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Review

The Chemical Biology of Reversible Lysine Post-translational Modifications

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Lysine (Lys) residues in proteins undergo a wide range of reversible post-translational modifications (PTMs), which can regulate enzyme activities, chromatin structure, protein-protein interactions, protein stability, and cellular localization. Here we discuss the "writers," "erasers," and "readers" of some of the common protein Lys PTMs and summarize examples of their major biological impacts. We also review chemical biology approaches, from small-molecule probes to protein chemistry technologies, that have helped to delineate Lys PTM functions and show promise for a diverse set of biomedical applications.

Introduction

The discovery of Lys side-chain acetylation and methylation over 50 years ago in the context of histone proteins initiated a transformation in our understanding of gene regulation and beyond (Ali et al., 2018; Luo, 2018). Subsequently, protein Lys ubiquitination has revolutionized the field of protein turnover (Brooks and Gu, 2006). More recently, a wide range of protein Lys acylations including propionylation (K_{Pr}), butylation (K_{Bu}), crotonylation (K_{Cro}), malonylation (K_{Mal}), succinylation (K_{Succ}), glutarylation (K_{Glu}), β-hydroxybutylation (K_{Bhb}), 2-hydroxyisobutyrylation (K_{Hib}), lactylation (K_{Lac}) (Zhang et al., 2019), and benzoylation (K_{Bz}) (Huang et al., 2018) have been reported, although the functions of these PTMs are minimally characterized (Huang et al., 2015) (Figure 1). Beyond ubiquitination (K_{Ub}), ubiquitin-like PTMs such as SUMOylation (K_{Sumo}), NEDDylation (K_{Nedd}), and others are becoming intensive areas of investigation. Altogether, more than 100,000 sites of Lys modifications in over 10,000 proteins have been mapped by mass spectrometry and other methods (Hornbeck et al., 2012). In many cases, particular Lys residues can be modified by a number of different PTMs allowing for the potential of a diverse pattern of biological outputs and complex regulation. Here, we provide a summary of how some of these Lys PTMs are attached and removed, examples of how they mediate their structural and functional effects on proteins, and how their dysregulation can contribute to diseases. We also cover a range of chemical approaches from small-molecule probes to protein chemistry techniques that have contributed to our understanding of protein Lys modifications and their cellular effects.

Writers, Erasers, and Readers for Lys PTMs

The two major classes of enzymes that add and remove Lys PTMs, also known as "writers" and "erasers," can determine the amount, distribution, site specificity, and pattern of particular Lys PTMs. A third group of proteins that are commonly recruited to the modified Lys residues are often called "readers" and can transmit some of the functional impacts of Lys PTMs (Lambert et al., 2019). Notably, some large proteins may contain both a "reader" domain and a "writer" or "eraser" domain for the same or different Lys PTM(s) (Cieniewicz et al., 2014). In this section, we discuss well-characterized examples of writers, erasers, and readers for Lys acetylation, methylation, ubiquitination, and atypical acylations.

Lys Acetylation Writers, Erasers, and Readers

Lys acetylation (K_{Ac}), exists very widely in the human proteome with more than 38,000 mapped sites and can influence many cellular activities including gene expression, cell growth, and differentiation (Hornbeck et al., 2012).

Lys Acetyltransferases. Lys acetyltransferases (KATs, also commonly known as histone acetyltransferases or HATs) use acetyl-coenzyme A (acetyl-CoA, Ac-CoA) as their obligatory co-substrates (Figure 2A). Acetyl-CoA contains a reactive thioester, which facilitates acetyl transfer to Lys amino groups, and the nonenzymatic rates can be appreciable. There are three main groups of well-characterized nuclear KATs: GCN5/PCAF, p300/CBP, and the MYST family (Dancy and Cole, 2015). These KAT families appear to be examples of convergent evolution, and there are few to no significant sequence or structural similarities between the three families. GCN5/PCAF are components of the SAGA multiprotein complex and are highly selective for acetylating histone H3, especially on Lys14 (Nagy and Tora, 2007), whereas p300/CBP are broad-spectrum acetyltransferases with over 1,000 protein Lys sites mapped by mass spectrometry (Hansen et al., 2019). p300/CBP are thought to be the major enzymes for acetylating Lys18 and Lys27 in histone H3 and have been tied to regulating enhancer elements in transcription (Weinert et al., 2018). MYST enzymes are found in multiprotein complexes and can acetylate histones and other proteins. Each of these KATs has been implicated in diseases, including cancer, with both loss-of-function and gain-of-function roles in neoplasia (Dancy and Cole, 2015). p300/CBP and MYST enzymes appear to be regulated by autoacetylation and multiple protein-protein interactions (Dancy and Cole, 2015).

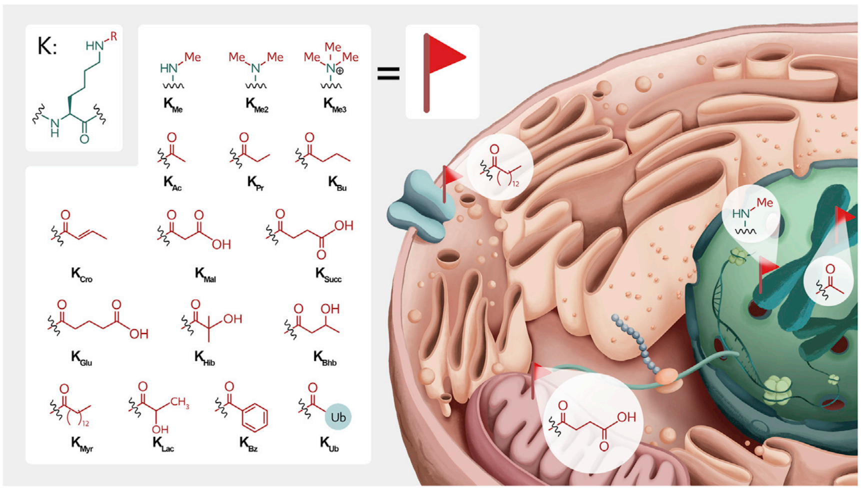


Figure 1. Structures and Locations of Various Lys PTMs in Eukaryotic Cells
Modifications include mono-, di-, and trimethylation ($K_{Me1,2,3}$); acetylation (K_{Ac}), propionylation (K_{Pr}), butyrylation (K_{Bu}), crotonylation (K_{Cro}), malonylation (K_{Mal}), succinylation (K_{Succ}), glutarylation (K_{Glu}), 2-hydroxyisobutyrylation (K_{Hib}), β-hydroxybutyrylation (K_{Bhb}), myristoylation (K_{Mys}), lactylation (K_{Lac}), benzoylation (K_{Bz}) and ubiquitination (K_{Ub}). Note that the cell structure is a cartoon to show the proposed locations of different protein Lys PTMs in the cell.

Lys Deacetylases. The removal of K_{Ac} is catalyzed by 18 different protein Lys deacetylases (KDACs, also commonly known as histone deacetylases or HDACs) in humans, which can be generally divided into four classes (Haberland et al., 2009). The 11 classical KDACs are distributed among class I, II, and IV HDACs, which are metallohydrolases that utilize a zinc(II) ion to catalyze the hydrolysis of acetyl groups (Figure 2B). However, some members of the class II HDAC family lack a key catalytic Tyr residue, which is replaced with a His, and appear to be pseudoenzymes. Class I HDACs (HDAC1, 2, 3, 8) are predominantly in cell nuclei and HDACs 1, 2, and 3 are found in a range of multiprotein complexes. Examples of these are CoREST (Humphrey et al., 2001), Sin3 (Laherty et al., 1997), NuRD (Xue et al., 1998), SMRT (Guenther et al., 2000), and MiDAC (Itoh et al., 2015). HDAC1 and HDAC2 possess about 80% sequence identity and show overlapping functions (Jamaladdin et al., 2014). Class II HDAC10 seems to be selective for hydrolyzing acetyl-polyamine metabolites rather than proteins (Hai et al., 2017). Class III HDACs are known as sirtuins (Wang et al., 2017c) and employ nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate (Figure 2C) (Jing and Lin, 2015). The sirtuins cleave acetyl-Lys by a complex mechanism that involves nucleophilic attack of the acetyl-carbonyl oxygen on the anomeric carbon of the nicotinamide ribose, displacing nicotinamide and generating *O*-acetyl-ADP-ribose.

K_{Ac} Readers. The major and best-characterized class of acetyl-Lys binding domains in proteins is the bromodomains (BRDs). To date, 61 BRD-containing proteins in humans have been annotated, and these are generally divided into eight families based on their structural and sequence similarity (Filippakopoulos et al., 2012). A heavily studied group of BRDs comprises the bromo- and extra-terminal (BET) family, which includes BRD2, BRD3, BRD4, and BRDT, possessing two independent BRDs at their N termini and showing distinct specificities for histone Lys acetylation sites (Fujisawa and Filippakopoulos, 2017). BRDs are found in several KATs, including p300/CBP and PCAF/GCN5, where they have regulatory as well as recruitment functions. BRDs are notable for their potent sensitivity to a range of small molecules that allow for acetyl-Lys protein-protein interactions to be antagonized (Filippakopoulos and Knapp, 2014).

A more recently discovered acetyl-Lys binding domain is the YEATs domain (Klein et al., 2018). This domain is found in several human proteins related to chromatin regulation, including GAS41 and AF9, and displays an immunoglobulin-like fold. In addition to acetyl-Lys, YEATs domains can also bind crotonyl-Lys (Li et al., 2018) and succinyl-Lys (Wang et al., 2018a), potentially serving as a reader for these modifications.

Lys Methylation Writers, Erasers, and Readers

As mentioned, Lys methylation can occur as monomethyl (K_{Me1}), dimethyl (K_{Me2}), and trimethyl (K_{Me3}) forms in proteins. Like Lys acetylation, Lys methylation is a common PTM, but it has been mostly closely associated with the regulation of chromatin structure and gene transcription (Luo, 2018). In histone H3, for instance, methylation exists on H3K9, H3K27, and H4K20 sites in human chromosomes and is a marker of heterochromatin and gene silencing (Huang et al., 2015). However, methylation on other sites such as H3K4, H3K36, and H3K79 sites are activation markers (Janssen et al., 2019). Lys methylation also plays roles in the regulation of different transcription factors, for example p53 (Chuikov et al., 2004) and nuclear factor κB (Lu and Stark, 2015).

Lys Methyltransferases. Attachment of methyl groups to Lys residues is catalyzed by Lys methyltransferases (KMTs) (Allis, 2018). KMTs utilize *S*-adenosylmethionine (SAM) as a methyl donor, a common chemical strategy for enzymatic methylation throughout metabolic and catabolic pathways (Figure 2D). Mechanistically, these enzymatic methylations are accepted to occur as S_N2-like displacement reactions. Two general classes of KMTs have been discovered, SET domain (about 50) and non-SET domain methyltransferases (a handful). SET domain proteins can be divided into seven subfamilies with limited sequence conservation and structural similarity (Dillon et al., 2005), and subfamily members show diverse substrate selectivity (Qian and Zhou, 2006). The first SET KMT identified was Su(Var)3–9 (Ebert et al., 2004), which methylates the histone H3 Lys9 site (Schotta et al., 2002). SET methyltransferases include EZH2, which can methylate histone H3 Lys27, and MLL methyltransferase, which targets Lys4 of histone H3. Dot1L was the first reported example of a non-SET KMT, and it specifically methylates Lys79 in a globular region of histone H3 and is linked to transcriptional activation (van Leeuwen et al., 2002). A novel N-terminal and C-terminal domain with a unique open α/β structure for SAM binding in Dot1L is different from

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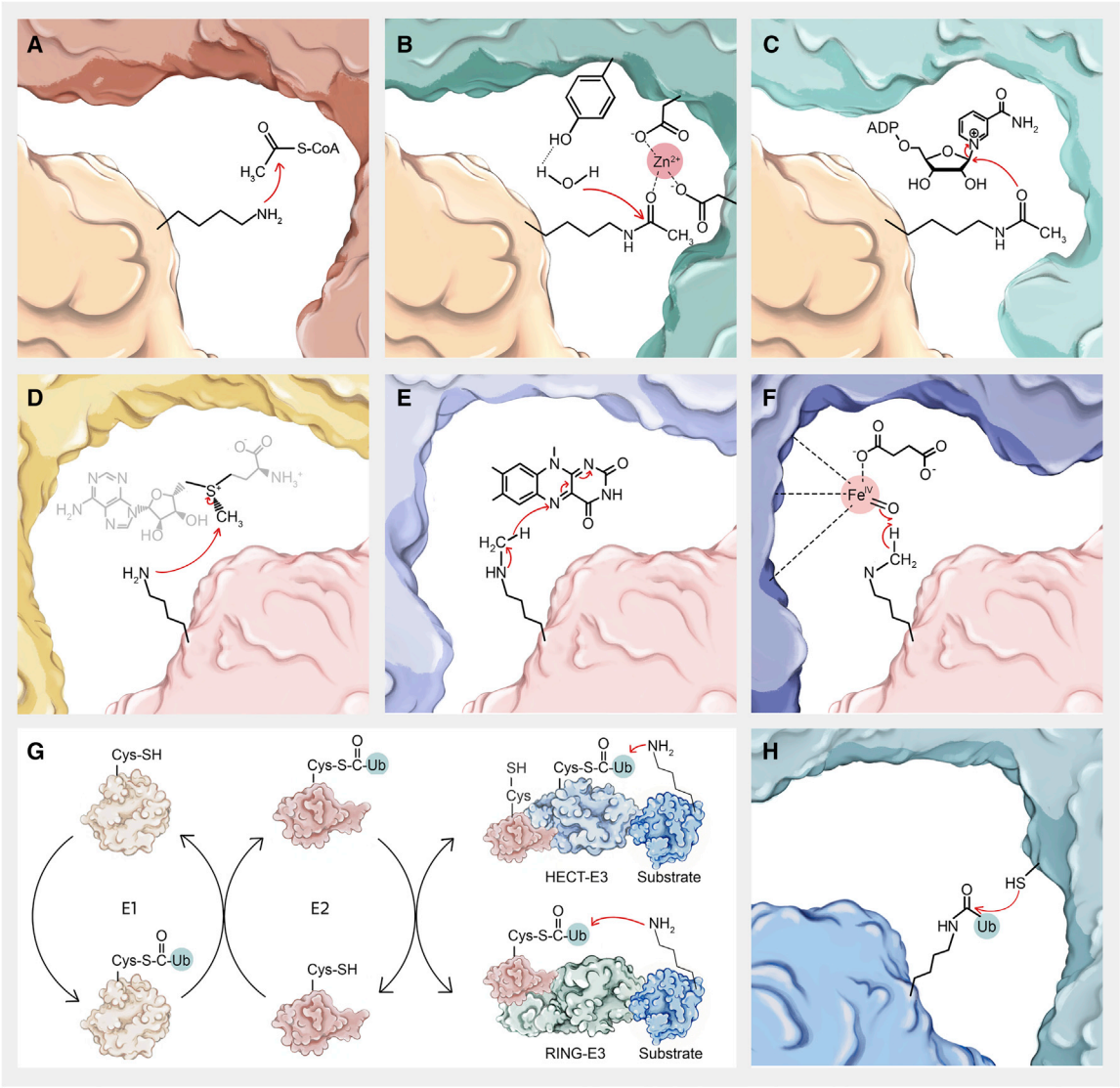


Figure 2. Writer and Eraser Enzymatic Features for Lys Acetylation, Methylation, and Ubiquitination
(A) HATs.
(B) Classical HDACs, showing typical chelating Asp residues and the key Tyr residue.
(C) Sirtuins. ADP, adenosine diphosphate.
(D) KMTs, showing nucleophilic displacement with SAM.
(E) LSD1, highlighting the oxidized flavin.
(F) Jmj demethylase, highlighting the active-site iron.
(G) E1-E2-RING/HECT-E3.
(H) DUBs. The water molecule and hydrolysis are omitted. Note that the images are cartoons of the stylized enzyme active sites instead of structurally precise crystal structures.

standard SET domain KMTs (Min et al., 2003), which show a conserved architecture that includes a pre-SET, SET, and post-SET domain organization (Trievel et al., 2002). It is believed that hyperactivity of KMTs may be disease drivers, especially in cancer. There is intense interest in finding KMT inhibitors as therapeutics, and there are promising candidates in development (Luo, 2018).

Lys Demethylases. Methyl-Lys cleavage events are catalyzed by Lys demethylases (KDMs) (Shi et al., 2004). Prior to their molecular identification beginning in 2004, the existence of KDMs was long questioned, perhaps because of the high stability of the carbon-nitrogen single bond of a methyl-Lys. We now know that there are two classes of demethylases, flavin-dependent enzymes Lysine-specific demethylase 1 (LSD1) and LSD2, and the non-heme iron-dependent Jumonji (Jmj) demethylases, a family of about ten enzymes (Kooistra and Helin, 2012). Both the flavin and Fe-dependent enzymes catalyze oxidative demethylation and use molecular oxygen as a cosubstrate. LSD1 is a polyamine oxidase paralog that removes electrons from the Lys side-chain amino group to afford an imine intermediate (Culhane

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and Cole, 2007). Hydrolysis of the imine to the demethylated Lys residue and formaldehyde is likely to be spontaneous. In addition, in each catalytic cycle, O_2 is used to regenerate the oxidized flavin and is converted to HOOH, which is a secondary by-product of LSD1-catalyzed demethylation (Figure 2E). LSD1 demethylation is highly selective for histone H3 Lys4me or Lys4me2 removal but is mechanistically incapable of processing Lys4me3. More details about the catalytic mechanism can be found elsewhere (Forneris et al., 2008).

The family of Jmj KDMs employs α-oxoglutarate (2-OG) and oxygen as cosubstrates and Fe as a metallocofactor. The Jmj KDMs generate a hypervalent iron oxo species by decarboxylation of α-ketoglutarate, affording succinate as a by-product (Figure 2F) (Tsukada et al., 2006). In a cytochrome P450-like reaction, the iron oxo species can hydroxylate the methyl attached to Lys, affording a hemiaminal, which decomposes to formaldehyde. More details about the enzyme mechanism are described elsewhere (Klose et al., 2006). In contrast to the flavoenzyme KDMs, Jmjs are mechanistically capable of acting on trimethyl-Lys in addition to monomethyl- and dimethyl-Lys substrates, although specific Jmjs show different selectivities with regard to preferred methyl-Lys forms and sequences. Representative Jmj examples are FBXL11 (JHDM1A) (Tsukada et al., 2006) that demethylates histone H3K36me and RBP2 (JARID1A) that demethylates histone H3K4me. There has been outstanding success in identifying highly selective LSD1 small-molecule inhibitors and more recent progress in finding pharmacologically active probes of the Jmj family (Shortt et al., 2017).

K_{Me} Readers. Many methyl-Lys binding protein domains have been discovered, including Plant Homeodomain (PHD) domains (about 100), chromodomains, Tudor domains, PWWP domains, WDR domains, and MBT domains (nine in human) (Teske and Hadden, 2017). These domains are often included in epigenetic enzymes or proteins found in histone-modifying complexes. Different members of these readers can show selectivity for amino acid sequence as well as the degree of methylation of Lys. The PHD domain, for example, is found in JARID1A KDM and is thought to regulate the substrate specificity of the enzyme (Wang et al., 2009). CDX7 is a drug target containing a chromodomain, whose overexpression leads to neoplastic transformation and cell proliferation (Pallante et al., 2015). Increasing numbers of inhibitors have been developed for these readers with a particular focus on the MBT domain of L3MBTL1 as an important regulatory protein that can serve as a "chromatin lock" (Trojer et al., 2007).

Lys Ubiquitination Writers, Erasers, and Readers

Lys ubiquitination is one of the most abundant PTMs in the human proteome. There is extensive protein machinery dedicated to adding, removing, and binding ubiquitin, in the range of 1,000 human genes. Below we highlight key aspects of ubiquitin writers, erasers, and readers.

Ub Ligases. The "writer" for Lys ubiquitination involves a cascade of three enzymes known as E1, E2, and E3 ubiquitin ligases. In a reaction with ATP, ubiquitin (Ub) is initially adenylated by the E1 enzyme and then generates an E1 Ub-C-terminal thioester intermediate, which is transferred to a catalytic Cys residue on an E2 enzyme (Scheffner et al., 1995). The Ub-E2 then collaborates with an E3 Ub ligase to transfer the Ub to a protein substrate Lys residue. There are two main E3 types. Members of the larger family of E3s, the RING-domain E3s, which number about 600 in humans, serve as a template that promotes the Ub-E2 to directly ubiquitinate a protein substrate Lys (Dikic et al., 2009). The second category of E3s including HECT domain (28 in human) and RBR (12 multidomain enzymes) (Spratt et al., 2014) form Ub-E3 thioester intermediates, and these Ub-E3 thioesters pass Ub to protein substrates (Figure 2G) (Chen et al., 2017).

Within Ub, there are seven Lys residues (K6, K11, K27, K29, K33, K48, and K63), as well as an N-terminal amino group, which allows the Ub to form linear or branched poly-Ub chains. Polyubiquitination on Lys48 is often a signal that leads to proteasomal targeting and degradation of the substrate. Polyubiquitination on Lys63 generally confers lysosomal targeting (Komander and Rape, 2012). Monoubiquitination of proteins is also common and can serve as a specific signal.

Interest in Ub ligases as drug targets has intensified with the discovery about a decade ago that thalidomide and related analogs act through modulating the Ub ligase cereblon as an allosteric modulator (Ito et al., 2010). Subsequently, researchers have attempted to exploit thalidomide functionality to target degradation of a protein of interest by recruitment to cereblon and other Ub ligases (Fisher and Phillips, 2018).

Deubiquitinases. Deubiquitinases (DUBs) can be considered as unconventional proteases, sometimes called isopeptidases, that sever the amide bond between a Lys side chain of one protein and the Ub C terminus. There are approximately 100 human DUBs and the overwhelming majority fall into the Cys protease family (Figure 2H), whereas smaller subsets are Ser hydrolases and metallohydrolases (Mevissen and Komander, 2017). Many DUBs can cleave Ub-Ub amide linkages whereas others show selectivity for particular ubiquitinated proteins (Komander et al., 2009). Within the DUB Cys hydrolase family, there are many subfamilies that show a wide range of catalytic and regulatory properties. As knowledge about their biological functions has expanded in normal and disease states, opportunities for targeting DUBs in drug-discovery programs have been identified (Schauer et al., 2020).

Ub-Binding Domains. As mentioned, different Ub linkages may specify distinct cellular functions. Over 20 different Ub-binding domains (UBDs) have been described, and these are thought to convey a diverse set of outputs. UBDs may lead to protein-protein interactions, influence enzymatic activities, modulate cellular localization, and affect protein stability. One such example of a UBD occurs in HECT-E3 ligases. Many of these enzymes have a nonsubstrate Ub-binding pocket known as the exosite. These HECT Ub exosites seem to allow catalytic activity and processivity of HECT enzymes to be controlled by noncovalent Ub levels (Zhang et al., 2016). In other cases, clusters of UBDs can bind concurrently to multiple Ubs via cooperativity, and this can serve as a signal for cellular machinery to act on poly-Ub chains (Dikic et al., 2009).

Atypical Lys Acylation Writers, Erasers, and Readers

Recently discovered "atypical" Lys acylations such as succinylation, malonylation, glutarylation, lactylation, and serotoninylation (Farrelly et al., 2019) have contributed to the idea that metabolism and protein modifications can be linked. It is not yet clear whether attachment of these Lys PTMs to proteins are enzyme catalyzed, although there are a few reports that this may be

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possible (Harmel and Fiedler, 2018). Physiological repurposing of some KATs as acyltransferases *in vivo* has been proposed, but this potential must be understood as difficult given the comparatively high concentrations of acetyl-CoA and more favorable catalytic parameters of KAT activity relative to the use of the atypical acyl-CoA substrates. Succinylation has been mapped on more than 1,000 protein Lys sites and is abundant on mitochondrial proteins (Xie et al., 2012). It is highly likely that nearly all of this occurs nonenzymatically, as succinyl-CoA is especially reactive with amine nucleophiles due to its propensity to form a cyclic anhydride (Weinert et al., 2013). Although Lys succinylation most likely occurs nonenzymatically, its removal has been well documented to be mediated by sirtuin 5 (Sirt5) (Du et al., 2011). Sirt5 can also act efficiently on other acyl-Lys PTMs including malonylation (Sadhukhan et al., 2016) and glutarylation (Tan et al., 2014). It seems plausible that Sirt5 helps to prevent the toxic effects of a buildup of acyl-Lys-modified proteins. Lys benzoylation appears to be driven by dietary sources of benzoate, while its removal is catalyzed by Sirt2 (Huang et al., 2018). Beyond the possibility of the Yeats domain for crotonyl-Lys (Li et al., 2018), specific reader domains for atypical acyl-Lys modifications have not yet been convincingly demonstrated.

Chemical Biology Approaches for Installing Lysine PTMs Site-Specifically into Proteins

To gain a detailed understanding of the structural and functional regulation of Lys PTMs, accessing pure and well-defined site-specifically modified proteins can be critical (Wang et al., 2015a). Isolation of Lys-modified proteins from endogenous sources typically affords heterogeneous materials with substoichiometric and mixed patterns of PTMs. For example, typical Lys acetylation stoichiometries across sites in the cellular proteome are less than 1%, indicating that obtaining a pure species is out of reach (Hansen et al., 2019). Efforts to overcome this with classical site-directed mutagenesis by replacing Lys with Gln or Arg to represent acetyl-Lys and unmodified Lys, respectively, are problematic because of the dissimilarity between these mimics and natural chemical structures. Moreover, there are not even crude natural mimics for methylation or larger acylation groups, let alone ubiquitination. It is also generally not feasible to use natural writer enzymes to introduce Lys PTMs at specific protein sites because of their insufficient site selectivity or because their catalytic powers are too low. Facing these challenges, chemical biologists have developed a range of approaches (Chin, 2017) to generate specifically modified proteins or protein complexes (Hoyt et al., 2019). Below we summarize several of the promising advances in this area.

Lysine PTM Mimics

Although the selective labeling of native Lys is accessible (Adusumalli et al., 2018), the thiol group of Cys is the most versatile among the proteinogenic amino acids and can undergo a wide range of "bio-orthogonal reactions" through alkylation as well as radical addition (Spicer and Davis, 2014). ThiaLys analogs containing *N*-methyl groups (Simon et al., 2007), and *N*-acyl modifications (Jing et al., 2018) have been generated in this fashion. These thiaLys analogs are structurally quite similar to a natural Lys, in size and geometry. In the standard alkylation procedure, a Lys-modified site of interest is mutated to Cys and then reacted with a bromoethylamino substituted reagent (Figure 3A). Alternatively, for the thiol-ene radical addition reactions, acyl-enamines are used (Trang et al., 2014). These approaches have been extensively used in the chromatin field to generate modified nucleosomes. These experiments have investigated the role of methyl-Lys to recruit particular readers and how these affect chromatin dynamics and remodeling (Voigt et al., 2012). Reductive hydrazide acyl-Lys mimics have also been generated at Cys residues, and these show special promise in generating Ub-modified proteins that serve as alternative substrates for DUBs (Figure 3B) (Bhat et al., 2018).

A related approach to install thiaLys analogs from Cys in proteins involves a reaction scheme of elimination generating dehydroalanine (Dha) (Bernardes et al., 2008). After Dha formation, these unsaturated residues can undergo Michael additions or radical coupling with thioethylamino groups. This approach has the potential to be quite versatile (Wright et al., 2016), but a limitation of the method is the scrambling of the stereochemistry of the α-carbon of the Lys analog upon thiol addition.

Fortunately, Cys is one of the lowest-abundance amino acids in natural proteins. Nevertheless, to avoid multiple modification sites, it is common practice to replace a small number of natural Cys with Ala or Ser, depending on the structural predictions about their functions. However, it should be stated that in some cases, the sulfur atom replacement of the γ-methylene in natural Lys to generate thiaLys may not be well tolerated due to altered conformational and electronic effects conferred by thiol substitution (Chen et al., 2018)

Noncanonical Amino Acid Incorporation

Noncanonical amino acid incorporation (ncAA) via amber codon suppression (Wang et al., 2001) is showing increasing promise for introducing modified Lys residues at specific sites in proteins (Tharp et al., 2018). The ncAA method has been used to install more than 100 unnatural amino acids into proteins via different tRNA-synthetase/tRNA pairs in various hosts (Figure 3C). Included in the ncAA roster of residues installed are K_{Ac}, K_{Pr}, K_{Bu}, K_{Cro}, and K_{Hib} (Yang et al., 2018). The direct incorporation of K_{Ac} into five different sites of the small guanosine triphosphate (GTP)-binding protein, Ran, as an example, revealed how Lys acetylation can influence Ran localization, GTPase activity, signaling functions, and receptor interactions (de Boor et al., 2015).

Some Lys acylations with more complicated structures or bulkier groups are not readily introduced in a direct fashion via the tRNA-synthetase/tRNA system due to the limited space and hydrophobicity of the enzyme active sites (Wan et al., 2014). This limitation has been overcome using a multistep approach, termed a "tag-and-modify" strategy (Chalker et al., 2011). In this fashion, a two-step introduction of K_{Succ} was achieved, whereby azidonorleucine (AznL) was installed and converted into different acyl-lysines via traceless-Staudinger ligation reactions (Wang et al., 2017b). By switching the acyl phosphinothioester, theoretically this strategy can be applied to any Lys acylation (Figure 3D) (Wang et al., 2015b).

The application of ncAA to produce methyl-lysine forms in proteins has also been pursued. Monomethyl-Lys precursors can be incorporated into proteins via amber codon suppression, and a deprotection step is needed to generate the native

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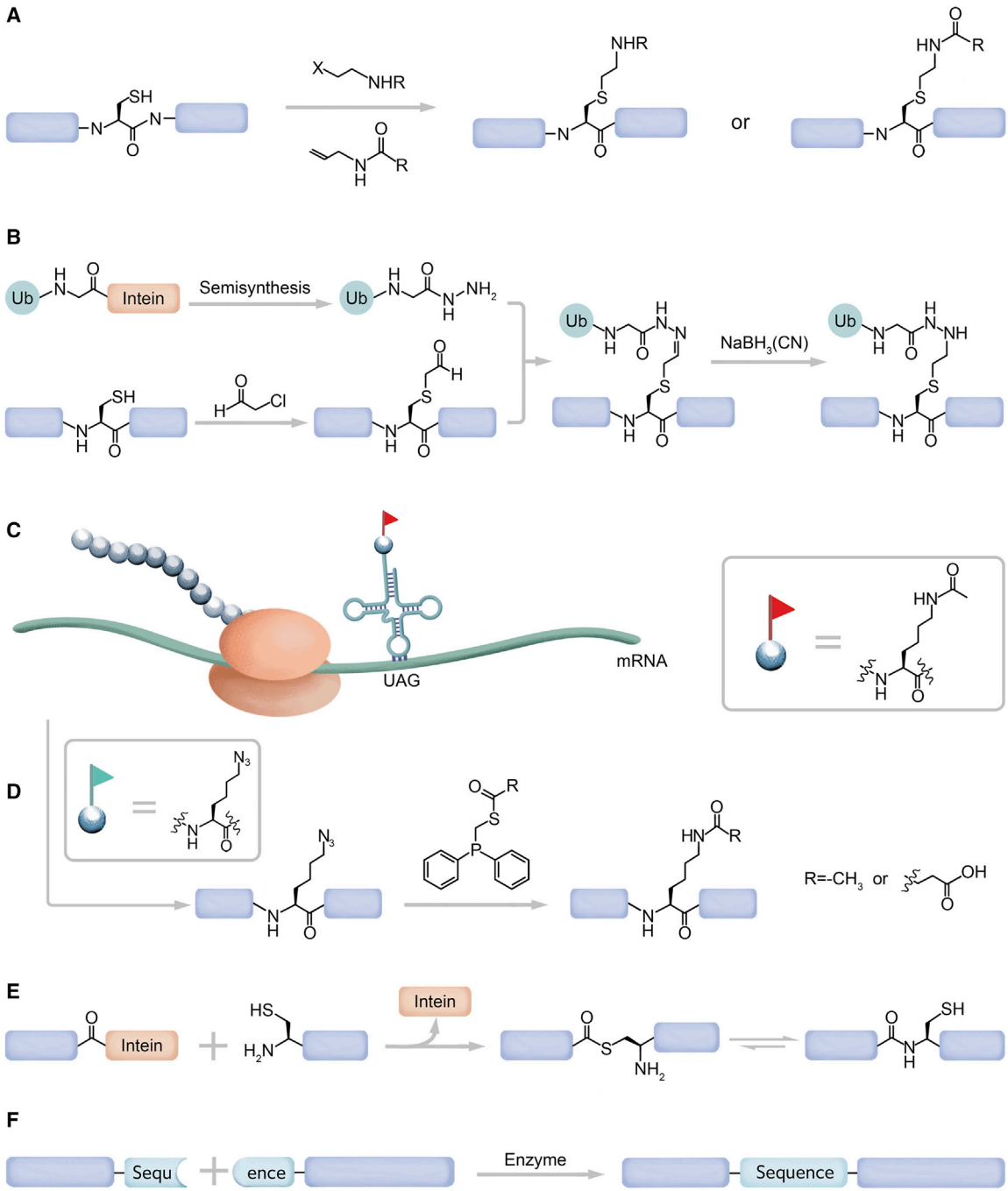


Figure 3. Chemical Biology Approaches for the Site-Specific Installation of Lys PTMs and Mimics into Proteins

(A) ThiaLys analogs generated by Cys modification.

(B) Introduction of Hydrazide acyl-Lys mimics.

(C) PTM installation by noncanonical amino acid incorporation, using acetyl-Lys as an example.

(D) A typical example for the "tag-and-modify" strategy: azidonorleucine (AznL) was first installed by noncanonical amino acid incorporation, followed by traceless-Staudinger ligation reactions with different phosphine reagents to generate acetyl-Lys and succinyl-Lys.

(E) Expressed protein ligation by chemoselective Cys/thioester reaction.

(F) Enzyme-catalyzed protein semisynthesis. The cartoon motif recognized by the enzyme is shown as "sequence."

methyl-Lys structure (Wang, 2019). However, for dimethyl-Lys, the situation is more complicated (Nguyen et al., 2010), but has been achieved via multistep chemical procedures. The reductive amination of allysine can generate mono- and di-methyl-Lys, and this approach was used to analyze the crosstalk between K372 methylation and K120 acetylation in p53 (Wang et al., 2017a). To date, we are unaware of success in generating tri-methyl-Lys via ncAA.

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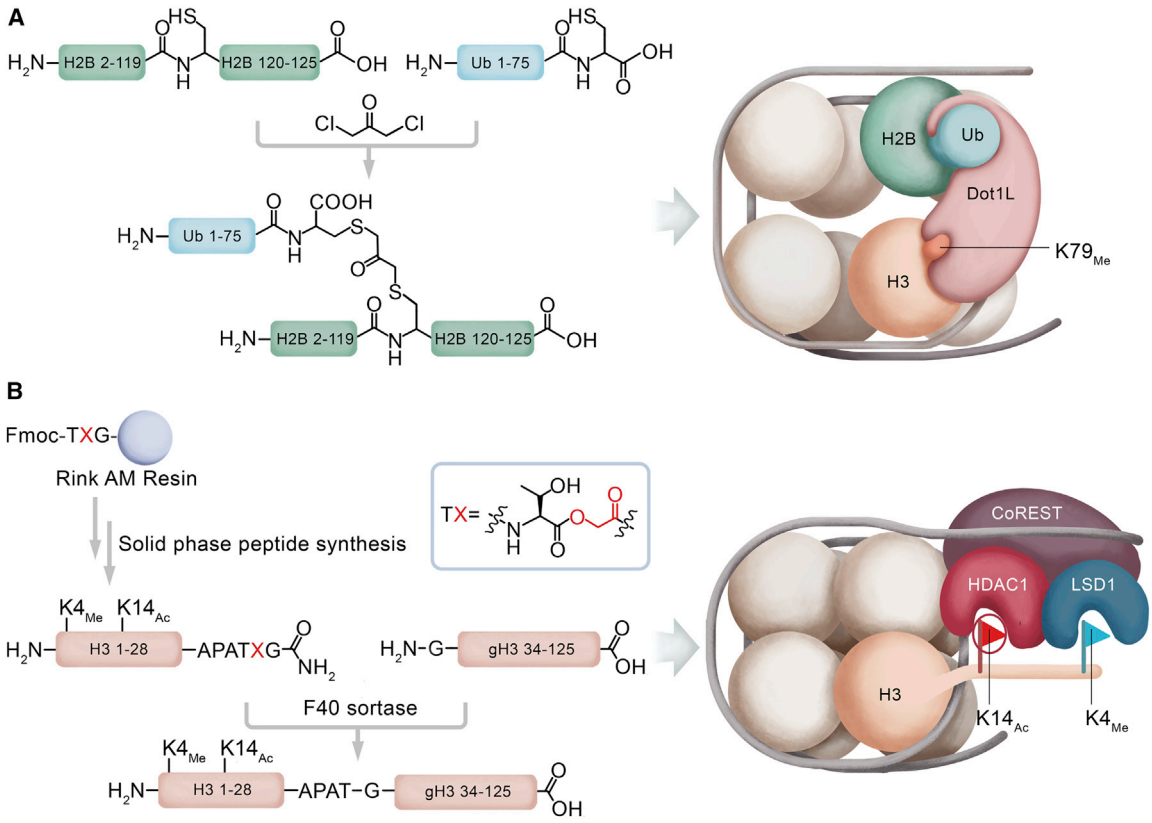


Figure 4. Examples of Biological Investigation Facilitated by the Chemical Preparation of Site-Specifically Modified Histones
(A) The recognition of H2BK120ub leads to H3K79 methylation as activation marker by Dot1L.
(B) H3K14ac blocks LSD1 of CoREST complex catalyzed demethylation at Lys4.

Chemoselective Ligations

Chemical preparation of proteins by total synthesis represents the ultimate method for complete control of the generation of a modified protein (Hackenberger and Schwarzer, 2008). The chemoselective reaction between peptides containing C-terminal thioesters and N-terminal Cys, known as "native chemical ligation (NCL)" (Dawson and Kent, 2000), has enabled the synthesis of small to medium-sized proteins (Zheng et al., 2013). Many Lys PTMs have been successfully installed, especially those two challenging groups as K_{Me3} and K_{Ub}, particularly in the context of histones (Qi et al., 2018).

These ligation strategies have been expanded to link recombinant protein fragments and synthetic peptides to generate larger proteins and circumvent technical challenges of protein refolding that can occur with total synthesis (Wang and Cole, 2020). The most popular version of this semisynthetic strategy, expressed protein ligation, involves using an intein to generate a recombinant protein fragment thioester that is ligated to an *N*-Cys synthetic peptide (Muir et al., 1998). A complementary semisynthetic approach utilizes an *N*-Cys recombinant protein generated by proteolysis with SUMO protease or tobacco etch virus and a synthetic peptide thioester. These companion semisynthetic methods have been extensively utilized to generate acetyl-Lys, methyl-Lys, and Ub-Lys containing proteins (Figure 3E). Several semisynthetic H2Bs with ubiquitination at Lys120 have been prepared with either native isopeptide bonds, mutated Ub(G76A), or structural mimics (Fierz and Muir, 2012). These protein chemical approaches have been employed in detailed functional and mechanistic characterization of ubiquitin-modified H2B (Müller and Muir, 2015). Examples of such analyses include the structural characterization of the crosstalk between H2B and H3K79 methylation by Dot1L (Figure 4A) (Worden et al., 2019), H3K4 methylation by COMPASS (Hsu et al., 2018), and H3K36 methylation by Set2 (Halic, 2019).

Beyond histones, applications of these semisynthetic approaches include generating modified full-length tau (Haj-Yahya and Lashuel, 2018), whose K280 acetylation showed its strong stimulation of tau aggregation, and *S*-adenosyl homocysteine hydrolase (Wang et al., 2014), whose acetylation was shown to regulate its catalytic activity in a manner that could lead to interplay with epigenetic methylation.

Enzyme-Catalyzed Protein Semisynthesis

The synthesis of thioesters and the requirement of *N*-Cys residues can be an impediment in some protein ligation applications. Enzymes for ligating peptides and proteins analogous to DNA ligases used commonly in molecular cloning are beginning to show promise. Several examples of such enzymes include sortase, subtiligase, butelase-1, and trypsiligase (Thompson and Muir, 2019). Sortase is a bacterial enzyme that catalyzes transpeptidation reactions involving cell-wall remodeling (Wu et al., 2017). Sortase and its engineered forms have been used in

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protein labeling at both the C terminus (Guimaraes et al., 2013) and N terminus (Theile et al., 2013) of proteins of interest. Natural sortase recognizes the epitope LPXTG for catalysis, and it has been engineered to expand its substrate scope to include "APXTG" (Piotukh et al., 2011). Conveniently, histone H3 contains an "APATG" sequence in amino acid residues 29–33, which enables the engineered sortase-catalyzed semisynthesis of H3 with Lys PTMs throughout the N-terminal tail (Figure 3F). Using this approach, histone H3 acetylated and methylated nucleosomes have been prepared and used for analysis of the site selectivity of the HDAC1-containing CoREST complex (Wu et al., 2018). These studies revealed that acetylation at Lys14 is especially resistant to deacetylation, which is notable because this modification also blocks CoREST complex catalyzed demethylation at Lys4 (Figure 4B). A similar approach has been applied to study all five known class I HDACs, which show different activity and selectivity over various nucleosome H3 acetylation sites (Wang et al., 2020). Histone H3 with K4 propargyl group has been prepared by this approach for the structural analysis of CoREST complex binding (Song et al., 2020). Compared with chemical ligation methods, enzyme-catalyzed protein semisynthesis strategies are generally more rapid in mild aqueous conditions and require less chemistry expertise. However, the need for specialized folded protein reagents and corresponding recognition sequences can prove an impediment to enzyme-catalyzed protein semisynthesis. For a more detailed discussion of the strengths and weaknesses of various approaches, please refer to a recent review (Wang and Cole, 2020).

Small-Molecule Probes for Lys Writers, Erasers, and Readers

The development of small-molecule pharmacological probes has greatly affected our understanding of the function of the writers, readers, and erasers of Lys PTMs. The earliest important tool compounds, trichostatin A and trapoxin, are natural products that are potent HDAC inhibitors. These were characterized as blocking histone acetyl-Lys removal before the molecular identification of any of the reversible Lys-modifying enzymes or readers (Furumai et al., 2001). Building on the understanding of these compounds, six HDAC inhibitors are now approved by the US Food and Drug Administration (FDA) for the treatment of various hematologic and cutaneous malignancies (Banerjee et al., 2019). Over the past decade, major progress has been made in identifying a range of small molecules that have sufficiently robust pharmacological properties to be reliable probes of writer, eraser, and reader functions of key proteins involved with Lys acetylation, methylation, and ubiquitination. Representative small-molecule probes are listed in Table 1 and highlighted based on a combination of established clinical impact, perceived pharmacological utility, and chemical novelty.

Small-Molecule Probes for Lys Acetylation

Lys Acetyltransferase Inhibitors. For many years, KAT enzymes were rather recalcitrant to the kind of cell-active robust chemical inhibitors that had been well established for KDACs. An incremental advance in this regard was the identification of C646 (Figure 5A) as a moderately potent and selective p300/CBP KAT inhibitor (Bowers et al., 2010). C646 was identified as an active-site p300 inhibitor through virtual screening and, despite limitations as a reversible covalent Cys modifier, has been widely used to assess p300/CBP function. Representative studies employed C646 to analyze p300/CBP in hematological, prostate, and cutaneous malignancies as well as basic transcriptional processes involving RNA polymerase regulation (Dancy and Cole, 2015).

However, the landscape of p300/CBP pharmacology was transformed with the development of A485 as a highly potent and specific p300/CBP acetyltransferase through a combination of virtual screening and extensive medicinal chemistry (Lasko et al., 2017). A485, a spiro-oxazolidinedione, was determined by crystallography to bind to the active site excluding acetyl-CoA interaction. A485 has become a powerful tool in the assessment of p300/CBP acetyltransferase activity in cellular pathways. Notably, comprehensive mass spectrometric proteomics experiments employing A485 revealed that p300/CBP behaves as an "acetyl-spray" in the nucleus, coating a vast swathe of the transcriptional machinery with Lys acetylation (Weinert et al., 2018). The kinetics analysis of these acetylation-mark changes with A485 revealed that many are on minute time scales. Unexpectedly, the tail of histone H2B showed a large cluster of dynamic p300/CBP acetylation sites (Weinert et al., 2018). In a cancer-related study, A485 was key in pinpointing the role of the transcription factor MITF in melanoma responsiveness to HAT inhibition (Kim et al., 2019). A485 has also helped enhance our understanding of p300/CBP in prostate cancer and other malignancies.

Another recent exciting advance in KAT inhibition has been the identification of high-quality inhibitors for the MYST family of KATs including MORF, MOZ, and HBO1 (MacPherson et al., 2020). These sulfonyl hydrazide compounds such as WM-1119/WM-3835 are potent against two or more MYST family members. Crystal structures have shown how they target the active sites of these enzymes by occluding acetyl-CoA binding. These compounds demonstrate the ability to block cancer cell proliferation with particular promise in hematological malignancies and show *in vivo* activity in mice (Baell et al., 2018). They have been used to demonstrate that HBO1 may modulate acetylation of Lys14 of histone H3 (MacPherson et al., 2020). These new MYST inhibitors promise to be useful tools for analyzing a wide range of functions of these KATs (Figure 5A).

Lys Deacetylase Inhibitors. Although potent pharmacologically active KDAC inhibitors have been established for three decades and have been reviewed previously, we will highlight several recent advances (Figure 5B). HDAC8 is a nuclear deacetylase and is unusual for a KDAC in having high catalytic activity in the absence of a corepressor binding partner (Castañeda et al., 2017). Although its detailed functions are still being elucidated, a range of highly selective inhibitors containing Zn^{2+}-binding warheads have been described in the past 5 years (Balasubramanian et al., 2008). These show effectiveness in a range of antitumor applications (Connolly et al., 2017). Likewise, cytoplasmic deacetylase HDAC6 has become a popular therapeutic target that plays key roles in protein homeostasis (Kalin et al., 2012). Preclinical studies in neurologic diseases such as Charcot-Marie-Tooth have been a recent direction in this area. Targeting HDAC1 in the context of a particular corepressor complex is a relatively new direction in the field. The designed CoREST complex inhibitor compound corin has dual warheads, one a phenyl-cyclopropylamine for LSD1 demethylase and the other

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Table 1. Small-Molecule Tools for Lys PTM Writers, Erasers, and Readers

Inhibitor	Target Enzyme/Domain	Features	Reference
A485	P300/CBP	binds to the active site, leads to a rapid global acetylation level drop in nuclear proteins	Lasko et al., 2017
WM-1119/WM-3835	HBO1 (KAT7 or MYST2)	competitive inhibitor of Ac-CoA causes the decrease of H3K14ac and shows promise in leukemias	MacPherson et al., 2020
WM-8014/WM-2474	KAT6A (MOZ) KAT6B (MORF)	competitive inhibitor of Ac-CoA leads to histone acetylation reduction	Baell et al., 2018
SAHA (vorinostat)	classical HDAC	Zn^{2+}-chelating hydroxamic acid for cutaneous T cell lymphoma treatment approved by FDA in 2006	Banerjee et al., 2019
FK228 (romidepsin)	classical HDAC	cyclic peptide natural product for the treatment of cutaneous T cell lymphoma approved by FDA in 2009	Banerjee et al., 2019
PXD101 (belinostat)	classical HDAC	used to treat peripheral T cell lymphoma approved by FDA in 2014	Banerjee et al., 2019
LBH589 (panobinostat)	classical HDAC	used for the treatment of relapsed multiple myeloma approved by FDA in 2015	Banerjee et al., 2019
HBI-8000 (chidamide)	classical HDAC	benzamide functions as Zn^{2+}-chelator approved by FDA in 2015 for relapsed or refractory peripheral T cell lymphoma and pancreatic cancer	Banerjee et al., 2019
SB939 (pracinostat)	classical HDAC	drug approved by FDA in 2016 for the treatment of acute myeloid leukemia	Banerjee et al., 2019
MS275 (entinostat)	HDAC1-3	benzamide, phase III candidate for cancer therapy	Connolly et al., 2017
PCI-34051	HDAC8	selectively inhibits HDAC8, causing caspase-dependent apoptosis in certain T cell leukemia cell lines	Balasubramanian et al., 2008
Mercaptoaceamide-7a	HDAC6	selective inhibitor for HDAC6 with neuroprotective effects	Kalin et al., 2012
Corin	CoREST complex	a chemical combination of entinostat and tranylcypromine analog inhibiting both HDAC1 and LSD1, shows antitumor effects	Kalin et al., 2018
TM	sirtuin2	thioacyl functionality, causes degradation of c-Myc	Jing et al., 2016
SirReal2	sirtuin2	induces enzyme active-site conformational change, leads to tubulin hyperacetylation in cancer cells	Rumpf et al., 2015
NPD11033	sirtuin2	binds and inhibits the deacetylase activity but not the defatty-acylase activity of sirtuin2 selectively, resulting in pancreatic cancer cell growth inhibition	Kudo et al., 2018
(+)-JQ1	BET/BET-BRD domain	pan-BET-BRD competitive inhibitor with antiproliferation effects and widespread epigenetic effects	Filippakopoulos et al., 2010
OTX015	BET/BET-BRD domain	pan-BET-BRD inhibitor with antiproliferative action	Lu et al., 2016
L-Moses	PCAF/GCN5/Non-BET-BRD	selective inhibitor for non-BET-BRD with no toxicity, good cell permeability, and high stability	Moustakim et al., 2017
I-CBP112	P300/CBP/Non-BET-BRD	allosterically activates p300/CBP acetyltransferase activity; can synergize with A-485 in blocking cancer cell growth	Zucconi et al., 2016; Zucconi et al., 2019
GNE-781	P300/CBP/Non-BET-BRD	highly specific Inhibition of BRD in p300/CBP leading to the decrease of Foxp3 transcription and antitumor activity in tumor models	Romero et al., 2017

(*Continued on next page*)

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Table 1. *Continued*

Inhibitor	Target Enzyme/Domain	Features	Reference
VTP50469	MLL (KMT2A)/SET domain	selectively inhibits Menin-MLL protein-protein interaction and causes drop in chromatin occupancy and cell differentiation or apoptosis for cancer treatment	Krivtsov et al., 2019
MS453	SETD8/SET domain	covalently modifies a Cys near the binding site of SET domain and blocks H4K20me	Butler et al., 2016
UNC0638/UNC0642	EHMT2(G9a, KMT1C); EHMT1(GLP, KMT1D)/ SET domain	blocks H3K9 methylation; UNC0642 shows improved pharmacokinetic properties	Liu et al., 2013
EPZ-6438 (tazemetostat)	EZH2/SET domain	SAM competitive inhibitor, studied in clinical trials	Italiano et al., 2018
EPZ-5676 (pinometostat)	Dot1L/Non-SET domain	selective inhibitor for Dot1L leading to the decrease of H3K79 methylation for the treatment of acute leukemia with MLL-rearranged gene	Campbell et al., 2017
GSK-LSD1	LSD1 (KDM1A)/amine oxidase	cyclopropylamine, mechanism-based irreversible inhibitor, shows antiproliferation against acute leukemia	Mohammad et al., 2015; Egolf et al., 2019
GSK-J1 GSK-J4 (prodrug)	JMJD3 (UTX)/Jmj domain	chelates Fe in the active site, modulates H3K27 methylation for pharmacological applications	Kruidenier et al., 2012
UNC1215	L3MBTL3/MBT domain	binds to the methyl-Lys pocket competitively	James et al., 2013
UNC2170	53BP1/Tudor domain	competitive active-site binding ligand for methyl-Lys	Perfetti et al., 2015
UNC4976	CBX7/Chromodomain	a peptidomimetic as a positive allosteric modulator (PAM) that blocks H3K27me3 binding, leads to the relocation of PRC1	Lamb et al., 2019
TAK-243	E1	binds to the active site and blocks Ub transfer to the E2 enzyme, leading to antiproliferation of cancer cells	Hyer et al., 2018
(S)-thalidomide and analogs	Cereblon (CRBN)/Ring E3	allosteric CRBN inhibitor, reduces ubiquitination of various transcription factor substrates	Liu et al., 2015
Nutlin1/2/3	MDM2-p53/DUB	competitively binds to MDM2 and blocks p53 ubiquitination	Vassilev et al., 2004
P5091	USP7/DUB	selective inhibitor with action against multiple myeloma	Chauhan et al., 2012
XL188	USP7/DUB	high selectivity based on structure-guided development, reversible with utility in cells and *in vivo*	Lamberto et al., 2017
IU1/IU1-47	USP14/DUB	allosteric inhibitor that blocks substrate binding, leading to an increase in proteasome substrate degradation	Wang et al., 2018b; Boselli et al., 2017

a benzamide that engages HDAC1 (Kalin et al., 2018). Corin shows high potency for both enzymatic activities in the CoREST complex and has a prolonged residence time in the HDAC1 active sites relative to monofunctional benzamide KDAC inhibitor. Corin has demonstrated promising antitumor behavior across melanoma, diffuse pontine glioma (Anastas et al., 2019), and colon cancer (Miller et al., 2020), and can enhance cancer immune surveillance (Xiong et al., 2020). These data underscore the concept that selectively targeting KDACs in the context of an individual corepressor complex may offer therapeutic advantages.

There has also been recent progress in the identification of sirtuin KDAC inhibitors. Although activators of sirtuins have long been discussed as a strategy to slow the aging process, sirtuin inhibitors are now being pursued as therapeutics for a range of applications. Thiomyristoyl derivative TM (Jing et al., 2016), thiopyrazine SirReal2 (Rumpf et al., 2015) and pyridone NPD11033 (Kudo et al., 2018) show high potency and selectivity as well as antitumor potential. Crystal structures have been determined that help define the binding mode of these compounds to Sirt2 via active-site interactions. NPD11033 is notable for blocking deacetylase activity of Sirt2 but not its less-well-studied fatty deacylase activity. For many Lys PTM targeting compounds, the precise mechanism of how Sirt2 inhibition leads to antitumor effects is not fully understood, although an impact on inhibiting Myc transcriptional activity has been described.

Bromodomain Inhibitors. Broad-spectrum BET bromodomain inhibitors from the early compounds such as (+)-JQ1 (Filippakopoulos et al., 2010) and OTX015 (Lu et al., 2016) (Figure 5C) to next-generation specific BET isoform inhibitors have been

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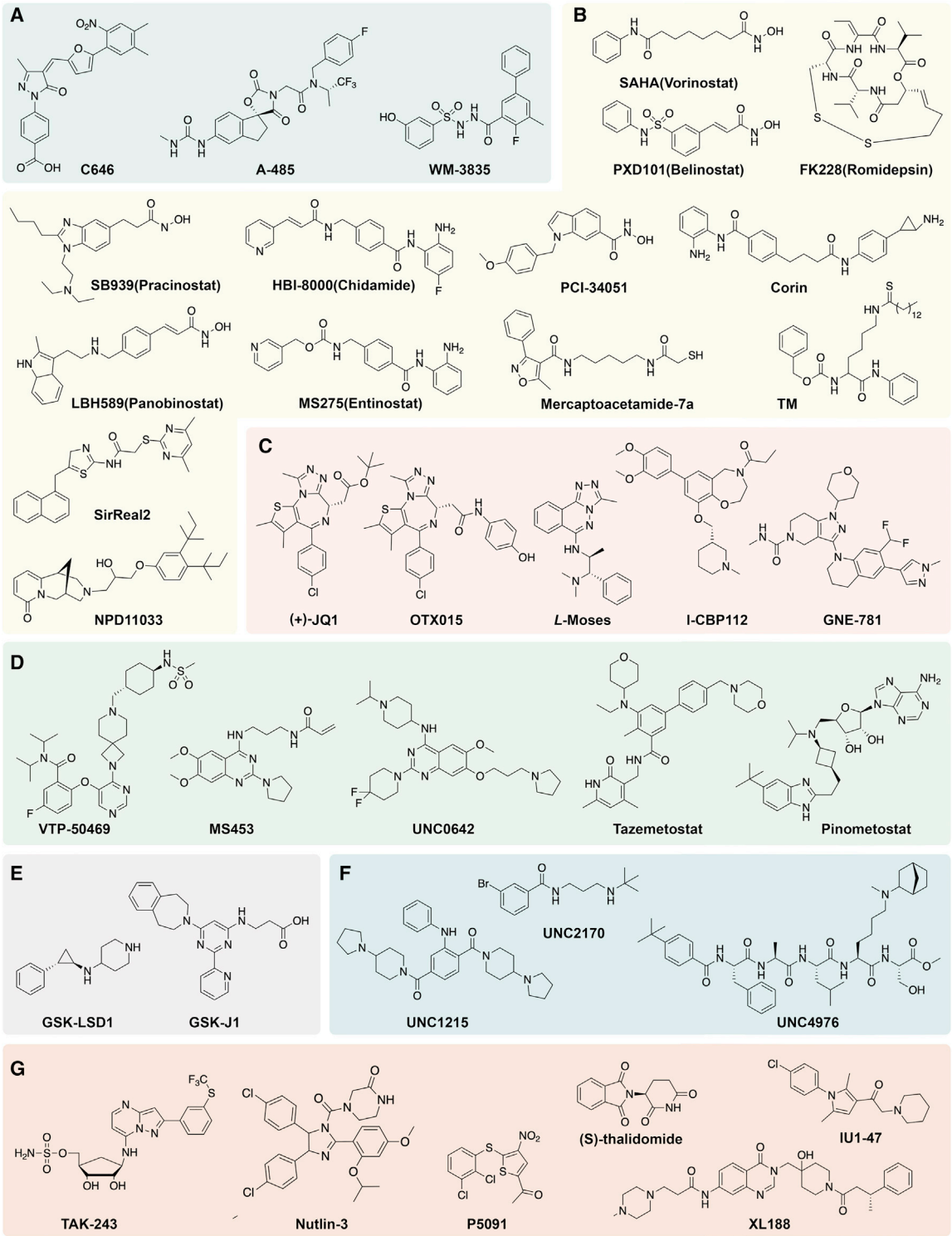


Figure 5. Examples of Small-Molecule Inhibitors Developed for Lys PTM Writers, Erasers, and Readers
(A) Acetylation writer inhibitors.
(B) Acetylation eraser inhibitors.
(C) Acetylation reader inhibitors.
(D) Methylation writer inhibitors.
(E) Methylation eraser inhibitors.
(F) Methylation reader inhibitors.
(G) Ubiquitination regulators.

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heavily applied in biological investigation. These agents have helped establish the role of BET bromodomain-acetyl-Lys interactions in transcriptional regulation and have shaped the concept of "superenhancers"(Lovén et al., 2013). Although they have shown tremendous promise in preclinical investigation in cancer studies and other disease models, BET bromodomain inhibitors have demonstrated toxicity in human trials, especially thrombocytopenia (Falchook et al., 2020). More recent compound discovery efforts to identify non-BET bromodomain antagonists have led to selective PCAF/GCN5 and p300/CBP bromodomain inhibitors. The triazolophthalazine compound dubbed *L*-Moses is a pharmacologically potent PCAF/GCN5 antagonist and interestingly shows anti-parasitic action (Moustakim et al., 2017). Selective p300/CBP bromodomain compounds including earlier generation I-CBP112 (Zucconi et al., 2016) and the more potent compound GNE-781 (Romero et al., 2017) have demonstrated antineoplastic activity. Interestingly, I-CBP112 has been shown to enhance p300-catalyzed nucleosome acetylation (Zucconi et al., 2019) while GNE-781 lacks this action (Morrison-Smith et al., 2020), suggesting that the allosteric regulation of p300/CBP is sensitive to precise bromodomain interactions (Figure 5C).

Small-Molecule Probes for Lys Methylation

Methyltransferase Inhibitors. Dramatic progress has been achieved in targeting a series of methyltransferases that methylate histone H3 on Lys4 (MLL), Lys9 (G9a/GLP), Lys27 (EZH2), and Lys79 (Dot1L). Many of the compounds target the SAM-binding site of these enzymes, which are sufficiently distinct to engender specificity (Butler et al., 2016). The identification of VTP50469 as a Menin-MLL protein-protein interaction disruptor rather than a SAM antagonist can prevent MLL from binding to chromatin and is showing promise in leukemias characterized by MLL gene translocations (Krivtsov et al., 2019) (Figure 5D). G9a/GLP inhibitor UNC0638/UNC0642 (Liu et al., 2013) has been used in a range of applications including a study to treat the Prader-Willi metabolic syndrome (Kim et al., 2017). EZH2 SAM antagonists such as tazemetostat have moved into advanced clinical trials for cancer and are indispensable tools for the analysis of the polycomb repressor complex 2 function (Italiano et al., 2018). Similarly, Dot1L SAM antagonists such as pinometostat (Daigle et al., 2013) have been investigated as epigenetic modulators in malignancy (Campbell et al., 2017) (Figure 5D).

Lys Demethylase Inhibitors. In the context of lysine demethylases, small-molecule inhibitors that are the furthest along are mechanism-based inactivators based on classical monoamine oxidase pharmacology. Selective phenyl-cyclopropylamine compounds, as exemplified by GSK-LSD1 (Figure 5E), show time-dependent covalent adducts with the tightly bound flavin cofactor resulting in irreversible inhibition (Mohammad et al., 2015). These compounds have shown antiproliferative activity against a subset of leukemias and lung cancer (Egolf et al., 2019). It was originally believed that such LSD1 inhibitors achieved their cell-growth effects exclusively through catalytic inactivation, but more recent studies indicate that the mechanism in some cases involves disruption of LSD1 and SNAG transcription factor binding (Vinyard et al., 2019).

Jmj Fe-dependent demethylases have proved more difficult to inhibit than LSD1, but there are now pharmacological tools of increasing potency and specificity for these enzymes. A combined JMJD3/UTX inhibitor GSK-J1 and its prodrug ester form GSK-J4 are pyridyl-pyrimidines (Kruidenier et al., 2012). As shown by X-ray crystallography, this family of compounds blocks H3K27me demethylase activity by coordinating the Fe and obstructing α-ketoglutarate interaction. These compounds show cellular activity. Nevertheless, further work is needed to advance chemical probe development for the family of Jmj demethylases (Figure 5E).

Methyl-Lys Reader Antagonists. There is a wide range of methyl-Lys protein reader domains that show distinct preferences for mono-, di-, and trimethyl-Lys levels as well as the proximal amino acid sequence surrounding the modified Lys. Small-molecule probes of these domains have been more difficult to identify than for acetyl-Lys binding bromodomains, but progress is ramping up. An important early step in this direction was the identification of the pyrrolidine derivative UNC1215 (Figure 5F) as an antagonist of the MBT domain of L3MBTL3 (James et al., 2013). This domain preferentially binds monomethyl and dimethyl-Lys and is functionally associated with gene silencing. A crystal structure of UNC1215 in complex with the MBT of L3MBTL3 revealed deep engagement of the small molecule in the methyl-Lys pocket. UNC1215 was shown to block L3MBTL3 in cell culture and led to the identification of the transcriptional repressor protein BCLAF1, a methylated protein, as a binding partner of L3MBTL3 (James et al., 2013). A fairly potent chemical probe UNC2170 has been developed for the methyl-Lys binding tandem Tudor domain of the DNA-repair-related protein 53BP1 (Perfetti et al., 2015). This *t*-butylamine was shown to be selective for 53BP1 and could block class switch recombination in cells. Most recently, a chromobox (CBX7) chromo-methyl-Lys reader domain antagonist UNC4976 was reported (Lamb et al., 2019). CBX7 binds to histone H3K27me3 and helps silence gene expression as part of the polycomb repressor complex 1 (PRC1). UNC4976 is a short peptide and allosteric modulator of CBX7 that can enhance nucleic acid interaction with the protein. It was shown that UNC4976 can induce gene reactivation of PRC1 target genes (Lamb et al., 2019) (Figure 5F).

Ub Chemical Probes

The discovery of thalidomide analogs as cereblon Ring E3 ubiquitin ligase allosteric modulators has led to an explosive interest in targeted protein degradation and has been heavily discussed in the literature (Liu et al., 2015). These glutaramide derivatives appear to steer the E3 toward certain protein substrates, such as key transcription factors, by enhancing their recruitment. Potent and selective E1 ubiquitin ligase and proteasome inhibitors have also been well characterized (Hyer et al., 2018), and several proteasome inhibitors are in clinical use for cancer (Rulina et al., 2016). In general, it has been challenging to identify active-site small-molecule probes of Ring or HECT domain E3 enzymes that act in a traditional inhibitor sense. This could reflect our limited understanding of the detailed catalytic mechanisms of Ub ligases. One early success story for the E3 ligase, MDM2, was the discovery of the nutlin compounds (Figure 5G), which block MDM2/p53 interaction and thereby show antitumor activity (Vassilev et al., 2004). It has been difficult to build on this finding, however. Moreover, there have not been specific probes reported of ubiquitin-reader interactions. However, there has

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been great recent progress in the development of DUB small-molecule inhibitors that show high potency and isoform selectivity. Perhaps the clearest example of this has been the development of promising inhibitors for the DUB USP7. Highly potent and selective USP7 inhibitors, including irreversible covalent inhibitors such as P5091 (Chauhan et al., 2012) and reversible compound XL188 (Lamberto et al., 2017), show antineoplastic activity in a mechanism that likely involves MDM2 destabilization with rescue of p53. Selective inhibitors for other DUBs (Wang et al., 2018b), such as USP14 (Boselli et al., 2017), suggest this to be a fertile field in coming years (Figure 5G).

Chemical Complementation of Mutant Methyltransferases and Acetyltransferases

An emerging area in the chemical analysis of KMTs and KATs has involved introducing point mutations into specific cosubstrate interacting residues in these enzymes, which relaxes their specificities for SAM and acetyl-CoA analogs, respectively. In this way, SAM and acetyl-CoA analogs containing "clickable" groups such as alkynes can be efficiently processed by these mutant KMTs and KATs in a fashion that makes them orthogonal to the natural enzymes. In this fashion, these clickable cosubstrates leave a handle that allows for efficient protein substrate identification (Han et al., 2017). These mutant enzyme cosubstrate pairs have been applied to the histone H3K9 KMTs G9a and GLP1, KATs GCN5, MOF, and p300, and are leading to the identification of novel substrate proteins modified by these enzymes (Luo, 2018).

Summary and Outlook

The biological, chemical, and therapeutics landscapes of reversible Lys PTMs have become increasingly rich and complex over the past 25 years since the molecular identification of the first KATs and KDACs (Allis and Jenuwein, 2016). Indeed, the integration of chemical tools and concepts with the functional and structural characterization of the writers, erasers, and readers of Lys PTMs has led to major advances in our understanding of the proteins and modifications involved. From the big chemistry on proteins and protein complexes to the small chemistry of probe development, we have learned how site-specific modifications of Lys regulate gene expression, cell growth, and a multitude of disease processes. It is gratifying that there are now a number of epigenetic therapies that have emerged from the marriage between the chemistry and biology of the study of Lys PTMs. However, despite this progress, we believe that we have barely scratched the surface in unraveling the details of the interconnected cellular pathways that give rise to the Lys PTM and their impacts. There are still major gaps in our knowledge surrounding Lys PTMs and the chemical tools needed to address them. We have a very limited understanding of how a Lys PTM at one site influences a PTM at a different site in the same protein, or another protein, and what this PTM interplay means biomedically. Increasingly sophisticated top-down and middle-down mass spectrometric approaches will be needed to help illuminate these PTM inter-relationships. We lack high-quality small-molecule probes for the large majority of enzymes that write and erase Lys PTMs in the multisubunit macromolecular complexes in which they reside, and are deficient in such chemical probes of most readers as well. Increasingly, protein chemical methods that can work inside the live cell will be needed to pinpoint the functional features of specific Lys PTMs. Despite these challenges, we are most sanguine that, given the creativity and commitment of the investigators who populate the epigenetics community, the next 25 years will revolutionize our understanding of reversible Lys PTMs even more than the last.

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DECLARATION OF INTERESTS

P.A.C. is a cofounder of Acylin and has been a scientific advisor for Abbvie, which has had therapeutics programs targeting p300 and CBP.

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