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# Lysine methylation regulators moonlighting outside the epigenome

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**ABSTRACT**

Landmark discoveries made nearly two decades ago identified known transcriptional regulators as histone lysine methyltransferases; since then the field of lysine methylation signaling has been dominated by studies of how this small chemical posttranslational modification regulates gene expression and other chromatin-based processes. However, recent advances in mass spectrometry-based proteomics have revealed that histones are just a subset of the thousands of eukaryotic proteins marked by lysine methylation. As the writers, erasers, and readers of histone lysine methylation are emerging as a promising therapeutic target class for cancer and other diseases, a key challenge for the field is to define the spectrum of activities for these proteins, beyond histones. Here we summarize recent discoveries implicating non-histone lysine methylation as a major regulator of diverse cellular processes. We further discuss recent technological innovations that are enabling the expanded study of lysine methylation signaling. Collectively, these findings are shaping our understanding of the fundamental mechanisms of non-histone protein regulation through this dynamic and multi-functional posttranslational modification.

## Introduction

Covalent, reversible posttranslational modifications (PTMs) greatly expand the functional diversity of proteins beyond that provided by amino acid composition and tertiary. Methylation on the ε-amine of a lysine sidechain was first reported on a bacterial flagellar protein in 1959 (Ambler and Rees, 1959) and shortly thereafter on histone proteins (MURRAY, 1964). However, the functional implications of lysine methylation were not broadly appreciated until the turn of the century when Jenuwein and colleagues revealed the molecular activity of a known transcriptional repressor, Suppressor of Variegation 3-9 Homolog 1 S(UV39H1), as a histone lysine methyltransferase (Murn and Shi, 2017; Rea et al., 2000). Since this landmark discovery, substantial progress has been made characterizing proteins that write (lysine methyltransferases; KMTs), erase (lysine demethylases; KDMs), and read (effector proteins) lysine methylation (**Figure 1**) (Murn and Shi, 2017). The studies of lysine methylation signaling have focused largely on the modification of histone proteins, owing to their abundance, evolutionary conservation, biochemical tractability, and established phenotypic assays. This body of work has firmly established that histone lysine methylation is a major contributor to fundamental chromatin-templated biological processes including transcription, DNA replication, and DNA repair (Black et al., 2012; Rothbart and Strahl, 2014).

Recent technical advances in mass spectrometry (MS)-based proteomics have revealed that the scope of lysine methylation extends far beyond histone proteins. To date, nearly 3,000 non-histone proteins from human cells have been reported to be modified with lysine methylation at approximately 5,000 unique sites (Hornbeck et al., 2015). The identification of new sites of lysine methylation has far outpaced functional annotation. In fact, only a small fraction of non-histone lysine methylation sites have been assigned a function (**Supplementary Table 1**) and the majority of these studies have been limited to a few proteins (e.g., p53, ER, RB1), which have been extensively reviewed elsewhere (Biggar and Li, 2015; Biggar et al., 2017; Carlson and Gozani, 2016). Here, we discuss key findings in recent years that have expanded our understanding and appreciation of the functional role of lysine methylation in molecular processes by fine-tuning non-histone protein function (**Figure 2**). We also reflect on technological innovations that have enabled these discoveries. We then end with a perspective of the perceived challenges and opportunities that lie ahead for the blossoming field of lysine methylation research.

## Lysine Methylation Facilitates Protein-Protein Interactions

Protein methylation, unlike acetylation or phosphorylation, does little to the charge properties of amino acid side chains(Luo, 2018). In the context of histone proteins, it is appreciated that lysine methylation functions primarily as a signal for the selective recruitment of effector proteins (Musselman et al., 2012). On non-histone proteins as well, lysine methylation can facilitate protein-protein interactions (**Figure 2**). For example, a chemical proteomics screen for substrates of the G9a/GLP (EHMT2/EHMT1) dimer in mouse embryonic stem cells led to the identification of Activating Transcription Factor 7 Interacting Protein (ATF7IP) lysine 16 as a novel substrate (Tsusaka et al., 2018). In the same study, immunoprecipitation of

ATF7IP followed by mass spectrometry revealed that the histone H3 lysine 9 (H3K9) methyl-effector protein MPP8 selectively binds ATF7IP K16 tri-methylation. ATF7IP and M-Phase Phosphoprotein 8 (MPP8) are both part of the HUman Silencing Hub (HUSH) complex which contains the histone H3 lysine 9 methyltransferase SET Domain Bifurcated 1 (SETDB1) (**Figure 3A**). The HUSH complex mediates gene silencing, and knockout of either ATF7IP or MPP8 impaired HUSH complex function. Interestingly, mutation or truncation of ATF7IP to prevent K16 methylation led to a delay in HUSH complex silencing of a virally introduced transgene, suggesting methylation of ATF7IP partially contributes to HUSH complex function (Tsusaka et al., 2018).

Like MPP8, the DNA methylation regulatory protein Ubiquitin-like with PHD and Ring Finger Domains 1 (UHRF1) also reads H3K9 methylation (**Figure 3B**). An interaction between UHRF1 and DNA Ligase 1 (LIG1) was recently reported in a lysine methylation-dependent manner (Ferry et al., 2017; Kori et al., 2019). While loss of LIG1 had no detectable impact on DNA methylation, deletion of the region surrounding the methylation site on LIG1 showed a DNA methylation defect (Ferry et al., 2017).

As can be seen from these examples, KMTs can have both histone and non-histone substrates. Likewise, it is likely that most effector proteins, KMTs, and KDMs will target both histone and non-histone proteins. Interestingly, as illustrated by the above-described studies, histone and non-histone lysine methylation can converge on shared biological outcomes (**Figure 3**). Deconvoluting the contribution of specific lysine methylation events on histone and non-histone proteins will be a crucial line of future inquiry.

In part because high-throughput approaches to identify methyllysine-driven protein-protein interactions are limited, the progress connecting reader proteins to non-histone protein lysine methylation has been slow. For histone lysine methylation, the use of histone peptide microarrays has been instrumental in making connections between reader proteins and histone lysine methylation (Rothbart et al., 2012; Wilkinson and Gozani, 2014). For non-histone proteins, most connections made thus far have been facilitated by low-throughput candidate-based approaches. For example, protein-domain microarrays displaying recombinant methyllysine reader domain libraries recently helped identify that the plant homeodomain (PHD) of PHD Finger Protein 20-Like protein (PHF20L1) reads methylation on DNA Methyltransferase 1 (DNMT1) (Estève et al., 2014) and Retinoblastoma Protein 1 (RB1) (Carr et al., 2017). Clearly, evidence is mounting that methyllysine readers domains recognize both methylated histone and non-histone proteins. Making additional connections between reader proteins and the vast non-histone lysine methylome is a major challenge and an important direction of future research.

### Lysine Methylation Regulates Protein-DNA Interactions

Lysine methylation has been associated with the regulation of protein-DNA interactions since early studies characterizing non-histone lysine methylation on the tumor suppressor protein p53 (Chuikov et al., 2004), and this has been confirmed to be a major mechanism by which lysine methylation impacts protein function (**Figure 2**). Recently, the multifunctional transcription factors Yin Yang 1 (YY1) and Yin Yang 2 (YY2) were

shown to be regulated by lysine methylation. Several KMTs interact with YY1, which prompted Zhang *et al*. to test whether YY1 might be directly methylated (Zhang et al., 2016). Indeed, YY1 is mono-methylated on lysines 173 and 411 by SET domain-containing protein 7/9 (SET7/9), resulting in increased YY1 DNA binding. YY2 was also shown to be mono-methylated by SET7/9. Mono-methylation of YY2 on lysine 247 (K247) enhanced YY2 binding to DNA, and knockout of SET7/9 phenocopied a K247 to arginine mutation of YY2. Furthermore, lysine-specific histone demethylase 1A (LSD1) removed YY2 K247me1 and knockout of LSD1 resulted in increased YY2 binding to DNA in cells. These recent examples and others (Ea and Baltimore, 2009; Liu et al., 2015; Xie et al., 2012) provide additional evidence in support of a role for lysine methylation in the regulation of protein-DNA interactions, particularly for transcription factors. Future structural and quantitative analyses of these interactions will be important to further understand this regulatory mechanism. Additionally, as evidence accumulates demonstrating transcription factor regulation by lysine methylation, so do questions about how upstream KMTs are themselves regulated. It is well appreciated that cascades of phosphorylation lead to transcriptional regulation. Whether KMTs fit into these cascades remains to be determined.

**Protein Stability is Regulated by Lysine Methylation**

Lysine methylation has been shown to regulate protein stability by directly competing with the conjugation of ubiquitin to lysine side chains (**Figure 2**). For example, SET7/9 mediated lysine methylation stabilizes both p53 and Estrogen Receptor alpha (ER$\alpha$) by blocking polyubiquitination-dependent proteasomal degradation (Chuikov et al., 2004; Subramanian et al., 2008). More recently, it was shown that a cystic fibrosis (CF)-associated mutation in the CFTR gene causes a shift from methylation to ubiquitination (Pankow et al., 2019). While functional studies are needed to determine whether the stability of this mutant CFTR ion channel is compromised, it is exciting to speculate that modulation of this lysine methylation site – perhaps through inhibition of a KDM – could offer a new treatment paradigm for CF by preventing degradation of the CFTR protein.

In addition to stabilizing proteins by blocking potential sites of ubiquitylation, lysine methylation can also play the opposite role as a signal for protein degradation; it accomplishes this by recruiting the ubiquitin ligase machinery, directly or indirectly, through adaptor proteins. Lysine methylation events that function in this way have been termed "methyl-degrons." The KMT Enhancer of Zeste 2 (EZH2) was reported to generate a methyl-degron on the nuclear receptor Retinoid-related Orphan Receptor Alpha (RORalpha/NR1F1) that is recognized by a putative chromodomain of the DDB1-CUL4-Associated Factor 1 (DCAF1) (Lee et al., 2012). DCAF1 recognition of EZH2-directed mono-methylation on NR1F1 lysine 38 leads to ubiquitylation of NR1F1 by the DDB1/2/CUL4 E3 ligase complex. A similar methyl-degron mechanism was recently shown for SET7/9 directed mono-methylation of SMAD7 lysine 70, which promotes ubiquitylation by the E3-ligase Arkadia and degradation of SMAD7 (Elkouris et al., 2016).

These studies raise questions regarding how E3 ligases recognize methylated lysines. It is intriguing to speculate that some E3-ligases might themselves contain cryptic methyllysine effector domains. Methyllysine effectors have also been shown to recruit E3 ligases. For example, SET Domain Bifurcated 1 (SETDB1) mono-methylates RAC-alpha serine/threonine-protein kinase (AKT) on lysine 64, leading to ubiquitylation by the E3 ligase TNF Receptor Associated Factor 6 (TRAF6) or the Skp, Cullin, F-box containing (SCF) complex (Wang et al., 2019). The interaction between AKT and these E3 ligases is mediated through Lysine-specific Demethylase 4A (KDM4A), which recognizes methylated AKT through its Tandem Tudor domain. However, KDM4A facilitates ubiquitylation independent of its KDM activity. Surely, the role of lysine methylation in regulating protein stability will be an exciting area of research in the coming years.

**Lysine Methylation can affect Protein Subcellular Localization**

Lysine methylation is a major determinant of protein localization within cells. For instance, the nuclear localization of Catenin beta-1 (β-catenin) was shown to be positively regulated by SET and MYND domain containing 2 (SMYD2), a KMT that catalyzes mono-methylation of β-catenin on lysine 133 (Matsuo et al., 2017). SMYD2 is found in the nucleus (**Figure 1**), and methylation of β-catenin may promote its nuclear accumulation by facilitating an interaction with the import machinery. The nuclear-localizing KMT SET Domain Containing 1A (SETD1A) has also been shown to regulate the localization of several proteins. Di-methylation of HSP70 on lysine 561 by SETD1A leads to enhanced HSP70 nuclear localization (Cho et al., 2012). Similarly, SETD1A-dependent mono-methylation on lysine 342 of Yes-Associated Protein 1 (YAP1), a key transcriptional coactivator in the Hippo signaling pathway, promoted its nuclear localization. Interestingly, it was found that YAP1 K342me1 blocks its interaction with Chromosomal Maintenance 1 (CRM1), a nuclear export protein (Fang et al., 2018), thus offering a methylation-dependent mechanism to trap YAP1 in the nucleus.

**Crosstalk between Lysine Methylation and other PTMs**

Histone PTMs regulate other chromatin modifications through dynamic crosstalk mechanisms. Lysine methylation is a major contributor to this signaling complexity on histone proteins (Rothbart and Strahl, 2014), and there is growing evidence it plays a similar role on non-histone proteins. Lysine methylation crosstalk with other PTMs occurs through a variety of mechanisms. One of the appreciated crosstalk mechanisms on histone proteins is the H3K9/S10 methyl-phospho switch that contributes to the ejection of methyllysine-reading Heterochromatin Protein 1 (HP1) from mitotic chromatin (Fischle et al., 2005; Hirota et al., 2005). Similar mechanisms for non-histone proteins have been described where methylation and phosphorylation at neighboring sites have opposing functions, and these have been reviewed elsewhere (Biggar and Li, 2015). Recent evidence suggests that additional mechanisms of crosstalk between lysine methylation and phosphorylation exist. The KMT SET Domain Bifurcated 2 (SETD2) was

shown to mono-methylate Signal Transducer and Activator of Transcription 1 (STAT1) on lysine 525, leading to enhanced phosphorylation of STAT1 on Y701. Phosphorylation of STAT1 Y701 leads to STAT1 activation and ultimately downstream activation of the interferon-mediated antiviral response (Chen et al., 2017). How STAT1 K525me1 leads to increased phosphorylation remains unclear. Methylation may alter the conformation of the protein or the responsible kinase. Alternatively, an unidentified effector protein may recognize this methylation site to facilitate phosphorylation.

It is clear that crosstalk between phosphorylation and lysine methylation occurs at neighboring sites, but lysine methylation has also recently been shown to directly and indirectly affect the activity of kinases and phosphatases. On the one hand, cell cycle-associated Polo-like Kinase 1 (PLK1) was recently identified as a KMT substrate for SET Domain Containing 6 (SETD6). SETD6 methylates two lysine residues on PLK1, and loss of SETD6 and PLK1 methylation resulted in an increase in PLK1 kinase activity (Vershinin et al., 2019). On the other hand, Interferon Regulatory Factor 3 (IRF3) was shown to be methylated by Nuclear Receptor Binding SET Domain Protein 3 (NSD3) on K366 (Wang et al., 2017). IRF3 is activated through phosphorylation on S396 and is deactivated by removal of this phosphorylation event by the Protein Phosphatase 1 Catalytic Subunit Gamma (PP1cc). Notably, PP1cc is unable to recognize the methylated form of IRF3, leading to the stabilization of activated IRF3. It is interesting to speculate that a demethylase exists to remove this methylation and subsequently allow PP1cc to dephosphorylate IFR3 and disable its function.

Lysine methylation also directly competes with other PTMs directed toward lysine residues. As outlined above, ubiquitin and lysine methylation can directly compete as a mechanism to regulate protein stability. Similarly, lysine methylation and acetylation can directly compete: for instance SETD2 tri-methylates α-Tubulin on lysine 40, preventing acetylation of this residue (Park et al., 2016) and contributing to cytoskeletal remodeling. As a result, loss of SETD2 leads to genomic instability, presumably through destabilization of microtubules. However, SETD2 also catalyzes tri-methylation of lysine 36 on histone H3 (H3K36me3), a binding site for the PWWP domain of the *de novo* DNA methyltransferase 3B (DNMT3B) (Baubec et al., 2015; Dhayalan et al., 2010). Mutations in the PWWP domain cause immunodeficiency, centromere instability, and facial abnormalities (ICF) syndrome (Ge et al., 2004), suggesting that the role of SETD2 in maintaining genomic stability may be through both histone and non-histone pathways (**Figure 3C**).

Evidence is mounting that lysine methylation on non-histone proteins functions as part of a dynamic network of PTMs to mediate signaling within cells. As shown exquisitely on histone proteins, lysine methylation can function to promote or prevent the addition of other PTMs. The examples outlined above demonstrate that similar mechanisms regulate PTMs on non-histone proteins. The concept that combinations of PTMs function together to mediate specific signaling events through histone proteins was coined the "histone code" hypothesis (Strahl and Allis, 2000). Inspired by this concept, a more broad "protein code" was hypothesized, encompassing both histone and non-histone proteins (Sims and

Reinberg, 2008). Future study of the many ways by which lysine methylation works with and opposed to other PTMs will certainly expand our understanding of the diverse functions of lysine methylation, but also of PTM signaling in general.

**Lysine Methylation Inside the Nucleus**

Most lysine methylation regulators localize to the nucleus at least some of the time (**Figure 1**) (Carlson and Gozani, 2016). However, beyond chromatin function, few studies have revealed mechanisms of lysine methylation signaling in the nucleus. Growing evidence suggests lysine methylation regulates RNA splicing. A proteome-wide screen for methylated proteins enriched for many factors involved in splicing, yet at the time, little was known about the function of these lysine methylation events or the responsible enzymes (Moore et al., 2013). The KMT SET Domain and Mariner Transposase Fusion Gene-Containing Protein (SETMAR), disputed for its function as an H3K36 methyltransferase (Carlson et al., 2015; Fnu et al., 2011), was shown to mono-methylate the splicing factor Small Nuclear Ribonucleoprotein U1 Subunit 70 (snRNP70) on lysine 130 (Carlson et al., 2015). Notably, SETMAR was the first identified KMT to target a splicing factor, but the functional significance of snRNP70K130me1 is unclear. The splicing factor RNA Binding Motif Protein 25 (RBM25) was shown to be mono-methylated on lysine 77 (Carlson et al., 2017). While the responsible KMT is elusive, functional studies suggest RBM25K77me1 may regulate splicing by blocking the interaction between RBM25 and another splicing factor, Serine- and Arginine-rich Splicing Factor 2 (SRSF2). With the identification of a large number of splicing proteins that undergo lysine methylation, continued study of the role lysine methylation plays in regulating RNA splicing will be an important area of future study.

In addition to regulating splicing, studies suggest lysine methylation may regulate the transcriptional machinery itself. Posttranslational modification of the RNA polymerase II (RNAPII) C-terminal domain (CTD) is an important regulatory mechanism of RNAPII function. The CTD consist of heptad-repeats of a consensus sequence (Try-Ser-Pro-Thr-Ser-Pro-Ser), and phosphorylation of serines 2 and 5 are associated with transcription elongation and initiation, respectively (Buratowski, 2009). Notably, vertebrates also contain a variable number of non-canonical repeats that contain lysine substituted for serine in the seventh position. These non-canonical K7-containing RNAPII CTD repeats were shown to be mono- and di-methylated (Dias et al., 2015; Voss et al., 2015). RNAPII CTD-K7me1/me2 was found to be associated with the early stages of transcription, but the function of these modifications and the responsible regulators are unknown.

Determining whether biological effects associated with a lysine methylation regulator are due to a histone or non-histone protein remains a significant challenge, particularly for non-histone lysine methylation occurring on proteins in the nucleus. As discussed above for effector proteins, evidence is mounting that lysine methylation regulators modulate cellular processes through methylation and/or recognition of both histone and non-histone proteins (**Figure 3**). Recent studies found this is true even for

proteins with appreciated biological functions associated with their histone targets. For example, Polycomb Repressive Complex 2 (PRC2) is well studied for its role in epigenetic regulation of facultative heterochromatin through KMT activity toward lysine 27 on histone H3 (H3K27) by the catalytic component of the complex, EZH2. Using a SPOT array to characterize the substrate selectivity of PRC2, it was discovered that the RNAPII transcription elongation factor Elongin A (EloA) is mono-methylated on lysine 754 by PRC2 (Ardehali et al., 2017). Mutation of the endogenous target lysine to methionine with CRISPR/Cas9 revealed that PRC2-directed methylation of EloAK754 helps reinforce suppression of a subset of PRC2 target genes. Details of this molecular mechanism involving EloAK754me1 are still unclear, but this recent study exemplifies the types of systematic approaches that will be necessary for delineating histone- versus non-histone-specific functions of nuclear KMTs. Notably, it was found that PRC2 activity toward H3K27 and EloAK754 both contribute to gene regulation by PRC2 (**Figure 3D**). PRC2 has other reported non-histone substrates (He et al., 2012), and EZH2 inhibitors are in clinical trials for hematologic malignancies. It is therefore fundamental to further study how the sum of all PRC2 substrates contribute to its chromatin regulatory and oncogenic functions.

**Lysine Methylation Outside the Nucleus**

Despite the majority of lysine methylation regulators being localized to the nucleus, several recent studies have begun to unveil an expansive role for lysine methylation in regulating processes in the cytoplasm. In particular, lysine methylation is emerging as a regulator of translation. Despite being first reported on the translation machinery nearly 50 years ago (Chang et al., 1976), it was not until recently that progress was made toward determining the function of this methylation or the responsible KMTs or KDMs. An example of the rapid progress that has recently been made are studies of Elongation Factor 1 Alpha (eEF1A), an essential GTPase associated with the translation machinery. Methylation of eEF1a was mapped on several lysine residues (Jakobsson et al., 2018a), and five unique KMTs were recently connected to methylation of eEF1a (Jakobsson et al., 2018b; Liu et al., 2019; Małecki et al., 2017). Two groups independently identified Methyltransferase-like Protein 13 (METTL13) as the KMT responsible for catalyzing di-methylation on lysine 55 of eEF1A (Jakobsson et al., 2018b; Liu et al., 2019). Notably, this methylation directly affects translation. It was mechanistically determined that METTL13-directed methylation of eEF1AK55me2 leads to an increased rate of eEF1A GTP hydrolysis and increased cellular protein synthesis (Liu et al., 2019). Indeed, investigating the role of lysine methylation in regulating translation may hold promise for new cancer treatments. Liu *et al.* showed the increased rate of translation mediated through METTL13 methylation of eEF1AK55 promotes tumorigenesis (Liu et al., 2019). Elevated METTL13 expression was also associated with poor survival in patients with pancreatic ductal adenocarcinoma (PDAC). METTL13 depletion reduced tumor size and extended life in a PDAC mouse model (Liu et al., 2019), supporting chemical inhibition of METTL13 as a rationale treatment approach.

Recent studies also support a role for KDM4A, a KDM appreciated for its activity toward H3K9 and H3K36 (Klose et al., 2006; Whetstine et al., 2006). KDM4A was found to associate with the translation machinery in the cytoplasm, and depletion or inhibition of its catalytic activity altered the distribution of translation factors in polysome fractions (Van Rechem et al., 2015). Intriguingly, this resulted in a net decrease in global protein synthesis. Substrates of KDM4A in the cytoplasm remain elusive, and their identification will be a decisive step toward mechanistic understanding of this protein's translation regulatory function.

**New Technologies Guiding the Study of Lysine Methylation**

The progress made to date mapping lysine methylation and defining its functions is intimately linked to the development of innovative technologies. Perhaps the most critical advance has been the ability to detect lysine methylation by mass spectrometry (MS). A combination of developments in state-of-the-art high-sensitivity MS instrumentation and optimized sample preparation techniques have rapidly expanded the lysine methylome in recent years. In general, robust PTM analysis by MS requires enrichment for peptides containing the modification of interest. Lysine methylation presents a unique challenge in this regard, since the modification is relatively small and does not modify the charge properties of the amino acid side chain. Enrichment strategies for methylated peptides have relied heavily on the use of "pan" methyllysine antibody reagents (Bremang et al., 2013; Cao and Garcia, 2016; Guo et al., 2014). However, creating a reagent that is truly "pan" has proven difficult (Levy et al., 2011). Essentially an antibody must be able to distinguish a single methyl group while also binding with high affinity to all possible sequences surrounding a target lysine – a tall task for an antibody whose epitope footprint is typically 5-8 residues. This enrichment step is a significant drawback of lysine methylation MS workflows, and as a result, several groups have proposed alternative approaches.

For example, as an alternative to antibody-based enrichment, effector protein domains with limited sequence selectivity have been employed. In one approach, the Malignant Brain Tumor (MBT) domain repeats from Lethal(3) MBT-like Protein 1 (L3MBTL1) were used to enrich for mono- and di-methylated lysine-containing peptides (Moore et al., 2013). The HP1β chromodomain was used in a similar approach that resulted in the identification of 30 methylated proteins (Wang et al., 2018). Other strategies follow in the footsteps of mapping phosphorylation, which was facilitated, in part, by chromatographic separation techniques to enrich for phospho-peptides (Roux and Thibault, 2013). A similar strategy for lysine methylation would be extremely useful, as it would bypass the sequence bias limitations stemming from the use of affinity reagents. Toward this goal, several chromatographic strategies to enrich for methyllysine peptides have recently been proposed (Ning et al., 2016; Wang et al., 2016; Wu et al., 2015). For example, a charge-suppression strategy has been implemented in which the charge on unmodified lysine and arginine residues after tryptic digestion is neutralized by a chemical reaction that has no effect on methylated residues (Ning et al., 2016). Coupling this approach to charge-based separation enriched for

methylated peptides and identified 399 unique lysine methylation sites (Ning et al., 2016). Overall, the combination of these alternative affinity reagents and novel chromatographic enrichment strategies has helped expand cartography of the lysine methylome in recent years.

The functional annotation of lysine methylation sites has lagged significantly behind their identification. However, despite the relatively slow progress compared to mapping efforts, the molecular functions for lysine methylation in a variety of cellular processes is becoming clearer, as discussed above (**Figure 2**). These findings were facilitated, in large part, by approaches that connect the regulators of lysine methylation to their substrates. Knowledge of specific KMT or KDM substrate sites has greatly facilitated investigation of the function of specific lysine methylation events. This includes several MS-based approaches (Carlson et al., 2015; Olsen et al., 2016), but these approaches are limited since proteome-wide analysis of lysine methylation by MS is still not robust.

Beyond MS-based approaches, a variety of techniques have been developed that aim to identify substrates *in vitro.* One example uses an array fabricated with thousands of proteins immobilized on a functionalized glass surface. The microarray is subjected to an *in* vitro KMT reaction, facilitating protein-level identification of substrates. This approach was used to identify Mitogen-Activated Protein Kinase(3) 2 (MAP3K2) as a novel KMT substrate of SET and MYND domain containing Protein 3 (SMYD3), connecting lysine methylation signaling to RAS-driven cancers (Mazur et al., 2014). A key advantage to the use of protein arrays is identification of substrates at the protein level, removing the possibility of false positives due to the use of unstructured peptides. While it is appreciated that SET domain-containing KMTs recognize linear motifs, recent evidence suggests contacts outside of the active site impact substrate selectivity (Kublanovsky et al., 2018). A limitation to the use of protein arrays for substrate identification is that the common surface chemistry for protein immobilization relies on charge interactions, which prevents control over uniformity of protein display and may mask lysine side-chains. Control over protein folding is also a concern that increases the likelihood of false negatives with this approach.

Synthetic peptides on membrane support (SPOT) arrays have also found common use for KMT substrate identification (Rathert et al., 2008). In this approach, iterative on-membrane synthesis of peptides with differing amino acids in positions near the target lysine of a known substrate helps identify the positional dependence of a KMT motif. This approach was used to identify new substrates for several KMTs (Ardehali et al., 2017; Lanouette et al., 2015; Rathert et al., 2008; Rowe and Biggar, 2018). A limitation of this tolerance-based approach is the requirement of starting with a known substrate for derivation. As many KMT family members are still considered ‘orphan’ enzymes with no known substrates (Murn and Shi, 2017), this is considered a major drawback of this assay platform. In addition, since peptide substrates are synthesized directly on the array, precise quality control over peptide composition being queried is lacking.

An assay querying a lysine-oriented peptide library (K-OPL) was recently reported for KMT substrate identification (Cornett et al., 2018). Unlike SPOT peptide arrays, the approach required no prior knowledge

of a substrate. The approach screened a large combinatorial peptide library to determine the sequence determinants of substrate selectivity plus and minus a defined number of residues from a target lysine. The resultant substrate selectivity profile was used in a variety of ways to guide downstream study of the lysine methylation regulator of interest. Novel substrates were revealed by ranking all the lysine residues in the proteome based on their propensity to be used as a substrate. The approach also has potential utility for evaluating how mutations in disease may rewire lysine methylation signaling networks, as was shown for phosphorylation (Creixell et al., 2015).

The demonstrated utility of these various techniques provides a comprehensive toolkit to map and annotate the function of lysine methylation. An important observation stemming from the use of these approaches is that the mapping of lysine methylation is likely incomplete. The use of substrate selectivity technologies (SPOT and K-OPL) reveal that the sequences most preferred by KMTs are difficult to detect by standard MS pipelines (Cornett et al., 2018). Likewise, as MS pipelines have improved with the use of newer affinity reagents, chromatographic strategies, and instrumentation, the lysine methylome has expanded. Overall, while the field has made progress developing new technologies to map lysine methylation and facilitate connections between lysine methylation sites and the enzymes responsible for regulating them, we predict there is still a lot of work ahead to comprehensively map and functionally annotate the lysine methylome. Future efforts integrating the use of these complementary technologies will help reveal the breadth of biological activities associated with lysine methylation and will be paramount for developing new strategies to exploit lysine methylation signaling for therapeutic benefit.

## Concluding Remarks

Remarkable progress has been made in the last decade to reveal how lysine methylation fine-tunes the function of proteins in a variety of ways. These findings were fueled by new tools and technologies focused on mapping lysine methylation and connecting sites of lysine methylation to their regulatory proteins. The field of lysine methylation is now entering a new era that will be dominated by studies detailing the functional significance of lysine methylation at the mechanistic and biological levels. As evidenced by recent studies highlighted in this review, we suggest the field is just beginning to appreciate the expansive role of lysine methylation in regulating protein function, cellular processes, and disease progression.

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## Figure Legends

**Figure 1. Lysine Methylation Regulators.** Wheel plots represent current knowledge of the localization, substrates, and status of small molecular inhibitors for each lysine methylation regulator. (A) Lysine methyltransferases (KMTs) (B) Lysine Demethylases (C) Proteins with PHD, BAH, CW, and SPIN lysine methylation reader domains. (D) Royal family proteins containing Tudor, MBT, PWWP, or Chromo lysine methylation reader domains.

**Figure 2. The molecular functions of lysine methylation.** Radar plot representing frequency of the reported molecular functions of lysine methylation as indicated.

**Figure 3. The cellular effects of lysine methylation are often due to the sum of both histone and non-histone targets.** (A) G9a/GLP methylation of H3K9 and ATF7IPK16 is read by the chromodomain of MPP8 to mediate gene repression. (B) G9a/GLP methylation of H3K9 and LIG1 is read by the tudor domain of UHRF1 to mediate maintenance of DNA methylation. (C) SETD2 methylation of $\alpha$TubulinK40 contributes to cytoskeletal and genomic stability. SETD2 also methylates H3K36 which plays a role in *de novo* DNA methylation and contributes to genomic stability. (D) The catalytic subunit of the PRC2 complex, EZH2, catalyzes the methylation of both H3K27 and ElonginAK754 to mediate repression of genes.

**Figure 4. Technologies used to identify new substrates of lysine methyltransferases.** Each technology compromises between immediate biological relevance and the number of sequences queried, as indicated. (A) Pipeline for identification of lysine methylation sites using mass spectrometry. (B) SPOT arrays for evaluating the substrate selectivity of KMTs. (C) Protein arrays for identifying KMT substrates. (D) Lysine oriented peptide library (KOPL) for identification of the sequence determinants of KMT substrate selectivity.

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**Figure 1 Cornett et al.**

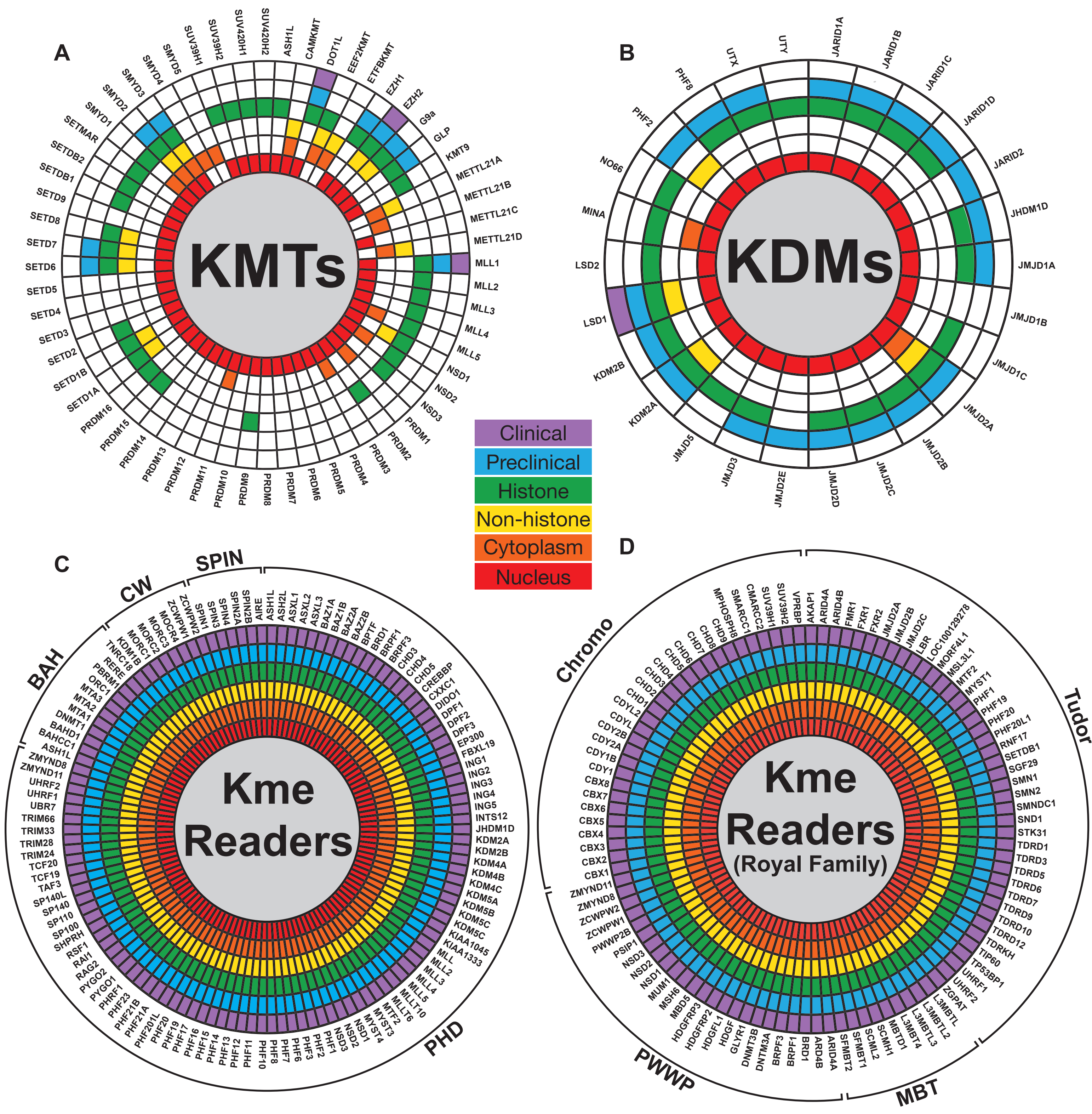


**Figure 2 Cornett et al.**

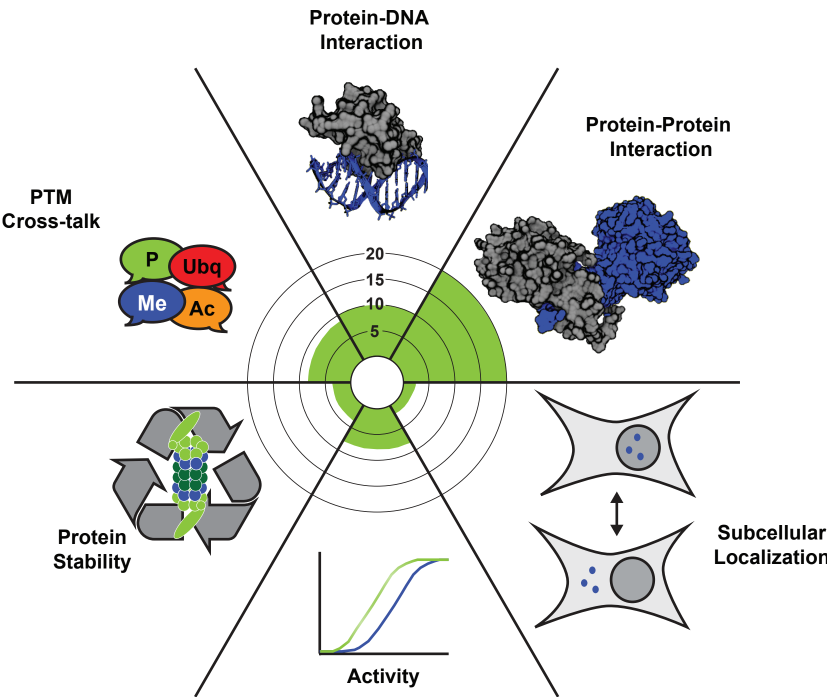


**Figure 3 Cornett et al.**

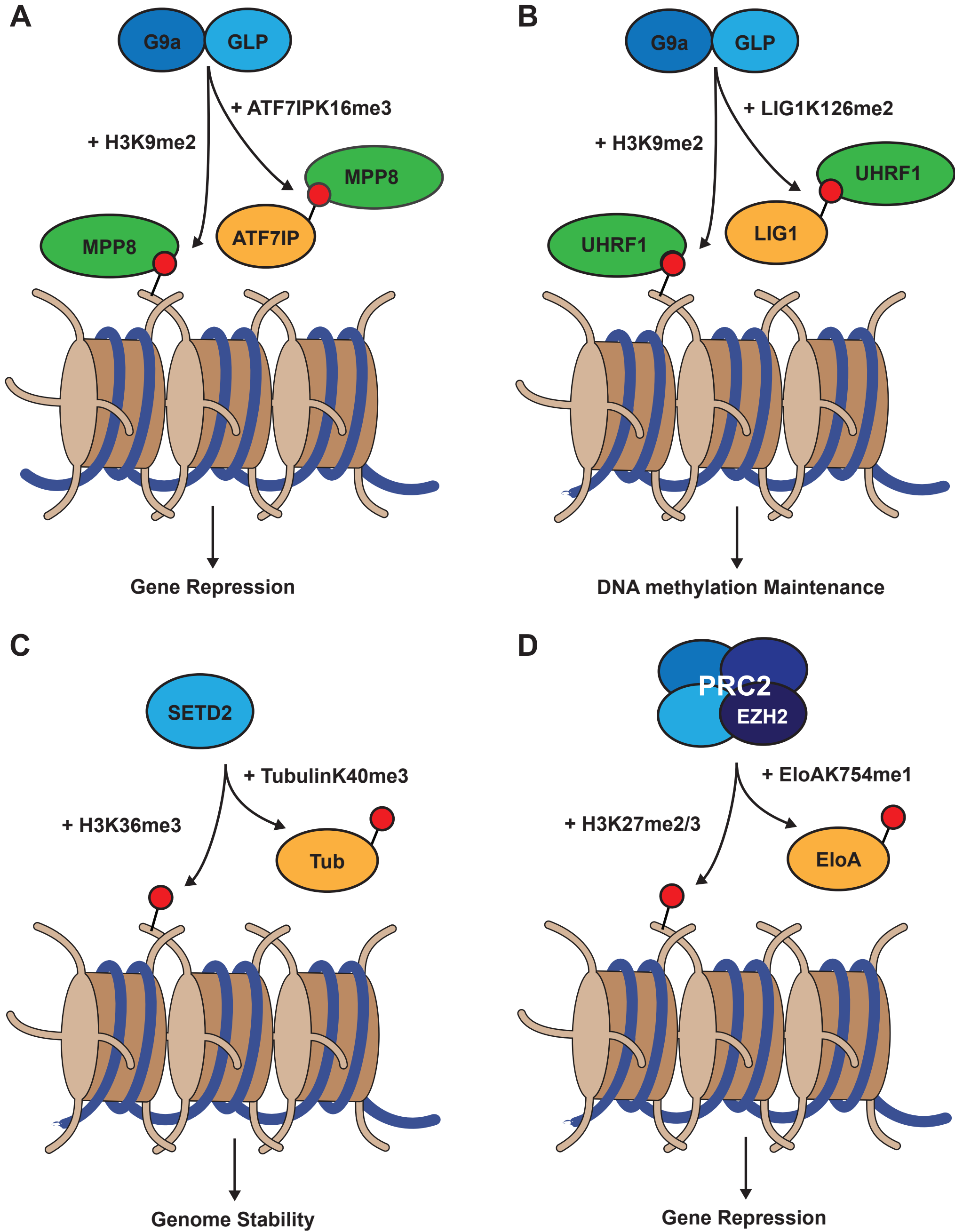
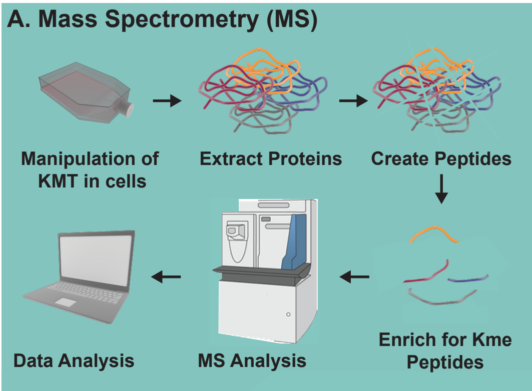
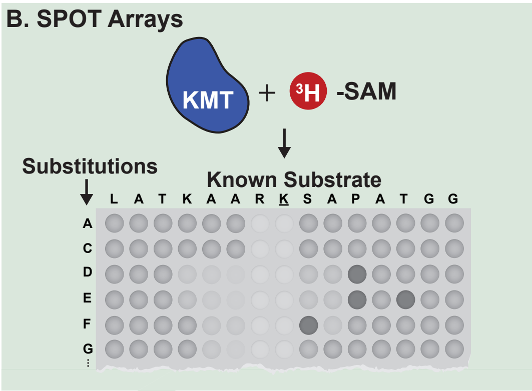


**Figure 4 Cornett et al.**

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