones yield from *E. coli* expression systems range from 10 to 40 mg per liter of culture, as reported in classical purification protocols (177,178). The yields obtained in our study (12 to 115 mg/L) fall within or exceed these values in most cases (Table 3.1). Both desalting and HPLC methods yielded comparable protein amounts per eluted volume. However, desalting demonstrated a clear

advantage in time efficiency, with histone purification time efficiency ranging from 12.17 to 66.74 mg/h, in contrast to 5.17 to 12.63 mg/h for HPLC (Table 3.1).

Table 3.1. Efficiency profile of our method of histone purification

Method	Histone	Total Yield (mg)	Total time (h)	Efficiency (mg/h)	Yield (mg/L)
HPLC	H2A	113.8	22	5.2	56.9
	H2B	24.2	11	2.2	12.1
	H3	231.7	17	13.6	115.9
	H4	202.0	16	12.6	101.0
Desalting	H2A	127.0	5	25.4	63.5
	H2B	42.6	3.5	12.1	21.3
	H3	233.6	3.5	66.7	116.8
	H4	84.5	4	21.1	42.3

3.3.2 Identification and Purity

Histone molecular weights were determined using LC-MS/MS. The experimental molecular weights closely matched the theoretical values predicted from the primary amino acid sequences, confirming the identity and integrity of the purified histones (Figure 3.2, Table 3.2). To evaluate protein purity, samples were subjected to SDS-PAGE, followed by imaging. Each histone yielded a single band, with some very low intensity contaminating bands indicating high sample purity (Figure 3.3). Gels were analyzed using ImageJ (183) to quantitatively assess band intensities and percentage purities were calculated with purity ranging from 87.76% to 93.94% (Table 3.3). There

were no statistically significant differences in purity between histones purified using HPLC and desalting.

```

Histone H2A
  10      20      30      40      50      60
SGRGKQGGKT RAKAKTRSSR AGLQFPVGRV HRLLRKGNYA ERVGAGAPVY LAAVLEYLTA

  70      80      90     100     110     120
EILELAGNAA RDNKKTRIIP RHLQLAVRND EELNKLLGRV TIAQGGVLPN IQSVLLPKKT

ESSKSAKSK

Histone H2B
  10      20      30      40      50      60
AKSAPAPKKG SKKAVTKTQK KDGKKRRKTR KESYAIYVYK VLKQVHPDTG ISSKAMSIMN

  70      80      90     100     110     120
SfVNDVFERI AGEASRLAHY NKRSTITSRE IQTAVRLLLP GELAKHAVSE GTKAVTKYTS

AK

Histone H3
  10      20      30      40      50      60
ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE IRRYQKSTEL

  70      80      90     100     110     120
LIRKLPPFQRL VREIAQDFKT DLRFQSSAVM ALQEASEAYL VALFEDTNLC AIHAKRVTIM

 130
PKDIQLARRI RGERA

Histone H4
  10      20      30      40      50      60
SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI YEETRGVLKV

  70      80      90     100
FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF GG

```

Figure 3.2. Amino acid sequence of histones from *Xenopus laevis*. Histone H2A Uniprot ID - Q6AZJ8, histone H2B Uniprot ID* - A0A8J0U496, histone H3 Uniprot ID - A0A310TTQ1, histone H4 Uniprot ID - P62799.

* Missing the first 3 residues – PEP.

Table 3.2. Observed mass and calculated mass via mass spectrometry

Histone	Method	Mass (Da)	
		Observed	Expected
H2A	HPLC	13,950.1	13,950.2
	Desalting	13,950.2	13,950.2
H2B	HPLC	13,493.5	13,493.7
	Desalting	13,493.6	13,493.7
H3	HPLC	15,273.0	15,270.9
	Desalting	15,273.5	15,270.9
H4	HPLC	11,235.7	11,236.2
	Desalting	11,236.2	11,236.2

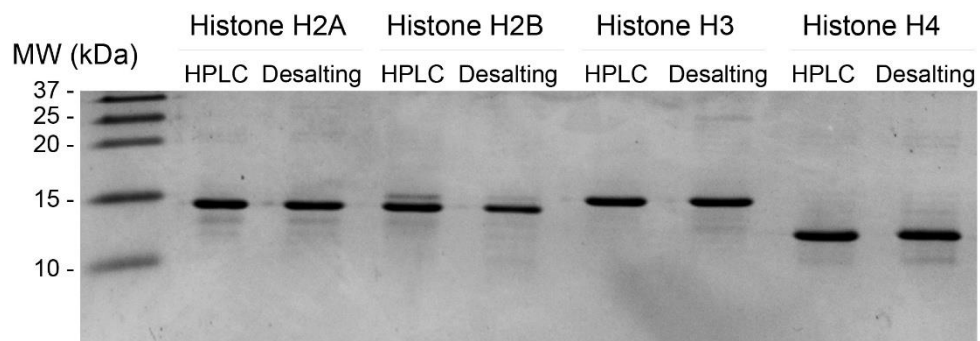


Figure 3.3. Histones purified by HPLC and Desalting. Histones purified by HPLC or desalting were resolved by SDS-PAGE and stained with Coomassie Blue. The gel was brightness- and contrast-enhanced. The full gel and the repeat gel used to generate the data in Table 3.3 are shown in Figure S3.3.

Table 3.3. Histone purity using densitometric analysis of SDS PAGE of core histones.

Histone	Method	Mean purity% $\pm$ SD [†]	p-value [*]
H2A	HPLC	89.0 $\pm$ 4.2	0.3
	Desalting	90.7 $\pm$ 3.3	
H2B	HPLC	87.8 $\pm$ 3.3	0.4
	Desalting	92.1 $\pm$ 3.9	
H3	HPLC	93.9 $\pm$ 6.6	0.6
	Desalting	91.9 $\pm$ 2.6	
H4	HPLC	92.7 $\pm$ 0.4	1.0
	Desalting	92.8 $\pm$ 1.5	

[†]Values are the means of 2 measurements from 2 gels and SD.

^{*}Student's t-test shows no statistically significant difference in purity between purification methods.

3.3.3 Methylation Reactions

The purified histones were used in enzymatic reactions with the protein methyltransferases PRMT1, PRMT6, and SET7/9 to see if the different histone purification methods influence enzymatic activity. Figure 3.4 shows the concentration of Rme and Rme2a and specific activity of total methyl-groups transferred to arginine residues on histones H2A, H2B, H3 and H4 by PRMT1 (Figure 3.4A-C) and PRMT6 (Figure 3.4D-F). Methylation of lysine residues on histones H2A, H2B, H3 and H4, is shown in Figure 3.4G and the specific activity of total methyl-groups transferred by SET7/9 is shown in Figure 3.4H. Enzymatic reactions were conducted in duplicate (n = 2) similar to our previous enzymatic studies and those of others (15,182,186). Although broadly similar, some significant differences in histone methylation were observed between HPLC-purified and desalting column-purified histones. The formation of Rme by PRMT1 was

significantly higher (~2-fold) with desalting-purified histone H2A compared to that purified by HPLC. Although PRMT1 exhibited higher production of Rme on HPLC-purified histone H4 compared to the desalting-purified histone H4, there was no significant difference in Rme2a and total arginine methylation specific activity (Figure 3.4C). Moreover, no significant differences were detected for PRMT1-mediated methylation of histone H2B or H3 purified by either method. Similarly, there were no significant differences in Rme, Rme2a, and total arginine methylation specific activity catalyzed by PRMT6 on histones purified by either method (Figure 3.4F). SET7/9 produced significantly more Kme on histone H2B, H3 and H4 purified by desalting column vs HPLC, but *vice versa* for histone H2A. It is worth noting that while these differences are statistically significant at most there is only a ~30% difference in methylation activity.

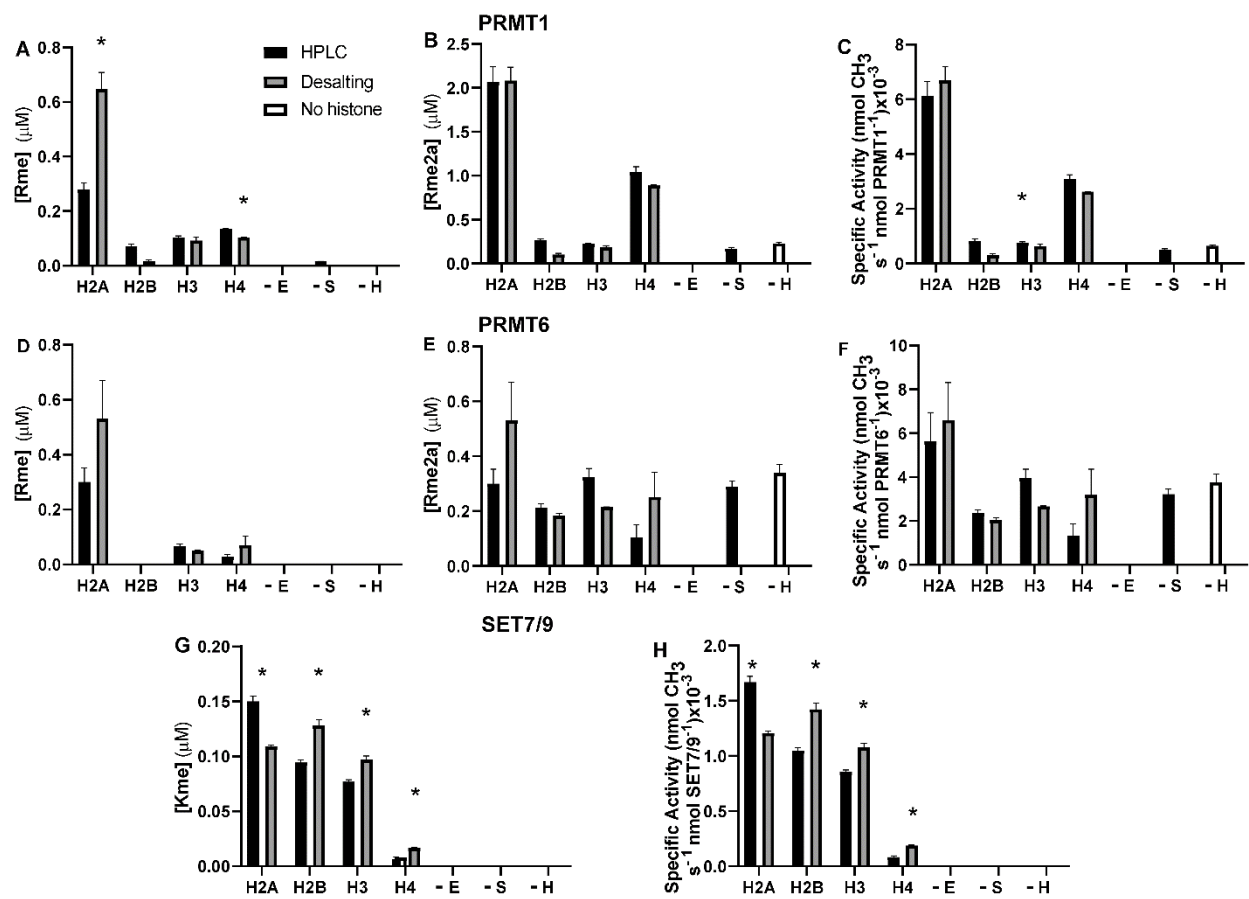


Figure 3.4. Methylation of histones purified by HPLC and desalting column with protein methyltransferases.

PRMT1 (A-C), PRMT6 (D-F), and SET7/9 (G-H) were used to methylate histones H2A H2B, H3, and H4 with 25 μM SAM. The total methyl groups transferred were calculated by the weighted sum of Rme and Rme2a (Rme + 2Rme2a) for PRMTs and the Kme for SET7/9 in nmol. This was used to calculate total specific activity as nmol of methyl-group (CH₃) per second (s) per nmol PRMT1 or PRMT6 or SET7/9. Each bar is the mean and SD for 2 repeats. An asterisk (*) indicates statistically significant differences using Student's t test. - E, - S and - H are no-enzyme, no-SAM and no-histone controls, respectively. Histone H2B purified by HPLC was used in no-enzyme and no-SAM controls.

3.4 Discussion

Histones expressed in *E. coli* accumulate as unfolded insoluble inclusion bodies, so they must be denatured to solubilize them and then refolded. The previous methods of histone purification and refolding utilize at least one step of dialysis against a refolding buffer (176,178,180). Although straightforward, dialysis is time-consuming, typically being performed overnight (~12-16 h). As such, there is the potential for proteolytic hydrolysis of the histones in addition to oxidation of amino acids. Moreover, histone refolding and purification techniques require multi-day protocols. Previously, we developed the first method to utilize the rapid dilution method of refolding histones that had a further step of purification via HPLC (181). Although this method yielded pure histone H4, it was not applied to all 4 core histones, and the HPLC purification step was time-consuming and required costly equipment. We improved on this method by purifying refolded histones using a desalting column rather than HPLC which was less time-consuming, more efficient and resulted in less mechanical loss in the column since the analyte theoretically does not even interact with the stationary phase in size exclusion chromatography. Moreover, as we do not use dialysis, our approach also reduces the risk of mechanical loss that can happen during dialysis which includes nonspecific adsorption of histones to the dialysis membranes (187). Since desalting chromatography is more rapid than any of the other histone purification techniques, it also minimizes the potential for proteolytic hydrolysis and oxidation. Importantly, desalting purification more efficiently removes denaturants such as urea than even multiple dialysis steps as evidenced by the resolution of the histone and salt peaks. This is important because residual denaturants can affect subsequent applications with histones such as enzymatic reactions.

Both HPLC and desalting chromatography purification methods presented here offer significant advantages over affinity purification of histones. Tanaka and colleagues expressed histones with an N-terminal 6His tag and purified histones denatured with 6 M urea with nickel-based IMAC followed by dialysis to remove the urea. The histone H2A/H2B and histone H3/H4 dimers were formed by mixing the denatured histones together and then dialyzing overnight, followed by thrombin cleavage overnight to remove the 6His tag. The next day, the resultant was purified using Sephadex 70 size exclusion chromatography to purify the histone H2A/H2B and histone H3/H4 dimers from the 6His tags, thrombin and histones that were not in the dimer form (179). We estimate that the tag-removal step and subsequent purification steps add at least 16 to 20 h to the protocol in comparison to methods that do not use affinity tags. The use of N-terminal affinity tags and sequences resulted in the inclusion of an initiator methionine that is not present in the amino acid sequences of any histones because such initiator methionine residues are naturally removed from histones expressed in bacteria if no affinity tags are used (176,177). In addition, the affinity tags used by Tanaka *et al* were removed using thrombin and they reported the thrombin recognition sequence Leu-Val-Pro-Arg-Gly-Ser-His, with cleavage after the Arg residue. Therefore, the histones purified using this method will have the additional N-terminal residues Gly-Ser-His-Met. Such additional sequences are typically insignificant in the context of large proteins with hundreds of amino acids. However, the relatively short sequences of histones (just over 100 residues) mean that such sequences could be significant. This is especially true because most histone modifications happen on their N-terminal tails. This additional sequence could, therefore, adversely influence the activity of reader, writer and eraser epigenetic enzymes that add and remove these modifications. Indeed, N-terminal affinity tags can affect the intrinsic disordered nature of the N-terminal tails of histones changing the conformation of those tails (188,189). From a strict

purification perspective, we don't see any need to use affinity tags for the purification of histones as they are very pure in the inclusion bodies (see below), nevertheless if affinity tags are deemed necessary, they would be much less disruptive to histone activity if they are placed on the C-terminus.

3.4.1 Comparing desalting chromatography and HPLC purification

Desalting chromatography yielded a greater efficiency advantage over HPLC as measured by amount of pure histone produced per h of the protocol (Table 3.1) with ~5 fold higher efficiency for histones H2A, H2B and H3 and >1.6-fold higher efficiency for histone H4. Desalting chromatographic purification produced histone purities between 91 and 93% whereas HPLC purification yielded between 88 and 94% purity. Although the resolution of HPLC should be superior to desalting chromatography, our results show that there is no significant increase in the purity of histones purified via HPLC vs desalting chromatography. Given that the desalting purification method does not result in any filtration of proteins greater than ~ 5kDa, this suggests that the histones in the purified inclusion body form are already >87% pure (Table 3.3). This is consistent with the findings of Lugar *et al.* (176,177). Our results suggest that the HPLC method of histone purification, while time-consuming and expensive, does not result in a significant reduction in protein impurities and only serves to remove the salts and buffers from denaturation and refolding. In fact, after the rapid refolding step of our methods the main "impurities" are small molecules such as the denaturation agent, DTT, and arginine which are in the molar concentration range. Desalting chromatography is ideally suited to remove such low molecular weight contaminants, even at very high injection volumes. This is confirmed by the resolution between the histone and salt peaks being ~5. Although we never injected more than 23 mL onto the HiPrep 26/10 desalting column, the maximum loading volume for a typical desalting column is 1 column

volume, which means that we could have injected ~50mL, potentially making the protocol even more efficient.

Several aspects of HPLC purification of histones make it less efficient at purifying refolded histones, including the limited injection volume and on-column mass, the longer overall runtime, the need to re-equilibrate the column after running the gradient program, and the potential for mechanical loss in the HPLC column. The reverse phase column and 5 mL loop we used, limited our maximum injection volume to no more than 5mL. The total runtime for the HPLC method was 60 minutes, and after this, it was necessary to re-equilibrate the column at the initial conditions of the gradient for a minimum of 20 minutes. Therefore, 5 mL could be injected and purified every 80 minutes. It was therefore feasible to inject a maximum of 6 to 7 times per day for a maximum of 30 to 40 mL of refolded histones purified per day.

The ion pairing reagent TFA was used to increase retention times for the histones purified via HPLC, but this was not needed for the desalting chromatography method. Concerns exist about potential downstream effects of residual TFA salts and histone TFA adducts. Our results suggest that in some cases histones purified via desalting chromatography were more readily methylated as seen for histone H2A for PRMT1, histones H2A and H4 for PRMT6 and histones H2B, H3 and H4 for SET7/9. Alternatively, we found only two instances where HPLC-purified histones yielded higher activity: histone H2B with PRMT1 and histone H2A with SET7/9. This will be discussed in more detail below.

3.4.2 Yield, purity and functional validation assessment

Both of our methods yielded similar quantities of each histone. Histone amounts were quantified by determining lysine content in hydrolyzed samples, while molecular weights were verified using mass spectrometry (Table 3.2). The molecular weights of histones produced using both of our

methods match the expected molecular weight of the desired histone indicating both methods preserved histone integrity throughout the purification process.

To determine if our two methods had any effect on the function of the histones as substrates for epigenetic enzymes, we evaluated the histones as substrates in methylation assays with SET7/9, PRMT1, and PRMT6.

Both PRMT1 and PRMT6 are known to methylate histone H2AR3, R11 and R29 (190). PRMT1 and PRMT6 are also known to methylate histone H4R3 producing histone H4R3me2a (191,192) where the modification results in chromatin remodeling. PRMT1 is not thought to methylate histone H2B or histone H3 but recent evidence suggests that heterodimerization of PRMT2 and PRMT1 boosts PRMT1 activity towards histone H2A (193). In addition to the histones listed above PRMT6 methylates histone H3R2, producing histone H3R2me2a (194). Our results with PRMT1 largely corroborate previous findings that PRMT1 primarily methylates histones H2A and H4. Although we find methylation of histones H2B and H3, their activity with PRMT1 is much lower than for histones H2A and H4. Moreover, the total methylation of histones H2B and H3 is consistent with the level of methylation of PRMT1 before the initiation of the reaction (Figure 3.4C). Similarly, we find that PRMT6 methylate histones H2A, H3 and H4 but not histone H2B. It is noteworthy that PRMT1 and 6 yield some Rme2a in the control samples lacking SAM which is indicative of automethylation before initiation of the reaction. Automethylation of PRMT enzymes is a well-documented phenomenon (195) and in some cases it is thought to influence PRMT activity (196). However, automethylation is also known to happen during expression in *E. coli* (197) and this is likely to explain the presence of Rme2a in the PRMT 1 and 6 reactions without SAM (Figure 3.4B and 3.4E). Alternatively, this behavior may be attributed to PRMTs intrinsic preference for methylating histone substrates when present (16). SET7/9-catalyzed lysine

methylation was significantly higher for desalting column purified histones except histone H2A, where HPLC-purified samples showed higher methylation. Unlike PRMTs SET7/9 showed no propensity for automethylation (Figure 3.4G), which we previously confirmed (182).

The differences in histone methylation between HPLC and desalting chromatography-purified histones reflect the potential influence of purification conditions on substrate suitability for enzymatic assays. We observed that in most cases with statistically significant differences, the histone purified via desalting chromatography exhibited higher methylation activity. Histone purification via HPLC was performed using TFA, an ion-pairing agent that may disrupt protein-protein interactions (198). In particular, TFA can form salts with histones specifically lysine or arginine $R-NH_3^+ CF_3COO^-$ salt adducts. This is critical since the mechanisms of both PRMTs and PKMTs like SET7/9, are thought to require a proton abstraction step on the target residue $R-NH_3^+$ before methylation (199). TFA ion pairing could prevent this proton abstraction and could sterically block the target residue from entering the enzyme active site. In contrast, the desalting purification process utilized 50 mM ammonium bicarbonate, a volatile buffer that is mostly removed by the process of lyophilization. Moreover, carbonate salts of histones are possible, but carbonic acid is a much weaker acid than TFA so it can be more easily removed and it is prone to decomposition into H_2O and CO_2 .

TFA salts of histones may also lead to site-specific structural distortion, impacting enzyme-substrate recognition. In the two cases where HPLC-purified samples showed slightly increased methylation it may be that TFA-induced conformational changes enhanced enzyme access to target histone residues. Otherwise, histones purified via HPLC and therefore likely containing TFA adducts generally exhibited reduced methylation or had no effect (200). Ultimately, the potential effects of TFA only appear to be important with particular histones and methyltransferases.

Densitometric analysis of SDS-PAGE gels revealed no statistically significant difference in histone purity between HPLC and desalting-column purified samples. Therefore, we can rule this out as a cause for the differences in methylation of histones purified via the different techniques.

3.5 Conclusion

In this study, we developed a rapid, cost-effective, and scalable method for the purification of recombinant core histones that significantly reduces the time, complexity, and resource demands compared to previous protocols. By combining rapid dilution refolding with single-step desalting size-exclusion chromatography, we eliminated the need for extensive dialysis and multiple chromatographic steps, without compromising protein yield, purity, or functional integrity. Comparative analyses with HPLC-purified histones confirmed that our desalting column-based approach produces histones of comparable quality, as evidenced by identical molecular masses, similar purity profiles, and similar enzymatic methylation profile. This method enables the high-throughput production of each of the four core histones within a single day and increases accessibility for laboratories lacking advanced chromatographic FPLC or HPLC equipment. Histones purified using this method could be used for other post-translational modification assays, including lysine acetylation. Moreover, such histones could potentially be used for the reconstitution of nucleosomes.

3.6 Supplementals

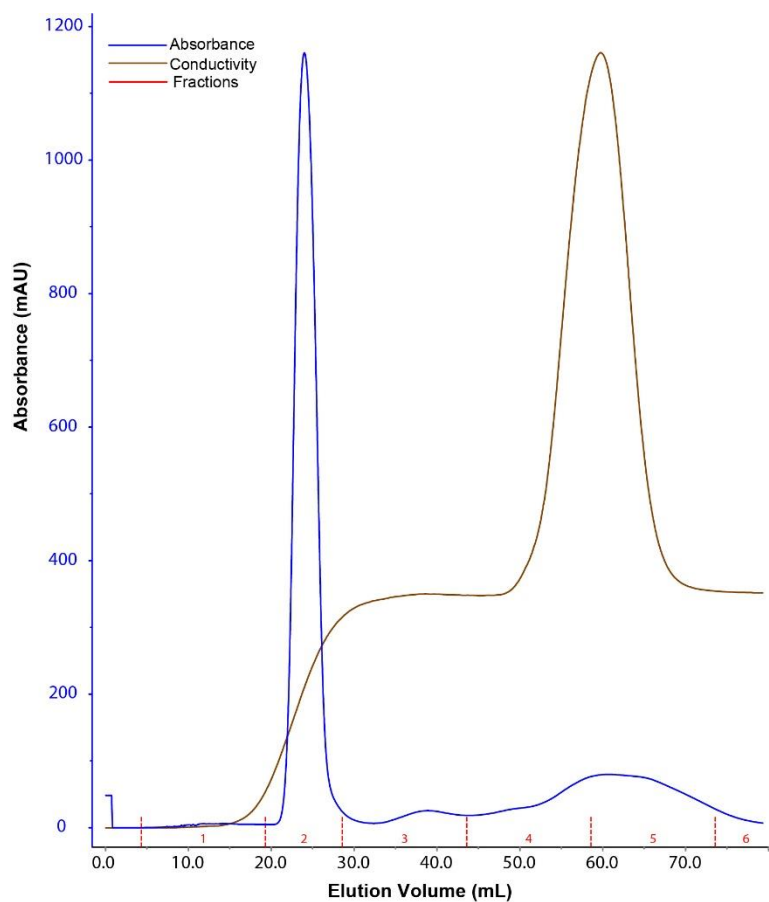


Figure S3.1. Chromatogram of histone purification with FPLC desalting method. UV absorbance at 280 nm and conductivity were monitored. The protein was eluted between 20 mL to 30 mL of elution while the impurities (mostly salts) were eluted later between 50 mL to 70 mL of the run.

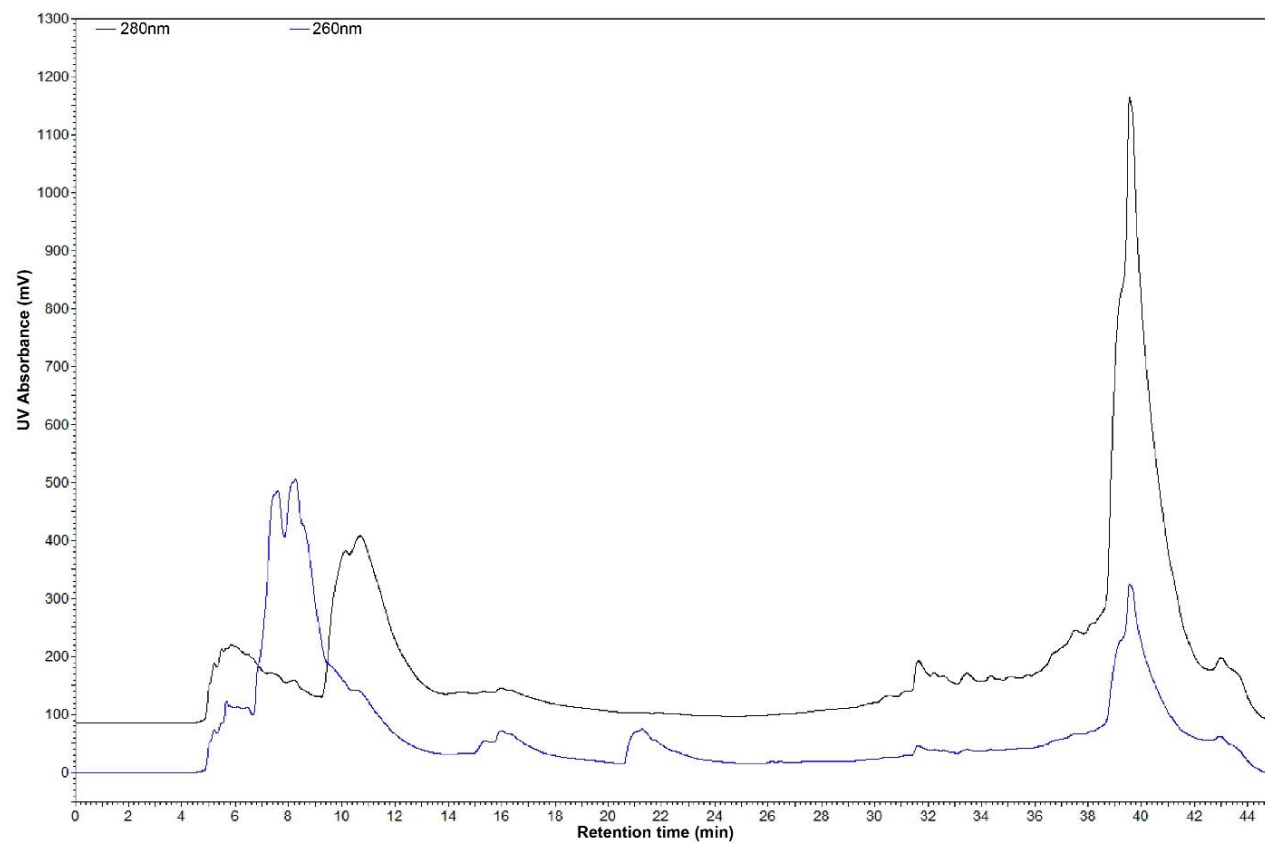


Figure S3.2. Chromatogram of histone purification HPLC method. The run was monitored at 260 and 280 nm.

The proteins were eluted at about 39 minutes of the run. The impurities were eluted before 12 minutes.

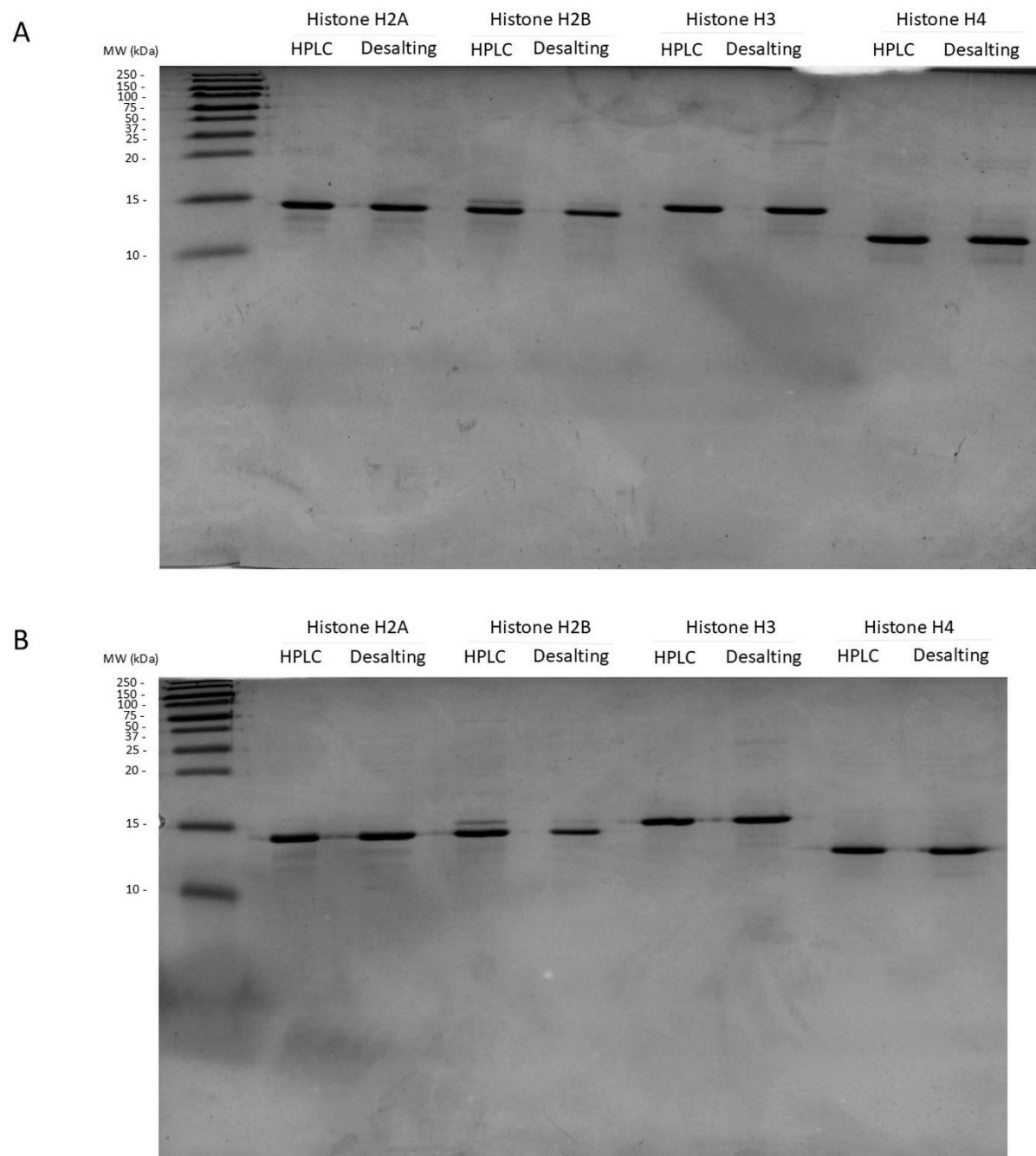


Figure S3.3. SDS-PAGE analysis of histone purity following HPLC and desalting purification. The images shown here are cropped, but the unmodified gel images were used to generate the data presented in Table 3.3. For alignment purposes, panel B was mirrored horizontally.

Table S3.1. Mass Spectrometry parameters: MRM transitions and collision energies

Methylated Amino Acids	Retention Time (min.)	MRM	Collision Energy (eV)
K	2.03	146.9>130.0	-14
Kme	3.34	161.2>84.0	-18
Kme2	3.42	174.8>84.0	-20
Kme3	3.5	189.2>83.9	-22
R	2.15	175.2>70.10	-14
Rme	3.41	188.8>70.0	-24
Rme2s	3.46	202.8>171.9	-14
Rme2a	3.46	203.0>45.9	-14
¹³ C6 ¹⁵ N2 lysine	2.07	155.0>90.2	-17

Chapter 4: SET7/9 exhibits sigmoidal kinetics on nucleosomes, hyperbolic kinetics on histones by an ordered sequential mechanism and methylates lysine and arginine

This chapter has also been published: Ige OO, Hendrickson-Rebizant T, Luo W, Cheung P, Lao Y, Zahedi RP, Davie JR, Lakowski TM. SET7/9 exhibits sigmoidal kinetics on nucleosomes, hyperbolic kinetics on histones by an ordered sequential mechanism, and methylates lysine and arginine. J Biol Chem. 2025 Oct;301(10):110639. doi: 10.1016/j.jbc.2025.110639. Epub 2025 Aug 28. PMID: 40885388; PMCID: PMC12514578.

4.0 Abstract

SET7/9 (SETD7) is a SET domain protein lysine methyltransferase (PKMT). We characterized its activity using a mass spectrometry (MS) assay, showing that it follows an ordered sequential enzyme kinetic mechanism where SAM is the first substrate to bind, followed by histone H3, and monomethylated histone H3 is the first product to dissociate, followed by SAH. Full-length histones H2A, H2B, and H4 are also substrates for SET7/9. We found that SET7/9 methylates nucleosome core particles (NCP), histone H2BK120 ubiquitinated nucleosome core particles (Ub-NCP) as well as histone octamers, exhibiting sigmoidal kinetics, and suggesting an allosteric interaction that was not observed with free histones, which follow hyperbolic (Michaelis–Menten) kinetics. Using low (25 nM) SET7/9, we only detect monomethyl-lysine with all substrates except Ub-NCP, which produces mono- and dimethyllysine at equal rates. Proteomic analysis shows that SET7/9 catalyzes multiple sites and types of, methylation on histones, depending on if they are free histones or within an octamer. Free histone H3 is methylated at multiple N-terminal sites (including histone H3K4) that are not methylated in the octamer. Some C-terminal methylation sites were discovered that can also be ubiquitinated including histone H2BK120. Since lysine

methylation and ubiquitination are mutually exclusive such methylation by SET7/9 may serve to prevent ubiquitination. At high enzyme and substrate concentrations and extended incubation times, we show that SET7/9 can catalyze the formation of dimethyl- and trimethyllysine on histones H2B, H3 and histone octamers and, most remarkably, the formation of monomethyl- and dimethylarginine on histone H3 and within octamers.

4.1 Introduction

The protein lysine methyltransferases (PKMTs) are a family of enzymes requiring two substrates and producing two products following a Bi-substrate and Bi-product (Bi-Bi) enzymatic mechanism. Accordingly, they transfer methyl groups from the substrate S-adenosyl-L-methionine (SAM) to the ϵ -amino group of lysine residues on protein substrates, producing the products S-adenosyl-L-homocysteine (SAH), and methyllysine residues (78). Perhaps the most famous PKMT substrates are histones where their methylation influences gene expression through epigenetic processes. Depending on the PKMT and protein substrate up to three methyl-groups can be transferred forming mono-, di- and trimethyllysine (Kme1, Kme2, and Kme3, respectively) (Figure 4.1) (201). Methylation of lysine does not affect its charge but may interfere with hydrogen bonding. Methyl-lysines bind to what are known as reader domains within effector proteins like transcription factors, co-activators, co-repressors, or other proteins that often change gene expression. Histone methyllysine recognition by reader domains is context-dependent as it can be based on the number of methyl-groups on the target lysine, the surrounding amino-acid sequence, and their potential post-translational modifications (202). Histone lysine methylation can be associated with heterochromatin or euchromatin and, low and actively transcribed regions, respectively. Generally, methylation of histone H3K4, H3K36, and H3K79 are associated with active transcription, while methylation on histones H3K9, H3K27 and H4K20 are associated with

decreasing transcription, depending on the level of methylation (203). However, the context of other histone modifications can influence the overall effect on expression. PKMTs typically methylate particular histone N-terminal tail residues, and a maximal number of methyl-groups are added. In addition to histone N-terminal tails, core and C-terminal lysine residues can also be methylated or be the site of other modifications such as acetylation and ubiquitination but not usually at the same time. Since such modifications cannot simultaneously be on the same lysine residue, they are said to be mutually exclusive. SET7/9 is a SET domain PKMT, so called because it contains a SET methylation domain (204). It was initially given the names SET7 (205) and SET9 (206). Although it is now formally known as SETD7, the name SET7/9 is more commonly used and will be used throughout this manuscript. SET7/9 is known for catalyzing mono-methylation of histone H3K4

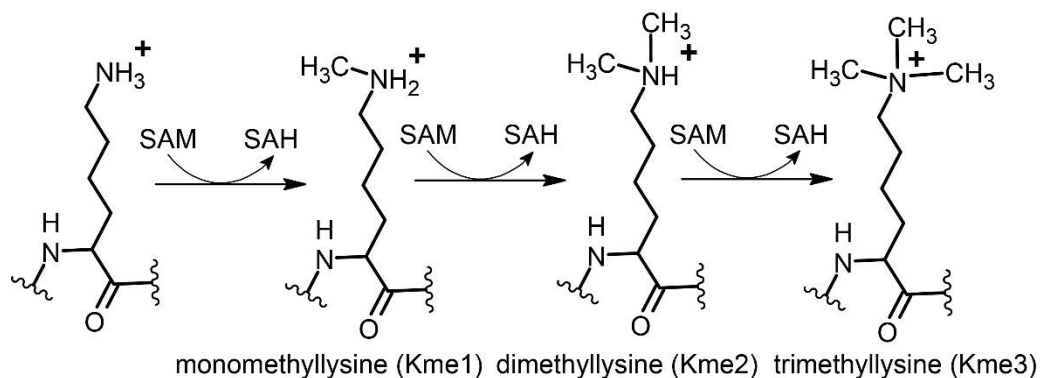


Figure 4.1. Reactions catalyzed by protein lysine methyltransferases. PKMT enzymes can add up to three methyl groups to lysine residues on their protein substrates.

(H3K4me1), where it is thought to play a role in active enhancers (207), but with the modification being added prior to nucleosome assembly, since SET7/9 was previously not known to methylate nucleosomes (206). SET7/9 has demonstrated limited ability to mono-methylate peptides representing the N-terminal tails of histones H2A and H2B (208,209), and histone H1 (210).

Although SET7/9 methylates nonhistone proteins, the focus of this study is on the histone substrates of SET7/9. SET7/9 binds the target lysine residue through a channel lined with conserved tyrosine residues (211). The hydroxyl groups of such conserved residues Y245 and Y305, form hydrogen bonds with the target lysine residue, and this dictates the maximal number of methyl-groups the enzyme can add. For example, wild-type SET7/9 is thought to only catalyze histone H3K4me1, but the mutation Y245A produces histone H3K4me1 and di-methylation (histone H3K4me2) (211), whereas Y305F produces histone H3K4me2 and small amounts of trimethylation (histone H3K4me3) (205). Here we investigated the methylation activity of SET7/9 on free histone and nucleosome substrates to determine enzymatic mechanism and to see if it can methylate nucleosomes. A validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assay was used to quantify the modified amino acids Kme1, Kme2, and Kme3 as well as mono-, and asymmetric and symmetric dimethylarginine (Rme1, Rme2a, Rme2s) derived from quantitatively hydrolyzed reactions containing SET7/9 and free histone and nucleosome substrates. We show that SET7/9 follows an ordered sequential Bi-Bi enzymatic mechanism where SAM is the first substrate to bind, followed by histone H3, and mono-methylated histone H3 is the first product to dissociate, followed by SAH. Full-length free histones H2A, H2B and H4 are also substrates for SET7/9. For the first time, we find that SET7/9 methylates, histone octamers, nucleosome core particles (NCP), and histone H2BK120 mono-ubiquitinated nucleosome core particles (Ub-NCP) following sigmoidal enzyme kinetics and suggesting an allosteric interaction that was not observed with free histones which follow typical hyperbolic (Michaelis–Menten) kinetics. Based on previous studies, we suggest that Membrane Occupation and Recognition Nexus MORN repeats of SET7/9 are the allosteric site for interactions with histone H3 in the context of a nucleosome. We conducted the first proteomic analysis of the methylation of histones

by SET7/9 discovering multiple sites, and types of methylation that differ depending on if the histones are free or within an octamer. Free histone H3 is methylated at multiple N-terminal sites but almost none of these sites, including the active mark histone H3K4, are methylated in the histone octamer. Many of the sites of histone lysine methylation are also sites of monoubiquitination (Ub), including histone H2BK120, and these modifications are mutually exclusive. We theorize that the concerted effect of SET7/9 on nucleosomes may be to decrease histone H3K4me1 and thereby reduce the activity of active enhancers. With high enzyme and substrate concentrations and extended incubation SET7/9 catalyzes the formation of Kme2 and Kme3 on histones H2B, H3, and histone octamers and, most remarkably, the formation of Rme1 and Rme2a on histone H3 and histone octamers.

4.2 Results

The method we used to measure the enzymatic activity of SET7/9 was adapted from our previous validated method except we utilized a volatile buffer $(\text{NH}_4)\text{HCO}_3$ to reduce background and ion suppression, and incorporated stable isotope labeled $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine as an internal standard to correct for loss during sample processing and injection (138,185,212). The details are briefly summarized in the Materials and Methods and supplementary materials. At high (25 μM) and low (5 μM) concentrations of histone H3, the rate of methylation (represented by rate of production of Kme1) by SET7/9 (25 nM) is linear with respect to time up to at least 60 min at 30 °C (Figure S4.1). The rate of production of Kme1 is linear with respect to enzyme concentration from 3.125 to 200 nM SET7/9 (Figure S4.2). The pH optimum was identified as 9 (Figure S4.3), and DTT did not influence enzyme rate (Figure S4.4). Therefore, all subsequent kinetic reactions were carried out for 20 min with 25 nM SET7/9 in a buffer with 100 mM $(\text{NH}_4)\text{HCO}_3$ at pH 9, and 200 nM $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine internal standard at 30 °C, unless otherwise stated. Previous studies apparently

showed SET7/9 catalysed methylation of itself (AKA auto-methylation) but this was only in the presence of the transcription factor Yin Yang 1. Auto-methylation experiments with only SET7/9 and SAM were never performed (213). Notably, our substrate control assays which contained SET7/9, and SAM only (no histone) showed no detectable methylation activity. This suggests that SET7/9 does not auto-methylate (Figure S4.5).

4.2.1 SET7/9 follows a sequential mechanism

The enzymatic mechanism of an enzyme determines how substrates bind to that enzyme and allows determination of its enzymatic parameters. To start determination of the enzymatic mechanism of SET7/9, we incubated the enzyme with a range of increasing concentrations of histone H3 and SAM. First, we estimated the V_{Max} and K_M values to determine these concentration ranges by varying each substrate in the presence of a fixed excess of the other. Based on these initial values (apparent values) the concentration ranges for SAM and histone H3 were selected for further experimentation. When histone H3 was varied in the presence of several constant SAM concentrations, the double reciprocal plots exhibit increasing slopes with decreasing SAM concentration, producing a pattern of lines intersecting to the left of the y-axis, above the x-axis (Figure 4.2). The pattern of lines suggests that the substrates bind sequentially, therefore following a sequential Bi-Bi substrate binding mechanism, rather than a double displacement Bi-Bi substrate enzymatic mechanism where the methyl-group from SAM would transiently attach to the enzyme before being transferred to the substrate. Such a sequential mechanism is consistent with most (214), but not all SAM dependent methyltransferases (215).

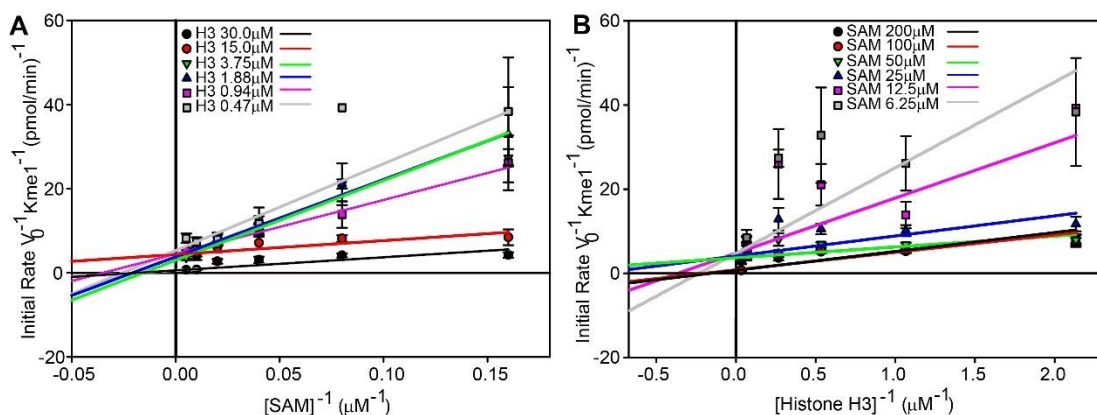


Figure 4.2. Sequential Bi-Bi substrate enzymatic mechanism for SET7/9. Reciprocal plots of the initial velocity (v_0) of SET7/9 with the substrates, full-length histone H3 and SAM. The concentration of SAM was varied with fixed histone H3 concentrations from 0.47 to 30 μM (A). These data were replotted where the concentration of histone H3 was varied with fixed SAM concentrations from 6.25 to 200 μM (B). The initial velocity was measured by quantifying the amount of Kme1 produced over 20 min. Each point is the mean and SD of three reactions. The kinetic parameters derived from these graphs and the replots in Figure S4.8, are shown in Table 4.1.

Table 4.1. Kinetic parameters for SET7/9 substrates

Compound	Type	K_M (μM)	K_{SA} (μM)	K_i (μM)	V_{Max} (pmol/min)	k_{cat} (s^{-1}) $\times 10^{-3}$	k_{cat}/K_M ($M^{-1}s^{-1}$)
SAM	Substrate	12.0 ± 3.2	37.9 ± 20		0.536 ± 0.13	5.95 ± 1.43	498 ± 32
Histone H3	Substrate	1.36 ± 0.22			0.472 ± 0.048	5.24 ± 0.54	$3,970 \pm 1,100$
SAH	Product			12.1 ± 3.1			

4.2.2 Product inhibition studies

The data in Figure 4.2 suggest a sequential mechanism of substrate binding to SET7/9 but cannot determine if the substrate binding and product release has to follow a strict order where one substrate must bind first before the other, and one product must dissociate first before the other (an

ordered sequential Bi-Bi mechanism), or if either substrate can randomly bind first and either product dissociate first (a random sequential Bi-Bi mechanism). Both mechanisms form a stable ternary complex composed of both substrates and the enzyme, whereas a special case of the ordered sequential Bi-Bi mechanism, known as the Theorell Chance mechanism, does not. To determine which of these mechanisms SET7/9 follows, product inhibition assays must be performed where the products of the reaction, called product inhibitors, are added to the reaction to determine what type of inhibition they produce, since this informs us about how the substrates bind to the enzyme. We used constant and variable substrates, SAM and histone H3, and increasing concentrations of the product inhibitors histone H3Kme1 methylated lysine analog (MLA) (H3K4me1MLA) or SAH. Preliminary measurements of the inhibition of SET7/9 by histone H3K4me1MLA (Figure S4.6) and SAH (Figure S4.7) were performed to find an appropriate concentration range for each product inhibitor. Figure 4.3A shows the inhibition of SET7/9 by SAH with a constant non-saturating concentration of SAM and variable histone H3 concentrations. The curves appear to converge to the left of the y-axis, indicating mixed inhibition. In contrast, Figure 4.3B shows the inhibition of SET7/9 by SAH with a constant non-saturating concentration of histone H3 and variable SAM, where the curves appear to converge on the y-axis. This suggests competitive inhibition by SAH, where SAM and SAH compete for the SAM/SAH binding site. Such inhibition suggests an ordered sequential Bi-Bi mechanism, in which SAM is the first substrate to bind and SAH is the last product to dissociate from SET7/9. These data also argue against a random sequential Bi-Bi mechanism, which would yield mixed inhibition. Mixed inhibition of SET7/9 is observed with increasing concentrations of histone H3K4me1MLA in Figure 4.3C, where histone H3 is varied with fixed SAM. Such inhibition is indicative of an ordered sequential Bi-Bi mechanism, but not a Theorell-Chance mechanism, which would exhibit

competitive inhibition. Figure 4.3D shows the inhibition of SET7/9 by histone H3K4me1MLA, where the SAM concentration varies with fixed histone H3. Most of the curves appear to converge to the left of the y-axis, suggesting a mixed type of inhibition and an ordered sequential Bi-Bi mechanism (214). Therefore, we conclude that SET7/9 follows an ordered sequential Bi-Bi mechanism, where SAM binds to the enzyme first, followed by histone H3, and the product methylated histone H3 dissociates from the enzyme first, followed by SAH (Figure 4.4). These substrates and products are conventionally referred to as substrates A and B, and products P and Q, respectively. The initial rate (v_o) of the reaction for an ordered sequential Bi-Bi mechanism can be described using Equation 4.1 and its reciprocal forms, Equation 4.2, where the first substrate A is varied and the second substrate B fixed, and Equation 4.3, where B is varied and A fixed. K_{SA} is the equilibrium dissociation constant for the first substrate to bind to SET7/9, K_{MA} and K_{MB} are the Michaelis constants for the substrates A and B, respectively, V_{Max} is the maximum rate of the reaction, and $[A]$ and $[B]$ are the concentrations of the substrates. The turnover number or k_{cat} is defined according to Equation 4.4 (216).

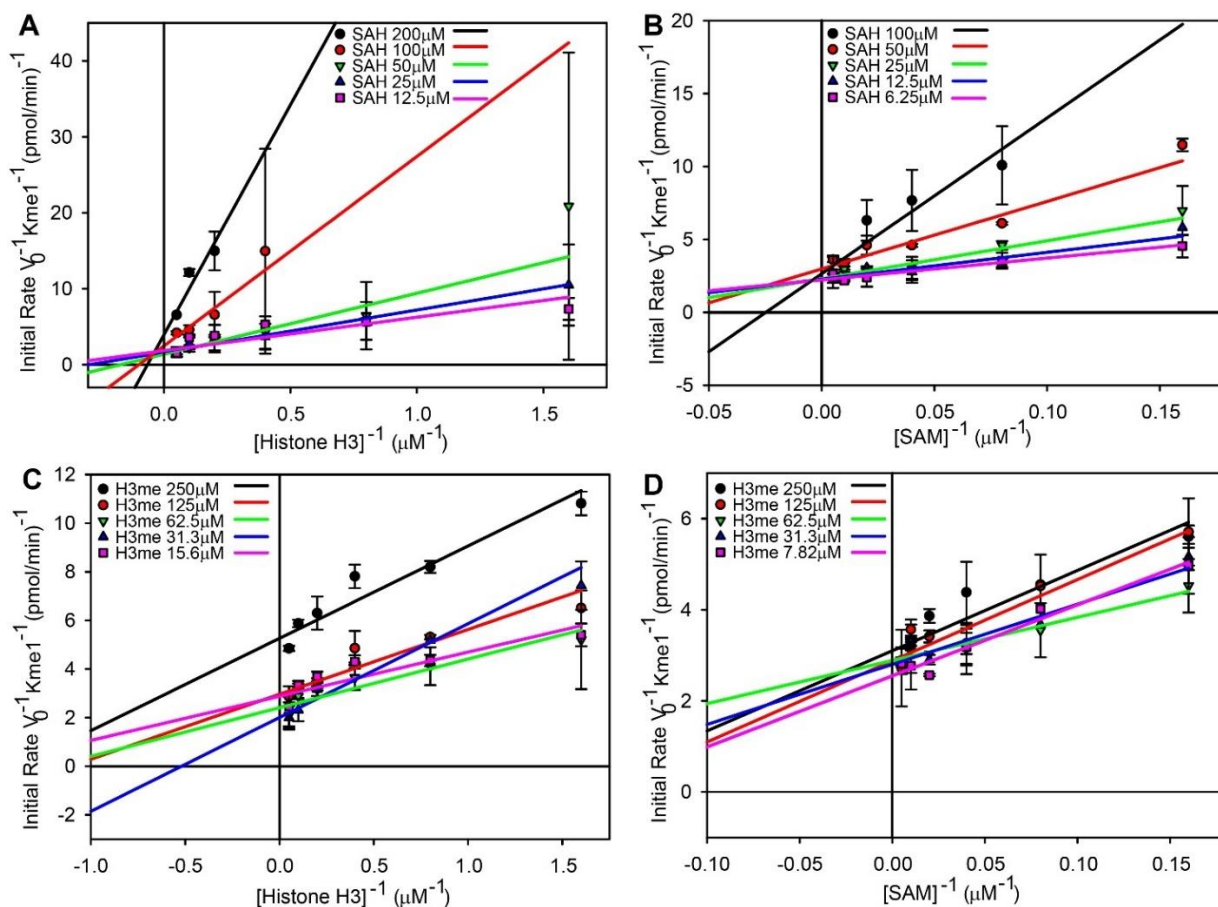


Figure 4.3. SET7/9 follows an ordered sequential Bi-Bi enzymatic mechanism. Reciprocal plots of the initial velocity (v_0) of SET7/9 with the substrates, full-length histone H3 and SAM, and the product inhibitors SAH and histone H3K4me1MLA (H3me). Plotted are fixed concentrations of the product inhibitor SAH with variable histone H3 concentrations and a fixed SAM concentration of 30 μM (A) or variable SAM and a fixed histone H3 concentration of 4 μM (B). Shown below them are fixed concentrations of the product inhibitor histone H3K4me1MLA with variable histone H3 concentrations and a fixed SAM concentration of 30 μM (C) or variable SAM and a fixed histone H3 concentration of 4 μM (D). The initial velocity was measured by quantifying the amount of Kme1 produced over 20 min. Each point is the mean and SD of two reactions. The replots of the slopes and intercepts from these graphs are shown in Figure S4.9. The product inhibitor constants and their mathematical descriptions are shown in Table S4.2 and supplemental Equations S4.1–S4.7, respectively.

$$v_0 = \frac{V_{\text{Max}}[A][B]}{K_{\text{SA}}K_{\text{MB}} + K_{\text{MB}}[A] + K_{\text{MA}}[B] + [A][B]} \quad [\text{Equation 4.1}]$$

$$\frac{1}{v_0} = \frac{K_{MA}}{V_{Max}} \left(\frac{K_{SA}K_{MB}}{[B]K_{MA}} + 1 \right) \frac{1}{[A]} + \frac{1}{V_{Max}} \left(\frac{K_{MB}}{[B]} + 1 \right) \quad [\text{Equation 4.2}]$$

$$\frac{1}{v_0} = \frac{K_{MB}}{V_{Max}} \left(\frac{K_{SA}}{[A]} + 1 \right) \frac{1}{[B]} + \frac{1}{V_{Max}} \left(\frac{K_{MA}}{[A]} + 1 \right) \quad [\text{Equation 4.3}]$$

$$k_{cat} = \frac{V_{Max}}{[E]_T} = \frac{k_3k_4}{k_3+k_4} \quad [\text{Equation 4.4}]$$

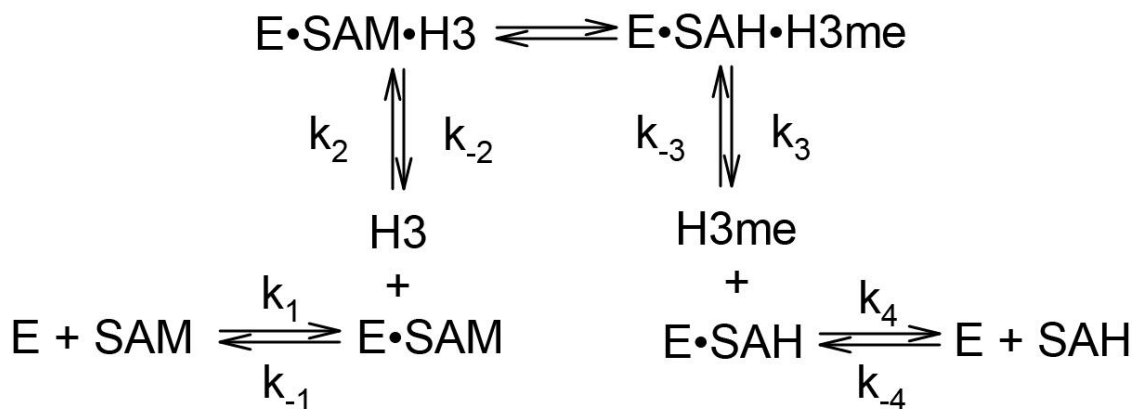


Figure 4.4. The kinetic mechanism of SET7/9. Shown is the ordered, sequential Bi-Bi enzymatic mechanism of SET7/9 (represented as E) where SAM is the first substrate to bind followed by histone H3 (H3) and the first product to dissociate is monomethylated histone H3 (H3me) followed by SAH. The microscopic forward k_n and reverse k_{-n} rate constants are shown for each step n of the reaction. With respect to equations 4.1 through 4.7, SAM is substrate A, histone H3 substrate B, monomethylated histone H3 product P, and SAH product Q.

Knowing that it follows an ordered sequential Bi-Bi mechanism, the kinetic parameters for SET7/9, V_{Max} , K_{SA} , K_{MA} , K_{MB} , and K_i (Table 4.1) were determined by replotting the slopes and intercepts from the double reciprocal plots from Figure 4.2 in Figure S4.8, and by replotting the slopes and intercepts from product inhibitor plots from Figure 4.3 in Figure S4.9. The turnover numbers, k_{cat} values, were calculated using Equation 4.4, and the specificity constants (k_{cat}/K_M) were calculated using the derived parameters. As expected, the V_{Max} for both histone H3 and SAM

is approximately the same. However, the K_M and k_{cat}/K_M for SAM are almost 9-fold and 8-fold higher than the corresponding parameters for histone H3. Since the reaction follows an ordered sequential Bi-Bi mechanism and we are not using saturating concentrations of either substrate, the K_i for SAH (Table 4.1, defined as K_{iQ} in the supplemental equation (Equation S4.5)) is the only true K_i derived from the product inhibitor studies (214). A full description of the product inhibitor slope and intercept inhibition constant equations (216), and their values, is available in the Supplementary material (Equations S4.1–S4.7, and Table S4.2).

4.2.3 Histone and nucleosome substrate profiling

The activity of SET7/9 against free histones, histone octamers, NCP, and Ub-NCP was compared using apparent kinetic parameters for an unbiased comparison among substrates. Plots of initial reaction rates as measured by the amount of Kme1 produced per unit time with increasing free histones H3, H2A, H2B, and H4 and fixed saturating SAM are shown in Figure 4.5. To calculate apparent kinetic parameters, we used saturating concentrations of substrate B (histones), where Equation 4.2 reduces to Equation 4.5, and similarly used saturating concentrations of substrate A (SAM), where Equation 4.3 reduces to Equation 4.6 (*i.e.* one substrate Michaelis–Menten kinetics, AKA hyperbolic kinetics). The rate data for SET7/9 against histones H3, H2A, H2B, and H4 were fit to Equations 4.5 and 4.6 to derive apparent kinetic parameters (Table 4.2). The apparent V_{Max} ($V_{Max App}$) for histone H3 and SAM are 20 to 30% higher than the actual values in Table 4.1, and the apparent K_M ($K_{M App}$) for histone H3 and SAM are 3 and 1.7-fold higher, respectively, than the actual values determined by our kinetic analysis (Table 4.1). Using the apparent specificity constant (k_{cat}/K_M)_{App}, the preference of SET7/9 for free histones is histone H2B > histone H2A > histone H3 >> histone H4. Contrary to others, we found that SET7/9 does methylate histone H4 but with $V_{Max App}$ values less than 1/4 to one-fourth of that for histones H3, H2A, and H2B.

$$V_{0 \text{ App}} = \frac{V_{\text{Max App}}[A]}{K_{\text{MA App}} + [A]} \quad [\text{Equation 4.5}]$$

$$V_{0 \text{ App}} = \frac{V_{\text{Max App}}[B]}{K_{\text{MB App}} + [B]} \quad [\text{Equation 4.6}]$$

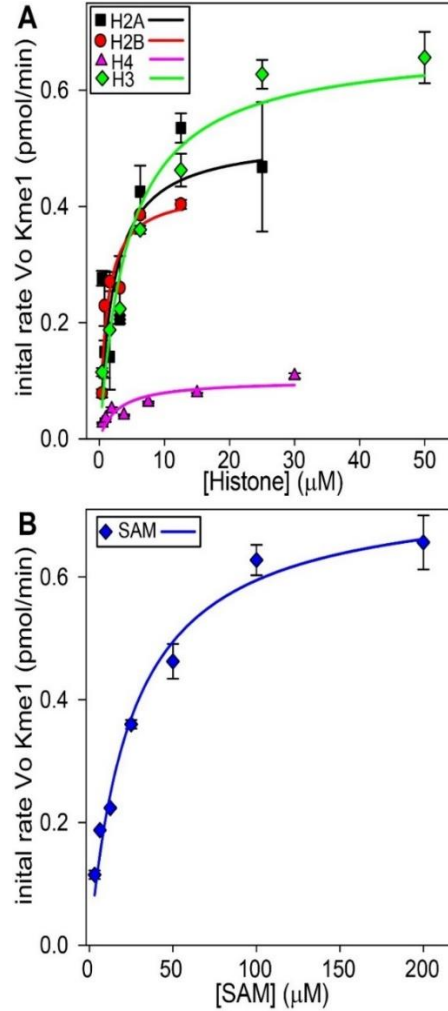


Figure 4.5. Substrate profiling for SET7/9 with free histones and SAM. The initial rate of production of Kme1 by SET7/9 with varying concentrations of histone H3, H2A, H2B, or H4 with a fixed SAM concentration of 200 μM (A) or varying SAM with a fixed histone H3 concentration of 30 μM (B). The initial velocity was measured by quantifying the amount of Kme1 produced over 20 min. Each point is the mean and SD of two reactions. The curves were fit to Equation 4.5 (A) and Equation 4.6 (B), and the apparent Michaelis–Menten kinetic parameters are shown in Table 4.2.

Table 4.2. Apparent Michaelis–Menten kinetic parameters for SET7/9 with histones

Variable	Saturating	K_M App	V_{Max} App	k_{cat} App	(k_{cat}/K_M)_{App}
Substrate	Substate	(μM)	(pmol/min)	(s⁻¹)x10⁻³	(M⁻¹s⁻¹)
SAM	Histone H3	20.5±1.8	0.686±0.030	7.62±0.34	373±38
Histone H3	SAM	4.38±0.42	0.653±0.046	7.25±0.51	1,660±41
Histone H2A	SAM	1.99±0.21	0.511±0.055	5.68±0.61	2,860±5.1
Histone H2B	SAM	1.07±0.26	0.429±0.024	4.76±0.27	4,570±84
Histone H4	SAM	2.79±0.025	0.101±0.0016	1.13±0.017	403±9.8

Since previous studies showed that SET7/9 cannot methylate NCP (31,217–220), we measured the activity of SET7/9 against NCP, Ub-NCP, and histone octamers. Interestingly, SET7/9 methylated histone octamers, NCP, and Ub-NCP exhibited sigmoidal (*i.e.* non-Michaelis–Menten) kinetics when measuring initial rate with increasing octamers or NCP (Figure 4.6A) or Ub-NCP (Figure 4.6, B and C) with saturating SAM. Such sigmoidal curves suggest an allosteric interaction between NCP, Ub-NCP, histone octamers, and SET7/9. We could not saturate the reaction since we could not reach concentrations higher than 4 μ M for NCP and histone octamers and 3 μ M for Ub-NCP. Nevertheless, even at these low substrate concentrations, the sigmoid shape is evident, and the V_{Max} App was at least 6.2 to 9-fold higher for NCP and histone octamers than the V_{Max} App for any of the free histones. Since the curves were sigmoidal, the data from Figure 4.6, A and B were fit to the Hill equation (Equation 4.7), and the apparent kinetic parameters are listed in Table 4.3. Interestingly, Ub-NCP is the only substrate that produces Kme1 and Kme2 at equal rates (Figure 4.6C). All other SET7/9 substrates only produce Kme1 under these conditions.

$$V_{0\text{ App}} = \frac{V_{\text{Max App}}[B]^{n_H}}{K_{B\text{ App}}^{n_H} + [B]^{n_H}}$$

[Equation 4.7]

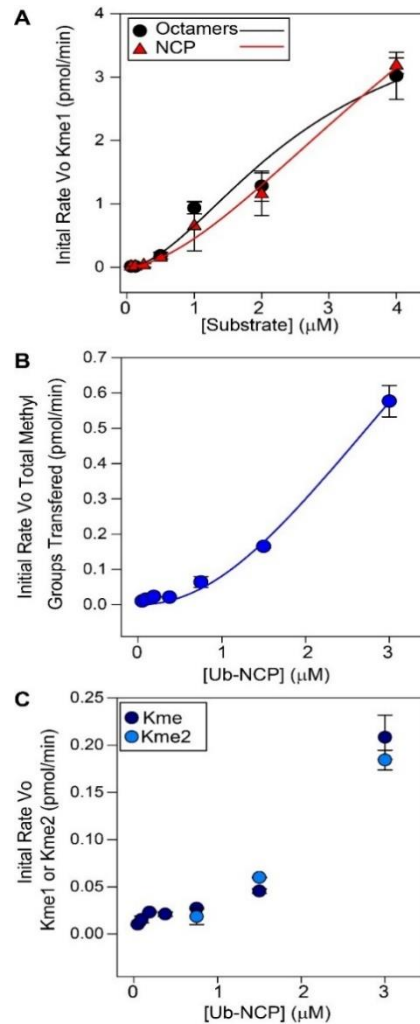


Figure 4.6. Substrate profiling for SET7/9 with histone octamers, NCP and Ub-NCP. The initial rate of production of Kme1) in pmol per min by SET7/9 with varying concentrations of histone octamers or NCP with a fixed SAM concentration of 200μM (A). The initial rate of addition of total methyl groups as measured by the weighted sum of Kme1 and Kme2 (Kme1+2Kme2) in pmol of methyl-groups per min by SET7/9 with a fixed SAM concentration of 200μM (B). The initial rate of production of Kme1 and Kme2 in pmol per min by SET7/9 with a fixed SAM

concentration of 200 μ M. Each point is the mean and S.D. of 2 repeats except for 6A NCP which is a mean and S.D. of 4 repeats. The curves in A and B were fit to Equation 4.7 and the kinetic parameters are shown in Table 4.3.

Table 4.3. Apparent sigmoidal kinetic parameters for SET7/9 NCP and histone octamers.

Variable	Saturating	K'_{MB} App	V_{Max} App	n_H	k_{cat} App	$(k_{cat}/K'_{MB})_{App}$
Substrate	Substate	(μ M)	(pmol/min)		(s^{-1}) $\times 10^{-3}$	($M^{-1}s^{-1}$)
NCP	SAM	3.61 $\pm$ 0.35	5.88 $\pm$ 0.16	2.06 $\pm$ 0.44	65.3 $\pm$ 1.8	18,200 $\pm$ 1,800
Octamers	SAM	2.69 $\pm$ 0.098	4.43 $\pm$ 0.56	1.75 $\pm$ 0.0071	49.3 $\pm$ 6.2.	18,300 $\pm$ 1,700
Ub-NCP	SAM	3.02 $\pm$ 0.33	1.18 $\pm$ 0.24	2.42 $\pm$ 0.019	13.1 $\pm$ 2.7	4,300 $\pm$ 410

4.2.4 Proteomic studies

Histone H3K4me1 has been assumed to be the only or primary histone product of SET7/9. However, there is some evidence that SET7/9 can methylate histone H2A and histone H2B tail peptides (208,209), and the C-terminal residues of the linker histone H1.4 (210). Therefore, we performed a proteomic analysis of the methylation of full-length free *Xenopus laevis* histones H3, H2A, H2B, and H4 (Figure 4.7A) as well as human histone octamers (Figure 4.7B) to determine all sites of methylation catalyzed by SET7/9. At the relatively low concentrations of histone substrates and SET7/9 in our kinetic studies, and irrespective of incubation time, we could not detect any methylation using proteomics methods. Moreover, under these conditions, our assay could detect only Kme1 in all histones and histone octamers (Figure 4.7C). To detect methylation and to get sufficient histone sequence coverage, we were forced to use substantially higher SAM and SET7/9 concentrations, in addition to longer incubation times. Under these conditions, SET7/9 catalyzed multiple sites of methylation on free histones, and for the first time, we show that the enzyme can catalyze the formation of Kme3 as well as methylated arginine species (Figure 4.7A). SET7/9 catalyzed broad-spectrum methylation of free histone H2B and H3, including mono and

di-methylation of histones H2BK20, and H3K14, H3K36, as well as trimethylation of histone H3K14. The human histone octamers exhibited different sequence coverage; however, where sequence coverage was congruent, there were many sites of differential methylation, including the lack of methylation on histone H3K4, H3K9, and H3K14 within octamers.

Most surprising is the finding of both mono and dimethylation of arginine on free histone H3R2, H3R8, H3R17, H3R35, and H3R42. We independently confirmed our proteomics studies showing the production of the methylated amino acids Kme2, Kme3, and Rme1 and Rme2a produced from reactions with SET7/9, free histones, and histone octamers using analytical LC-MS/MS, which is different from the proteomic methods used in Figure 4.7, A and B. Reactions were prepared similar to the proteomic study with high SAM, enzyme concentrations and extended incubation times but at the end of the incubation, the reactions were hydrolyzed into component amino acids. We quantified Kme1, Kme2, and Kme3, as well as Rme1, primarily on free histone H3 but also to some extent on octamers and free histone H2A (Figure 4.7, D and E). We did not detect any Rme2s but could detect Rme2a; however, it was below our low limit of quantification (LLOQ), so we could not quantify it.

As a third independent method of confirmation that SET7/9 can catalyze the formation of Kme3 and Rme1, we recorded characteristic fragment ion masses derived from product ion mass spectra of Kme3 and Rme1 using analytical LC-MS/MS. Reactions were prepared similar to the proteomic study but were hydrolyzed into component amino acids to record product ion spectra for Kme3 and Rme1. We recorded the product ion mass spectra of standard pure Kme3 and Rme1 (Figure 4.7F) and compared these to the hydrolyzed products of reactions with SET7/9, histone H3, and SAM (Figure 4.7G). Both Kme3 and Rme1 have similar precursor ion mass (189 m/z), so the product ion spectra of the hydrolyzed products of the reactions with SET7/9, histone H3, and SAM

would be expected to have characteristic product ion masses from both Kme3 and Rme1. Indeed, we found characteristic product ion masses 130, 84, and 60 m/z corresponding to Kme3 and 74, 70, and 57 m/z corresponding to Rme1, confirming the presence of Kme3 and Rme1 in our reaction samples. As noted, we could detect Rme2a, but the intensity was too low to produce a product ion spectrum. It is important to note that the product ion mass spectra of acid hydrolyzed reactions in Figure 4.7, F and G represents a distinct and independent confirmation of the production of Kme3 and Rme1 by SET7/9 from the proteomic studies in Figure 4.7, A and B.

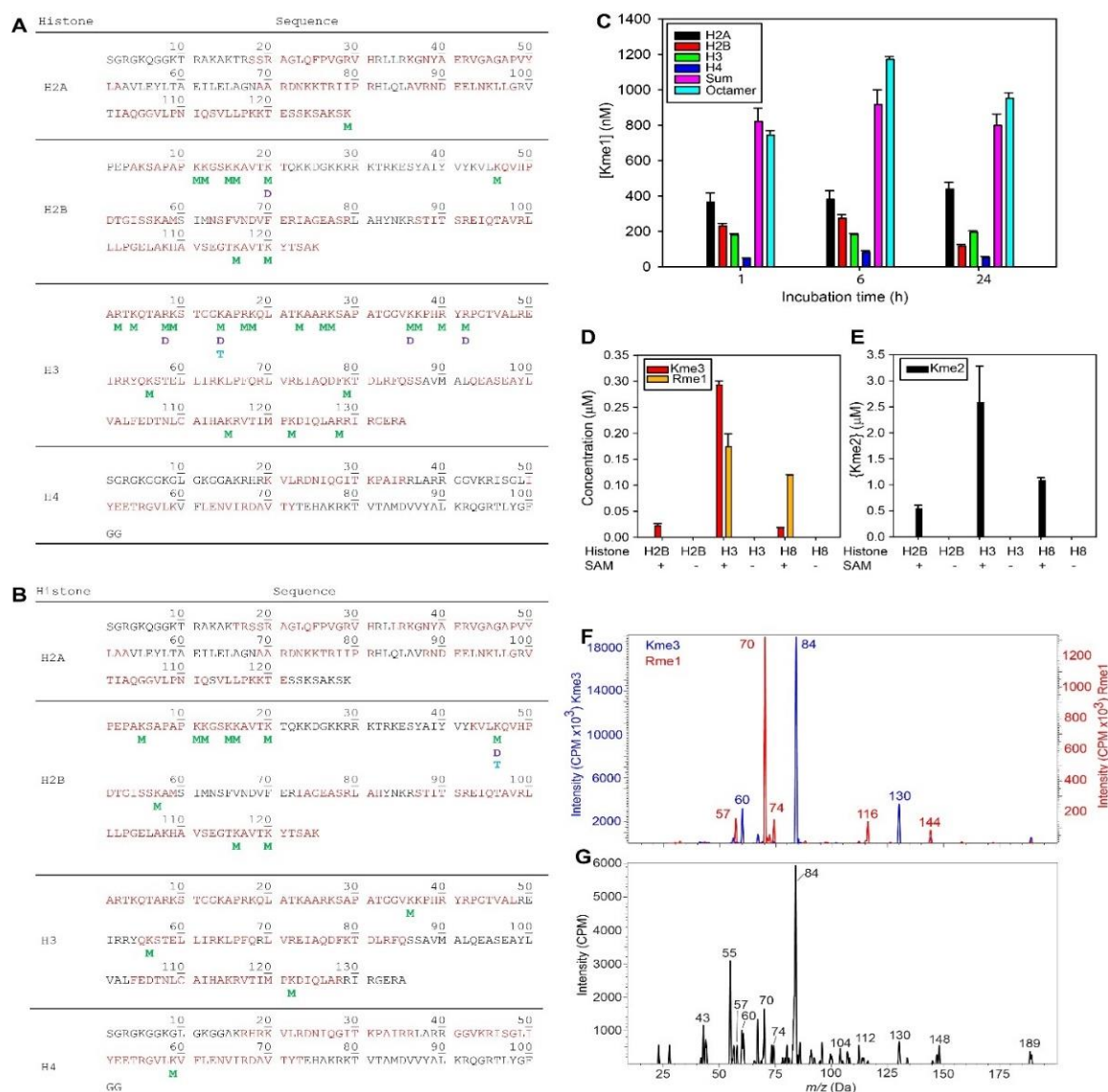


Figure 4.7. Analysis of sites and types of methylation on free histones H2A, H2B, H3, and H4 and histone octamers. The sites of methylation on free *Xenopus laevis* histones H2A, H2B, H3, and H4 (A), where M (green), D (purple), and T (cyan), are mono-di and tri-methylation, respectively. Mono- and di-methylation of arginine can be detected on histone H3. The sites of methylation on human histones H2A, H2B, H3, and H4 as part of histone octamers (B), where M (green), and D (purple), are mono-di-methylation, respectively. The peptide sequence coverage is shown in red and the sequence numbering above each line. Methylation of free histones H2A, H2B, H3, and H4 and histone octamers (C) at 25 nM SET7/9 and 500 nM of substrate. “Sum” is the sum of methylation of free histones H2A, H2B, H3, and H4. Exhaustive methylation of free histones H2A and H3 and histone octamers (H8) with SET7/9 0.5 μM and SAM 500 μM. Shown is the concentration of Kme3 and Rme1 (D) and Kme2 (E). Product ion spectra of standards

Kme3 (blue) and Rme1 (red) (F). The product ion spectra of a hydrolyzed reaction of histone H3 SET7/9 0.5 μ M and SAM 500 μ M, recording a precursor ion 189 m/z which corresponds to both Kme3 and Rme1 (G). This confirms the presence of Kme3 and Rme1 on histone H3. Ion intensity is in counts per minute (CPM) and mass as mass/charge (m/z).

4.3. Discussion

4.3.1 SET7/9 mechanism and product inhibition studies

Protein lysine or arginine methyltransferases typically fall into one of two enzymatic mechanisms: ordered sequential Bi-Bi or random sequential Bi-Bi. We find that SET7/9 follows an ordered sequential Bi-Bi mechanism (Figure 4.4). Similarly, the histone H3K4 methyltransferase MLL4 (221) and protein arginine methyltransferases (PRMT) one and six must bind SAM before the protein substrate. Therefore, these enzymes also follow an ordered sequential Bi-Bi mechanism (15,184,222,223). For many SAM-dependent protein methyltransferases, the SAM/SAH binding site has a higher affinity than the methyl acceptor binding site. The histone H4K20 mono-methyltransferase SET8 has a SAM K_M of 16 μ M and a histone H4 K_M of 40 μ M (224). Similarly, the SAM K_M values for PRMT one and six are lower than the K_M values for their respective protein substrates (15,184). In comparison, SET7/9 has a relatively weak SAM/SAH binding site as measured by the SAH K_i and SAM K_S and K_M , whereas the histone H3 K_M is 8.8-fold lower (Table 4.1). Such weak SAM/SAH binding may suggest that SET7/9 has relative insensitivity to high intracellular SAH concentrations and sensitivity to low SAM concentrations (225).

4.3.2 Stability of histone octamers

Histone octamers are most stable at high ionic strength (*e.g.*, ~0.8–2 M NaCl) and high pH, whereas NCPs are most stable at lower ionic strength, 0.1 to 0.4 M NaCl (226,227). To make our results comparable, we chose a single condition (buffer, pH, temperature, *etc.*) to perform all experiments with free histones, histone octamers, NCP, and Ub-NCP. We also had to consider the

potential for high salt concentrations to suppress signal with analytical mass spectrometry and proteomic experiments (a phenomenon known as “ion suppression”), and the pH maximum for SET7/9 activity (Figure S4.3). We therefore used a buffer with 100 mM $(\text{NH}_4)\text{HCO}_3$ at pH 9. Moreover, given the difficulty we had getting useful data and broad sequence coverage with our proteomics experiments with free histones, we used histone octamers in place of NCP because they lack DNA, which might have interfered with the proteomics experiments. We also had a greater quantity of histone octamers, which we found that we needed for proteomics experiments.

Histone octamers exist in an equilibrium with a histones H3 and H4 tetramer $((\text{H3-H4})_2)$ and two histones H2A, H2B dimers $(2(\text{H2A-H2B}))$ (226). Salt concentrations between ~ 0.8 to 2 M NaCl displace this equilibrium to the octamer, whereas concentrations lower than 0.6 M NaCl displace the equilibrium to $(\text{H3-H4})_2 + 2(\text{H2A-H2B})$ (226,227). In addition, $10.5 > \text{pH} < 5$ displaces the equilibrium to $(\text{H3-H4})_2 + 2(\text{H2A-H2B})$ but $7 > \text{pH} < 9.5$ promotes the formation of the octamer, with pH 9.5 yielding maximum octamer stability (226). Given our conditions of low salt and pH 9, the histone octamers in our experiments likely exist as an equilibrium of at least equal parts histone octamers + $(\text{H3-H4})_2 + 2(\text{H2A-H2B})$. This is corroborated by the similar sigmoidal rate versus substrate curves and comparable K'_B , $V_{\text{Max App}}$, and specificity constants $(k_{\text{cat}}/K'_B)_{\text{App}}$ with NCP and histone octamers (Figure 4.6 and Table 4.3). However, it is important to note that the sites and types of methylation we identified on histone octamers (Figure 4.7B) may not be the same as those in NCP.

For the experiments involving free histones, the sequences were derived from *Xenopus laevis*, whereas for histone octamers, NCP, and Ub-NCP experiments, the histones had the human sequence. Human histones H2A, H2B, H3.1, and H4 have 93.8%, 93.4%, 98.5% and 100% residue identity, respectively, with the corresponding histone sequences in *Xenopus laevis*. Moreover,

none of the minor sequence differences involves lysine or arginine residues that we identified as being methylated. Although we cannot rule out these variations as causing any of the differences we observed between free histones and histone octamers, NCP, or Ub-NCP, we think that this is unlikely given their nearly identical sequences.

4.3.3 Sigmoidal dependence of rate on NCP, Ub-NCP, or histone octamers

A study by Chuikov *et al.* failed to detect SET7/9 catalyzed NCP methylation, leading to the assumption that SET7/9 cannot methylate nucleosomes *in vivo*. Since this study, many have repeated this claim without further experimentation (217–220). However, we show for the first time that SET7/9 can methylate NCP and Ub-NCP (Figure 4.6). Care must be taken in the interpretation of the kinetic parameters in Table 4.3 based on Figure 4.6. Since we could not saturate SET7/9 with either histone octamers, NCP, or Ub-NCP, the determination of $V_{\text{Max App}}$ using Equation 4.7 is based on the non-linear least squares fitting. $K'_{\text{B App}}$ and $K_{\text{M App}}$ are not equivalent but as others have done, we have calculated an apparent $(k_{\text{cat}}/K'_{\text{B}})_{\text{App}}$ as a surrogate of the apparent specificity constant $(k_{\text{cat}}/K_{\text{M}})_{\text{App}}$ (228–231). With these caveats in mind, we note that the $V_{\text{Max App}}$ for NCPs is at least 9-fold greater, and the $V_{\text{Max App}}$ for Ub-NCP is at least 1.8-fold higher than the $V_{\text{Max App}}$ for any of the free histones. The specificity constant $(k_{\text{cat}}/K'_{\text{B}})_{\text{App}}$ for NCP and octamers is 11-; 6.4-; 4.0-; and 45-fold higher than the $(k_{\text{cat}}/K_{\text{M}})_{\text{App}}$ values for histones H3, H2A, H2B, or H4, respectively. Combined with the sigmoidal curves showing allostery, this suggests not only that SET7/9 catalyzed methylation of NCP is possible *in vivo* but that nucleosomes (with and without Ub) are the preferred substrates. Depending on their value, Hill coefficients can suggest the presence of an allosteric interaction. However, Hill coefficients cannot be related to a specific number of binding sites but are only a rough indicator of an allosteric interaction where $n_{\text{H}} > 1$ is indicative of a positive allosteric interaction, $n_{\text{H}} = 0$ indicates no

allostery, and $n_H < 1$ indicates a negative allosteric interaction. The Hill coefficients (n_H) for NCP, Ub-NCP, and histone octamers are 2.062, 2.418, and 1.748, respectively. Therefore, these Hill coefficients suggest a positive allosteric interaction that is only observed between SET7/9 and NCPs (with or without Ub) or octamers. Moreover, the similarity in (k_{cat}/K'_B) App for NCP and histone octamers suggests that the DNA in the NCPs is not involved in this interaction (Table 4.3).

The sigmoidal rate vs substrate curves might be explained by cooperativity between sites in a random sequential Bi-Bi mechanism or a multimeric enzyme. However, this cannot explain the behavior of SET7/9, as it exhibits an ordered sequential Bi-Bi mechanism, and to the best of our knowledge, SET7/9 exists as a monomer. Furthermore, we cannot attribute this activity to the presence of multiple sites of methylation, as sigmoidal curves were not observed with the free histones, where we also observed multiple sites of methylation. Sigmoidal rate vs substrate curves have been observed with monomeric enzymes where a slow conformation change occurs, such as with hexokinase (232) and the histone acetyltransferases p300 and CBP (228,229). This mechanism assumes a random ordered Bi-Bi enzymatic mechanism manifesting as sigmoidal substrate versus rate curves with one substrate fixed and the other varied, and hyperbolic curves when the fixed and varied substrates are reversed (232). We did not find a random ordered Bi-Bi mechanism for SET7/9, nor did we observe sigmoidal curves in our kinetic analysis when histone H3 was fixed and SAM varied, nor when SAM was fixed and histone H3 varied, nor with changing concentrations of any of the free histones in the presence of fixed saturating SAM. Accordingly, we rule out the slow conformation change mechanism as explaining the apparent allosteric activity of SET7/9 with histone octamers, NCP, and Ub-NCP. Instead, our data suggest that there is an allosteric site on SET7/9 that binds to one histone while the target lysine of another histone that is part of an octamer, NCP, or Ub-NCP is in the active site. A possible candidate for this allosteric

site is the three MORN domains of SET7/9, which bind positively charged amino acids (233). In fact, the MORN domains of SET7/9 were shown to bind to a sequence from K14 to H39 on a peptide of the N-terminus of histone H3, where the interaction positions SET7/9 for methylation of histone H3K4 following hyperbolic kinetics (234). We speculate that, in the context of the histone octamer, NCP or Ub-NCP, binding of the SET7/9 MORN domains to the histone H3 N-terminal tail results in an allosteric interaction and methylation of neighboring histones following sigmoidal kinetics. The implications of this will be discussed in more depth below.

4.3.4 Methylation sites on free histones and histone octamers

We determined specific sites where SET7/9 can methylate free histones and histone octamers. Our study differs from others since we used full-length histones and octamers and proteomic analysis rather than N-terminal tail peptides and scanning alanine mutagenesis (208,209). We confirmed that SET7/9 produces histone H2BK15me1 (208), but found that it also produced histone H2BK11me1, H2BK12me1, H2BK15me1, H2BK16me1, and H2BK20me1/me2 in the free histone H2B tail, H2BK46me1 in the core, and H2BK116me1 and H2BK120me1 in the C-terminus. In the context of an octamer, slightly different histone H2B methylation was observed, H2BK5me1/me2, and dimethylation of histone H2BK11, 12, 15, and 16, but a lack of histone H2BK20me2. The role of this histone H2B N-terminal tail methylation is unclear, but CPB/p300 catalyzed acetylation of histone H2BK5, 12, 15, and 20 (235,236) is referred to as histone H2B N-terminus multisite lysine acetylation and marks active enhancers (236). Such acetylation would be mutually exclusive with SET7/9 catalyzed methylation at the same sites. Interestingly, histone H2BK20ac has been identified as the “most predictive mark of active enhancers” and, in some cases, was considered more important than histone H3K27ac (237). Thus, histone H2BN-terminal

tail methylation by SET7/9 may prevent histone H2B N-terminus multisite lysine acetylation, reducing the activity of active enhancers.

Histone H3 has the highest number of sites of methylation of all free histones. Every K and R residue in the first 42 residues of histone H3 is at least mono-methylated. In contrast, we detected no sites of methylation in the first 17 residues of histone H3 within an octamer, only detecting histone H3K18me1, H3K36me1, H3K56me1, and H3K122me1. The lack of histone H3K4me1 in the octamer may explain why others have failed to detect SET7/9 catalyzed methylation of NCP, since this specific site is assumed to be the only or most important methylation site for SET7/9 (217–220). Therefore, if one was using Western blotting for SET7/9 catalyzed histone H3K4me1 on NCP, our results suggest that histone H3K4me1 would not be detected. The difference in methylation between free histone H3 and histone H3 in an octamer may be mediated by the MORN domains of SET7/9. The MORN domains may bind to the solvent-exposed histone H3 N-terminal tail in a nucleosome in an orientation that positions the catalytic domain to methylate the C-terminus of histone H3 and neighboring histones but not histone H3K4. This might explain why there is no methylation in the first 17 residues of histone H3 when it is part of an octamer. Moreover, our results confirm that SET7/9 catalyzed histone H3K4me1 can happen prior to nucleosome assembly but not after (206,207).

Many of the SET7/9 methylation sites we identified on histones are known sites of ubiquitination, including histone H2AK129, histone H2BK120, and histone H3K122. We found histone H2AK129me1 in free histone H2A, but in the octamer, our coverage did not include this residue, so it is unclear if this modification exists in the octamer. Interestingly, histone H2AK129 is part of a R/KSK motif, which is a methylation consensus sequence for SET7/9 (208). Histone H2A has several sites of monoubiquitination, but relevant to this discussion, BRCA1/BARD1

monoubiquitinates histone H2AK127 and H2AK129 (238). Since lysine methylation and ubiquitination are mutually exclusive, our results suggest that SET7/9 may methylate histone H2AK129, preventing subsequent monoubiquitination at that site (239).

Histone H2BK120Ub is found in actively transcribed genes (233) and induces changes in nucleosome structure that result in increased active marks, histone H3K79 and H3K4 mono and di-methylation catalyzed by DOT1L (240) and MLL1 (241), respectively. Histone H3K4 methylation catalyzed by MLL1 or its yeast cognate Set1 appears to be dependent on prior histone H2BK120Ub (241,242). Histone H2BK120Ub prevents chromatin compaction, yielding open chromatin, increasing the interaction between MLL1 and nucleosomes, and increasing histone H3K4 methylation (241). SET7/9 catalyzed histone H2BK120me1 may block subsequent histone H2BK120Ub, leading to reduced MLL1-mediated histone H3K4me3.

Active enhancers can be superficially characterized on a histone level by the presence of histone H3K4me1 and H3K27ac or histone H2BK20ac. Previously, SET7/9 was thought to play a role in constructing active enhancers by catalyzing histone H3K4me1, prior to nucleosome assembly (207). Our results confirm the formation of histone H3K4me1 only on free histone H3, but we also reveal many other sites and types of methylation on free histones and nucleosomes. In the context of the nucleosome, we show that the net effect of SET7/9 may be to reduce the activity of active enhancers by, not catalyzing histone H3K4me1, by preventing histone H2B N-terminus multisite lysine acetylation, including histone H2B20ac, and by blocking histone H2BK120Ub, thereby leading to reduced MLL1-mediated histone H3K4me3.

The ubiquitination of histone H2BK120, in Ub-NCP blocks subsequent methylation at that site, and this could explain why the Ub-NCP $V_{\text{Max App}}$ is $\frac{1}{5}$ of that for NCP. Nevertheless, SET7/9 exhibits sigmoidal kinetics with Ub-NCP and, unlike any other substrate, the production of Kme1

and Kme2 at equal rates (Figure 4.6, B and C) with low enzyme and short incubation times, suggesting it has *in vivo* relevance. It was previously thought that wild-type SET7/9 only catalyzes the formation of Kme1, and initial structural studies suggested that SET7/9 cannot catalyze the formation of Kme2 or Kme3 because of restricted volume in the active site (211). As noted, mutations in the active site may increase this volume, allowing the formation of Kme2 and limited Kme3 (205,211). Absent such a mutation, it's difficult to understand how Ub-NCP can yield Kme2. However, structural studies with MLL1, MLL3, or DOT1L bound to Ub-NCP show structural changes in the enzyme that permit higher methylation activity on Ub-NCP and cause distinct changes in binding compared to NCP (240,241). Similar structural changes in binding between SET7/9 and Ub-NCP may alter active site architecture to allow formation of Kme1 and Kme2 without the need for the high SET7/9 and SAM and extended incubation times needed to produce Kme2 and Kme3 in Figure 4.7, A and B, D and E.

Most surprising is the finding of SET7/9 dependent formation of Rme1 and Rme2a (known as Type I arginine methylation). This is the first finding that Rme1 and Rme2a formation can be catalyzed by any enzyme other than PRMTs. Given the exhaustive methylation conditions needed to observe this, the biological relevance of Rme1 and Rme2a formation by SET7/9 is unclear. However, recent selectivity studies with inhibitors of PRMT1 (a Type I PRMT) show that some PRMT1 inhibitors are also low-potency SET7/9 inhibitors (243,244) suggesting that there is sufficient similarity between the active sites of SET7/9 and PRMT1 that allows these inhibitors to bind to both enzymes (199).

4.4 Experimental procedures

4.4.1 Protein expression and purification

The constructs for *Xenopus laevis* histones H2A, H2B, H3, and H4 in the pET3a vector were a generous gift from Dr Karolin Luger at the University of Colorado, which were transformed into BL21(DE3) pLysS *E. coli* cells, grown, and induced according to previous methods (177). Inclusion bodies were prepared from the pellets and histones denatured, renatured, and purified according to previous methods (181) (see supplemental methods for additional details). Full-length human SET7/9 in the pET28a-LIC vector was a generous gift from the laboratory of Dr Masoud Vedadi of the Ontario Institute of Cancer Research. Details on the expression of SET7/9 can be found in the supplemental methods.

4.4.2 Methylation reactions

Methylation reactions contained SET7/9, free *Xenopus laevis* histones, human recombinant histone octamers (EpiCypher EP16–0001), human recombinant NCP (EpiCypher EP160009), or human recombinant Ub-NCP (EpiCypher EP160396) and SAM (Cayman Chemical Co.) in a buffer with 100 mM (NH₄)HCO₃ (pH 9), and 200 nM internal standard stable isotope labeled ¹³C6 ¹⁵N₂ lysine, at 30 °C. The NCP, Ub-NCP, and octamers have been used by many groups, having been validated by SDS-PAGE, mass spectrometry, and Western blot previously (245–247). The details for the various methylation reactions are in the supplemental methods. Dried samples were acid hydrolysed in the vapor phase using 6M HCl at 110 °C for 24 h, and the hydrolyzed samples were dried in a vacuum centrifuge and reconstituted in 100 µL of 0.05% formic acid for analysis by LC-MS/MS (see below). The concentration of free histones, histone octamers, NCP, Ub-NCP, and SET7/9 was determined by acid hydrolysis and quantification of K and R using LC-MS/MS (below). SAM stock concentrations were determined by UV absorbance and the molar extinction

coefficient $15,400 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 260 nm (248). Details on the methylation reactions can be found in the supplemental materials.

4.4.3 Analytical LC-MS/MS

We previously developed methods to quantify multiple post-translational modifications including lysine (K) acetyllysine (Kac), mono- (Kme1) di- (Kme2) and trimethyllysine (Kme3), arginine (R) monomethylarginine (Rme1), asymmetric-dimethylarginine (Rme2a), and symmetric dimethylarginine (Rme2s) that were validated using full length histones with a single site of modification (138,185,212). This method was modified to quantify methylated lysines and arginines with the internal standard $^{13}\text{C}_6 \text{ }^{15}\text{N}_2$ lysine for enzymatic reactions. Hydrolyzed samples were analysed using a Nexera UHPLC connected to a Shimadzu 8040 triple quadrupole mass spectrometry system. Chromatographic separation was achieved using a Primesep200 (SIELC) $4.6 \times 150 \text{ mm } 5 \mu\text{m } 100 \text{ \AA}$ HPLC column at 40°C . Mobile phases (A) 0.05% formic acid in water and (B) 1% formic acid in 50% aqueous acetonitrile were used at a flow rate of 0.4 ml/min. Initial conditions were 0% (B) for 1.5 minutes, increasing to 85% over 30 s and held for 3 minutes. Positive ion analytes were quantified using multiple reaction monitoring (MRM) and dual ESI, APCI (DUIS) ionization. The precursor and product ion m/z values collision energies and other parameters are in Table S4.1. The nebulizing gas was set to 2 L/min, drying gas was 15 L/min, desolvation line temperature was 250°C and the heating block 400°C . The assay can quantify K, Kme1, Kme2, Kme3, R, Rme1, Rme2a, and Rme2s.

4.4.4 Data analysis

Enzymatic rate data and substrate histone H3 and SAM concentrations for the kinetic analysis of SET7/9 and product inhibitor studies were reciprocal transformed and fit to a straight-line equation with $1/Y^2$ weighting (to account for bias due to reciprocal transformation) using SigmaPlot 14.5.

The hyperbolic rate data for the activity of SET7/9 against free histones H2A, H2B, H3, and H4 were fit to the Michaelis–Menten equation (Equation 4.5 and 4.6), and the sigmoidal rate data for SET7/9 activity on NCP with and without histone H2BK120 ubiquitination and histone octamers were fit to the Hill equation (Equation 4.7) using SigmaPlot 14.5.

4.4.5 Proteomic experiments

Extended incubation experiments were performed with 0.5 μM SET7/9, 100 mM $(\text{NH}_4)\text{HCO}_3$ buffer (pH9), 200 nM internal standard $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine, 10 μM histones H2A, H2B, H3, H4 or 1 μM histone octamer substrate, with and without 500 μM SAM. The reactions were carried out at 30 °C with an incubation time of 24 h.

Per sample, 3 pmol were incubated with 10 mM dithiothreitol to reduce disulfide bonds, and free cysteines were alkylated with 10 mM iodoacetamide for 30 minutes at RT in the dark. Samples were digested by adding the broad specificity protease subtilisin (Sigma Aldrich) at a 1:10 ratio (protease: protein) for 20 minutes at 56 °C (249). The digestion was quenched by the addition of 1% TFA, and 500 fmol per digest were analyzed by nanoflow LC-MS/MS using an Easy-nLC 1200 system coupled to an Exploris 480 (Thermo Fisher Scientific).

Per samples, 500 fmol aliquots were separated on a selfpacked C18 analytical column (Luna C18(2), 3 μm particle, 100 μm ID x 30 cm, Phenomenex, Torrance, CA) using water with 0.1% FA (mobile phase A) and 80% ACN with 0.1% FA (mobile phase B) using a binary gradient from 2 to 6% B over 3 minutes, 6 to 30% B over 36 minutes, 30 to 50% B over 5 minutes, 50 to 100% B over 1 minutes at a flow rate of 300 nl/min. MS acquisition was conducted in data-dependent acquisition mode (DDA) with a top 10 method. Full MS scans were acquired at a resolution of 45,000 from 380 to 1500 m/z (AGC 300%, 45 ms max injection time). Precursor ions with charge states between +2 and +5 were isolated with a m/z 1.6 window and fragmented with a normalized

collision energy of 30%. MS/ MS spectra were acquired at a resolution of 45,000 (AGC 100%, max injection time set to auto). Dynamic exclusion was set to 5 s. For free histone samples, Proteome Discoverer 3.01.27 was used for data analysis with unspecific digestion, 10 ppm was allowed a maximum precursor mass deviation, and 0.02 Da for fragment ions. A subset of the human proteome database with 6516 protein entries without histone sequences was used, and the *Xenopus* histone sequences were appended for free histones. Mono-, di-, and tri-methylation of Lys and Arg, as well as oxidation of Met residues, were allowed as variable and carbamidomethylation of Cys was set as a fixed modification. All matched peptide sequences passing a 5% false discovery rate (target/decoy approach with Percolator) and matching only the expected histone protein (uniqueness) were considered for data analysis. Only lysine and arginine modifications that could be localized with >90% site probability to a specific Lys residue were considered. Peptide intensities (area under the curve) were determined using label-free quantitation (LFQ) and compared between +SAM and –SAM samples, where required, these were normalized to reflect equal overall histone target intensities.

Octamer samples were searched using FragPipe 23.0 and a human Swissprot database (downloaded April 2025). To avoid ambiguous assignment of octamer-histone peptides, all histone sequences other than human histone H2A (H2AC4 (P04908)), histone H2B (H2BC12 (O60814)), histone H3.1 (H3C1 (P68431)) and histone H4 (H4C1 (P62805)) were removed, resulting in a total of 20,383 target sequences, including common contaminants. Nonspecific cleavage was selected, peptide length was set from 7 to 25 amino acids, precursor and product ion mass tolerances were set to 20 ppm. Oxidation of Met, as well as mono-, di-, and trimethylation of Lys residues were allowed as variable modifications. These settings resulted in a total of 1,677,783 modified peptides considered in the search. As above, because of the low probability of having random peptide-

spectrum-matches to the four octamer proteins, a false-discovery-rate of 5% was allowed on the peptide level. LFQ was performed using default IonQuant settings including the default minimum site-localization probability of 0.75.

4.5 Supplemental methods

Histones expression and purification of Histones

E. coli BL21(DE3) pLysS cells containing the genes for *Xenopus Laevis* histones H2A, H2B, H3 and H4 in the pET3a vector were a generous gift from Dr Karolin Luger at the University of Colorado that were transformed into *E. coli* BL21(DE3) pLysS *E. coli*, grown, and induced according to previous methods Briefly, bacteria were cultured in 2 × LB both at 37 °C and induced with 0.2 mM IPTG for 2 h, then centrifuged at 15000 × g for 20 minutes at 4 °C. The pellet was suspended in a lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM PMSF, 1 mM BME, 2 protease inhibitor tablets (Life Technologies), 20 U DNase, 0.33 mg/mL lysozyme (Amresco)) and sonicated using a Branson Sonifier with 30 s and breaks on ice. Inclusion bodies were prepared from the pellets and histones denatured, renatured, and purified according to previous methods. The resulting suspension was washed with lysis buffer but with the addition of 1% Triton X-100. It was then sonicated centrifuged at 4 °C at 12,000 × g for 20 minutes and the supernatant is decanted. The pellets wash steps were repeated at least 3 times until inclusion bodies were obtained. The inclusion bodies were denatured in a buffer containing 20 mM Tris, 10 M guanidine hydrochloride and 10 mM DTT for 1 h and centrifuged at 1200 × g to separate the pellets. The inclusion bodies refolded in a buffer containing 100 mM Tris, 400 mM arginine, 2 mM EDTA, 2 mM DTT and 2 mM PMSF. The solution containing the denatured buffer was added at around 1 mL/min on ice. The resulting solution is concentrated with Amicon® Ultra Centrifugal Filter, 10 kDa MWCO at 4000 × g for 45 minutes. The concentrate was collected and injected into a

Shimadzu 10A VP series HPLC. Mobile phase A is 0.1% Trifluoroacetic acid in H₂O, B is 0.1% Trifluoroacetic acid in acetonitrile at 2mL/min. Initial conditions were 5% (B) for 5 minutes, increasing to 65% (B) over 28 minutes and then increased to 90% (B) for 2 minutes and held for 3 minutes. The run was monitored at 260 and 280 nM. The column was a semi preparative ZORBAX C8 9.4 × 250mm. The peaks were collected, analyzed with Protein SDS Gel electrophoresis and freeze dried with a Labconco FreeZone 4.5 Liter Benchtop Freeze Dry System and then stored at -80 °C.

SET7/9 expression and purification

Full-length human SET7/9 in the pET28a-LIC vector was a generous gift from the laboratory of Dr. Masoud Vedadi of the Ontario Institute of Cancer Research. SET7/9 was expressed in BL21(DE3) pLysS cells (Invitrogen) induced with 1 mM IPTG and incubated at ambient temperature (~27 °C) with shaking at 300 rpm overnight (16 h), centrifuged at 15000 × g for 20 minutes at 4 °C. The pellet was resuspended in 20 mM NaHPO₄, (pH7.5) 100 mM NaCl, 1 mM PMSF, 7 mM β-mercaptoethanol, 0.1% Triton X-100, 0.5 mg/mL lysozyme with an EDTA-free protease inhibitor tablet (Life Technologies) and 10 U DNase, and sonicated on ice using a Branson Sonifier, centrifuged and the supernatant loaded onto a 5 mL HisTrap FF column (GE Healthcare) connected to an AKTÄ Purifier FPLC at a flow rate of 1 mL/min. SET7/9 was purified with a step gradient of A: 20 mM NaHPO₄, pH7.5 100 mM NaCl, 1 mM PMSF, 7 mM β-mercaptoethanol and B: 20 mM NaHPO₄, pH 7.5 100 mM NaCl, 1 mM PMSF, 7 mM β-mercaptoethanol with 250 mM imidazole. The column was washed with 70 mL of A, then 10 mL of 10% B, then SET7/9 eluted with 15 mL of 100% B, all flow rates were 3 mL/min. The eluted fraction was concentrated with a Spin-X UF20 concentrator 10 kDa NMWCO concentrator (Corning) and dissolved in a storage buffer with 50 mM Tris pH 7.5 with 5% glycerol and re-concentrated.

Details of methylation reactions

To determine where the reaction rate (in pmol/min) for SET7/9, SAM and histone H3 is increasing linearly over time, SET7/9 (100 nM) was incubated with high and low concentrations of substrates SAM (25 μ M and 5 μ M) and histone H3 (5 μ M and 1 μ M). The samples were analyzed at 5, 10, 20, 30, 40, 60, 120, 240 and 360 minutes in a buffer with (NH₄)HCO₃ buffer (pH9) (Sigma-Aldrich) (100 mM), internal standard stable isotope labeled ¹³C6 ¹⁵N2 lysine (Cambridge Isotopes Laboratories Inc.) (200 nM) (Figure S4.1).

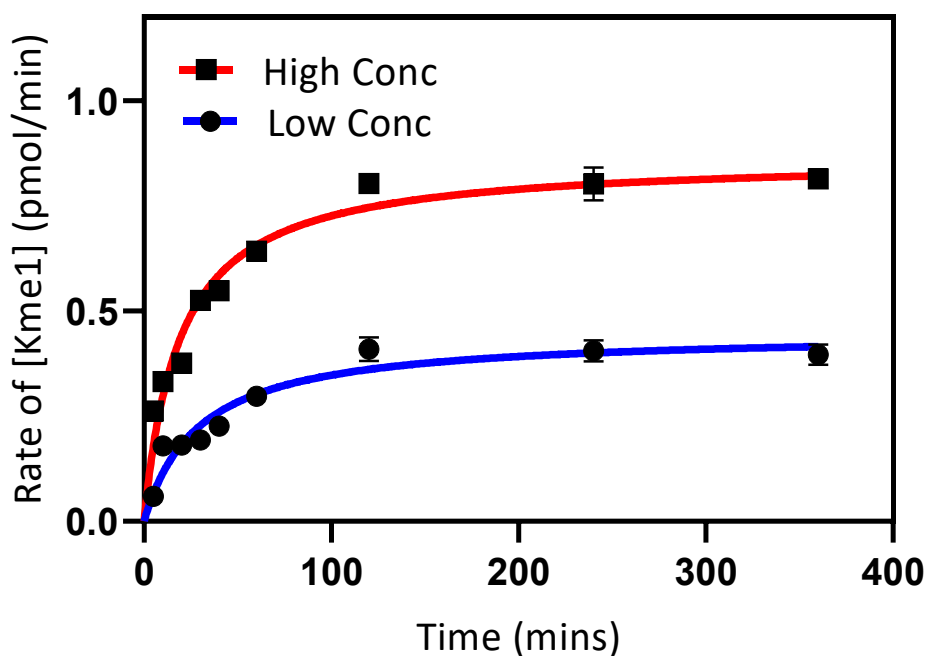


Figure S4.1. Time course activity of high and low concentrations of SAM (25 μ M and 5 μ M) and histone H3 (5 μ M and 1 μ M) with 100 nM of SET 7/9. This is used to determine where the reaction rate for SET7/9, SAM and histone H3 is increasing linearly over time. The Mass Spectrometry parameters are listed in Table S4.1.

Table S4.1. Mass Spectrometry parameters: MRM transitions and collision energies.

Analyte	Retention Time (min.)	Precursor>product ion (<i>m/z</i>)	Collision Energy (eV)
K	2.03	146.9>130.0	-14
Kme1	3.34	161.2>84.0	-18
Kme2	3.42	174.8>84.0	-20
Kme3	3.5	189.2>83.9	-22
R	2.15	175.2>70.10	-14
Rme1	3.41	188.8>70.0	-24
Rme2s	3.46	202.8>171.9	-14
Rme2a	3.46	203.0>45.9	-14
¹³ C ₆ ¹⁵ N ₂ lysine	2.07	155.0>90.2	-17

To determine the range over which the rate of reaction for SET7/9 with SAM and histone H3 increases linearly with enzyme concentration, SET7/9 was incubated with SAM and histone H3 (1 μM with 3.125, 6.25, 12.5, 25, 50, 100 and 200 nM SET7/9 (Figure S4.2).

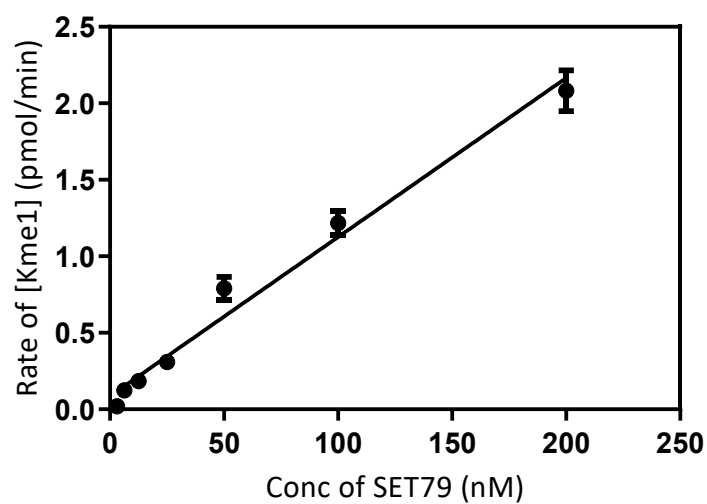


Figure S4.2. The activity of increasing concentrations of SET 7/9 with 5 μ M SAM and 1 μ M histone H3. This shows the rate of reaction for SET7/9 with SAM and histone H3 increases linearly with enzyme concentration up to at least 200 nM.

Effect of pH on the activity of SET7/9 was determined with NH_4HCO_3 buffer (100 mM) varying from pH 6 to pH 10 with 5 μ M SAM and 1 μ M histone H3. The samples were stopped at 30 minutes (Figure S4.3).

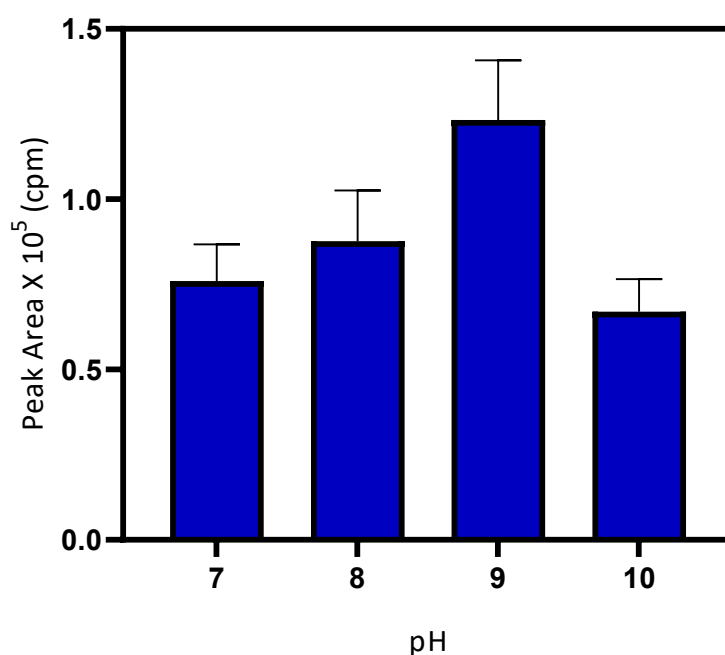


Figure S4.3. The activity of SET 7/9 at various pH points. SET 7/9 shows its highest activity at pH 9. Each bar is the mean and S.D. of 2 repeats.

The effect of DTT on the enzymatic activity of SET7/9 was determined by incubating the enzyme in one group with SAM histone H3 (5 μ M and 1 μ M respectively) and (NH₄)HCO₃ buffer (pH 9) (100 mM), internal standard stable isotope labeled ¹³C6 ¹⁵N2 lysine (200 nM) and 2.5 mM DTT and another group that did not contain DTT. The samples were analyzed at 15, 30 and 60 minutes (Figure S4.4).

Given the results from these experiments, all subsequent kinetic methylation reactions were carried out for 20 minutes with 25 nM SET7/9 in a buffer with 100 mM (NH₄)HCO₃ at pH 9, and 200 nM ¹³C6 ¹⁵N2 lysine internal standard, unless otherwise stated.

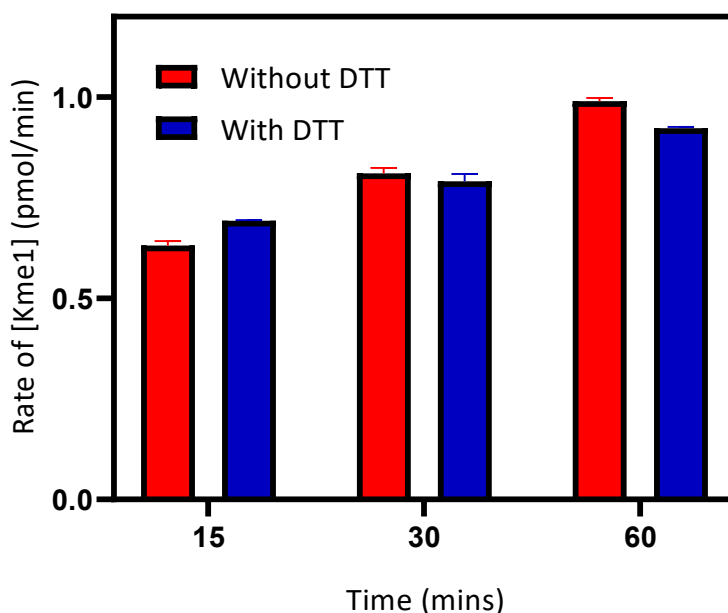


Figure S4.4. The activity of SET 7/9 with and without DTT. Analysis using paired t-test shows the differences due to the presence of DTT are not statistically significant ($p > 0.05$) up to 60 minutes.

Substrate control and SET7/9 auto-methylation experiments

To assess the potential auto-methylation activity of SET7/9, enzymatic reactions were performed under different conditions in the presence and absence of histone H3 and SAM. The reactions included a complete reaction containing 30 μM histone H3, 50 nM SET7/9, and 100 μM SAM; a control reaction with only SET7/9 and SAM (50 nM SET7/9 and 100 μM SAM); another control with only SET7/9 and histone H3 (30 μM histone H3 and 50 nM SET7/9); and a final control with histone H3 and SAM only (30 μM histone H3 and 100 μM SAM). Following a 30-minute incubation, the samples were analyzed to evaluate auto-methylation. The reactions were carried out in 100 mM $(\text{NH}_4)\text{HCO}_3$ pH9, 200 nM $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine (Figure S4.5)

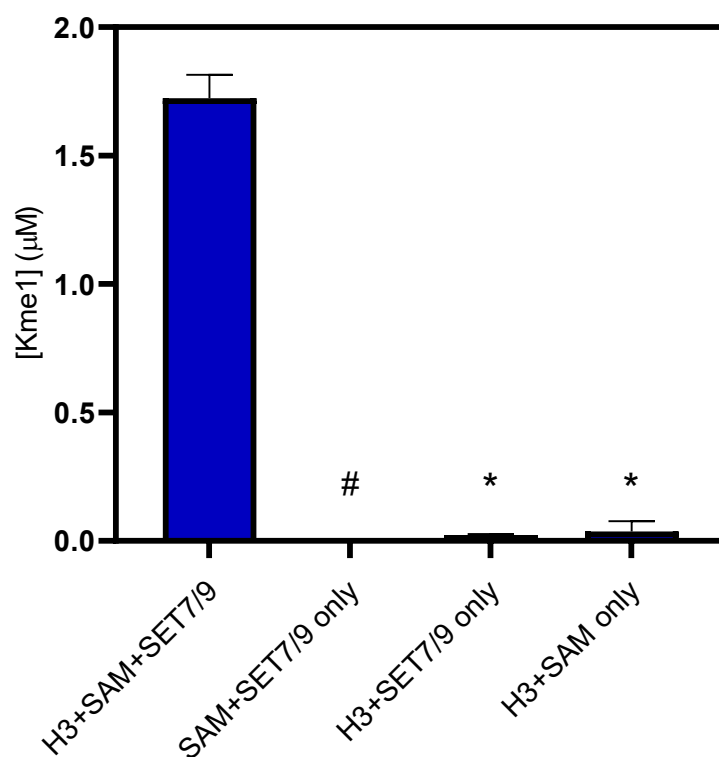


Figure S4.5. Substrate control assay for SET7/9. To determine whether SET7/9 exhibits auto-methylation. Enzymatic reactions were conducted in the absence of histone H3 and/or SAM. The methylation signals detected under these conditions were below the limit of quantification, indicating that SET7/9 does not undergo auto-methylation. These results confirm that the methylation activity of SET7/9 is strictly dependent on the presence of a viable substrate and SAM. Each bar is the mean and S.D. of 2 repeats.

indicates the methylation signals detected under these conditions were below the limit of detection.

* indicates the methylation signals detected under these conditions were below the limit of quantification.

Preliminary product inhibitor experiments

Xenopus laevis histone H3Kme1 methylated lysine analog (MLA) (H3K4me1MLA) was purchased from ActiveMotif (31719) and SAH >98% from Cayman.

Preliminary measurements of the inhibition of SET7/9 by histone H3K4me1MLA were determined by incubating 25nM SET7/9, 20 μ M SAM, 6 μ M histone H3 and histone H3K4me1MLA at concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μ M (Figure S4.6).

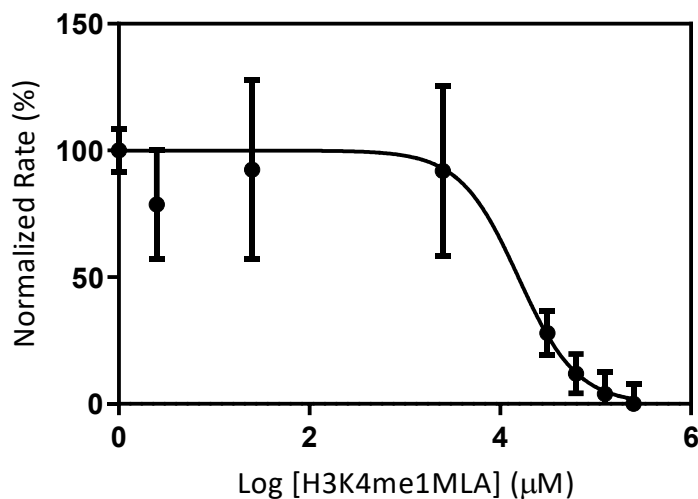


Figure S4.6. IC₅₀ curve of the inhibition of SET7/9 by histone H3K4me1MLA with 25 nM of SET 7/9. 5 μ M SAM and 1 μ M histone H3. The IC₅₀ value for histone H3K4me1MLA is 15.485 μ M.

Preliminary measurements of the inhibition of SET7/9 by SAH were determined by incubating 20 μ M SAM, 6 μ M histone H3 and SAH at concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000 μ M (Figure S4.7).

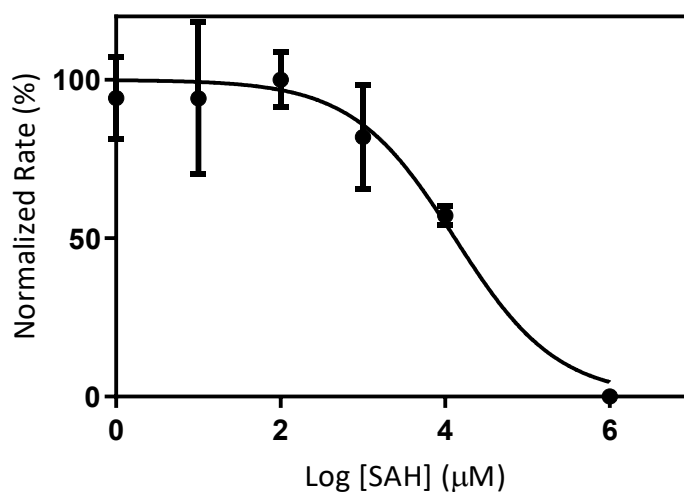


Figure S4.7. IC₅₀ curve of the inhibition of SET7/9 by SAH. The IC₅₀ value for SAH is 13.081 μM.

Determination of V_{Max} and K_M

The experiments to determine V_{Max} and K_M values for histone H3 and SAM were as follows 0.47, 0.94, 1.88, 3.75, 15 and 30 μM μM of histone H3 were incubated with SAM concentrations of 6.25, 12.5, 25, 50, 100 and 200 μM and for a total of 36 samples. Replots from Figure 4.2 (Figure S4.8) are shown for the slopes from the reciprocal V_o vs reciprocal histone H3 plotted against reciprocal SAM, the reciprocal intercept ($V_{Max App}$) for the plots of reciprocal V_o vs reciprocal histone H3, plotted against reciprocal SAM, and the reciprocal intercept ($V_{Max App}$) for the plots of reciprocal V_o vs reciprocal SAM, plotted against reciprocal histone H3.

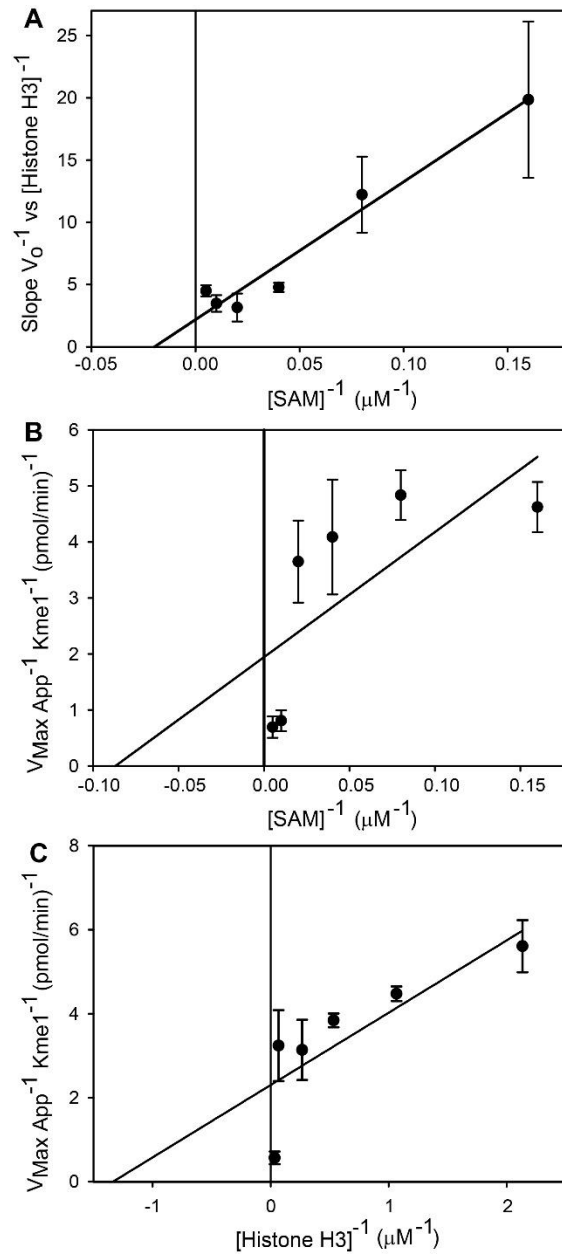


Figure S4.8. Replots from Figure 2 for the slopes from the reciprocal V_o vs reciprocal histone H3 plotted against reciprocal SAM (A) the reciprocal intercept ($V_{\text{Max App}}$) for the plots of reciprocal V_o vs reciprocal histone H3, plotted against reciprocal SAM (B), and the reciprocal intercept ($V_{\text{Max App}}$) for the plots of reciprocal V_o vs reciprocal SAM, plotted against reciprocal histone H3 (C).

Product inhibitor experiments

The product inhibitor experiments for SAH against histone H3 (Figure 4.3A) were performed with a fixed 30 μM of SAM with 0.625, 1.25, 2.5, 5, 10 and 20 μM histone H3 and 12.5, 25, 50, 100 and 200 μM of SAH. 4 samples were not plotted at the highest concentrations of SAH and lowest of histone H3: 200 SAH and 0.625, 1.25 and 2.5 μM histone H3 and 100 μM of SAH and 0.625 μM histone H3 because the measured Kme1 was below the calibrated range or LLOQ of our assay.

The product inhibitor experiments for SAH against SAM (Figure 4.3B) were performed with fixed concentrations of 4 μM of histone H3 with 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM of SAM and 6.25, 12.5, 25, 50, 100 and 200 μM of SAH.

The product inhibitor experiments for histone H3K4me1MLA against histone H3 (Figure 4.3C) were performed with a fixed 30 μM of SAM with and 0.625, 1.25, 2.5, 5, 10 and 20 μM of histone H3 and 15.6, 31.2, 62.5, 125, 250 μM of histone H3K4me1MLA.

The product inhibitor experiments for histone H3K4me1MLA against SAM (Figure 4.3D) were performed with fixed 4 μM histone H3 with 6.25, 12.5, 25, 50, 100 and 200 μM of SAM and 7.8125, 31.25, 62.5, 125, 250 μM of histone H3K4me1MLA. The replots from Figure 4.3 are shown in Figure S4.9.

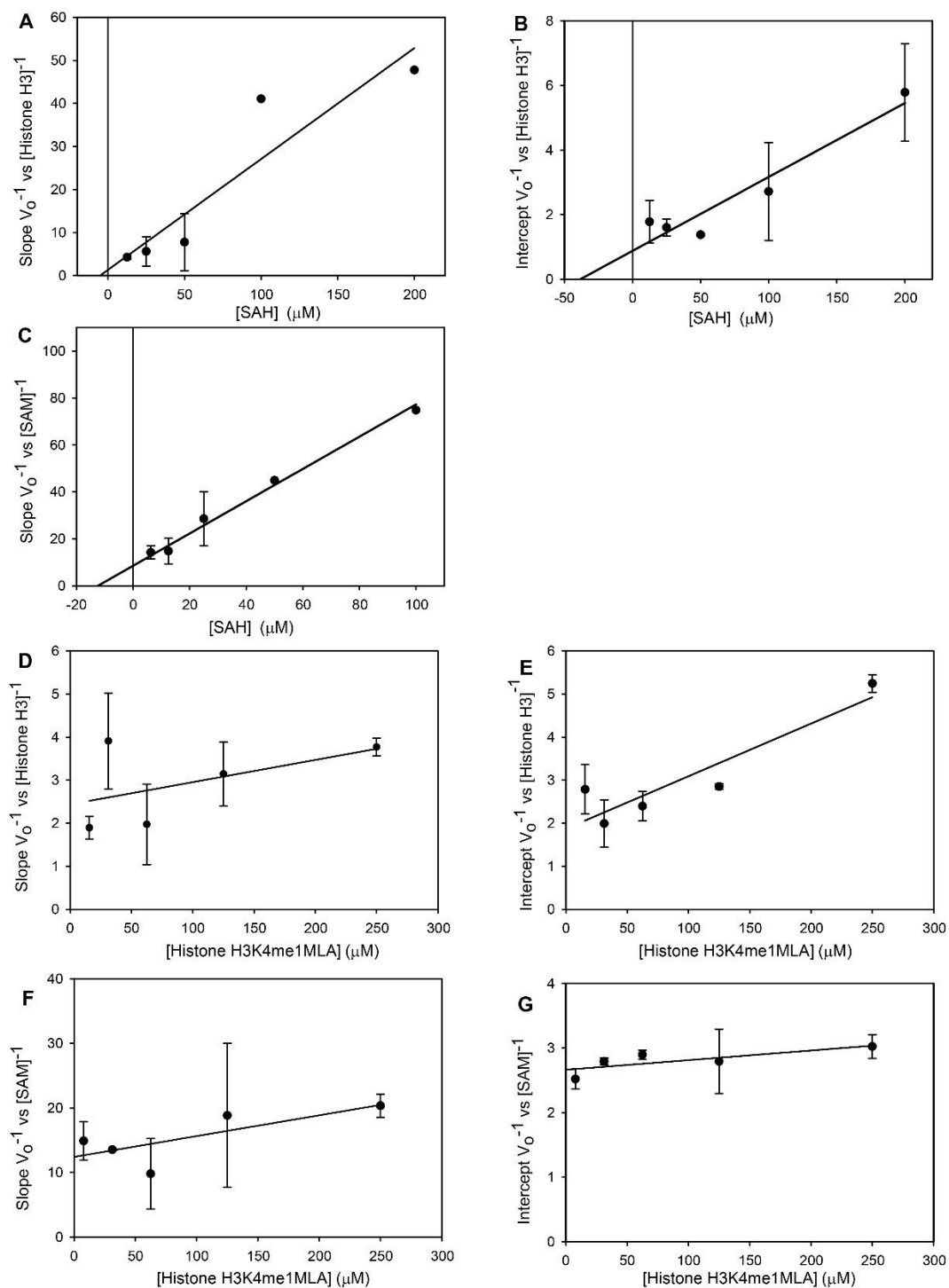


Figure S4.9. The replots from Figure 4.3 for slope (A) and intercept (B) where histone H3 is the substrate and SAH is the product inhibitor, slope (C) where SAM is the substrate and SAH the product inhibitor. The replots for slope (D) and intercept (E) where histone H3 is the substrate and histone H3K4me1MLA is the product inhibitor, the slope

(F) and intercept (G) where SAM is the substrate and histone H3K4me1MLA the product inhibitor. The product inhibitor constants are summarized in Table S4.2.

Table S4.2. Product inhibitor constants for SET7/9.

Substrate	Product	K _{i slope} (μM)	K _{i Int} (μM)
A (SAM)	P (H3K4me1)	428 ±210	1940±900
B (H3)	P (H3K4me1)	531±290	167±96
A (SAM)	Q (SAH)	12.1±3.1	NA ^a
B (H3)	Q (SAH)	85.2±99	50.9±49

^a Cannot be calculated with a sequential ordered model.

Other than what is explicitly stated above no more than 5 points from each group of 30 samples for each set of product inhibitor experiments were rejected for being lower than the calibrated range, below the LLOQ or deviating too much from linearity. The following equations were used to determine the inhibition constants.

$$K_{iSlope}^{P/A} = \frac{K_{iQ}K_{MP}}{K_{MQ}} \left(1 + \frac{K_{MA}[B]}{K_{SA}K_{MB}} \right) \quad [\text{Equation S4.1}]$$

$$K_{iInt.}^{P/A} = \frac{K_{iP} \left(1 + \frac{K_{MB}}{[B]} \right)}{\left(1 + \frac{K_{MQ}K_{MB}K_{iP}}{K_{iQ}K_{MP}[B]} \right)} \quad [\text{Equation S4.2}]$$

$$K_{iSlope}^{P/B} = \frac{K_{iQ}K_{MP}}{K_{MQ}} \quad [\text{Equation S4.3}]$$

$$K_{iInt.}^{P/B} = K_{iP} \left(1 + \frac{K_{MA}}{[A]} \right) \quad [\text{Equation S4.4}]$$

$$K_{iSlope.}^{Q/A} = K_{iQ} = \frac{k_4}{k_{-4}} \quad [\text{Equation S4.5}]$$

$$K_{iSlope}^{Q/B} = K_{iQ} \left(1 + \frac{[A]}{K_{SA}} \right) \quad [\text{Equation S4.6}]$$

$$K_{iInt.}^{Q/B} = K_{iQ} \left(1 + \frac{[A]}{K_{MA}} \right) \quad [\text{Equation S4.7}]$$

Apparent Michelis-Menten values of histone H3 and SAM

The apparent Michelis-Menten parameters $V_{\text{Max-App}}$ and $K_{\text{M-App}}$ for histone H3 was determined with 25 nM of SET7/9 incubated with 200 μM of SAM and histone H3 at 0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15 and 30 μM . While the apparent Michelis-Menten parameters $V_{\text{Max-App}}$ and $K_{\text{M-app}}$ for SAM were determined with 30 μM histone H3 and SAM at 3.125, 6.25, 12.5, 25, 50, 100 and 200 μM .

Histone and nucleosomal substrate profiling

The apparent Michelis-Menten parameters $V_{\text{Max-App}}$ and $K_{\text{M-App}}$ for other substrates were determined with 25 nM SET7/9 with SAM fixed at 200 μM . The concentrations of histone H2A were 0.78, 1.56, 3.125, 6.25, 12.5 and 25 μM . The concentrations of histone H2B were 0.78, 1.56, 3.125, 6.25 and 12.5 μM . The concentrations for histone H4 were at 0.47, 0.94, 1.88, 3.75, 7.5, 15 and 30 μM . The concentrations of histone octamers were 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 μM . The concentrations of NCP were 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 μM . The concentrations of Ub-NCP were 0.047, 0.094, 0.188, 0.375, 0.75, 1.5 and 3 μM . For histone octamers, NCP and Ub-NCP the concentration range was dictated by the maximum concentration supplied.

Chapter 5: A Kinetic Study of DOT1L Reveals Activity Dependent on Prior Nucleosome Modification

5.0 Abstract

DOT1L is the methyltransferase responsible for histone H3 lysine 79 (H3K79) methylation, a chromatin modification essential for transcriptional regulation, genome stability, and leukemogenesis. Despite structural and cellular studies, the enzymatic mechanism of DOT1L and changes in its activity with nucleosomal post-translational modifications like ubiquitination and acetylation remain unresolved. Here, we show that human DOT1L follows sequential Bi-Bi mechanism and demonstrates that DOT1L requires the nucleosome for catalysis, methylating intact nucleosomes and octamers but not individual histones.

We show that histone H2BK120 monoubiquitination is a potent activator that enhances catalytic efficiency, lowers apparent K_M , and uniquely enables progression to the trimethylated state, particularly under SAM-limiting conditions. By contrast, histone H4K16 acetylation modestly increases catalytic efficiency. Together, these data suggest that DOT1L integrates nucleosomal architecture, chromatin modifications, and cofactor availability through a system in which ubiquitination primes DOT1L for high methylation activity while acetylation provides secondary modulation. This gives an idea of how chromatin PTMs encode control of histone H3K79 methylation states and establishes a quantitative foundation for therapeutic targeting of DOT1L in cancer.

5.1 Introduction

Histone methylation in part controls chromatin structure and gene expression, influencing transcription, DNA repair, and cell identity. Among lysine methylation marks, methylation of histone H3 lysine 79 (H3K79) is unusual because it occurs within the globular core of the

nucleosome rather than on histone tails, and to the best of our knowledge is catalyzed exclusively by the only human non-SET domain methyltransferase Disruptor of Telomeric silencing 1-Like (DOT1L) that catalyzes histone H3K79 mono- di- and tri- methylation (histone H3K79me1/me2/me3) (250). Histone H3K79 methylation is enriched in actively transcribed chromatin and contributes to transcriptional elongation, boundary element formation, and lineage-specific gene-expression programs (250). Dysregulation of DOT1L activity is a hallmark of a form of acute myeloid leukemia (AML), mixed-lineage leukemia which is KMT2A-rearranged leukemia, where aberrant histone H3K79 hypermethylation sustains oncogenic transcriptional circuits (125,251). In mixed-lineage leukemia (MLL), DOT1L is aberrantly recruited by KMT2A fusion proteins such as AF4/FMR2 family member 1 (AFF1) and MLL Translocated To 3 (MLLT3) (formerly known as ALL1-Fused gene from chromosome 4 (AF4) and ALL1-Fused gene from chromosome 9 (AF9) respectively) to oncogenic genes such as *HOXA9*, leading to sustained histone H3K79 hypermethylation and transcriptional dysregulation. Genetic knock down of, and pharmacological inhibition of DOT1L, suppresses leukemic gene expression, restores differentiation, and impairs leukemic cell survival. This establishes DOT1L as a critical driver of leukemogenesis. Therefore, DOT1L is a prominent epigenetic drug target, stimulating the development of selective small-molecule inhibitors. These inhibitors can disrupt DOT1L catalytic activity, and therefore histone H3K79 methylation, and offering a therapeutic strategy for mixed lineage leukemia. (126,252,253).

Unlike many SET-domain lysine methyltransferases, which primarily modify accessible unfolded histone tails, DOT1L acts on the folded globular part of histone H3 and only in the intact nucleosome (254). Structural studies have revealed that DOT1L engages a composite surface of the nucleosome including the acidic patch (240), the histone H4 tail, and DNA geometry to

position histone H3K79 within its active site (255). However, a complete understanding of how DOT1L binds cofactors, engages the nucleosome, and progresses through mono-, di-, and potential trimethylation states remains incompletely defined. Given the increasing interest in the potential use of SAM-competitive DOT1L inhibitors such as pinometostat (EPZ-5676) (140,252,256) for AML and the metabolic sensitivity of SAM/SAH homeostasis (252), establishing DOT1L's catalytic mechanism is of both biological and therapeutic relevance.

Post-translational modifications (PTMs) on the nucleosome critically modulate DOT1L. Among these, monoubiquitination of histone H2B at lysine 120 (H2BK120Ub) is the most widely studied activator of DOT1L activity. It represents a fundamental epigenetic modification that serves as a hallmark of active gene transcription. Unlike many histone marks that remain strictly localized to specific regulatory elements like enhancers or promoters, histone H2BK120Ub plays a dynamic role in facilitating the passage of RNA polymerase II through the chromatin. It opens the DNA template and coordinates the installation of downstream histone modifications through complex biochemical crosstalk.

Monoubiquitination of histone H2B follows a classic enzymatic cascade initiated by the activation of ubiquitin by the E1 enzyme UBA1. This molecule is then passed to the E2 conjugating enzymes, primarily UBE2A or UBE2B, which work in concert with the central E3 ligase machinery. In mammals, the heterodimeric RNF20/RNF40 complex is the primary E3 ligase responsible for site-specificity at K120. This complex does not operate in isolation; it is recruited to chromatin by the PAF1 complex and the adaptor protein WAC. These regulatory factors link the ubiquitination machinery directly to the RNA Polymerase II C-terminal domain, ensuring that histone H2B is modified exclusively at genes undergoing active transcription. While RNF20/40 is the canonical

writer, specialized enzymes such as PHF6 and UBR7 provide additional layers of regulation during stem cell differentiation and tumor suppression (257–260).

Histone H2BK120Ub is enriched within the gene body rather than the promoter region. While the promoter is defined by initiation factors and marks like histone H3K4me3, histone H2BK120Ub appears at the critical transition to transcriptional elongation. Because the RNF20/40 complex travels physically with the elongating polymerase, the mark is deposited co-transcriptionally. High-resolution mapping shows that histone H2BK120Ub levels typically peak shortly after the transcription start site and maintain a presence throughout the coding region, serving as an accurate proxy for the rate of polymerase passage. It is virtually absent from the core promoter and inactive genes, reinforcing its status as a dedicated marker of the elongation phase (261).

The biological significance of this gene-body localization is rooted in both physical and signaling properties. The addition of a bulky ubiquitin moiety to the histone H2B tail physically hinders the dense folding of the chromatin, maintaining a "decondensed" state that is more accessible to the transcriptional machinery. Beyond this structural role, histone H2BK120Ub acts as a prerequisite for other essential histone marks. Its presence is required for the recruitment and activation of the COMPASS (Complex of Proteins Associated with Set1) complex and DOT1L, which catalyze the deposition of histone H3K4me3 and histone H3K79me2/3, respectively. This hierarchy establishes histone H2BK120Ub as a central pillar in the epigenetic landscape, ensuring that the transition from gene recognition to active protein synthesis is executed with precision (262). Cryo-EM studies reveal that ubiquitin engages a hydrophobic cradle on DOT1L, stabilizing higher methylation activity (240,263). Moreover, loss of histone H2B ubiquitination diminishes histone H3K79 methylation *in vivo* (264,265).

While the relationship between histone H2BK120Ub and DOT1L is the canonical model for trans-histone crosstalk, recent structural and biochemical studies have identified histone H2BK34Ub as a distinct and potent stimulator of histone H3K79 methylation (266). Although both modifications activate DOT1L, they do so through fundamentally different molecular mechanisms, illustrating the diverse ways in which the ubiquitin moiety can manipulate the nucleosomal landscape to facilitate enzymatic activity. Unlike histone H2BK120Ub, which stimulates DOT1L through direct physical contact between the ubiquitin molecule and the enzyme's C-terminal hydrophobic helix, histone H2BK34Ub does not make direct contact with DOT1L. Because K34 is located near the DNA entry and exit site and is partially buried within the nucleosome core, its attached ubiquitin is positioned far from the nucleosome disk surface where DOT1L typically binds (266,267). Instead, histone H2BK34Ub activates DOT1L through a sophisticated process of nucleosome distortion. The presence of the bulky ubiquitin molecule at the K34 position acts as a wedge between the DNA super-helix turn around the histone core, causing a distortion of this DNA. This movement conformationally restructures the adjacent histone H3K79 loop and because K79 is normally tucked within the histone octamer core, this structural repositioning leaves the side chain and its surrounding residues in a state that is more accessible and energetically favourable for DOT1L binding. By reshaping the nucleosome surface in this manner, histone H2BK34Ub essentially locks the methyltransferase into its active or productive orientation toward the histone H3 target without requiring the direct ubiquitin-enzyme bridge seen in the K120 pathway (266,267).

The biological context of this modification further distinguishes it from the RNF20/40-mediated K120 pathway. Histone H2BK34Ub is typically deposited by the MSL (Male-Specific Lethal) complex, specifically MSL2, a core component of the MOF (males absent on the first)-

MSL complex with E3 ubiquitin ligase activity. This specific modification appears to play a specialized role in helping the transcriptional machinery navigate particularly dense or stable chromatin environments. Interestingly, histone H2BK34Ub and histone H2BK120Ub can work either independently or synergistically to ensure the high-order methylation states, such as histone H3K79me₃, required for robust gene expression. Wu et al. reported that depletion of the MSL complex reduced RNF20 and RNF40 transcript levels by ~30% and diminished their chromatin association, suggesting that histone H2BK34Ub may indirectly regulate histone H2BK120 ubiquitylation through modulation of RNF20/40 activity (267).

Also, the MSL complex contains the acetyltransferase KMT8, also called MOF, which acetylates the histone H4 tail at K16. This modification serves a dual purpose: it prevents chromatin from folding into dense fibers, ensuring an open template, and it stimulates DOT1L by promoting chromatin accessibility and counteracting chromatin compaction and silencing factors. Altogether, these results indicate that the MOF-MSL complex possesses two distinct chromatin-modifying activities: histone H4K16 acetylation and histone H2BK34 ubiquitylation (267,268).

The flexible N-terminal tail of histone H4 interacts with DOT1L and is necessary for the methylation of histone H3K79 in humans and yeast (255). The dependency of DOT1L on the histone H4 tail centers on a specific region known as the basic patch, spanning residues R17 to R19. This basic patch interacts directly with a conserved acidic surface on the DOT1L catalytic domain. This binding is not merely for recruitment; it acts as a mechanical tether that precisely orients the enzyme over the nucleosome core. Because K79 is located within the globular domain of histone H3 rather than on a flexible tail, it is relatively inaccessible. The binding of the histone H4 tail to DOT1L is thought to induce a subtle conformational shift in the nucleosome, positioning the histone H3 target site and guiding the K79 side chain into the enzyme's active site for

methylation (269). Furthermore, the histone H4 tail serves as a platform for chemical modifications that tune DOT1L activity. A prominent example is the acetylation of histone H4K16 (H4K16ac). In both yeast and higher eukaryotes, histone H4K16ac has been shown to have stimulatory effects on DOT1L. It is also thought to modulate DOT1L catalysis by destabilizing higher-order chromatin structure and increasing nucleosome accessibility (270,271). This creates a synergistic regulatory loop: while histone H2BK120Ub provides the initial anchor for DOT1L recruitment, the histone H4K16ac, stabilizes the enzyme in a productive conformation (240,242,270).

This requirement for the histone H4 tail also facilitates a critical biological switch between active chromatin and gene silencing. In yeast, silencing proteins such as SIRT3 compete with the DOT1L homolog (Dot1) for the same basic patch on the histone H4 tail. When SIRT3 occupies the histone H4 tail, it physically blocks Dot1, preventing the methylation of histone H3K79 and promoting the formation of heterochromatin. Conversely, when the histone H4 tail is acetylated at K16, SIRT3 is displaced, allowing DOT1L to bind and establish the histone H3K79me mark characteristic of open, transcribed chromatin. Thus, the histone H4 tail acts as the primary gatekeeper, integrating multiple epigenetic signals to control the methylation state of the histone H3 core (272). However, a comprehensive study on how nucleosome PTMs affect the enzyme activity of DOT1L has yet to be done.

To address these gaps, we developed a high-sensitivity LC–MS/MS analytical method to quantify Kme1, Kme2, and Kme3 using isotope-labeled lysine internal standards. Using this assay, we performed rate measurements, varied SAM and nucleosome concentrations, and attempted product inhibition analyses to define the order of substrate binding. In parallel, we compared DOT1L activity on unmodified nucleosome core particles (NCP), histone H2BK120-ubiquitinated

nucleosomes (Ub-NCP), and histone H4K16-acetylated nucleosomes (Ac-NCP), revealing how these PTMs reshape both catalytic efficiency and ultimate methylation state catalyzed by DOT1L. We demonstrate that DOT1L follows a sequential Bi-Bi mechanism, histone H2BK120 ubiquitination enhances catalytic efficiency, increases affinity, and showing for the first time that it uniquely enables histone H3K79me3 trimethylation. Furthermore, we show that histone H4K16 acetylation produces modest activity enhancement while surprisingly slightly reducing affinity. We show that nucleosome modifications encode regulatory information that modifies DOT1L activity, advancing our understanding of histone H3K79 methylation.

5.2 Results

The method used to measure the enzymatic activity of DOT1L was adapted from our previous methods (182,273). Here we utilized a volatile buffer (NH₄)HCO₃ to reduce background and ion suppression and incorporated stable isotope labeled ¹³C6 ¹⁵N2 lysine as an internal standard to correct for loss during sample processing and injection (182)

At high (200 nM) and low (40 nM) concentrations of NCP, the rate of total methylation by DOT1L (40 nM) is linear with respect to time up 1h at 30 °C (Figure S5.1). The rate of total methylation is linear with respect to enzyme concentration up to 200 nM DOT1L (Figure S5.2). The pH was maintained as 8 and DTT was not found to influence enzyme rate (Figure S5.3). Based in these preliminary studies, all subsequent kinetic reactions were carried out at 30 °C with 40 nM DOT1L in a buffer with 100 mM (NH₄)HCO₃ at pH 8, and 200 nM ¹³C6¹⁵N2 lysine internal standard for 30 min, unless otherwise stated. Substrate control assays which contained only DOT1L and SAM, but no NCP showed no detectable methylation activity suggesting that DOT1L does not auto-methylate (Figure S5.4). We also showed that at high concentrations, DOT1L can produce Kme1, Kme2 and Kme3 with nucleosomes *in vitro* (Figure 5.1A). With histone octamers, only Kme1 was

detected. The individual histones H2A, H2B, H3 and H4 exhibited methylation that was below the low limit of quantification for our method (Figure 5.1B). The ability to resolve Kme3 by our LC–MS/MS methods provided clear and unambiguous difference from Kme2, which can be challenging with immunoblotting methods where cross-reactivity can be a problem (274).

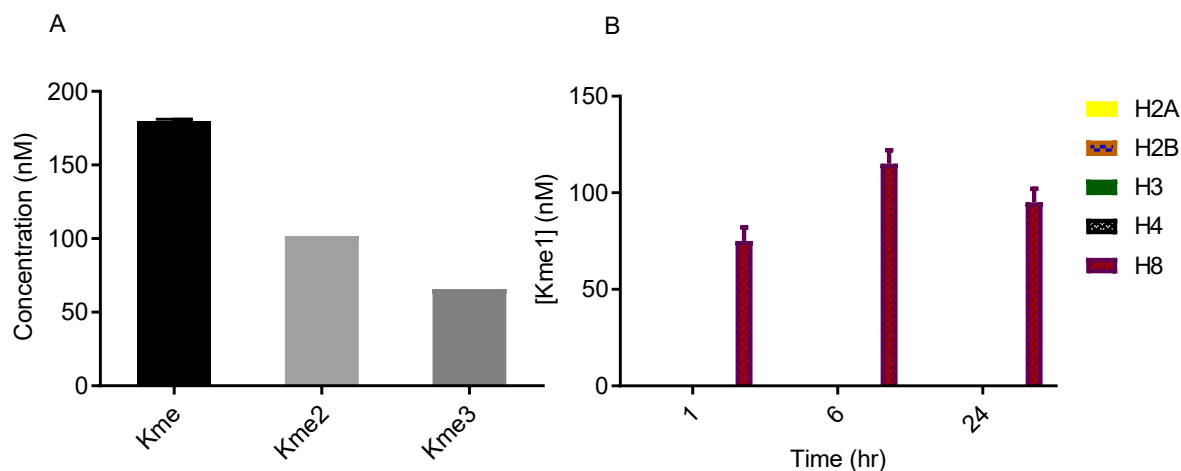


Figure 5.1. DOT1L methylates nucleosomes and octamers but not individual histones. DOT1L catalyzes mono-, di-, and trimethylation of lysine residues on nucleosomes (A), produces only monomethylation on histone octamers, and shows no detectable activity toward individual histones (B). Each bar is the mean and S.D of 2 reactions.

5.2.1 DOT1L follows a sequential mechanism

To determine the DOT1L Bi-Bi substrate enzymatic mechanism, we incubated increasing concentrations of NCP with increasing SAM concentrations. The V_{Max} and K_M values for these substrates were initially estimated by varying each in the presence of fixed excess of the other. Based on these values the concentration ranges for SAM and NCP were selected such that the estimated apparent K_M was within the range.

When NCP was varied in the presence of constant SAM concentrations, the double reciprocal plots exhibit producing a pattern of lines intersecting to the left of the y- axis (Figure 5.2).

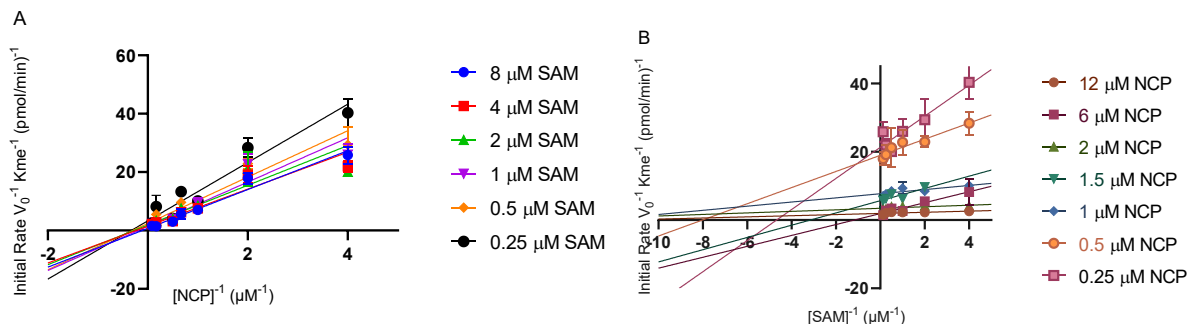


Figure 5.2. Sequential Bi-Bi substrate enzymatic mechanism for DOT1L. Reciprocal plots of the initial velocity (v_o) of DOT1L with the substrates, NCP and SAM. The concentration of NCP was varied with fixed SAM from 0.125 to 4 μ M (A). These data were re-plotted where the concentration of SAM was varied with NCP concentrations from 0.083 to 4 μ M. The initial velocity was measured by quantifying the amount of Kme1 produced over 30 min. Each point is the mean and S.D of 2 reactions. The kinetic parameters are shown in Table 5.1.

This pattern of lines is indicative of a sequential, but not a double displacement (ping-pong) Bi-Bi substrate enzymatic mechanism for DOT1L (275). The initial rate (v_o) of a sequential enzymatic mechanism can be described using Equation 5.1 and its reciprocal forms, Equation 5.2 where substrate A is varied and B fixed, and Equation 5.3 where B is varied and A fixed. K_{SA} is the equilibrium dissociation constant for the first substrate to bind to DOT1L, K_{MA} and K_{MB} are the Michaelis constants for the substrates A and B, respectively, V_{Max} is the maximum rate of the reaction, and $[A]$ and $[B]$ are the concentrations of the substrates. For an ordered sequential mechanism where substrate A must bind before B, K_{MA} and K_{MB} are the Michaelis constants for the substrates A and B, respectively. In the case of a rapid equilibrium random sequential mechanism, these are equilibrium dissociation constants where $K_{MB}=[EA][B]/[EAB]$,

$K_{MA}=[EB][A]/[EAB]$ and $K_{SB} =K_{SA} K_{MB}/K_{MA}$. and Equation 5.2 becomes 5.3. The turnover number or k_{cat} is defined according to Equation 5.5 (276). The slopes and intercepts from the double reciprocal plots from Figure 5.2 were replotted in Figure S5.5 and these were used to determine the values for V_{Max} , K_{SA} , K_{MA} , and K_{MB} and will be described below (Table 5.1).

$$v_0 = \frac{V_{Max}[A][B]}{K_{SA}K_{MB}+K_{MB}[A]+K_{MA}[B]+[A][B]} \quad [\text{Equation 5.1}]$$

$$\frac{1}{v_0} = \frac{K_{MA}}{V_{Max}} \left(\frac{K_{SA}K_{MB}}{[B]K_{MA}} + 1 \right) \frac{1}{[A]} + \frac{1}{V_{Max}} \left(\frac{K_{MB}}{[B]} + 1 \right) \quad [\text{Equation 5.2}]$$

$$\frac{1}{v_0} = \frac{K_{MA}}{V_{Max}} \left(\frac{K_{SB}}{[B]} + 1 \right) \frac{1}{[A]} + \frac{1}{V_{Max}} \left(\frac{K_{MB}}{[B]} + 1 \right) \quad [\text{Equation 5.3}]$$

$$\frac{1}{v_0} = \frac{K_{MB}}{V_{Max}} \left(\frac{K_{SA}}{[A]} + 1 \right) \frac{1}{[B]} + \frac{1}{V_{Max}} \left(\frac{K_{MA}}{[A]} + 1 \right) \quad [\text{Equation 5.4}]$$

$$k_{cat} = \frac{V_{Max}}{[E]_T} \quad [\text{Equation 5.5}]$$

5.2.2 Product inhibition studies

Figure 5.2 suggests a sequential mechanism of substrate binding to DOT1L, but it cannot differentiate between an ordered sequential where substrate binding and product release has to follow a strict order where one substrate “A” must bind first before the other “B”, and one product “P” must dissociate first before the other “Q”, or random sequential where either substrate “A” or “B” can randomly bind first and either product can first dissociate, Bi-Bi enzymatic mechanisms.

To determine which of these mechanisms DOT1L follows, product inhibition assays must be performed where the products of the reaction (product inhibitors) are added to the reaction to elucidate what type of inhibition they produce. Product inhibition analysis is a classical approach for determining the order of substrate binding in multi-substrate reactions (16,182). This articulates

how the substrates bind to the enzyme. We used substrates, SAM and NCP, and increasing concentrations of the product inhibitor SAH.

We first determined the IC_{50} of SAH to obtain the range of concentration for the curves (Figure S5.5). When the SAM concentration was maintained at a fixed level and NCP was varied, the double reciprocal plots converged on the right side of the y-axis, and most of the lines, particularly those obtained at higher concentrations of SAH, intersected the y-axis at negative values (data not shown). This pattern is not characteristic of any kind of inhibition and may result from the low level of substrate NCP used for this experiment. However, methylated nucleosome product-inhibition experiments were not performed because, as demonstrated earlier, the methylated nucleosome product can undergo further rounds of methylation, potentially complicating kinetic interpretations. Ultimately these data are inconclusive with respect to kinetic mechanism.

Table 5.1. Kinetic parameters for DOT1L substrates.

V_{MaxB} (pmol/min)	V_{MaxA} (pmol/min)	K_{MB} (μM)	K_{MA} (μM)	K_{SA} (μM)	k_{cat} (s^{-1}) $\times 10^{-3}$
0.871 $\pm$ 0.17	0.912 $\pm$ 0.24	5.70 $\pm$ 0.17	0.531 $\pm$ 0.037	0.133 $\pm$ 0.0082	7.43 $\pm$ 0.24

5.2.3 DOT1L Activity on Nucleosomes and Modified Nucleosomes

To dissect how histone post-translational modifications affect DOT1L catalysis, we analyzed the initial velocity of Kme1 formation using the substrates unmodified NCP, Ub-NCP, and Ac-NCP and SAM. To calculate apparent kinetic parameters, we performed reactions for unmodified and modified nucleosomes at a fixed, saturating SAM concentration (200 μM). The reactions for varying SAM were carried out at a fixed 2 μM NCP. At concentrations below 2 μM of NCP, Ub-NCP, and Ac-NCP, DOT1L displayed characteristic hyperbolic saturation kinetics with

nucleosomal substrates (Figure 5.3A-B). Data was fit to Equation 5.7 or 5.8 and the apparent parameters listed in Table 5.2.

H2BK120 Ubiquitination produced the strongest effect, increasing both k_{cat} and K_M resulting in nearly a two-fold rise in catalytic efficiency relative to unmodified NCP. Acetylation also enhanced the apparent V_{max} by approximately 60%, but there was a three-fold increase in K_M , indicating likely weaker enzyme–substrate binding and resulting ~ half the catalytic efficiency for NCP (Table 5.2). Thus, acetylation confers a minor stimulatory effect on maximum reaction rate but does not facilitate substrate recognition to the same degree as ubiquitination. The data suggest that acetylation may slightly improve catalysis once DOT1L is bound, whereas ubiquitin directly stabilizes productive enzyme–nucleosome complexes and enhances methylation.

At higher concentrations of Ac-NCP and Ub-NCP (Figure 5.3C), both substrates show increased initial velocity of Kme1. Collectively, these findings suggest that DOT1L activity is differentially tuned by chromatin modifications: ubiquitination functions as a potent activator that increases both catalytic rate and binding affinity, while acetylation yields only a modest kinetic enhancement that may arise from local structural relaxation of the nucleosome surface.

$$v_{0\ App} = \frac{V_{Max\ App}[A]}{K_{MA\ App} + [A]} \quad \text{[Equation 5.7]}$$

$$v_{0\ App} = \frac{V_{Max\ App}[B]}{K_{MB\ App} + [B]} \quad \text{[Equation 5.8]}$$

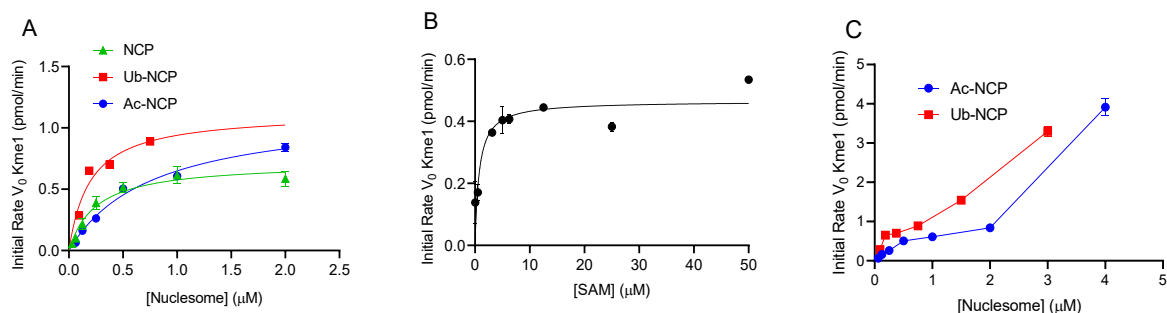


Figure 5.3. Substrate profiling for DOT1L with nucleosomes and modified nucleosomes. The initial rate of production of Kme1 in pmol per min by DOT1L with varying concentrations of NCP, Ub-NCP and Ac-NCP with a fixed SAM concentration of 200 μM (A), with varying concentrations of SAM with fixed NCP concentration of 1 μM (B). The amount of Kme1 produced by DOT1L with varying concentrations of Ub-NCP and Ac-NCP with a fixed SAM concentration of 200 μM . The initial velocity was measured by quantifying the amount of Kme1 produced over 30 min. Each point is the mean and S.D. of 2 repeats.

Table 5.2. Apparent Michaelis–Menten kinetic parameters for DOT1L with various substrates

Variable Substrate	Saturating Substrate	$V_{\text{Max App}}$ (pmol/min)	$K_{\text{M App}}$ (μM)	$k_{\text{cat App}}$ (s^{-1}) $\times 10^{-3}$	$(k_{\text{cat}}/K_{\text{M}})_{\text{App}}$ ($\text{M}^{-1} \text{s}^{-1}$) $\times 10^4$
NCP	SAM	0.718 ± 0.045	0.248 ± 0.049	5.98 ± 0.38	2.41 ± 0.50
Ub-NCP	SAM	1.12 ± 0.12	0.196 ± 0.055	9.36 ± 0.96	4.78 ± 1.4
Ac-NCP	SAM	1.15 ± 0.068	0.772 ± 0.10	9.58 ± 0.57	1.24 ± 0.18
SAM	NCP	0.465 ± 0.026	0.755 ± 0.28	3.87 ± 0.21	0.51 ± 0.19

5.2.4 DOT1L Catalysis is Stimulated and Redirected by Histone H2B Ubiquitination in a SAM-Dependent Manner

We wanted to find out how histone H2B monoubiquitination influences the catalytic behavior of DOT1L, we performed a substrate-profiling analysis using recombinant NCP and Ub-NCP. For

an unbiased comparison of catalytic output, initial velocities were calculated from the early linear phase of product accumulation and were expressed either as total methyl equivalents, defined as $Kme1 + 2 \times Kme2 + 3 \times Kme3$, which reflects the overall number of methyl groups transferred per unit time, or as the number of modified lysine residues, defined as $Kme1 + Kme2 + Kme3$, which counts each modified lysine once regardless of methyl state.

When reactions were performed at low SAM concentration, DOT1L activity on Ub-NCP was strongly enhanced compared with unmodified NCP. Both the rate of Kme1 formation and the rate of Kme2 production were significantly higher with the ubiquitinated substrate (Figure 5.4A-B). The increase was also evident when output was expressed as total methyl equivalents, with Ub-NCP supporting a substantially greater overall methyl-group transfer rate (Figure 5.4C), and when the number of modified lysine residues was considered, showing that more histone H3 sites were methylated in the presence of ubiquitin (Figure 5.4D). These observations indicate that under limiting SAM, the presence of histone H2B ubiquitination provides a general catalytic boost, stimulating the initiation of methylation and promoting progression to dimethylated products.

At high SAM concentration, overall DOT1L activity increased on both substrates, but the qualitative pattern of methylation diverged. Histone H3K79me3 was detected exclusively in reactions containing Ub-NCP, whereas unmodified NCP failed to produce histone H3K79me3 state even with saturating cofactor. Ub-NCP also accumulated more histone H3K79me2 and proportionally less histone H3K79me1 than NCP (Figure 5.5A-C). Although total kme rose for both substrates at saturating SAM, the fold-difference between Ub-NCP and NCP was reduced compared with the low-SAM condition (Figure 5.5D-E). This may suggest that ubiquitination is critical for maintaining catalytic efficiency when methyl donor is scarce, but even under abundant SAM it uniquely promotes methylation beyond the monomethyl state.

Our findings demonstrate that H2BK120Ub modulates DOT1L in two distinct but complementary ways. When SAM availability is low, ubiquitination broadly accelerates catalysis, enhancing both the rate of methylation initiation and the enzyme's capacity to produce histone H3K79me₂. When SAM is saturating, ubiquitination primarily alters product distribution, enabling the enzyme to achieve trimethylation and shifting the equilibrium away from histone H3K79me₁ products towards histone H3K79me₂ and histone H3K79me₃. This SAM-dependent change in catalytic behavior shows that histone H2B ubiquitination is not merely a rate enhancer but a key regulator of DOT1L methylation-state outcome.

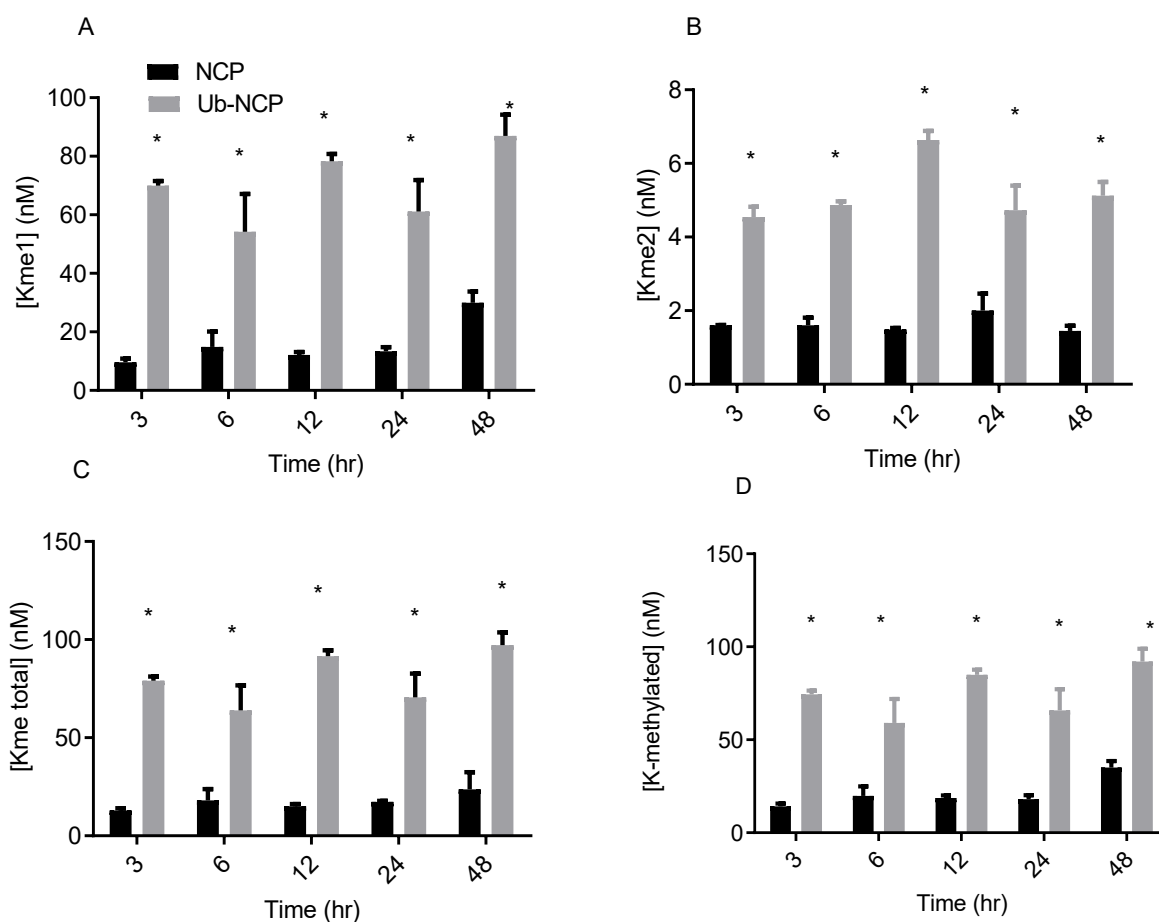


Figure 5.4. Substrate profiling for DOT1L with NCP and Ub-NCP at low SAM. The production of modified lysines by DOT1L with NCP and Ub-NCP (0.2 μ M each) with 5 μ M of SAM (Kme1 (A) Kme2 (B) Total methyl groups transferred - measured by the weighted sum of Kme1 and Kme2 (Kme1+2Kme2) in nM of methyl-groups (C) Number of lysine residues modified - measured by the plain sum of Kme1 and Kme2 (Kme1+Kme2) in nM of methyl-groups (D). Each point is the mean and S.D of 2 reactions. An asterisk (*) indicates statistically significant differences using 2-way ANOVA test.

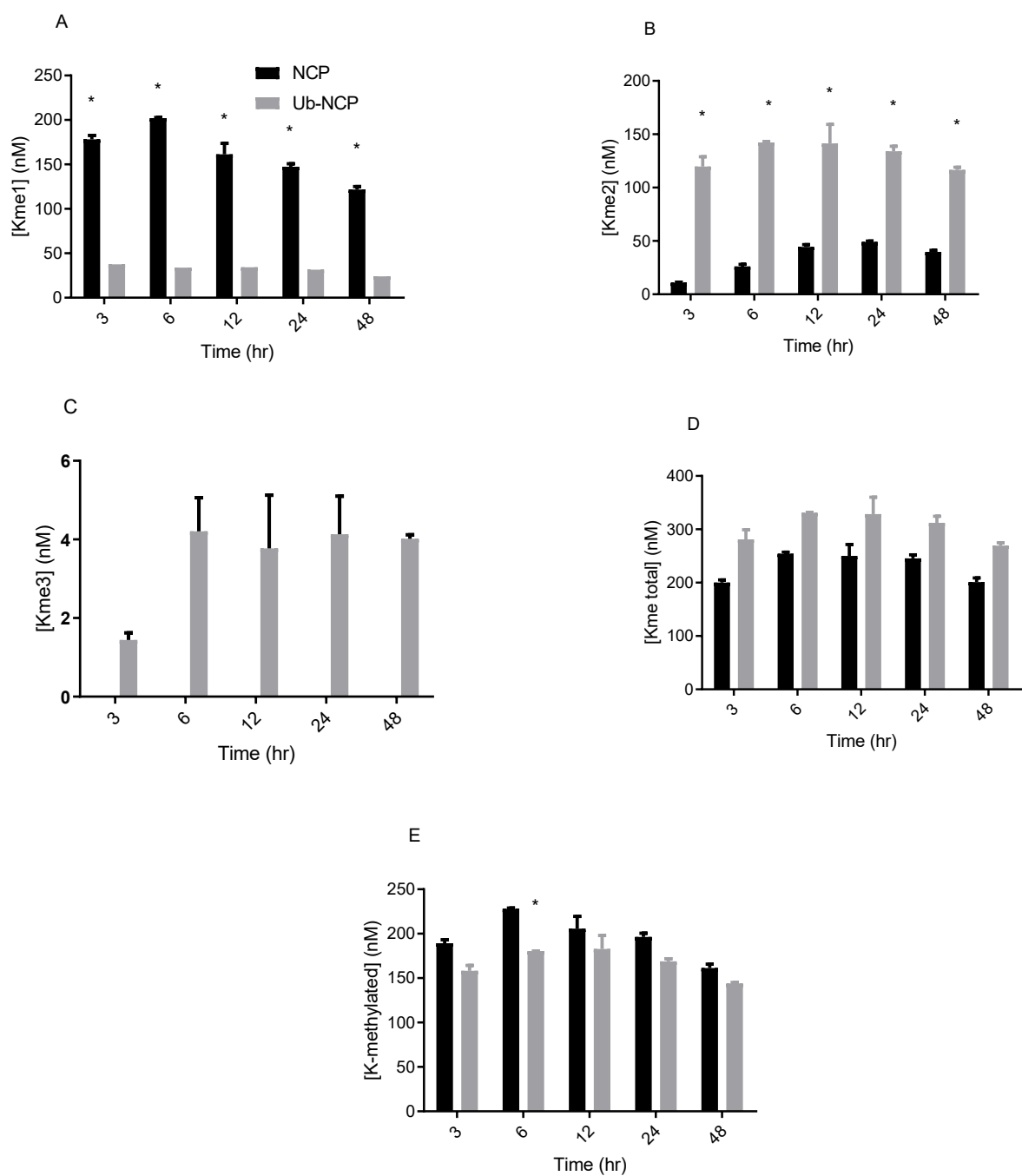


Figure 5.5. Substrate profiling for DOT1L with NCP and Ub-NCP at high SAM. The production of modified lysines by DOT1L with NCP and Ub-NCP (0.2 μ M each) with 200 μ M of SAM. Kme1 (A) Kme2 (B) Kme3 (C) Total methyl groups transferred - measured by the weighted sum of Kme1, Kme2 and Kme3 ($Kme1+2Kme2+3Kme3$) in nM of methyl-groups (D) Number of lysine residues modified- measured by the plain sum of Kme1 and Kme2

(Kme1+Kme2) in nM of methyl-groups (E). Total methyl groups as measured by the weighted sum of Kme1 and Kme2 (Kme1+2Kme2) in nM of methyl-groups per min. Each point is the mean and S.D. of 2 repeats. An asterisk (*) indicates statistically significant differences using 2-way ANOVA test.

5.3 Discussion

5.3.1 Sequential Bi-Bi Mechanism of DOT1L

The double-reciprocal kinetic analysis revealed that DOT1L conforms to a sequential Bi-Bi mechanism, consistent with the patterns observed for other methyltransferases such as SET7/9 and PRMT1 (16,182). DOT1L has a Rossman fold like SAM binding motif similar to arginine and DNA methyltransferases and not a SET domain like the majority of KMTs (277,278). It is a non-SET domain lysine methyltransferase (279,280).

DOT1L's low apparent K_M for SAM suggests the enzyme remains catalytically competent under all physiological relevant SAM concentrations (10-50 μ M). The variations can also affect the outcome of DOT1L catalysis depending on the presence and type of modifications on the nucleosome.

5.3.2 DOT1L Requires the Nucleosome for Efficient Catalysis

We observed robust mono-, di-, and trimethylation on intact nucleosomes, monomethylation on histone octamers, but no detectable activity on individual histones. This strict dependence on nucleosomal structure confirms earlier studies (281), but provides stronger chemical evidence due to the ability of LC-MS/MS to unambiguously distinguish K79 methylation states (274).

Mechanistically, this requirement reflects the necessity of multiple nucleosome-embedded contacts, including the acidic patch, the histone H4 tail, and the histone H3 globular domain, to position histone H3K79 deep within the catalytic channel. Structural studies (240,270) show that DOT1L adopts different conformations during the catalytic cycle (282), and our kinetic data

reinforce these structural observations by demonstrating that isolated histones cannot productively orient the lysine substrate.

The finding that histone octamers are methylated suggests that interaction between nucleosomal DNA and DOT1L is not essential for its enzyme activity.

5.3.3 SAM Dependence and Cofactor Affinity

Our titration of SAM concentrations revealed classical Michaelis–Menten kinetics and confirms that DOT1L has a reasonable affinity for the methyl donor at physiological SAM levels. The estimated intracellular concentration of SAM is around 10 μ M (283), but can vary ~10–100 fold under physiological conditions (284–286). Therefore, the low apparent K_M value for SAM suggests that DOT1L will have sufficient SAM to work at V_{max} under most physiologic conditions.

5.3.4 Ubiquitination of Histone H2B as a Potent Activator of DOT1L

Among the post-translational modifications tested, histone H2BK120 ubiquitination had the strongest effect. At both low and saturating SAM concentrations, Ub-NCP exhibited higher V_{max} and lower K_M values relative to unmodified NCP, yielding a two-fold increase in catalytic efficiency (V_{max}/K_M). More importantly, ubiquitination enables accumulation of H3K79me3, a state that unmodified nucleosomes fail to reach even under saturating SAM. This enhancement persisted across donor conditions, confirming that ubiquitination affects substrate binding and catalytic turnover.

Our results agree with cell-based observations linking histone H2B ubiquitination to higher histone H3K79 methylation states. The activation of DOT1L by ubiquitinated nucleosomes has been attributed to structural stabilization of the enzyme–nucleosome interface, which optimally positions histone H3K79 within the active site (263,265). Cryo-EM reconstructions of the DOT1L–Ub-NCP complex show that the ubiquitin engages the enzyme’s “hydrophobic cradle”,

locking it into a catalytically more favorable configuration (240,282). Our kinetic analysis reveals the functional consequence of this structural stabilization: ubiquitination both reduces K_M and increases V_{max} , increasing the specificity constant, promoting efficient transition from mono- to di- and trimethylation, with effects more pronounced when SAM is limiting. Thus, ubiquitination regulates DOT1L higher order methylation to the histone H3K79me2 and me3 form promoting transcriptionally active chromatin.

5.3.5 Acetylation as a Modest Modulator

While histone H4K16 acetylation (Ac-NCP) enhanced DOT1L activity compared with unmodified NCP, the effect was modest compared to that of ubiquitination. Kinetic parameters revealed an approximately 60% increase in V_{Max} accompanied by a three-fold rise in K_M , suggesting improved apparent k_{cat} but reduced apparent K_M which may indicate reduced enzyme affinity.

5.3.6 Integration of Substrate Modifications and Cofactor Availability

The combined analysis of unmodified, acetylated, and ubiquitinated nucleosomes under variable SAM concentrations reveals a hierarchical model of DOT1L regulation. Histone H2BK120 monoubiquitination primarily increases apparent k_{cat} presumably by stabilizing the productive conformation of the enzyme–nucleosome complex consistent with structural studies. Histone H4K16ac modulates local chromatin accessibility and may fine-tune reaction rates under specific structural contexts. Limited SAM availability, in turn, may act as a metabolic regulator, shifting the equilibrium of methylation states ($Kme1 \rightarrow Kme2 \rightarrow Kme3$). Based on this we propose that ubiquitination primes DOT1L for high-order methylation (i.e. histones H3K79me2/3, and H4K16ac modulates substrate accessibility.

5.3.7 Biological Implications and Chromatin Crosstalk

Our results may have implications for the epigenetic control of gene expression and chromatin state maintenance. Histone H3K79 methylation catalyzed by DOT1L is a hallmark of active chromatin and participates in transcriptional elongation, DNA damage response (287), and hematopoietic lineage commitment (288). The sequential mechanism and modification-dependent kinetics revealed in this study provide mechanistic insight into how DOT1L integrates local chromatin cues with cellular metabolic status.

5.3.8 Physiological and Therapeutic Relevance

DOT1L is the only known methyltransferase targeting histone H3K79 in mammals, and aberrant DOT1L activity is causally linked to KMT2A-rearranged leukemia and other malignancies (253,289). Understanding the enzymatic basis of its regulation is therefore critical for designing selective inhibitors. Our results further suggest that nucleosome-targeted disruption, for example via modulation of histone H2B monoubiquitination, could synergize with DOT1L inhibitors for better therapeutic outcomes.

5.4 Materials and Methods

5.4.1 Protein expression and purification

Wild-type DOT1L catalytic domain (a.a. 1-416) in the pGEX6p vector was a generous gift from the laboratory of Dr. Ji-Joon Song of the Department of Biological Sciences, Korea Advanced Institute of Science and Technology. DOT1L was expressed as a GST-fusion in BL21(DE3) pLysS cells (Invitrogen) *E. coli* cells induced with 1 mM IPTG and incubated at 16 °C while shaking at 300 rpm overnight (16 h). The pellet was centrifuged at $15000 \times g$ for 20 minutes at 4 °C. The pellet was resuspended in lysis buffer which has 1 $\times$ PBS, 0.5 mg/mL Lysozyme, 0.5 M EDTA, DTT, 1 mM PMSF, 0.1% Triton X-100, 10 U DNase and protease inhibitor tablets, and sonicated

on ice using a Branson Sonifier, centrifuged and the supernatant loaded onto a 5 mL GStrap FF column (Cytiva) connected to an AKTÄ Purifier FPLC at a flow rate of 1 mL/min. DOT1L was purified with a step gradient of A: PBS, EDTA, DTT, 1 mM PMSF and B: 50 mM Tris pH 8, 20 mM Glutathione, 300 mM NaCl, and 1 mM DTT. The column was washed with 70 mL of A, then 10 mL of 10% B, then SET7/9 eluted with 15 mL of 100% B, all flow rates were 3 mL/min. The eluted fraction was concentrated with a Spin-X UF20 10 kDa NMWCO concentrator (Corning), dissolved in storage buffer with 50mM Tris pH 7.5 with 5% glycerol, and re-concentrated.

5.4.2 Methylation reactions

Methylation reactions contain DOT1L, NCP, Ac-NCP and Ub-NCP (EpiCypher) and SAM (Cayman Chemical Co.) in a buffer with 100 mM $(\text{NH}_4)\text{HCO}_3$ (pH 8), and 200 nM internal standard stable isotope labelled $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine, at 30°C. The details of the various methylation reactions are in the supplemental methods. Dried samples were acid hydrolyzed in the vapor phase using 6M HCl at 110 °C for 24 h and the hydrolyzed samples were dried in a vacuum centrifuge and reconstituted in 50 μL of 0.05% formic acid for analysis by LC-MS/MS (see below). The concentration of NCP, Ac-NCP and Ub-NCP, and DOT1L were determined by acid hydrolysis and quantification of lysine using LC-MS/MS. SAM stock concentrations were determined by UV absorbance and the molar extinction coefficient $15400 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 260 nm(290). For reactions with NCP, Ac-NCP and Ub-NCP DOT1L (40 nM) was incubated with each substrate (0.2 μM) under two cofactor conditions designed to model methyl donor limitation and saturation: a low S-adenosyl-L-methionine (SAM) concentration of 5 μM and a high, saturating concentration of 200 μM . Reaction products were quantified by LC-MS/MS as monomethylated (Kme1), dimethylated (Kme2) and trimethylated (Kme3) lysines on histone H3.

5.4.3 LC-MS/MS

We previously developed methods to quantify multiple post-translational modifications including lysine (K), mono- (Kme1) di- (Kme2) and trimethyl lysine (Kme3), that were validated using nucleosomes with a single site of modification (182,273,291). This method was modified to quantify methylated lysines with the internal standard $^{13}\text{C}_6$ $^{15}\text{N}_2$ lysine for enzymatic reactions. Hydrolyzed samples were analyzed using a Nexera UHPLC connected to a Shimadzu 8040 triple quadrupole mass spectrometry system. Chromatographic separation was achieved using a SIELC Primesep200 at 40 °C. Mobile phases (A) 0.05% formic acid in water and (B) 1% formic acid in 50% aqueous acetonitrile were used at a flow rate of 0.4 mL/min. Initial conditions were 0% (B) for 1.5 minutes, increasing to 85% (B) over 30 seconds and held for 3 minutes. Positive ion analytes were quantified using multiple reaction monitoring (MRM) and dual ESI, APCI (DUIS) ionization. The precursor and product ion m/z values collision energies and other parameters are in Table S1. The nebulizing gas was set to 2 L/min, the drying gas was 15 L/min, the desolvation line temperature was 250 °C and the heating block was 400 °C. The assay can quantify K, Kme1, Kme2, Kme3.

5.4.4 Data analysis

Rate data and substrate histone NCP and SAM concentrations for the kinetic analysis of DOT1L and product inhibitor studies were reciprocal transformed and fit to a straight-line equation with $1/Y^2$ weighting (to account for bias due to reciprocal transformation) using Graph pad. The hyperbolic rate data for the activity of DOT1L against nucleosomes and modified nucleosomes were fit to the Michaelis–Menten and double reciprocal plot equations were fit using GraphPad Prism 10.

5.5 Supplemental

DOT1L expression and purification

Wild-type DOT1L catalytic domain (a.a. 1-416) in the pGEX6p vector was a generous gift from the laboratory of Dr. Ji-Joon Song of the Department of Biological Sciences, Korea Advanced Institute of Science and Technology. DOT1L was expressed as a GST-fusion form in BL21(DE3) pLysS cells (Invitrogen). Bacteria were grown at 37 °C until an OD600 of 0.8 was reached. Then, isopropyl-b-D thio-galactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the protein for 16 h at 20 °C. The cells were harvested by centrifugation at $4800 \times g$ for 30 minutes. The cell pellet recovered from 1 L culture was resuspended in 50 mL lysis buffer which has 1 $\times$ PBS, 0.5 mg/mL Lysozyme, 0.5 M EDTA, DTT, 1 mM PMSF, 0.1% Triton X-100, 10 U DNase and protease inhibitor tablets, and sonicated on ice using a Branson Sonifier. The supernatant was then centrifuged at $30,700 \times g$ for 30 minutes, the supernatant containing proteins with nucleic acid contamination was poured into a beaker in an ice bath. Then, the proteins were separated by precipitating the nucleic acids using 5% polyethylenimine (PEI). Five percent PEI stock solution was added progressively into the extract with gently stirring until the final concentration of PEI was 0.8%. During this process, the nucleic acids were precipitated. The precipitate was removed by centrifugation at $17,300 \times g$ for 10 minutes, leaving the proteins in the supernatant. The supernatant was loaded onto a 5 mL GStap FF column (Cytiva) connected to an AKTÄ Purifier FPLC at a flow rate of 1 mL/min. DOT1L was purified with a step gradient of A: PBS, EDTA, DTT, 1 mM PMSF and B: 50 mM Tris pH8, 20 mM Glutathione, 300 mM NaCl, and 1 mM DTT. The column was washed with 70 mL of A, then 10 mL of 10% B, then SET7/9 eluted with 15 mL of 100% B, all flow rates were 3 mL/min. The eluted fraction was

concentrated with a Spin-X UF20 10 kDa NMWCO concentrator (Corning), dissolved in storage buffer with 300 mM, 25 mM Tris pH 8 with 5% glycerol, and re-concentrated.

Details of methylation reactions

To determine where the reaction rate (in pmol/min) for DOT1L, SAM and NCP is increasing linearly over time, DOT1L (80 nM) was incubated with high and low concentrations of substrates SAM (5 μ M and 1 μ M) and NCP (0.2 μ M and 0.08 μ M). The samples were analyzed at 5, 10, 20, 30, 40, 60, 120, 240 and 360 minutes in (NH₄)HCO₃ buffer (pH 8) (Sigma-Aldrich) (100 mM), internal standard stable isotope labeled ¹³C₆ ¹⁵N₂ lysine (Cambridge Isotopes Laboratories Inc.) (200 nM) (Figure S5.1).

To determine the range over which the rate of reaction for DOT1L with SAM and NCP increases linearly with enzyme concentration DOT1L was incubated with SAM (5 μ M) and NCP (0.2 μ M) with 3.125, 6.25, 12.5, 25, 50, 100 and 200nM DOT1L (Figure S5.2).

The effect of DTT on the enzymatic activity of DOT1L was determined by incubating the enzyme in one group with SAM, NCP (5 μ M and 1 μ M respectively) and (NH₄)HCO₃ buffer (pH 8) (100 mM), internal standard stable isotope labelled ¹³C₆¹⁵N₂ lysine (200 nM) and 2.5 mM DTT and another group that did not contain DTT. The samples were analyzed at 15, 30 and 60 minutes (Figure S5.3).

Given the results from these experiments, all subsequent kinetic methylation reactions were carried out for 30 minutes with 40 nM DOT1L in a buffer with 100 mM (NH₄)HCO₃ at pH 8, and 200 nM ¹³C₆ ¹⁵N₂ lysine internal standard, unless otherwise stated.

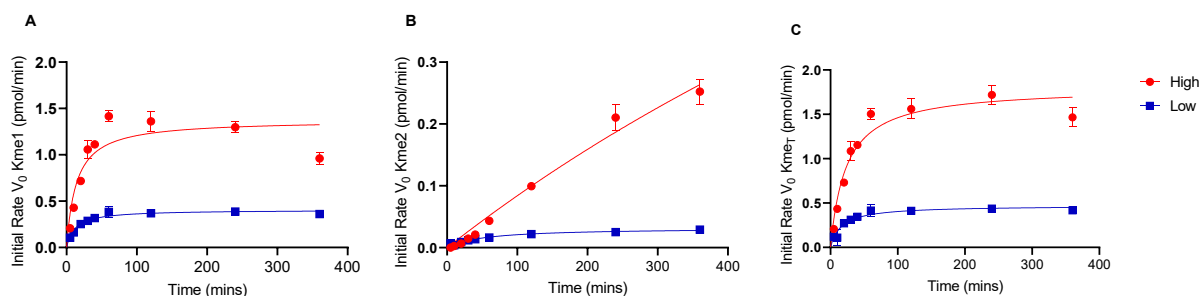


Figure S5.1. Time course activity of high and low concentrations of SAM. Time course activity of high and low concentrations of SAM (5 μ M and 1 μ M) and NCP (0.2 μ M and 0.08 μ M) with 80 nM of DOT1L. This was used to determine where the reaction rate for DOT1L, SAM and NCP is increasing linearly over time. (A) Monomethyl lysine (B) Dimethyl lysine (C) Total methyl groups transferred - measured by the weighted sum of Kme1 and Kme2 (Kme1+2Kme2) in nM of methyl-groups. Each point is the mean and S.D of 2 reactions.

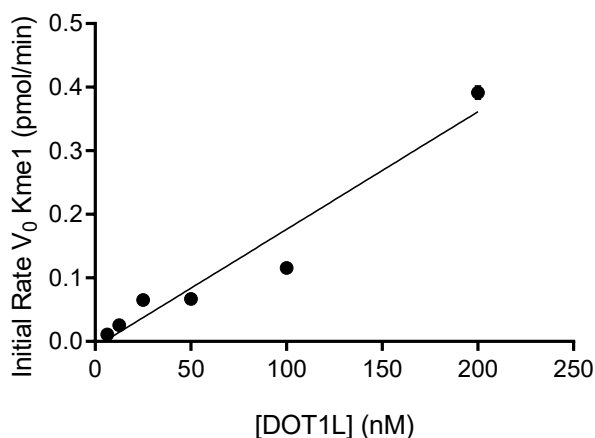


Figure S5.2. The activity of DOT1L at various concentrations at 5 μ M and 0.2 μ M of AdoMet and NCP respectively. This shows the rate of reaction for DOT1L with SAM and NCP increases linearly with enzyme concentration up to 200 nM.

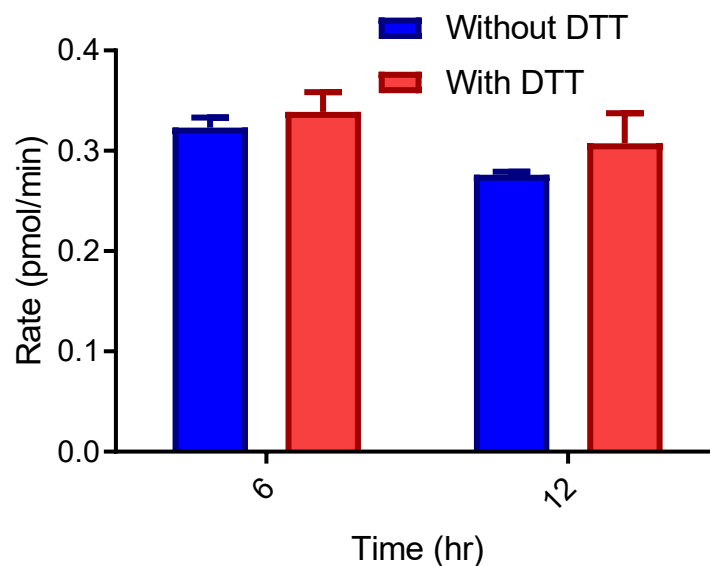


Figure S5.3. The activity of DOT1L with and without DTT. Analysis using paired t-test shows the differences due to the presence of DTT were not statistically significant ($p > 0.05$) up to 12 hours.

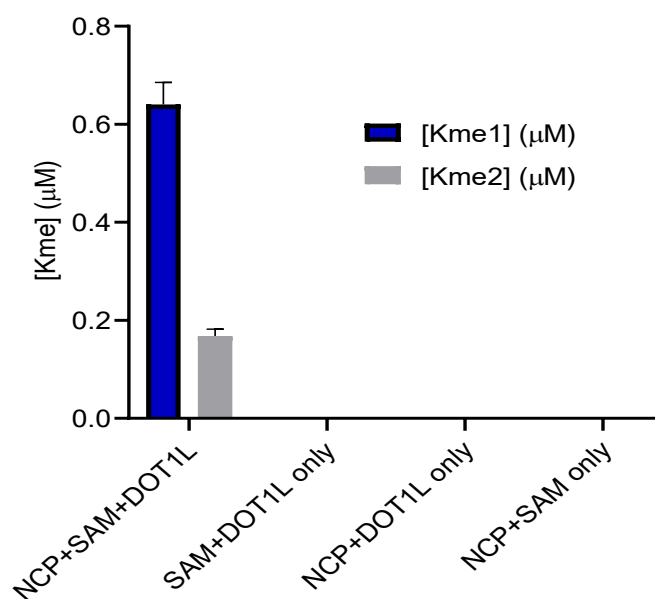


Figure S5.4. Substrate control assay for DOT1L. To determine whether DOT1L exhibits auto-methylation. Enzymatic reactions were conducted in the absence of NCP and/or SAM. The methylation signals detected under these conditions were below the limit of quantification, indicating that DOT1L does not undergo auto-methylation. These

results confirm that the methylation activity of DOT1L is strictly dependent on the presence of a viable substrate and SAM. Each bar is the mean and S.D. of 2 repeats.

Preliminary product inhibitor experiments

Preliminary measurements of the inhibition of 40 nM DOT1L by SAH were determined by incubating 1.5 μ M SAM, 0.5 μ M NCP and SAH at concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000 μ M (Figure S5.5).

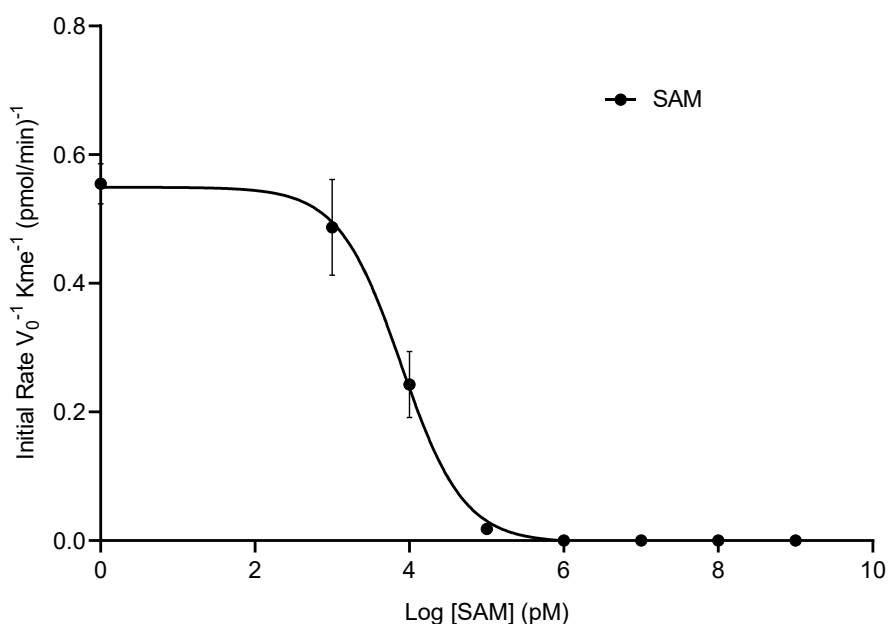


Figure S5.5. IC₅₀ curve of the inhibition of DOT1L by SAH. The IC₅₀ value for SAH is 0.007829 μ M.

Determination of V_{Max} and K_M

The experiments to determine V_{Max} and K_M values for NCP and SAM were as follows 0.25, 0.5, 1, 1.5, 2, 6 and 12 μ M of NCP were incubated with SAM concentrations of 0.25, 0.5, 1, 2, 4 and 8 μ M and for a total of 42 samples. Replots from Figure 5.2 (Figure S5.6) are shown for the slopes from the reciprocal V_0 vs reciprocal NCP plotted against reciprocal SAM, the intercept for the

plots of reciprocal V_o vs reciprocal histone NCP, plotted against reciprocal SAM. The slopes from the reciprocal V_o vs reciprocal SAM plotted against reciprocal NCP and the intercept for the plots of reciprocal V_o vs reciprocal SAM, plotted against reciprocal NCP.

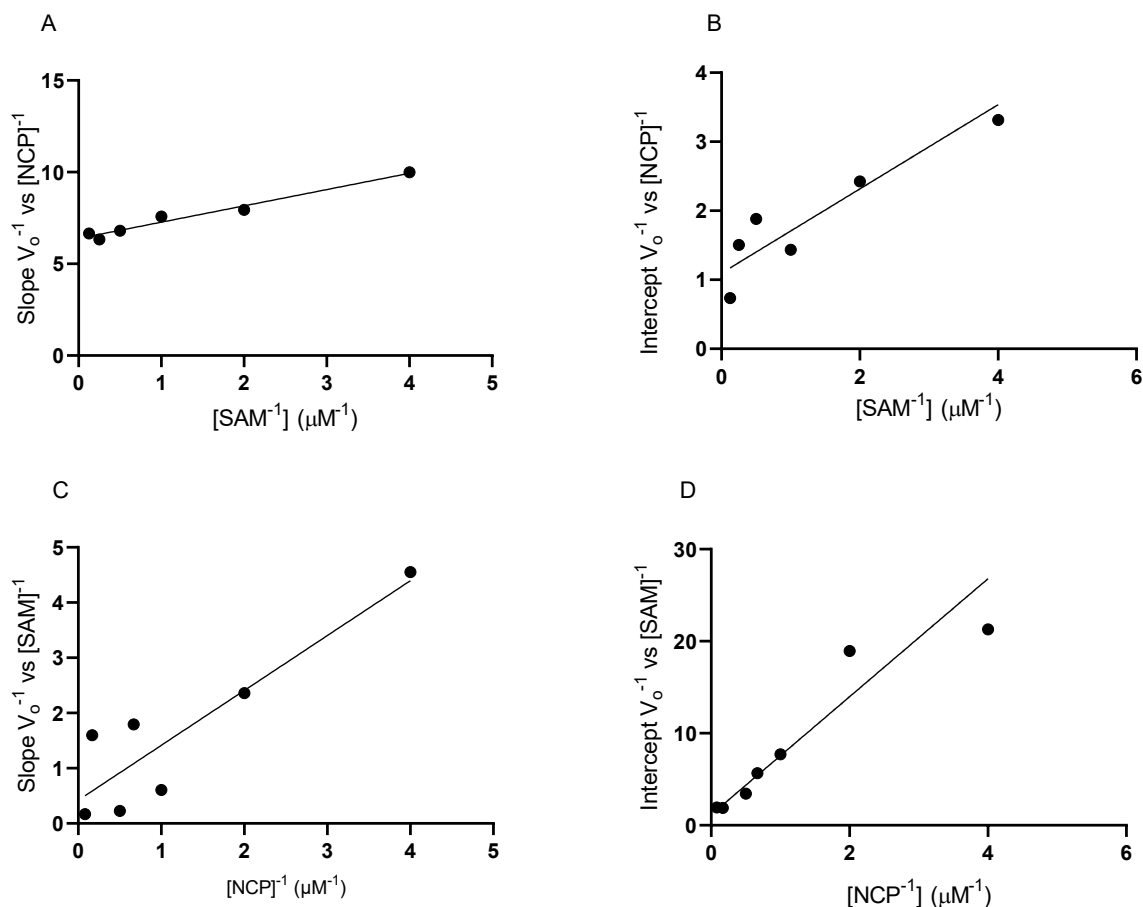


Figure S5.6. Replots from Figure 5.2 for the slopes from the reciprocal V_o vs reciprocal NCP, plotted against reciprocal SAM (A). The intercept for the plots of reciprocal V_o vs reciprocal histone NCP, plotted against reciprocal SAM (B). The slopes from the reciprocal V_o vs reciprocal SAM plotted against reciprocal NCP and the intercept for the plots of reciprocal V_o vs reciprocal SAM, plotted against reciprocal NCP.

Chapter 6: Conclusion and Future Work

5.0 Summary

This thesis set out to examine the activity and mechanisms of epigenetic enzymes, with a particular focus on histone methyltransferases, to understand how these enzymes function at the molecular level and why their pharmacological targeting remains both promising and problematic. Through a combination of biochemical method development, enzymology, and kinetic studies, this work demonstrates that histone methyltransferases are not merely chromatin modifiers but dynamic, context-dependent catalysts. Their kinetic behavior and substrate specificity fundamentally shape normal physiology, disease aetiology, and therapeutic response.

The limitations of epigenetic therapies arise not primarily from inadequate inhibitor design, but from the intrinsic pleiotropic properties of histone methyltransferase. Unlike receptors, which can often be targeted through tissue localization or biophysical compartmentalization, histone methyltransferases operate genome-wide and participate in multiple regulatory programs simultaneously. Consequently, global inhibition frequently produces both therapeutic and deleterious effects through the same underlying molecular mechanisms.

At the biochemical level, the development of a rapid, single-step histone purification and refolding method provides a practical foundation for accurate epigenetic enzymology. High-purity histones are essential for reproducible kinetic and proteomic analyses, and the desalting protocol described here removes a major technical barrier that has historically limited access to well-defined histone substrates. By reducing purification time, cost, and technical complexity, this method enables broader and more systematic interrogation of histone-modifying enzymes and their substrates.

Using these refined substrates and more, this thesis establishes that SET7/9 operates as an ordered sequential bi-substrate enzyme whose catalytic behavior is strongly dependent on chromatin context. The discovery that SET7/9 follows hyperbolic Michaelis–Menten kinetics on free histones but displays sigmoidal, cooperative kinetics on nucleosomes reveals that nucleosome architecture actively regulates enzymatic activity. This finding challenges the assumption that histone methyltransferases behave identically on isolated substrates and within chromatin, and highlights that the nucleosome may be an allosteric regulator of epigenetic enzymes. Moreover, the demonstration that SET7/9 can catalyze both lysine and arginine methylation and generate higher-order methylation states under specific conditions, expands its functional range beyond established definitions and show the flexibility of histone-modifying enzymes.

This thesis also defines the catalytic mechanism of DOT1L, a methyltransferase central to mixed-lineage leukemia. DOT1L was shown to function through a sequential mechanism and to exhibit strict dependence on nucleosomal substrates. Its activity is strongly enhanced by histone H2B ubiquitination and histone H4 acetylation. We show that it exhibits some form of ubiquitin-dependent processivity and works in a cofactor-dependent manner. These findings demonstrate that DOT1L activity is governed primarily by chromatin architecture, histone modification crosstalk, and cofactor availability, rather than enzyme abundance alone.

5.1 Experimental Limitations

This study used *Xenopus laevis* histones as a model system. Although histone proteins are highly conserved, small sequence differences may influence modification dynamics and enzyme recognition compared with human histones. Finally, the absence of cellular and *in vivo* validation is an important limitation. While *in vitro* assays provide mechanistic clarity, chromatin organization and regulatory factors in cells may significantly influence enzyme activity. In

addition, nucleosomes were not reconstituted using the histones made using desalting columns. While the purified histones were suitable for biochemical assays, assembling nucleosomes from these preparations would have allowed direct validation of their structural integrity.

One limitation of this thesis is that the enzymatic assays for SET7/9 were performed at pH 9, which is more alkaline than physiological conditions. This pH was necessary to optimize the activity of SET7/9, but it also changes the electrostatic properties and structure of the nucleosome. At pH 9, histidine residues ($pK_a \approx 6.0$) become neutral, and as the pH approaches the pK_a of lysine (≈ 10.5), the positive charge on histone tails begins to decrease. Because histone–DNA interactions depend on electrostatic attraction, this loss of positive charge can weaken DNA binding and increase nucleosome flexibility, a process often described as nucleosome breathing.

Alkaline conditions may also affect higher-order chromatin structure. The histone H4 tail contains a basic patch that stabilizes interactions between neighboring nucleosomes and promotes chromatin compaction. At elevated pH, reduced protonation weakens these interactions, leading to chromatin fiber swelling and a more open configuration. As a result, enzymes may encounter a substrate that is more accessible than in physiological chromatin. Therefore, increased enzymatic activity observed at pH 9 may reflect not only higher efficiency but also artificially increased substrate accessibility.

In addition, alkaline conditions may alter the flexibility and solubility of histone tails. Because histone tail dynamics regulate enzyme recruitment and modification accessibility, this structural fluidity could mask the regulatory effects of specific epigenetic marks. Modifications such as histone H2BK120 ubiquitination or histone H4K16 acetylation normally increase chromatin accessibility. If nucleosomes are already partially destabilized by the alkaline buffer, the stimulatory effects of these modifications may be underestimated. Thus, while pH 9 assays reveal

maximal catalytic potential, the nucleosome template likely exists in a more relaxed state than chromatin *in vivo*.

The allosteric behavior observed for SET7/9 with nucleosomal substrates was not mechanistically investigated. The sigmoidal kinetic profiles suggest cooperative or conformational regulation that requires further structural and biophysical study. In addition, the impact of histone acetylation on SET7/9 activity was not examined, even though acetylation can influence lysine charge and chromatin accessibility.

Although kinetic data support a sequential Bi-Bi mechanism for DOT1L, the exact substrate binding order remains unclear. Determining whether the mechanism is ordered or random will be important for understanding substrate coordination and for designing inhibitors. Several additional factors limit the interpretation of these findings. The combined effects of H2BK120 ubiquitination and histone H4K16 acetylation on DOT1L activity were not tested, although both modifications independently enhance chromatin accessibility and transcription. Other regulatory modifications, including histone H2BK34 ubiquitination, were also not examined. Recent studies suggest that histone H2BK34Ub can stimulate DOT1L activity by altering nucleosome structure, and its omission limits a full understanding of histone crosstalk.

5.2 Future Directions

Future studies should use human histones for nucleosome reconstitution to confirm that the observed findings apply directly to human chromatin. Investigating the effects of histone acetylation on SET7/9 activity will help clarify how chromatin accessibility and lysine charge influence enzyme function. Further exploration of arginine methylation and its interaction with lysine methylation may reveal additional regulatory layers in chromatin signaling. Structural and

biophysical studies are also needed to understand the allosteric behavior observed for SET7/9 on nucleosomal substrates.

For DOT1L, future work should examine the combined effects of histone H2BK120 ubiquitination and histone H4K16 acetylation, as well as additional modifications such as histone H2BK34 ubiquitination. Studying nucleosomes carrying multiple modifications will be essential to understand how chromatin integrates regulatory signals. These modifications may act additively, synergistically, or hierarchically to regulate enzyme activity.

Further kinetic studies are required to determine whether DOT1L follows an ordered or random sequential binding mechanism. Cryo-electron microscopy, single-molecule approaches, and molecular dynamics simulations could provide insight into conformational changes induced by nucleosome engagement and histone modifications.

From a therapeutic perspective, strategies are shifting from targeting catalytic activity to disrupting enzyme recruitment. Proteolysis-targeting chimeras (PROTACs) and small molecules designed to block the MLLT3(AF9)–DOT1L interaction may offer improved specificity by silencing leukemogenic transcriptional programs. Evaluating DOT1L inhibitors in nucleosome systems that include H2B ubiquitination will be essential to determine whether compounds differentially affect catalytic efficiency and higher methylation states.

Future integration of high-resolution cryo-EM structures with computational simulations will allow prediction of how poly-modified nucleosomes, bearing modifications such as histone H2BK120ub, H2BK34ub, and H4K16ac, cooperatively lower the energetic barrier for histone H3K79 methylation. These approaches will help move the field toward predictive models in which transcriptional output can be inferred from combinatorial histone modifications.

Ultimately, the field is shifting from studying individual enzymes and histone marks toward a systems-level understanding of multivalent chromatin signaling. By integrating structural biology, enzymology, computational modeling, and cellular studies, future research may enable predictive frameworks that link chromatin modifications to gene regulation and therapeutic response. These advances also support the development of strategies that incorporate genomic localization or modulate enzyme activity, rather than relying on indiscriminate enzyme inhibition, thereby offering greater selectivity and potentially more durable clinical benefit.

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