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Review

An unexpected journey: Lysine methylation across the proteome ☆

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ABSTRACT

The dynamic modification of histone proteins by lysine methylation has emerged over the last decade as a key regulator of chromatin functions. In contrast, our understanding of the biological roles for lysine methylation of non-histone proteins has progressed more slowly. Though recently it has attracted less attention, ε-methyl-lysine in non-histone proteins was first observed over 50 years ago. In that time, it has become clear that, like the case for histones, non-histone methylation represents a key and common signaling process within the cell. Recent work suggests that non-histone methylation occurs on hundreds of proteins found in both the nucleus and the cytoplasm, and with important biomedical implications. Technological advances that allow us to identify lysine methylation on a proteomic scale are opening new avenues in the non-histone methylation field, which is poised for dramatic growth. Here, we review historical and recent findings in non-histone lysine methylation signaling, highlight new methods that are expanding opportunities in the field, and discuss outstanding questions and future challenges about the role of this fundamental post-translational modification (PTM). This article is part of a Special Issue entitled: Methylation multifaceted modification — Looking at transcription and beyond.

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1. Introduction

1.1. Not so new: a historical perspective on non-histone methylation

As with histone methylation, non-histone methylation was discovered long before the field was prepared to study the post-translational modification. R.P. Ambler and M.W. Rees first reported the presence of ε-N-methyl-lysine in a natural protein in flagellin from *Salmonella typhimurium* in 1959 [1]. Five years later, Kenneth Murray reported methyl-lysine in histones. Murray based his analysis in part on comparison of the unusual amino acid he found in histones to methyl-lysine from flagellin [2]. Early reports of lysine methylation focused on chemical identification of the amino acid within a small subset of proteins, including ribosomal proteins, actin, myosin and myofibrillar proteins, and cytochrome c [3–8] reviewed in [9]. Rubisco, the most abundant protein on the planet [10], was also shown to be methylated [11].

Though today it is established that lysine methylation is a PTM, a great deal of work went into showing that the methyl group was added to lysine post-translationally, rather than directly incorporated as methyl-lysine during translation. Stocker, McDonough, and Ambler argued that methylation of flagellins occurred after lysine was incorporated into the protein because the presence of methyl-lysine was controlled by a gene other than the gene for flagellin [12]. Incorporation of radioactive methylation into methyl-lysine residues was not inhibited by puromycin, a translation inhibitor, again suggesting that methylation is a PTM [13,14]. Finally, methyl-lysine could not be conjugated to tRNA, which would be necessary if it were to be incorporated during translation [14].

If the methyl group was incorporated post-translationally, where did it come from? Using radiolabeling experiments, it was shown that either methionine or S-adenosyl-methionine could serve as the source of the methyl group on lysine [2,3,14,15]. Murray proposed that this methylation might be sequence specific [2], and by 1965, Kim and Paik argued that methylation in calf thymus nuclei was enzymatic [14]. In the years that followed, the field continued to focus on identifying new methylated proteins individually and on purifying a limited number of methylating enzymes (reviewed in [16]). As Woon Ki Paik, David C. Paik, and Sangduk Kim described it, the field made little progress in identifying the biological function of this methylation [16]. Without a downstream biological outcome for lysine methylation, the broader significance of adding this moiety was unclear.

Biological insight into histone methylation was fueled by discoveries like the one from the lab of Thomas Jenuwein identifying the epigenetic regulator Suv39H1 as a histone lysine methyltransferase [17]. Notably, comparison with a known non-histone methylation pathway aided the identification of this histone methylation pathway; Rea et al. hypothesized that Suv39h1 might be a methyltransferase based on its homology to Rubisco large subunit methyltransferase [17,18]. The availability of

Abbreviations: PTM, post-translational modification; KMT, lysine methyltransferase; KDM, lysine demethylase; SAM, S-adenosyl methionine; MBT, malignant brain tumor; RNP, ribonucleoprotein; MS, mass spectrometry. Post-translational indications are indicated with protein name, site of modification, and modification type/extent, without spaces (for example, RelAK310me1 is monomethylation of lysine 310 on the protein RelA)

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techniques to study chromatin biology allowed quick bridging of methylation events with downstream phenotypic assays. New assays for chromatin biology grew along with the field of histone methylation—for example, chromatin immunoprecipitation (ChIP) coupled with microarrays (ChIP-chip) [19–21] and more recently ChIP coupled with deep sequencing (ChIP-seq) [22,23]. Just as Paik, Paik, and Kim pointed out that the advent of modern molecular biology opened up the long struggling field of lysine methylation in general [16], tools for studying gene regulation and chromatin naturally shifted the focus of the field toward histones and other chromatin-related proteins. Tool development, as will be discussed later, is likely to be important for studying lysine methylation on non-histone proteins as we move forward.

2. Key examples of non-histone methylation

Non-histone methylation has experienced a moderate renaissance with the finding that several histone lysine methyltransferases also methylate other substrates (see [24] for a recent review of lysine methyltransferases (KMTs) and corresponding histone and non-histone targets). Below we describe regulatory lysine methylation on several key non-histone proteins. As the number of lysine methylation events has greatly expanded over the last few years, this review is by no means an exhaustive discussion, but rather focuses on some specific methylation events to highlight regulatory trends and concepts.

2.1. p53 methylation

p53 is a central tumor suppressor and a decision node for triggering apoptosis [25], which is regulated by lysine methylation (reviewed in [24,26–28]). p53 methylation was first reported by Reinberg and colleagues. In 2004, they showed that Set7/Set9 monomethylates p53 at lysine 372 (p53K372me1) [29]. This methylation event was found to promote the stability of nuclear p53, expression of the p53 target p21, and DNA-damage induced p53-mediated apoptosis [29], potentially through preceding and promoting acetylation of p53 [30]. Several methylation events have also been reported to negatively regulate p53 activity. Smyd2 monomethylates p53 at lysine 370 [31]. This methylation event represses p53 activity by decreasing its ability to bind promoters of target genes [31]. Huang et al. proposed that K370me1 is a repressive mark due to a decrease in the fraction of total p53 bearing the mark at a target promoter upon DNA damage and because Smyd2 expression was inversely correlated with p53-target gene expression [31]. Interestingly, prior to activating K372 monomethylation inhibits subsequent K370 monomethylation, but not vice versa, in part by decreasing the ability of Smyd2 to bind p53 [31]. SET8-mediated monomethylation at lysine 382 was also found to repress p53 transcriptional activation, decreasing expression of p53 target genes [32]. More recently, G9a and GLP were reported to dimethylate p53 at lysine 373 [33]. As levels of p53K373me2 do not increase with DNA damage despite a large increase in total p53 levels, this mark is postulated to repress p53 activity [33]. Consistent with this idea, knockdown of G9a and GLP increased apoptosis, with and without DNA damage [33].

p53 was the first non-histone protein for which regulatory demethylation was reported. In 2007, Berger and colleagues showed that the lysine demethylase LSD1 removes the p53K370me2 mark [34]. Dimethylation of K370 by a yet to be identified KMT activates p53 transcriptional activity through the recruitment of 53BP1, a protein involved in DNA damage signaling and initially identified as a p53 co-activator [35,36]. LSD1 demethylation of K370me2 disrupts the interaction between p53 and 53BP1 and thus acts to repress p53 function [34].

2.2. RelA

RelA/p65 is a component of the canonical NF-κB signaling pathway that translocates to the nucleus to bind target genes upon signaling activation. Several lysines in RelA have been reported to be methylated, including K37, K218, K221, K310, K314, and K315 [37–41]. Levy et al. provided an example of regulation of NF-κB signaling by methylation. In a biochemical screen with forty candidate KMTs, SETD6 was identified as a novel methyltransferase of the chromatin-associated fraction of the NF-κB subunit RelA [38]. SETD6-mediated methylation of RelA at lysine 310 (RelAK310me1) rendered RelA inert and attenuated RelA-driven transcriptional programs, including inflammatory responses in primary immune cells. The ankyrin repeat from GLP – an H3K9 methyltransferase involved in transcriptional repression – functioned as a recognition motif for RelAK310me1. Under basal conditions, SETD6-dependent binding of GLP to RelAK310me1 promoted a repressed chromatin state at RelA target genes through GLP-mediated H3K9 methylation. This repressive pathway was overridden by NF-κB activation-linked phosphorylation of RelA by PKCζ at serine 311 (RelAS311ph), which blocked GLP binding to RelAK310me1 and drove target gene expression [38]. These series of molecular events were the first description of a lysine methylation-signaling cascade and demonstrated a new mechanism for how integrated crosstalk between modifications on transcription factors and histones can modulate gene expression programs.

2.3. Dam1

Sharon Dent and colleagues have reported the Set1-dependent methylation of Dam 1 in yeast [42,43]. Dam1 forms part of a complex that regulates chromosome segregation, and is itself regulated through phosphorylation by the Aurora kinase Ipl1 [42]. Zhang et al. showed that loss of Set1 catalytic activity suppresses temperature-sensitive inactivation of Ipl1 [42]. They also found that this genetic interaction can be tied to Set1-dependent dimethylation of Dam1 at lysine 233 [42]. This methylation event interacts genetically with Ipl1 phosphorylation at nearby serine residues—K233 mutation can be rescued by subsequent mutation at S232, S234, or S235 [42]. The authors proposed that K233 methylation inhibits phosphorylation of S232 and S234 [42]. The group later showed that just as H2BK123 ubiquitination regulates Set1 methylation of H3K4, H2BK123ub is required for Set1 methylation of Dam1K233, establishing that both the histone and non-histone substrate of Set1 are regulated through the same initial histone modification pathway [43].

2.4. Spliceosomal proteins

The spliceosome is known to be a significant target of arginine methylation, which plays a role in snRNP assembly (see [44] for a review). Recent proteome-wide studies indicate that a significant portion of the spliceosome is also subject to lysine methylation [45–48]. The role of this methylation is as-yet unexplored. It is enticing to think that lysine methylation might modulate splicing through mechanisms such as spliceosome assembly or splice site selection, but experimental evidence in this area is currently lacking.

2.5. Ribosomal proteins

Methyl-lysine was discovered in ribosomal proteins of *S. cerevisiae* in 1984 [49]. Enzymes which methylate yeast ribosomal proteins were first identified in 1989 [50]. These enzymes were dubbed M23 and M32 because they methylate Y23 and Y32, respectively [50] (the enzymes are now referred to as Rkm2 and Rkm1, methylating Rpl12 and Rpl23, respectively [51]). A number of papers from Steven Clarke and colleagues describe a series of methylation events on ribosomes and the corresponding KMTs. The KMT Rkm1 dimethylates Rpl23ab at lysine 105 and 109 [52,53] and Rkm2 trimethylates Rpl12ab, most likely at lysine 3 (potentially at lysine 10) [54,55]. Rpl42ab is monomethylated at lysines 40 and 55 by Rkm3 and ySET7, respectively [55]. Rkm5 monomethylates Rpl1K46 [56]. As discussed in [56], it is unclear whether these yeast methylation events translate to homologous mammalian

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ribosomal methylation. However, methylated ribosomes have been independently identified in mammalian samples (for example, see [45–48,57], reviewed in [51]).

2.6. Translational elongation factors

Methylated elongation factors have been studied in bacteria, fungi, and mammalian systems since the late 1970s [51], with recent proteomic studies of lysine methylation identifying a large number of additional modified translation elongation factors [45,48]. Though the function of elongation factor methylation is still largely unknown, in *E. coli* methylation of EF-Tu tracks growth phase and may modulate EF-Tu's GTPase activity [58] (reviewed in [51]. Among more recent examples, Lipson et al. described monomethylation of eEF1A by YHL039W (dubbed Efm1) and dimethylation of eEF1A by See1 in *S. cerevisiae* [59]. Couttas et al. later localized these sites of methylation to K30 and K316, respectively [60]. They also described trimethylation of EF3AK187 by YBR271W (named Efm2) [60]. For a comprehensive review of elongation factor methylation, see [51].

2.7. Heat shock proteins

Heat shock proteins represent another class of cytosolic methylated proteins. Two groups reported Smyd2-mediated HSP90 methylation [61,62]. Abu-Farha et al. argued that Smyd2 methylates two sites on HSP90, the predominant one being K615 in the dimerization domain [61]. They also proposed that HSP90 might be demethylated by LSD1 [61]. Donlin et al. reported Smyd2-mediated monomethylation of K615 (numbered K616 in their study) [62]. They showed that Smyd2 and methylated HSP90 colocalized with titin and potentially regulate sarcomeres (and therefore muscle) through this interaction [62].

Two KMTs have been reported to methylate HSP70s: SETD1A and METTL21A/HSPA-KMT [63,64]. Cho et al. reported SETD1A-dependent HSP70-K561me2 [63]. They showed that this methylated version of HSP70 localizes to chromosomes, rather than the cytoplasm, and that this methylation event may promote an association with Aurora kinase B that stimulates kinase activity [63]. Jakobsson et al. showed that METTL21A trimethylates the same residue in several human HSP70s [64]. Though trimethylation of K561 does not appear to alter basic HSP70 functions, it does affect the interaction of the HSP70 isoform HSPA8s with α-synuclein, which is implicated in Parkinson's disease [64]. Methylation of this conserved lysine was also observed by Cloutier et al., who showed that several enzymes from METTL21A's family have lysine methylation activity [65]. Cloutier et al. also described methylation of several other HSP70 isoforms, as well as the proteins KIN and VCP [65].

3. Transducing non-histone methylation signals: "reader proteins"

Lysine methylation pathways are essentially signaling processes. In response to a signal, a KMT methylates a substrate. This signal is then transduced by state- and sequence-specific effector proteins, which read out the methylation event catalyzed by the KMT and translate that methylation event into downstream responses. As might be expected, these "reader" proteins have been most extensively studied as methyl-histone binders. However, several readers have been shown to bind to methylated lysine in non-histone proteins. Below, we discuss several of the most-studied non-histone methyl-lysine binders. Examples of methylation signaling pathways are shown in Fig. 1.

3.1. L3MBTL1

L3MBTL1 was originally studied as an H4K20me1/2 and H1bK26me1/2 binder [66]. L3MBTL1 binds methylated lysine through its 3 malignant brain tumor (MBT) domain repeats [66–68] (reviewed in [69]). L3MBTL1 was known to interact with a transcription regulatory complex involving Rb [66], and Saddic et al. showed that L3MBTL1 binds Smyd2-catalyzed Rb monomethylation at lysine 860 [70]. L3MBTL1 also interacts with SET8-catalyzed p53K382me1, leading to decreased expression of p53-regulated genes [71]. While full-length L3MBTL1 in these examples has specific biological functions, the MBT domains of L3MBTL1 appear to have very little amino acid sequence specificity [45,66,72].

3.2. 53BP1

p53-binding protein 1 (53BP1) was isolated in a two-hybrid screen for p53 interacting proteins [35]. 53BP1 has been reported to bind dimethylation of two residues within p53: K370 and K382 [34,73]. At K370, 53BP1 binding is thought to function as a transcriptional co-activator at p53-target genes [34]. For K382me2, the binding of 53BP1 is proposed to serve as a molecular mechanism by which DNA double strand break signals are transduced to activate p53 during the DNA damage response [73]. The sequence surrounding K382 of p53 is very similar to that surrounding H4K20. 53BP1 and L3MBTL1 both bind to methylated H4K20 and p53K382, raising the idea that sequences in non-histone proteins have evolved to mimic histone sequences and might therefore be regulated by conserved reader domains [28,66,71,73–75].

3.3. HP1

The chromodomain of HP1 was the first known methyl-lysine reader domain and helped establish the molecular mechanism and structural basis by which histone methylation marks are transduced [76–78]. The KMT G9a methylates H3K9 [79,80] and has also been reported to methylate other residues on histones as well as non-histone substrates [79,81–94]. Several groups therefore asked whether HP1, as a methyl-H3K9 binder, could also bind non-histone methylation at similar sequences. Sampath et al. found that HP1γ interacts with G9a dependent on an intact G9a automethylation site at lysine 165 [81]. Chin et al. also found that mouse HP1 interacts with G9a dependent upon the K239 automethylation site [82]. Rathert et al. generalized this idea, showing that HP1β can bind to methylated peptides representing G9a sites on WIZ, CDYL1, HDAC1, ACINUS, Kruppel, and G9a [83]. Expanding to a proteomic scale, Liu et al. assumed that HP1β would have many binding partners and set out to describe its methyl-dependent "interactome" [47]. They found that, among other proteins, HP1β binds to methylated DNA-PKcs [47].

4. Functional consequences of non-histone methylation

A recent expansion in methods for proteomic analysis has led to a large increase in the number of known methylated non-histone proteins. From larger scale "methylomics" studies [45–48], several cellular processes are emerging as hubs of potential regulation by lysine methylation. This information facilitates asking functional questions about what lysine methylation does on a broad scale to establish regulatory themes.

4.1. Interactions between PTMs on non-histone proteins

On histones, post-translational modifications act in a combinatorial or antagonistic manner to regulate signaling [69]. Naturally, this concept also applies to methylation of non-histone proteins. For example, crosstalk has been reported between several PTMs on p53. Huang et al. showed that methylation at K372 inhibits the interaction of p53 with Smyd2 and subsequent K370 methylation [31] and p53 methylation at K370 influences p53 acetylation [30].

"Methyl-phospho" switches represent a distinct class of PTM crosstalk. The classic model of a methyl-phospho switch is H3K9 methylation and S10 phosphorylation, wherein S10 phosphorylation serves to inhibit binding of HP1 to methylated K9 [17,95,96]. When Fischle,

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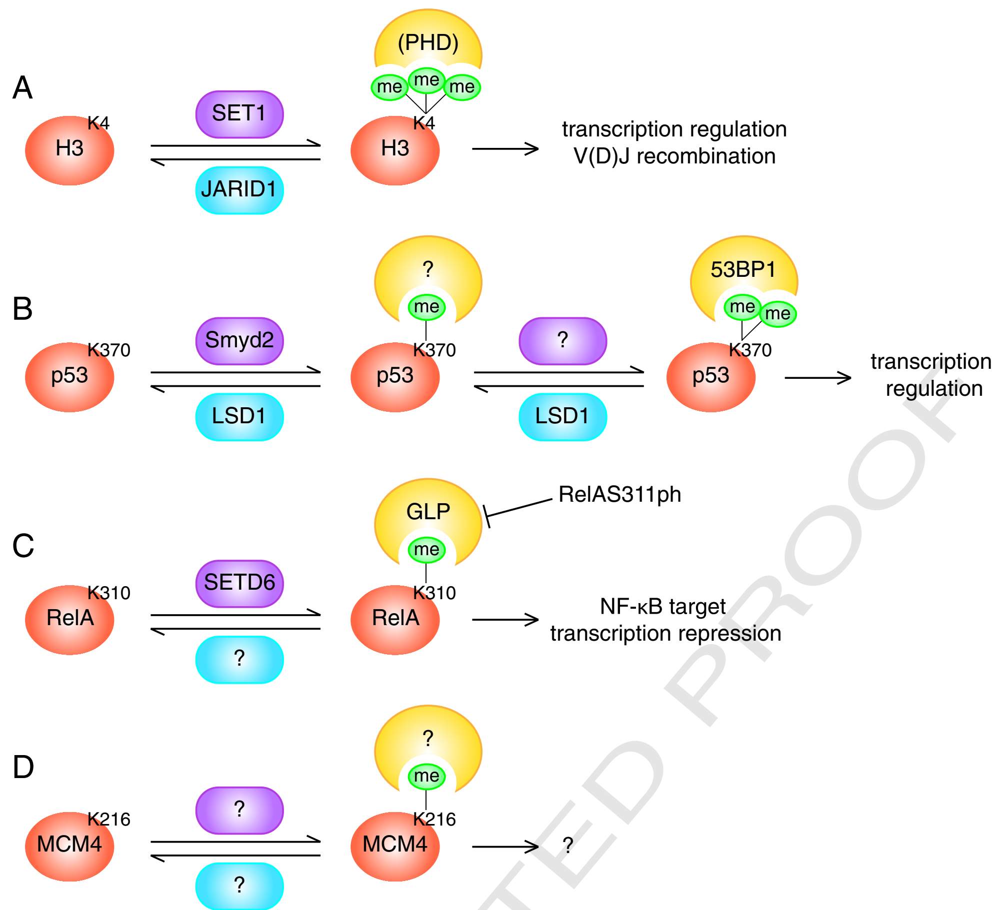

Fig. 1. Examples of methylation signaling pathways. A. Methylation signaling via H3K4me3. Trimethylation of H3K4 by the SET1 family of KMTs leads to recognition of the methylated histone by PHD finger-containing proteins and downstream regulation of transcription and V(D)J recombination [133–135] (reviewed in [69,123,136,137]). This methylation mark is removed by JARID1 family of demethylases (reviewed in [123,124,137]). B. Methylation signaling via p53K370 methylation. p53 is monomethylated at lysine 370 by Smyd2 [31]. Dimethylation by an unknown methyltransferase recruits 53BP1 and regulates transcription [34]. These methyl marks can be removed by LSD1 [34]. C. Methylation signaling via RelAK310me1. SETD6 catalyzes addition of one methyl group to lysine 310 of the NF-κB subunit RelA [38]. This mark is recognized by GLP and leads to tonic repression of target genes [38,39]. GLP binding is inhibited by phosphorylation of an adjacent serine residue [38,39]. D. Methylation signaling via MCM4K216me1. Monomethylation of the replication licensing factor MCM4 at lysine 216 was identified in a proteomic screen for methylated proteins [45]. The writer, reader, eraser, and function of this mark are unknown; it provides an example of methylation for which the signaling pathway has not yet been established.

Wang, and Allis formally proposed the idea of methyl-phospho switches as "local binary switches," they also suggested that the concept might extend beyond histones [97]. Similar switches exist between G9aK165 methylation and T166 phosphorylation [81] and between RelAK310 methylation and S311 phosphorylation [38]. Dam1K233 methylation also interacts with nearby phosphorylation at residues S232, S234, and S235 [42]. Another form of interaction between PTMs on non-histone proteins is through methylation "blocking," the concept that methylation could prevent acetylation or ubiquitination (or other lysine modification) of the same residue and thus suppress outcomes associated with those PTMs [98]. However, given that it is unlikely for a lysine to be 100% methylated – and indeed methylated species are likely in the minority – it is not clear that signaling through competition of modification systems for a specific residue would be an effective mechanism.

4.2. Methylation and protein-nucleic acid interactions

Of methylated non-histone proteins identified so far, a large portion form part of a protein–nucleic acid complex (also noted in [99]). For example, the ribosome and the spliceosome, both large RNP machines, are well-represented among methylated proteins [45–48]. Perhaps this shouldn't be surprising, given that histones largely function in complex with a nucleic acid. This trend may give us clues as to broader regulatory functions of methylation within the cell. In his review on the subject, Steven Clarke suggested that one function of methylation of ribosomal proteins is to encourage their interaction with rRNA [100]. Clarke also notes that many non-histone methylated proteins are related to transcription, again linking the modification to proteins that interact with a nucleic acid [100].

4.3. Biophysical functions

Unlike phosphorylation and acetylation, methylation does not alter the charge of the modified residue. The methyl group constitutes are relatively small change in steric bulk and hydrophobicity of lysine side chains. These observations suggest that lysine methylation would be less likely to play a direct chemical or biophysical role in protein regulation. One possible direct role for the modification might be to increase protein stability, through mechanisms such as decreasing susceptibility to proteases and increasing heat tolerance (as discussed in [11,56,100–102]), potentially through promoting intra-molecular interactions that stabilize the polypeptide. Two groups have also proposed that methylation can negatively regulate stability, citing decreased DNMT1 half-life when methylated, perhaps through a proteasome-mediated mechanism [103,104].

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5. Methods for uncovering non-histone methylation

The field of non-histone methylation faces a methods hurdle: most current studies rely on candidate-based approaches. However, recent work has focused on the problem of proteome-wide identification of methylated proteins. Two major proteomic approaches have been used: peptide and protein array-based assays, and affinity or chemical enrichment coupled with mass spectrometry.

5.1. Array-based approaches

Peptide and protein arrays have been used to identify non-histone substrates of KMTs. In the case of peptide arrays, the array often serves to define a consensus sequence for the methylation-related enzyme in question, whereas protein arrays are often used to directly test for enzymatic targets in a high-throughput manner. Albert Jeltsch and colleagues used permutation arrays of peptides based on the previously known G9a target, H3K9 [79,80], to identify a consensus methylation sequence for G9a [83]. They then predicted potential G9a substrates using two methods: searching the proteome for proteins bearing the consensus methylation motif, and searching known G9a-interacters for a reduced "RK" motif [83]. Using this method, they identified several non-histone targets of G9a, including G9a itself, WIZ, ACINUS, CDYL1, and others [83], supplementing previous reports of non-histone G9a targets identified by lower throughput methods [81,82]. They also found that HP1β could bind to trimethylation at several of these non-histone sites [83]. Using this method, Jeltsch and colleagues also identified a number of new Set7/9 substrates, including MINT, IRF1, MeCP2, ZHD8, and PPARBP, among others [105]. Peptide arrays have also been used to describe substrate specificity for SET8 [106] and Dim-5 [107].

Short peptides represent a minimal in vitro target for KMTs, but might not present an appropriate recognition surface for enzymes. Protein (rather than peptide) arrays represent a potentially more physiological, yet still in vitro, panel of potential KMT substrates. This approach was validated in Levy et al., which used Invitrogen ProtoArrays® as a substrate for methylation reactions in order to identify substrates of Set7/9 and SETD6 [108]. Several of the novel SETD6 substrates identified through the array were validated in cells [108].

5.2. Affinity enrichment coupled to mass spectrometry

Mass spectrometry (MS) is an excellent method for proteomic scale studies of PTMs. This method has significant advantages over arrays: it can identify the site, state, and stoichiometry of methylation directly and it can measure the results of in vivo methylation reactions representing physiological conditions. However, MS analyses are biased towards detecting the most abundant proteins, and given the relatively low abundance of methylation events, few methylation events tend to be "seen" in a given MS run. This can be circumvented in part through an enrichment step, which has been the focus of several recent method development efforts [45–48,84,85].

The most familiar approach for enriching a PTM is affinity enrichment using antibodies specific for the PTM but not specific to a particular amino acid sequence ("pan-specific" antibodies). This strategy has proven successful for phosphorylation, ubiquitination, acetylation, and arginine methylation [109–112], but has been difficult to adapt to lysine methylation due to the challenge in developing high-affinity "pan-methyl" antibodies. The first attempt at using pan-methyl antibodies for enrichment before mass spectrometry analysis found only four known methylation events on two lysines, both in histones [112]. In that particular study, pull-down with the pan-lysine-methyl antibody actually led to identification of a large number of arginine methylation events [112]. Levy et al. subsequently tested several commercially available "pan-methyl" antibodies on arrays of post-translationally modified peptides and found cross-reactivity with other PTMs and insufficiently broad sequence specificity [108]. More recently, Bremang et al. compared the enrichment activity of pan-methyl antibodies with the increase in proteomic coverage from additional sample fractionation and concluded that the antibodies contributed little in the way of identifying additional methylation events [46]. The failure of commercial antibodies has prompted others to generate their own polyclonal pan-methyl antibodies. For example, Ben Garcia's group developed polyclonal mono-, di-, and tri-methyl-lysine antibodies which enriched methylated peptides from tryptic digest of cell lysates, and found a significant number of novel methylation events [48].

A related approach employs natural methyl-lysine binding domains to affinity enrich methylated proteins. Liu et al. used precipitation by the chromodomain of HP1β, coupled with mass spectrometry, to uncover the interactome of the protein and novel methyl-lysine-mediated biology. They first established the consensus binding sequence of the HP1β chromodomain using peptide arrays, and then used this to predict methylation sites within proteins specifically precipitated by the chromodomain, validating the sites by mass spectrometry [47]. They directly identified 29 methylated HP1β-interacting proteins [47].

Using a similar approach, Moore and Carlson et al. specifically precipitated proteins that bound to the 3 malignant brain tumor domain repeats (3xMBT) of the L3MBTL1 protein. Several groups had previously suggested that 3xMBT had sequence promiscuity [67,68,72]. Moore and Carlson et al. further established that while 3xMBT binds mono- and di-methyl-lysine specifically, the sequence and modifications surrounding the methylated residue had little effect on 3xMBT binding [45]. Comparison of proteins pulled-down by 3xMBT and a binding-null point mutant, $3xMBT_{D355N}$ [67,68] was used to eliminate non-specific protein–protein interactions. In total, this report identified several hundred potentially methylated proteins, with methylation directly observed and validated on 26 lysines [45].

Together, these recent proteomic studies have identified RNA processing, transcription, chromatin remodeling/organization, and helicase activity, among others, as processes where methylation appears to play a significant role [45–48].

5.3. Chemical enrichment coupled to mass spectrometry

Rather than rely on an antibody or methyl-lysine binding domain to enrich for methylated proteins, chemical labeling methods also allow for subsequent enrichment of methylated proteins. In general, these methods functionalize the methyl-donor, S-adenosyl-methionine (SAM), with a bioorthogonally-reactive chemical moiety which is transferred to the substrate protein in place of the methyl group. While this approach has been conducted with a ketone-functionalized donor [113], by far the most common method has been functionalizing the donor with an alkyne- or azide-containing group [84,85,114–116]. Transfer of either of these particular moieties is preferred because azides and alkynes react in a bioorthogonal, copper-catalyzed cycloaddition, which can later be used to attach affinity tags or other probes to the "methylated" target. Other related reactions, including copper-free, strain promoted cycloadditions, have also been used [84,117]. (These reactions are generally referred to as "click" chemistry, though the term itself indicates a broader class of reactions.) Several native methyltransferases, including Dim5, MLL4, and SETDB1, can transfer alkyne groups to their substrates [114,115]. However, not all native KMTs accept these cofactors [115].

To generalize this labeling method, Minkui Luo and colleagues screened a panel of alkyne-functionalized SAM analogs against a set of G9a mutants to find an active analog–mutant pair [116]. This "bump hole" approach [118,119] allowed them to access the activity of a protein which would not, as wildtype, accept a functionalized cofactor. After validating the concept, they then coupled the bump-hole/click approach to mass spectrometry [84]. Bump-hole mutants of the related methyltransferases G9a and GLP were given their cognate analogs in cell lysate, labeled substrates were "clicked" to a biotin conjugate, and

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then "methylated" substrates were precipitated with streptavidin beads for mass spectrometry analysis [84]. They identified 82 and 64 substrates, respectively, for G9a and GLP [84]. Additionally, this work helped define the separate substrate repertories of G9a and GLP, which share several substrates (reviewed in [120])— 64 and 46 of the substrates identified by Islam et al. were unique, respectively [84]. In more recent work, Islam et al. analyzed structure–activity-relationships of active enzyme mutant/modified cofactor pairs, identifying many more potential G9a and GLP substrates [85]. For a review of chemical labeling methods used to study lysine methylation, see [121]. A comparison of methods for studying lysine methylation is summarized in Table 1 and reviewed in [98].

6. Moving forward: open questions

6.1. How many proteins are methylated? What is the specificity of various KMTs?

There are currently more than 60 predicted lysine methyltransferases and 30 predicted lysine demethylases in the human genome [122–124]. In total, recent reports have shown that hundreds of proteins may be methylated, some at multiple sites, and to different degrees (mono-, di-, tri-) at a given site [45–48,84,85]. It seems likely that many more methylation events will be uncovered soon. This suggests a comparison between the number of methylation events and the number of methyltransferases, and raises questions about the degree of specificity of these enzymes. How many substrates should we suspect a methyltransferase to have?

For several characterized KMTs, the answer is clearly "many"; in vitro, enzymes like Set7/9 and G9a are promiscuous [79–94,105,108] (reviewed in [24,26,27,125]). For example, on protein arrays, Set7/9 methylates over 300 proteins [108]. Other KMTs appear quite specific, with only one reported target [24]. KMTs may even be specific for particular substrate states—for example, NSD2/MMSET/WHSC1 has little activity on free histones but robust activity as an H3K36 dimethyltransferase on nucleosomes [126,127]. However, for many KMTs and KDMs, there has been no unbiased proteomic assessment of multi-substrate potential. Methyl-regulating enzymes are currently being examined as potential therapeutic targets (reviewed in [128]), so careful analysis of substrate repertoires and potential promiscuity could have profound impact on drug development research.

If KMTs and KDMs are not especially specific, we might also raise the question of the relative biological importance of multiple methylation events catalyzed by the same KMT. For example, Set7/9 methylates multiple sites on histones, including H3K4, as well as many non-histone substrates [29,30,105,108,129,130] reviewed in [24,26,27,125] (our unpublished observations), and the biological relevance of any particular catalyzed methylation event in vivo is difficult to discern. Despite Set7/9's many reported substrates, loss of Set7/9 activity in mice has a minimal phenotype, suggesting that the combined effect of losing multiple methylation events in conjunction with compensation mechanisms will complicate predictions about in vivo phenotypes of specific methylation reactions [131,132]. In the terminology of Erce et al., we need to describe the "'weighted' networks" of KMT activity [98]. With respect to pharmaceutical development, understanding the relative biological functions of methylation events is necessary to ensure that the relevant methylation events are targeted for intervention.

Another question raised by the large number of global methylation sites is whether we have accounted for enough enzymes to catalyze the growing number of methylation events. Without a thorough evaluation of KMT specificity, this is difficult to assess. Might there be another family of KMTs yet unaccounted for? A core group of enzymes with well-defined SET and seven-β-strand domains currently dominate the KMT field, but enzymes bearing more distant homology or other domain structures might be KMTs. For example, the seven-β-strand METTL21-related KMT family was identified only recently, and appears to be related to arginine methyltransferases [65,122]. The possibility that other such families may exist adds an interesting dimension to the assignment of methylated proteins to their cognate KMTs.

6.2. Methylation in transcription and translation: shared regulatory mechanisms?

Transcription and translation both appear as repeated themes among methylated proteins [45,46,48]. It is intriguing to speculate that these two fundamental processes could be regulated by similar mechanisms through lysine methylation. Comparatively little is known about the function of methylation of translation factors, but in both cases the ability of methylation to mediate protein–protein interactions could play a role in fine-tuning protein expression and activity. Even if methylation acts through different molecular functions in transcription and translation, feedback or co-regulation of the processes through regulation of the same or related KMTs presents an interesting possibility.

7. Conclusion

We now know that lysine methylation occurs broadly across the proteome—at chromatin, elsewhere in the nucleus, and in the cytoplasm. It regulates such core cellular processes as transcription and translation, influencing protein–protein and potentially protein–nucleic acid interactions. Recent proteomic studies have developed methods to more efficiently uncover lysine methylation events, elucidating the "where" of lysine methylation. However, the locations of the methylation events themselves are only part of the story. To truly understand lysine methylation biology, we need to understand the full signaling pathways that utilize this PTM. This will require experimental analysis of the enzymes that catalyze addition and removal of methyl marks, of

Table 1
Comparison of methods for proteomic analysis of lysine methylation.

Method	Pros	Cons	References
Peptide array	• Generate consensus methylation sequence • Relative simplicity of *in vitro* method	• Non-physiological • Enzymes may require cofactors not present *in vitro* • Not all enzymes methylate peptides	[83,105–107]
Protein array	• Full-length protein represented • Relative simplicity of *in vitro* method	• Non-physiological • Enzymes may require cofactors not present *in vitro* • Missing, incomplete, or incorrectly folded proteins	[108]
Pan-methyl antibody- MS	• *In vivo* biological methylation • Mass spectrometry analysis	• Current commercially-available antibodies unreliable • Polyclonal antibodies difficult to independently replicate	[46,48,112]
Domain affinity-MS	• *In vivo* biological methylation • Reproducible, engineerable monoclonal affinity reagent • Mass spectrometry analysis	• Protein, rather than peptide, pull-down identifies fewer PTM sites directly	[45,47]
Chemical affinity (click)-MS	• *In vivo* biological methylation • Mass spectrometry analysis	• Enzyme must accept functionalized cofactor	[84,85]

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the effectors that bind to methylation events, and of the direct biophysical and biochemical consequences of methylation. The ability of lysine methylation to regulate vast swathes of physiological and pathological biology provides a remarkable example of the biological utility of one small PTM.

Competing interests disclosure

Stanford University has filed a preliminary patent application on the use of 3xMBT as an affinity reagent. O.G. is a co-founder of Epicypher, Inc.

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