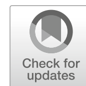
The Role of Protein Lysine Methylation in the Regulation of Protein Function: Looking Beyond the Histone Code



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Abstract Histone proteins and their diverse array of post-translational modifications have been subject to exquisite evolutionary conservation in eukaryotes. Accordingly, the factors that control the deposition, removal, and interpretation of histone modifications are themselves deeply conserved, with many strongly impacting development and disease in humans. Of these modifications, lysine methylation has in recent years emerged as a prevalent modification occurring on histone proteins. However, although numerous lysine methyltransferase and demethylase enzymes have been extensively characterized with respect to their ability to control methylation at

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specific histone residues, their known targets have been rapidly expanding to include the methylation of non-histone proteins as well. These findings extend the role of lysine methylation well-beyond the established histone code and its role in epigenetic regulation. To date, this lysine methylation has been found to directly regulate protein sub-cellular localization, protein-protein interactions, and has also been found to interplay with other post-translational modifications. As a result, lysine methylation is now known to coordinate protein function and be a key driving of a growing list of cellular signaling events, including apoptosis, DNA damage repair, protein translation, cell growth, and signal transduction among others. This chapter will provide insight into the role of protein lysine methylation and its role in regulating protein function and its impact on human development and disease.

Keywords Lysine methylation · Non-histone methylation · Methyllysine proteomics

1 Preface

There is a kink (shoulder) on [the] Lys peak... Richard P. Ambler (1959)

These words marked the initial discovery of lysine methylation and introduced a segue into a brand new field of scientific research. At the time, Ambler was a graduate student working in the laboratory of Dr. Maurice W. Reese at the University of Cambridge, working on the amino acid composition of bacterial flagellin (Ambler and Rees 1959). Through ion-exchange and 2D-chromatography experiments, a unique "kink" in a chromatograph was interpreted as a new amino acid, the ε-N-methyl-lysine. This new amino acid was discovered from the hydrolysates (proteins digested into smaller fragments, peptides, and amino acids) of *Salmonella typhimurium* flagellin, and provided the first insight that protein lysine methylation occurs amongst living cells. Although initially sparking a surge of research interest for a number of years, focus on lysine methylation quickly faded as a result of the inherent difficulty and lack of suitable technologies to study this very small, uncharged protein modification. Consequently, the functional implications of lysine methylation have only now begun to be established.

By the time protein methylation emerged as a field of interest, research into other post-translational modifications (PTMs; a chemical modification made to proteins that alter the host protein fate or function) more recently discovered was already firmly underway. For example, the discovery of lysine methylation (Kme) predates tyrosine phosphorylation by two decades following its discovery on v-Src-associated kinase (Anderson et al. 1990).

This chapter will discuss the expanding field of lysine methylation, along with its historical context and some of the key discoveries that have set the stage for a greater understanding of this intriguing post-translational modification. This chapter will also introduce several key examples of how lysine methylation is currently known to

regulate protein function, drive in disease pathologies, and finally, new technologies utilized for its discovery.

2 Lysine Methylation: A Brief History in Its Discovery

The initial discovery hallmarked by Ambler and Rees' observation of methyllysine in the flagellin of *Salmonella typhimurium*, provided the scientific community with its first evidence of protein methylation in living cells (Ambler and Rees 1959). Additional to this pivotal discovery, subsequent findings also led to the identification of a separate gene that influenced the presence, or absence, of the methyllysine modification—this demonstrated that methylation was a modification that occurred post-translationally (Stocker and McDonough 1961). It was then further reasoned that a specific enzyme must act to add the methylation modification directly to protein lysine residues. Impressively, these early theories posited the fundamental principles of which future revelations have been realized within the field.

Indeed, the lysine methylation of proteins have since been established to regulate many cellular processes, including protein interactions and cellular signaling transduction (Biggar and Li 2015; Wu et al. 2017). However, although the first lysine methylation event was found to occur in a non-histone protein, the methylation of histone proteins and its role in regulating chromatin structure became the impetus in driving the lysine methylation research for the following decades. It has now been established that hundreds of proteins are methylated at lysine residues and that this PTM is involved in regulating a growing number of cellular events, including growth signaling and DNA damage response (Carlson and Gozani 2016; Cao and Garcia 2016).

Although the physiological and regulatory roles of other PTMs, such as phosphorylation, were already being established, the 1960s brought important contributions to the most basic understanding of methylation. For example, in 1964 Kenneth Murray discovered the presence of methyllysine modified histone proteins (Murray 1964). Others have demonstrated that methyllysine could not be conjugated to tRNAs, thus resolving a persisting question on when the methylation of lysine occurred (Kim and Paik 1965). This discovery confirmed that histones were methylated after translation and not through the tRNA-mediated incorporation of a modified lysine residue. Building on these insights, Vincent Allfrey, and fellow researchers posited what, at the time, would have been a truly insightful hypothesis: that methylation of histones could regulate gene transcription (Allfrey et al. 1964).

Following these initial discoveries, there was a precipitous drop in research in subsequent decades. Throughout the 1960s and 1970s, Kim and Paik had diverted their focus towards the identification of the enzymes proposed to be involved in methylation. This was a fortunate detour, as they were able to establish the first methyltransferase activity, which involved the transfer of a methyl group from S-adenosylmethionine (SAM) to lysine, arginine, aspartic acid or glutamic acid

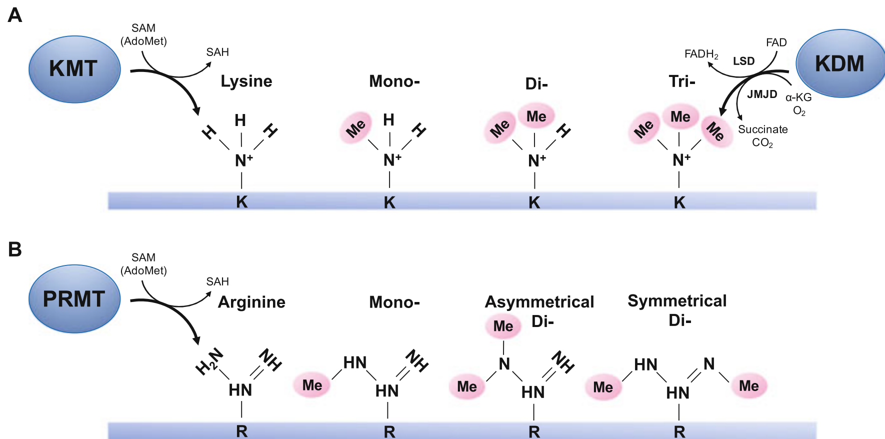

Fig. 1 Mechanism of lysine (K) and arginine (R) methylation. Lysine methyltransferase enzymes (KMTs) facilitate methylation through the use of S-adenosyl-L-methionine (SAM/AdoMet) as a methyl donor, yielding a methylated lysine residue and S-Adenosyl homocysteine (SAH). Specific to lysine, up to three methylation groups can be added a single lysine residue resulting in the formation of mono-, di- or tri-methyllysine. Lysine demethylases (KDMs) facilitate the removal of these methyl groups. Lysine specific demethylases (LSDs) target mono- and di-methylated lysines, reducing FAD to $FADH_2$ in the process. Jumonji domain containing demethylases (JMJDs) target mono-, di- and tri-methylated groups, carrying out oxidative decarboxylation and hydroxylation reaction with their associated co-factors, α-KG and Fe^{2+}. Arginine methyltransferases (PRMTs) facilitate arginine methylation through the use of SAM/AdoMet as a methyl donor, yielding mono-, asymmetrical di-, and symmetrical di-methylation

residues (Kim and Paik 1965). In the case of lysine methyltransferase (KMT) enzymes, it was determined that these enzymes were able to add a maximum of 3 methyl groups to the ε-nitrogen of the lysine residue (Fig. 1). It was not until several decades later that hints of a functional role for lysine methylation were finally beginning to be resolved, driven through advancements in genetics and molecular biology; notably through the study of gene expression and chromatin biology.

Methylation is the smallest PMT with little steric bulk and not contributing charge. This modification can occur on the side chains of at least 9 out of 20 amino acids, with lysine and arginine the most commonly methylated residues. To help direct the function of methylated protein, methylated lysine/arginine residues can also be recognized by proteins which “read” the adjacent amino acid sequence and the aromatic cage pockets of the methylated residues (Gayatri and Bedford 2014; Lachner et al. 2001); these modular protein domains are collectively referred to as methyl-binding domains (MBDs) and will be discussed periodically throughout the following sections of this chapter. These methyl-dependent interactions are stabilized through the strong attractive forces of the cation and the negative π-surface of the aromatic ring. Conversely, a non-methylated lysine residue displays acidic residues thus allowing for readers to be selective based on the ratio of aromatic to acidic residues.

Although it was first discovered in 1959, only in recent decades has our knowledge of protein methylation as a PTM has become a more prolific area of discovery. What we know of its properties and significance in biological functions leaves many unanswered questions, which makes it all the more intriguing for researchers to explore.

3 Protein Lysine Methylation: A Dynamic Post-Translational Modification

Estimated at over 21,000 different genes, the human genome provides greater proteome diversity through alternative mRNA splicing, giving rise to a number of proteins from a single gene. However, due to the myriad biochemical reactions present within a cell, even more protein diversity is required. Provided through the covalent addition of small moieties to specific amino acids, PTMs provide variations to protein function through modifications in electrostatic and structural properties, in addition to affecting the protein-protein interaction (PPI) that may be associated with the particular protein. As a result, this provides a diverse number of functions and interactions for a single protein, affecting a series of biochemical pathways and reactions within the cell (Duan and Walther 2015).

The nucleosome (i.e., the fundamental subunit of chromatin) is subjected to various PTMs (including phosphorylation, methylation, ubiquitylation, sumoylation, and acetylation) that work together to comprise what is known as the “histone code” for regulation of gene expression. Through various dynamic combinations of these PTMs, each cell can differentiate with unique morphology and biochemistry associated with its function. Among the most abundant of these PTMs, histone methylation has been established to play a critical role in transcriptional activation or repression—with the methylation of histone H3 at lysine 4 (H3K4me) known as a marker of gene activation, and both H3K9me and H3K27me as markers of gene repression (Arrowsmith et al. 2012). The dynamics of histone methylation and its control over gene expression can be reviewed in Hyun et al. (2017). Expanding beyond this ‘histone code’, sequence similarities between histone and other non-histone substrates have allowed for the novel identification of many other dynamically methylated substrates; in recent years, this has resulted in the methyllysine proteome expanding beyond histone methylation and chromatin regulation (Biggar and Li 2015). This expanded role of methylation has now been shown to include neoplastic growth and development, shedding light on the effects of methylation with regards to apoptosis, hypoxia, cell cycle arrest, and various other stress stimuli.

Predominantly favoring lysine and arginine residues in eukaryotic organisms (Clarke 2013), the addition of a methyl group to an amino acid requires the presence of a methyl-donor. The metabolite SAM (AdoMet), acts in this capacity and donates a methyl (CH_3) group to the recipient amino acid in a reaction that is facilitated by a

methyltransferase enzyme (Fig. 1). Dependent on the substrate, the methylation reaction may occur in a sequential fashion—adding one, two, or three methylation groups. In the presence of SAM and KMTs, methylation of the ε-amino group on lysine residues is open to mono-, di- or tri-methylation modification. While a similar process occurs with protein arginine methyltransferases (PRMTs) and the guanidine nitrogen of arginine residues, modification level is limited to mono- or di-methylation, with di-methylation expressed as either an asymmetrical or symmetrical modification. The ability to interact and recognize with specific lysine and arginine methylation events is separated within the methyltransferase families, with each class expressing specificity towards particular amino acids—thereby aiding in substrate specificity. As a result of the chemical nature of methylation, no effect has been observed towards protein integrity, as the addition of a methyl group itself provides minor size change and no direct charge difference. However, the modification leads to an increase in lysine basic nature, leading to an increased hydrogen bonding potential and thus increase recognition by other proteins (Hamamoto et al. 2015).

Following the discovery of KDM1A (LSD1), a histone-specific demethylase enzyme, the process of lysine methylation began to be understood as a dynamic modification—a modification that could be readily written (by KMTs) and removed (by KDMs) to regulate function (Shi et al. 2004). Similar to their methyltransferase counterparts, the demethylase family is subdivided into two main classes based on its catalytic domain, mechanism of demethylation, and interacting partners. Discovered as the first group of active demethylases, lysine-specific demethylases (LSDs) mainly target mono- and di-methyl substrates. In contrast, greater substrate diversity is observed with jumonji-domain containing demethylases (JmjCs), further subdividing the family of lysine demethylases (Accari and Fisher 2015). Utilizing α-ketoglutarate (αKG) and Fe^{2+} cofactors, methyllysine binding of JmjC-domain-containing enzymes follows a distinct mechanism involving the formation of a hydrogen bond network between the oxygen atoms of the catalytic residues and the methyl groups of the substrate. This non-classical methyl-binding mechanism allows correct positioning of the tri-methylated substrates to the Fe^{2+} cofactor, allowing ideal reaction conditions and the demethylation reaction to occur. In contrast, LSD enzymes require the presences of a lone electron pair at the methylated amine, opting out the possibility of LSD-catalyzed demethylation of tri-methyl substrates (Hou and Yu 2010).

3.1 SET Domain (Class V) Methyltransferases

Perhaps the most well-studied KMTs, lysine methylation is carried out by a class V methyltransferase that each contain a conserved catalytic SET domain (Fig. 2), consisting of four conserved active motifs GXG, YXG, NHXCXPN and ELXFDY that are composed of eight, curved β-sheet pseudo-knot-like structures (Fig. 2a). During the methyl-transfer reaction, the GXG motif aids in the correct positioning of the methyl donor SAM, while the hydrophobic pocket formed by the NHXCXPN

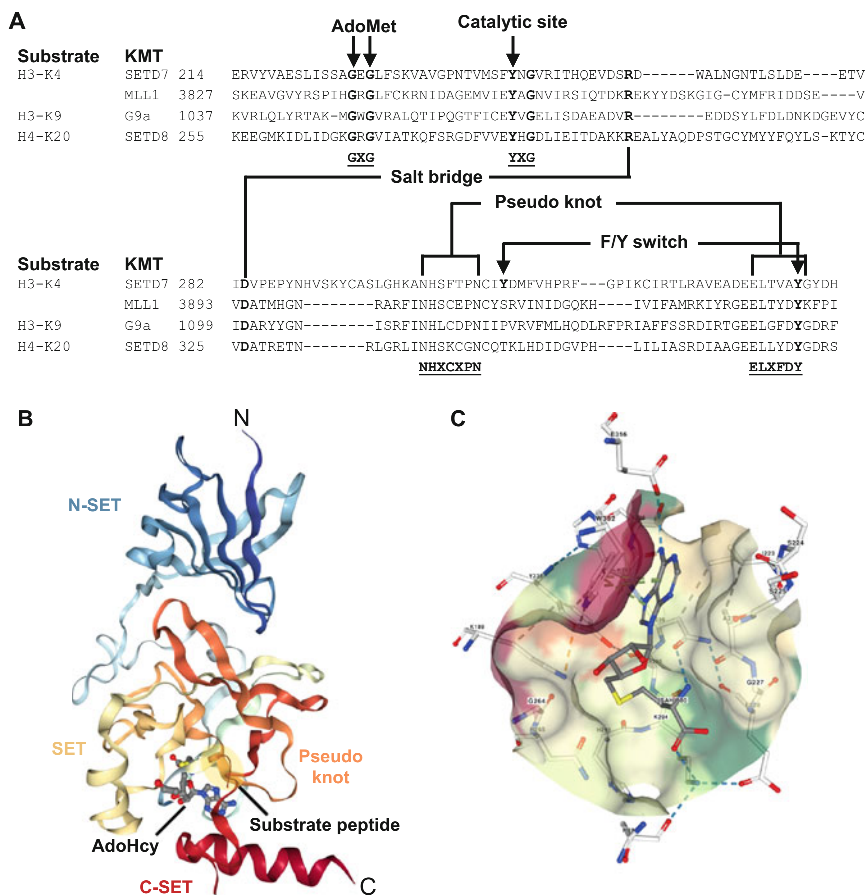

Fig. 2 SET domain lysine methyltransferases. (**a**) A protein sequence alignment of SET domains from several SET-domain containing lysine methyltransferase enzymes (KMTs). The involvement of residues in binding to AdoMet, catalysis, the structural pseudo knot, an intra-molecular interacting salt bridge, and an F/Y switch controlling whether the product is a mono-, di-, or tri-methylated lysine are indicated. (**b**) Representative structure of SETD7 KMT (3M53.pdb). The N-SET, SET, C-SET, pseudo knot, AdoHcy and substrate binding pockets in SET7/9 are indicated. (**c**) Structure of the co-factor AdoMet/AdoHcy binding site of SETD7 KMT

and ELXFDY motif aid in the recruitment and positioning of the methyl substrate on each side of its methyl transfer channel (Fig. 2b, c). This correct orientation allows for the SN_2 reaction (Helin and Dhanak 2013) carried out by the catalytic tyrosine present at the YXG motif (Petrossian and Clarke 2009a), transferring a methyl group from SAM to the ε-amine group of the lysine (Petrossian and Clarke 2009b). While sequence similarity is shared by all SET proteins at both N and C-terminal ends, it is the knot-like structure located at the C-terminal that is hypothesized to determine substrate specificity (Fig. 2b), in addition to the type of methylation carried out by the particular methyltransferase (Petrossian and Clarke 2009a).

3.2 Seven β-Strand (Class I) Methyltransferases

Found within all three domains of life (Lanouette et al. 2014), the class I methyltransferases, or seven β-strand (7BS) methyltransferases, comprise a larger superfamily of methyltransferases known to methylate a large variety of substrates such as DNA and RNA, in addition to a variety of amino acids such as arginine, glutamine, aspartate, histidine and lysine (Clarke 2013; Lanouette et al. 2014). The enzymes possess the conserved Rossmann fold characterized as several twisted beta sheets sandwiched between a series of alpha-helices with a C-terminal beta-hairpin (Petrossian and Clarke 2009a). Separated into four motifs (I, Post I, II and III), the first two motifs contain a conserved aspartate amino acid for charge stabilization and proper orientation, while the last two take part in methyl-substrate recruitment and binding (Zhang et al. 2000).

Forming a subdivision within the 7BS methyltransferase family, PRMTs catalyze the methylation of arginine residues resulting in either mono- or di-methylation. Unlike KMTs, arginine di-methylation through PRMTs can result in either symmetric or asymmetric methylation conformations (Smith and Denu 2009). Dependent on the type of PRMT catalyzing the reaction, further division can be made based on the type of di-methylation form that is facilitated by the enzyme. The most common, type I PRMTs, recognize terminal nitrogen atoms facilitating asymmetric di-methylation through the addition of two methyl groups (Kim et al. 2016a, b), or mono-methylation (Debler et al. 2016). In contrast, type II PRMTs carry out symmetric di-methylation though the addition of a single methyl group to terminal nitrogen groups, in addition to mono-methylation (Debler et al. 2016). While type III PRMTs are able to facilitate the production of the mono-methylated arginine (Kim et al. 2016a, b). Whereas types I–III are found in all life lineages, type IV PRMTs are specific to yeast and plants (Debler et al. 2016), catalyzing mono-methylation of internal nitrogen atoms. Similar to lysine methylation, such modifications are often involved in signal transduction, DNA damage and repair, protein interaction, translocation, cellular proliferation, chromatin remodeling and RNA splicing (Kim et al. 2016a, b).

Until recently, the histone-specific methyltransferase, DOT1L, was the only identified eukaryotic 7BS KMT (Singer et al. 1998). However, a number of novel 7BS KMTs have now been discovered. For example, the methyltransferase-like (METTL) protein family, containing METTL21D, METTL22, and METTL21A KMTs, has been found to methylate a number of different non-histone substrates (Falnes et al. 2016). The type II ATPase VCP/p97 has been shown to be tri-methylated by METTL21D (also known as VCPKMT) at lysine K315, negatively regulating VCP/p97 function including ubiquitin-dependent protein degradation (Kernstock et al. 2012). METTL21A has been reported to tri-methylate the HSP70 family (including HSPA1, HSPA8, and HSPA5) of chaperone proteins at an unknown site (s). This methylation event is especially interesting, as has been shown to interfere with the interaction between HSPA8 and alpha-synuclein (Jakobsson et al. 2013)—the main protein aggregate found in Parkinson's disease (Spillantini et al. 1997). Additionally, the association of the DNA/RNA binding protein, KIN17, with chromatin is thought to be influenced through lysine K135 tri-methylation by METTL22 (Cloutier et al. 2014). Together these findings collectively showcase the ability of 7BS KMTs in

the regulation of a broad range of non-histone protein targets and implication in diverse cellular functions.

3.3 Lysine-Specific Demethylases

Comprising the first group reported to function in histone lysine demethylation, lysine-specific demethylases (LSDs) comprise a sub-class of the amine oxidase superfamily (Smith and Denu 2009). Including only two members, LSD1 and LSD2, the pair share a conserved SWIRM domain located at the enzymes N-terminal. These domains form a globular core structure with the two amine oxidase domains (AOD) that contain the substrate and Flavin adenine dinucleotide (FAD) binding sites (Hou and Yu 2010; Liu et al. 2017). Specific to LSD1, a tower domain is formed between the two AODs by two antiparallel helices that function as a binding site for the binding partners CoREST, MTA2/NuRD, AR and AML (Marabelli et al. 2016; Yang et al. 2017). Utilizing a redox reaction, the mechanism results in the formation of an imine intermediate through FAD reduction and methyllysine oxidation. In order to produce the demethylated lysine, the imine intermediate is hydrolyzed to form a hemiaminal that breaks down to form an amine and formaldehyde. However, as the mechanism requires the presence of a methyllysine nitrogen lone electron pair, demethylation is limited to mono- and di-methylated substrates (Smith and Denu 2009). Nevertheless, recognition and binding to tri-methylated substrates persist with greater affinity than favored mono- and di-methyllysine (Hou and Yu 2010). While similar to LSD1 in catalytic mechanism and active structure, LSD2 expresses slight differences in function, structure, and kinetics. Lacking the tower domain, thus expressing no interaction with CoREST, LSD2 modifies its substrate binding core through interaction with the protein NPAC/GLYR1 (Fang et al. 2013; D'Oto et al. 2016). Binding in close proximity to the active site, the putative oxidoreductase allows tighter binding of substrate N-terminal residues through enlargement of the interaction surfaces (Marabelli et al. 2016; Fang et al. 2013). Additionally, LSD2 has been reported to feature a zinc-finger domain (Marabelli et al. 2016) and favors binding to transcribed coding regions, unlike its LSD1 counterpart which favors promoter regions (Chen et al. 2017).

3.4 Jumonji Domain Demethylases

Part of the 2-oxoglutarate (2OG)—and ferrous iron (Fe^{2+}) oxygenase superfamily, the JmjC-KMDs comprise the larger, second family of demethylases (Kooistra and Helin 2012). Sharing the characteristic JmjC domain, consisting of a jellyroll like β-fold homologous to the cupin metalloenzymatic superfamily, the enzymes maintain structural integrity and substrate specificity through a series of structural elements further surrounding the domain. Buried at its core, the domain carries the catalytic domain, in addition to the Fe^{2+} and α-ketoglutarate (α-KG) binding sites

and three essential residues, H188, E190, and H276 (as found in KDM4D), which in combination with α-KG, aid in the coordination of Fe^{2+}. Unlike their counterparts, JmjC-KDMs carry out an oxidative decarboxylation and hydroxylation reaction with their associated co-factors, α-KG, and Fe^{2+} (Fig. 1; Klose et al. 2006; Chen et al. 2006a, b). This leads to the production of an unstable hemiaminal intermediate, which then breaks down to produce the demethylated substrate and formaldehyde. As the mechanism lacks the requirement of lone pair electrons, demethylation of tri-methylated substrates by the majority of JmjC enzymes is possible. During the reaction, binding to the methyllysine substrate occurs through a distinct mechanism that involves the formation of a hydrogen bond network between the oxygen atoms of the catalytic residues and the methyl groups of the substrate (Hou and Yu 2010). This non-classical methyl-binding domain allows correct positioning of the tri-methylated substrate to the Fe^{2+} cofactor, allowing ideal conditions for reaction. Due to the reduced size of the mono- and di-methylated substrates, the formed hydrogen bonds separate the Fe^{2+} from the methyl groups limiting catalytic reaction. However, through rotational movement of the di-methylated substrates, interaction with Fe^{2+} becomes possible, allowing the demethylation reaction to occur. In the case of mono-methylated substrates, rotational movement produces no changes in orientation preventing their demethylation by some family members, such as KDM4A (Ng et al. 2007; Cloutier et al. 2014). However, due to steric hindrance from space limitation at the active core, other family members such as PHF8 and KDM7A express substrate specificity towards di-methylated lysine solely, while similar limitations as those associated with KDM4A govern their recognition of mono-methylated substrates. These slight differences in the JmjC core not only govern the substrate specificity of the enzymes, but also allow for their subdivision based on homology of the catalytic core (Horton et al. 2010; Yang et al. 2010). In addition to the characteristic JmjC domain, the majority of members possess other functional domains, including MBDs such as PHD, Tudor, as well as protein interaction F-box and TPR domains and DNA binding domains BRIGHT/ARID and Zn^{2+} fingers that further aid in substrate specificity, family subdivision and recruitment of the enzymes to specific loci (Klose et al. 2006).

Although the discovery of the KDMs helped establish lysine methylation as a dynamic process (Biggar and Li 2015), reports of arginine demethylases (PRDMs) are limited and often controversial. While in recent years the JmjC family member JMJD6 has been reported to express PRDM activity (Poulard et al. 2014); such functions for the enzyme remain unconfirmed as equal reports express lack of PRDM activity (Walport et al. 2016).

4 Non-Histone Methylation: Functional Methylation and Regulation of Cellular Processes

Kenneth Murray for the first-time reported lysine methylation on bovine histone proteins of mammals (Murray 1964). Following this discovery, most research on lysine methylation had followed suit and focused on histone methylation due to its

clear importance in chromatin biology and gene regulation. Until recently, research on non-histone lysine methylation was limited as there were no strategies to identify lysine methylation across the entire proteome. Starting in 2013, several research groups developed techniques to identify methylated proteins (Cao and Garcia 2016; Carlson and Gozani 2016; Liu et al. 2013). These proteomic studies have each revealed several hundreds of new methylated proteins and lysine residues. As a result, there is now an abundance of evidence demonstrating that, in addition to histones, lysine methylation also occurs on various non-histone proteins that are important for signal transduction events and epigenetic regulation of transcription and chromatin in eukaryotes.

Since its discovery in 1959, the role of histone and non-histone methylation has not only advanced our understanding of cellular regulation, but has also provided new target substrates for the development of therapeutics (Arrowsmith et al. 2012). The last decade has seen great advancement in substrate identification, enzyme characterization and functional characterization, such as those methylation events associated with the functional regulation of the p53 tumour suppressor protein (Scoumanne and Chen 2008). However, further research is still necessary to gauge the breadth of the methyllysine proteome and the cellular roles that it fulfills.

While the role of lysine methylation in histones was already being elucidated, in 1998 the discovery of methylation of RNA binding proteins (RBPs) began to expand the scope of protein methylation (Brahms et al. 2001). The methylation of RBPs was shown to have a regulatory role in ribonucleoprotein (RNP) assembly, pre-mRNA splicing, and mRNA stability. An important function of lysine methylation in the p53 tumor suppressor protein has been observed (Chuikov et al. 2004). In particular, SET domain-containing protein 7 (SETD7)-dependent methylation of lysine in p53 resulted in enhanced transcriptional activity, nuclear stability as well as apoptosis (Fig. 3). Subsequent studies revealed that p53 could function as an activator or repressor in response to the methylation of four other lysine and three arginine residues (Huang et al. 2006). In 2007, it has been found that p53 could also be demethylated and is a reversible protein modification. Specifically, LSD1 demethylated lysine K370 di-methylation thereby disrupting the methyl reader abilities of p53-binding protein 1 (53BP1) and decreasing its transcriptional activity (Huang et al. 2007). This discovery started a segue into the dynamic lysine methylation of non-histone proteins, a PTM with functional implications existing beyond epigenetics and chromatin organization.

Although the methylation of histone proteins have comprised the majority of lysine methylation research, the lysine methylation of non-histone proteins is also being realized to facilitate critical roles in the regulation of cellular stress, cell proliferation, and angiogenesis. A prototypical example includes the methyl-regulation of non-histone substrates by the SMYD3 KMT enzyme, which has been reported to have a significant role in oncogenic cell proliferation. The first insights into SMYD3 methylation of non-histone proteins were reported in 2007 (Kunizaki et al. 2007). They revealed that SMYD3 was able to methylate the vascular endothelial growth factor receptor 1 (VEGFR1) at lysine K831, a conserved residue located within the tyrosine kinase domain and proposed to regulate VEGFR1

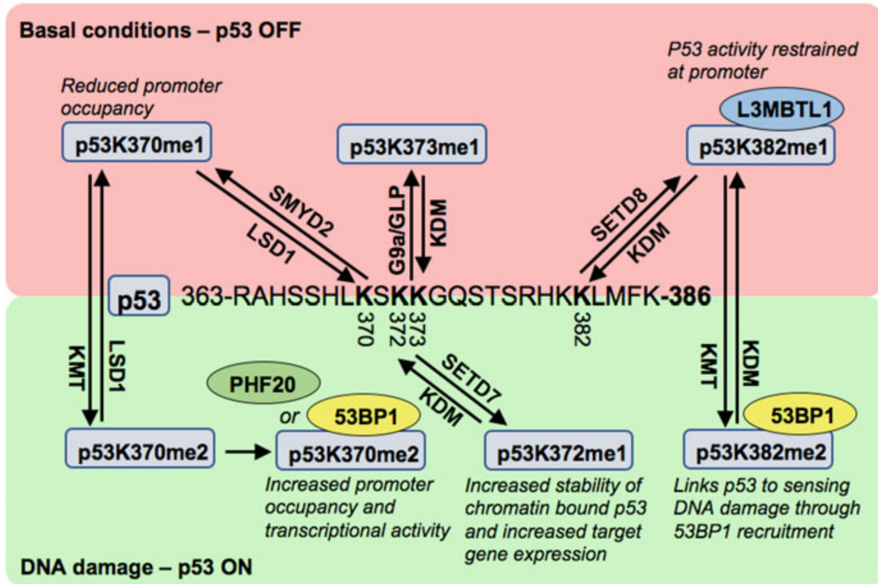

Fig. 3 Control of p53 signaling network through dynamic lysine methylation. Lysine methyltransferase enzymes (KMTs) methylate p53 at several C-terminal locations, acting to differentially activate or inhibitor p53 transcriptional activity and/or signaling. Red shading indicates methylation events that are known to negatively influence p53 activity, whereas green indicated methylation events that are currently thought to promote p53 signaling in response to periods of DNA damage

kinase activity. Following this discovery, it was shown that SMYD3 mediated the methylation mitogen-activated protein-3 kinase2 (MAP 3K2) at lysine K260 to promote ERK1/2 signaling (Mazur et al. 2014). These events increased Ras signaling leading to increased cell proliferation and pancreatic tumorigenesis in an SMYD3-dependent manner.

4.1 Control Over p53 Transcriptional Activity by Combinatorial Methylation Signals

The complexity of non-histone protein methylation in the regulation of protein function can be highlighted by the regulation of p53 by SET domain-containing KMTs (Fig. 3). The p53 tumor suppressor is currently known to be differentially regulated by a number of different KMT and KDM proteins (West and Gozani 2011). The function of p53 is controlled through at least four C-terminal lysine methylation sites, including K370, K372, K373, and K382. Collectively, these methylation events are controlled through the combined action of five KMTs, which include mono-methylation by SETD7 (K372me1) (Chuikov et al. 2004), SETD8 (K382me1) (Shi et al. 2007) and

SMYD2 (K370me1) (Huang et al. 2006), and di-methylation by G9a/GLP (K373me2) (Huang et al. 2010) KMT enzymes. Importantly, there still remains several methylation events with the direct implication in the regulation of p53 function with yet to be identified KMTs. For example, the di-methylation of p53 at K370 (i.e., p53K370me2) creates an interaction site for the tandem Tudor MBDs within 53BP1, increasing p53 promoter occupancy and increasing p53-dependent transcript of target genes. Although the KMT responsible for this di-methylation event at K370, it can be dynamically removed by the demethylase action of the LSD1 KDM, returning the K370 site back to mono-methylation status and resetting p53 activity by preventing the di-methylation-dependent 53BP1 association (Huang et al. 2007). This creates a simple 'switch'-like system that yields control of protein activity through the opposing action of KMT and KDM enzymes, however, this system fails to present the complexity of the overall methyllysine-regulatory system that acts to influence p53 transcriptional activity and cell fate.

Expanding upon the example of dynamic p53K370me2 methylation in the control of p53 transcriptional activity, several other methylation sites within p53 also exert regulatory influence over p53. For example, the mono-methylation of K370 by SMYD2 has been shown to be a methylation status correlated with reduced p53 promoter occupancy and lower p53 activity (Huang et al. 2006). Similarly, the mono-methylation modification imparted by SETD8 at K382 has been shown to restrain transcriptional activity at promoter sites through the mono-methylation-dependent interaction with the MBT MBD domains (3xMBT) of the L3MBTL1 protein (West et al. 2010). In contrast, nuclear mono-methylation at K372 by SETD7 has been shown to enhance activity through the stabilization of chromatin-bound p53 and has also been linked with the promotion of p53 acetylation in response to periods of cellular DNA damage (Chuikov et al. 2004). Lastly, the di-methylation of K373 by G9a/Glp has been classified as an inhibitory mark, reducing p53 activity in a methylation-dependent manner (Huang et al. 2010). Overall, p53 is an intriguing example of how dynamic lysine methylation events can exert regulatory control, how these methyl-modifications are sensed by MBD-containing proteins (such as 53BP1 and L3MBTL1), and how the act to modulate p53 function.

4.2 HIF Regulation by Dynamic Lysine Methylation

Research in recent years have begun to outline an important role for lysine methylation in tumorigenesis (Hamamoto et al. 2015), however much remains unknown regarding the mechanisms of which methylation mediates the initiation and progression of such diseases. As the microenvironment of malignant solid tumors is characterized by insufficient oxygen delivery, investigation of oxygen deprivation on the regulation of disease-relevant methylation events is essential for developing enhanced combination therapeutic strategies. Identified as a biomarker of a number of different carcinomas, interest in understanding how the LSD1 KDM contributes to cancer development has gained over the years as we continue to uncover its repertoire

of non-histone substrates (Nagasawa et al. 2015). Following di-methylation at lysine K271, the receptor of activated protein C kinase 1 (RACK1) mediates ubiquitination-mediated degradation of the low oxygen sensor, hypoxia-inducible factor 1 alpha (HIF-1α), in an oxygen-independent manner through the RACK1-Hsp90 pathway (Yang et al. 2017). This di-methylation modification at K271 mediates the RACK1-HIF-1α interaction, an interaction that facilitates HIF-1α degradation. However, under hypoxic conditions LSD1 is reported to demethylate the K271 residue in RACK1, diminishing the methyl-dependent interaction between RACK1 and HIF-1α. In contrast, the activity of LSD1 deceases under chronic hypoxia as the biosynthesis of the FAD co-factor decreases. Characteristic in triple-negative breast cancer (TNBC), is a greater LSD1 gene expression, in addition to an altered FAD biosynthetic gene, has been shown to provide insight into patient prognosis. As a result, TNBC patients with increased LSD1 activity were found to correlate with a poor prognosis (Nagasawa et al. 2015; Marabelli et al. 2016).

Similar to the rather complex methyl-regulation that associated with p53 function, various lysine residues on HIF-1α have been found to be subject to dynamic methylation and demethylation, several with documented impact on HIF-1α cellular function. For example, both the SETD7 KMT and the LSD1 KDM have been found to work together to mediate the methylation of lysine K32 and K391 (Liu et al. 2015; Kim et al. 2016a, b; Lee et al. 2017). Occurring primarily within the nucleus, the K32 methylation is subjected to increase methylation under normoxia and prolonged hypoxia, while increased demethylation is observed during the early hypoxic transition (Liu et al. 2015). This methylation site has been proposed to regulate HIF-1 stability under normoxia and during late hypoxia, when activity is minimal. Speculated as a recruitment signal for an unknown E3 ligase, this methylation event is thought to function as a fine-tuning mechanism modulating “leaky pools” of remaining HIF-1 proteins under normoxia and late hypoxia. As such, it is theorized that remaining pools of HIF-1α that avoid cytosolic degradation undergo SETD7-mediation methylation once localized to the nucleus, leading to their ubiquitination induced proteasomal degradation (Kim et al. 2016a, b; Baek and Kim 2016).

4.3 Dynamic Lysine Methylation of FOXO Protein

The activity of the FOXO subfamily of transcriptional factors has been shown to be largely mediated through a number of different PTMs. In addition to the currently known regulatory phosphorylation, acetylation, ubiquitination, and arginine methylation PTMs that are associated to occur within the FOXO3a protein, methylation of lysine K270 by SETD7 has been shown to mediate oxidative stress-induced apoptosis (Xie et al. 2012). Interestingly, once methylated by SETD7, FOXO3a does not show any change in protein stability, localization, or other PTMs/interactions associated with its normal signaling pathways (i.e., PI3K/Akt) (Zhu 2012). Instead, methylation by SETD7 has been found decreasing the DNA-binding capability of FOXO3a, thus preventing expression of its target gene, *Bim*, a BH3-only

protein. Due to the conserved nature of the K270 methylation site within the FOXO family, other family members, such as FOXO1, have also been shown to undergo SETD7 mediated methylation at their respective corresponding lysine residues; however, the functional outcome of this conserved methylation event has yet to be reported (Xie et al. 2012).

4.4 DNA Damage Repair Signaling Cascade

As the most important bio-macromolecule in the cell, DNA is subject to damage induced by ionizing radiation, UV and other chemical environmental agents which induce double-strand breaks (DSBs). If this damage is not repaired in a timely fashion, this damage can signal cellular autophagy (controlled digestion of damaged organelles within a cell), apoptosis (programmed cell death), aging and can result in the progression of cancer. Therefore, upon the detection of DNA damage, it is necessary for the cell to immediately identify any DSB and initiate appropriate repair mechanisms. To accomplish this, eukaryotes have two major pathways to repair damaged DNA: (a) homologous recombination repair (HRR) and (b) non-homologous end joining (NHEJ) (Ciccia and Elledge 2010). The tumor suppressors 53BP1 and BRCA1 are the two factors that are enriched at sites of DSBs and are emerging as pivotal regulators of repair by either NHEJ and HRR, respectively. DSBs that occur within G1 phase of the cell cycle are repaired by NHEJ. Repair is initiated through the recruitment of the Ku70-Ku80 heterodimer, followed by ATM-related DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Importantly, DNA-PKcs is responsible for maintaining the broken DNA ends within close proximity to each other, which is beneficial for recruiting end processing factors followed by re-ligation by DNA ligase complex. Previous studies have shown that DNA-PKcs undergoes active lysine methylation at K1150, K2746, and K3248 in response to DNA damage, and that loss of these methylation events impact repair capacity (Liu et al. 2013). Furthermore, the methyllysine interactions of the chromo MBD of heterochromatin protein (HP)1β are enriched with proteins involved in DNA damage repair (DDR), suggesting a central role for HP1β and methyl-dependent interactions in DDR. In this model, the HP1β chromo MBD interacts with DNA-PKcs in a methyllysine-dependent manner and regulates DNA-PKcs function in response to DNA damage.

On the other hand, HRR pathway is activated in response to DNA damage on S/G2 phase of the cell cycle. MRE11-Rad51-NBS1 (MRN) complex binds to the broken DNA ends followed by recruitment of CtIP (C-terminal binding protein interacting protein) and several nuclease machines to promote high throughput process of DNA end resection. Replication protein A (RPA) coat the generated 3′ ssDNA following resection. RAD51 displaces RPA to form a RAD51-ssDNA nucleofilament induced by BRCA1-PALB2-BRCA2 complex and RD51 paralogs. Finally, RAD51 nucleofilament searches for the complementary DNA template in the genome to synthesize and synapse to form a mature recombination product. HRR pathway is critically important for the cells to regulate normal cell behaviour. Mutations in the signature proteins of the pathway (BRCA1/2-PALB2-RAD51),

fuel cancer and chemoresistance where cell loses the choice between the two pathways and continue repair with error prone NHEJ.

Emerging evidence indicate that the lysine methylation of histone and non-histone proteins can play important role in determining the repair pathway of choice, whether the cell should undergo HRR or NHEJ repair (Chen and Zhu 2016). Differentially methylated lysine on histone and non-histone proteins are currently thought to serve as the docking sites for HRR or NHEJ-related proteins, influencing the signaling of a particular repair pathway. For example, tri-methylated H3K36 is required for HR repair, while di-methylated H4K20 have been shown to recruit the 53BP1 for NHEJ repair (Ng et al. 2009; Freitag 2017). In recent years, it has become increasingly clear that methylation entails remodeling chromatin, and plays a major role in regulating DDR singling cascade which is quite obvious in disease like cancer.

4.5 Lysine Methylation and Disease

Lysine methylation on histone tails is a common PTM and is pivotal in the regulation of chromatin structure and gene transcription, spanning from growth and proliferation in physiological and pathological conditions such as cancer and neurodegenerative diseases (Esteller 2007; Greer and Shi 2012; Hamamoto et al. 2015). For example, an up-regulated expression of SMYD2 in oesophageal squamous cell carcinoma and bladder cancer cells has been observed (Cho et al. 2012), and further, an overexpressed SMYD3 in breast carcinoma has been shown to correlate with tumor proliferation (Hamamoto et al. 2006). Additionally, the KMT G9a is overexpressed in hepatocellular carcinoma and contributes to the invasiveness of lung and prostate cancer (Casciello et al. 2015). Correspondingly, lysine methylation has been reported to influence oncogenic pathways and hence provides a rationale for the involvement of KMTs in cancer.

SETD8 (also known as KMT5A), member of the SET domain family known to catalyze the mono-methylation of histone H4K20 (Nishioka et al. 2002). This methylation event is believed to be necessary in the methylation-dependent recruitment of signalling proteins like 53BP1 to site of double-strand DNA breaks (Dulev et al. 2014), or state of chromatin compaction (Lu et al. 2008; Jørgensen et al. 2007). SETD8 has also been reported to have implications in breast cancer through the dynamic methylation of Numb protein at lysine K158 and K163 (Dhami et al. 2013). Normally, the Numb protein exhibits tumor-suppressive ability through a direct with p53, stabilizing and promoting p53 transcriptional activity and cellular apoptosis. Interestingly, this stabilizing interaction with p53 is dynamically disrupted through the tandem methylation of Numb at K158 and K163 within its phosphotyrosine binding domain (PTB); the domain responsible for recruitment and p53 binding. Following the treatment of breast cancer cells with a chemotherapeutic agent (doxorubicin), the expression of SETD8 was found to be significantly reduced, decreasing Numb methylation and enhancing Numb-p53 mediated cellular apoptosis. Collectively, this work demonstrated SETD8-mediated Numb-p53 interaction as an important regulatory axis in breast cancer, and further highlighting one of the

currently known roles that methyllysine facilitates in normal and disease cell biology.

Intriguingly, lysine methylation also has been proposed to play a role in bacterial pathogenicity. Vaccination efforts against typhus' agent *Rickettsia typhi* target the immunodominant antigen OmpB (Chao et al. 2004, 2008). The chemical methylation of lysine residues re-establishes serological reactivity of the OmpB fragment on a recombinant peptide (Chao et al. 2004). *Mycobacterium tuberculosis* adhesions (HBHA and LBP) important for adhesion to host cells are also heavily methylated (Biet et al. 2007; Delogu et al. 2011). Contemporary, methylation of *P. aeruginosa* Ef-Tu at K5 has been reported to mimic the ChoP epitope of human platelet-activating factor (PAF) further allowing association with PAF receptor and contributes to bacterial invasion and pneumonia onset (Barbier et al. 2013).

Taken together such findings demonstrate the infancy of the lysine methylation field on methyl-regulation function outside of epigenetics and chromatin biology. As a result, a number of questions still remain to be answered. For example, how many substrates do methyl-modifying enzymes regulate, and how expansive are the lysine methylation proteome and the cellular processes that it influences?

5 Methyllysine Proteomics: Methods to Discover Lysine Methylated Protein

Within the last decade of lysine methylation research, we have begun to define new and complex roles for this modification. Such cellular functions for this modification now include the facilitation of crosstalk between signaling cascades and connecting cellular signaling to nuclear effectors and chromatin regulation. Despite the rapid growth in our understanding of the function of lysine methylation, the field of lysine methylation has historically experienced limited growth as a result of a lack of suitable identification technology. Arginine has not experienced the same stunted growth as the identification of arginine methylation sites has been facilitated through the use of methylarginine-specific antibodies, enriching for arginine methylated proteins to be mapped and identified by mass spectrometry (Guo et al. 2014). In contrast, it has been difficult to develop suitable methyllysine-specific antibodies that are able to enrich for the lysine methylation modification without a high degree of non-specific interaction for unmodified protein. As a result, the identification and mapping of new lysine methylation sites have not undergone the same growth as that of arginine methylation.

5.1 Immunoaffinity-Based Annotation of Lysine Methylation Events

Initially, efforts towards the global identification of lysine-methylated proteins utilized methyl-specific antibodies for the initial enrichment of methylated peptides

prior to mass spectrometry-based detection and analysis. Although the first studies utilizing this approach were only able to identify several lysine methylation sites on histones H3 and H4, recent advancements in the development antibodies that display higher specificity towards methyllysine have begun to overcome the technical issues that previously plagued enrichment and identification. As methylation exists as a relatively small uncharged protein modification, it has been difficult to develop antibodies that do not suffer from low affinity and poor specificity, or that do not maintain specificity for the amino acid sequences surrounding the modified lysine. To overcome these technical issues, several labs have worked towards the development of methyllysine-specific antibodies with affinity and specificity appropriate to be used in methyllysine identification by immunoaffinity purification followed by tandem mass spectrometry (IP-MS/MS) (Fig. 4a). For example, one study utilized a panel of antibodies each specific against either mono-, di-, or tri-methylated lysine (Cao et al. 2013). They used these antibodies for immunoaffinity of trypsin-digested lysine-methylated peptides to be used for mass spectrometry, identifying 323 mono-methylation, 127 di-methylation, and 102 tri-methylation lysine modification sites within 413 proteins. Importantly, this study documented that it is possible to develop and utilize methyllysine-specific antibodies to be used in the IP-MS/MS identification of new lysine methylation sites.

5.2 Methyl-Binding Domains for the Identification of Methyl-Directed Protein Interactions

Although the use of antibody-based enrichment methods has begun to provide significant growth in the number of lysine-methylated sites that exist in the human proteome, a number of studies have begun to use MBDs for methyllysine enrichment. The use of these MBDs (such as the chromo, PHD, MBT, PWWP, WDR and Tudor domains) provide a means of natural methyl-specific affinity as a mechanism to enrich for lysine-methylated peptides prior to identification by mass spectrometry (Fig. 4b). This method has been successful in the mapping of the methyllysine proteome on a large scale by several labs (Liu et al. 2013; Carlson et al. 2014). As methyl-specific antibodies cannot provide information of direct physical interactions that may occur in the cell, this approach has been utilized for the mapping of methyl-depended complexes with MBDs, a collection of interactions referred to as the methyl-interactome (Liu et al. 2013). For example, Liu and colleagues use the chromo MBD from the HP1b protein to identify 29 methylated proteins. The associated HP1b methyl-interactome included a group of 14 proteins involved in the DNA damage response (including the aforementioned methylation of DNA-PKcs at lysine K1150 necessary for DNA-damage repair from Sect. 4.4), a cluster of 39 proteins involved in RNA splicing, and a group of eight ribosomal proteins (Liu et al. 2013). Another study successfully utilized the triple modular MBT domains (3xMBT) from the L3MBTL1 protein in an attempt to purify methyllysine modified proteins with

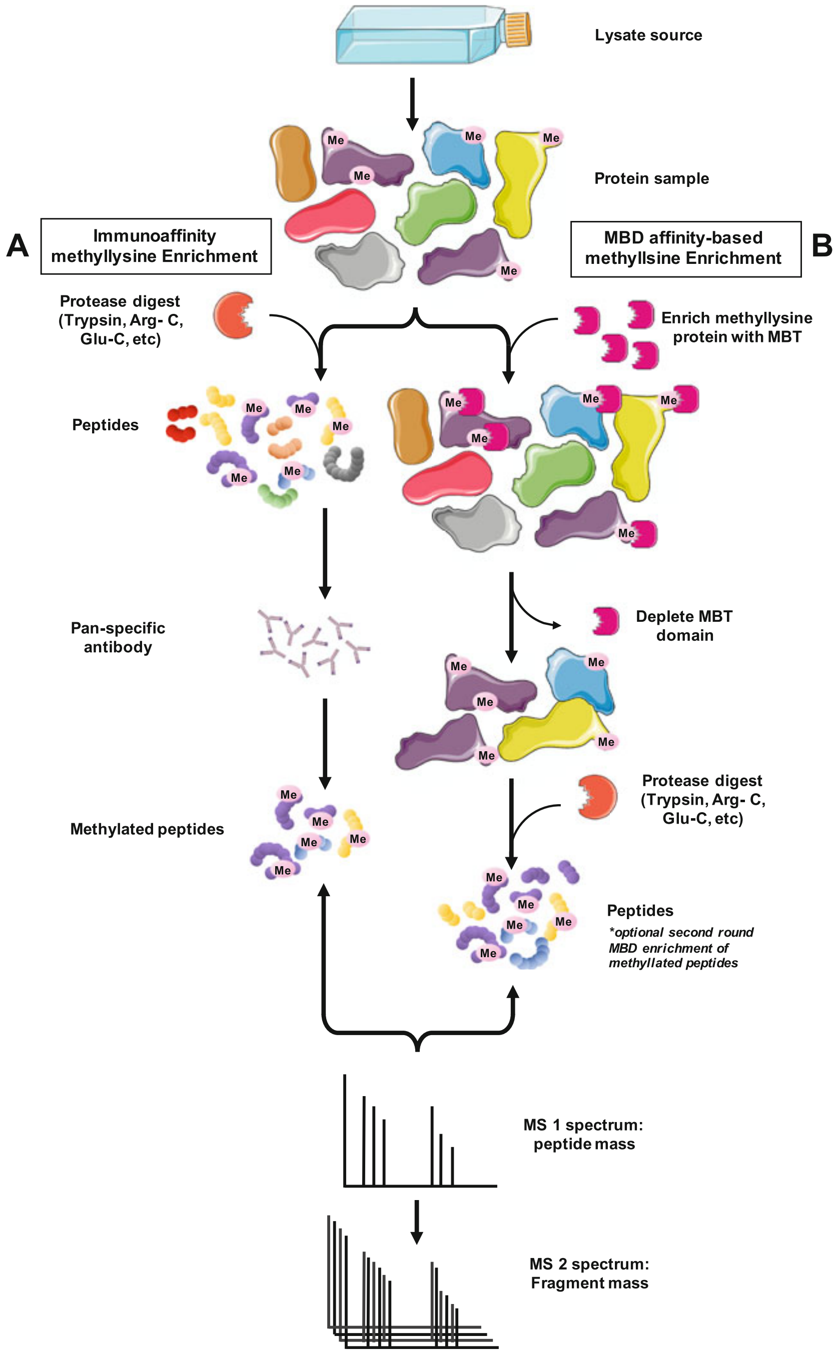

Fig. 4 Identification of new methylated proteins. (**a**) Following protein isolation, peptide fragments are obtained through a digestion by specific proteases. Methylation-specific antibodies are then used to isolated methylated peptide fragments from their unmodified counterparts, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for identification.

little specificity for amino acid sequence neighboring the modified lysine residue (Carlson et al. 2014). Collectively, these two studies demonstrate the utility of using MBDs for the enrichment and annotation of lysine methylation sites, providing a deeper understanding of how methylation can integrate into broader biological processes through methyl-dependent protein interactions with MBD.

5.3 Computational Predictions the Methyllysine Proteome

As previously mentioned, one of the largest challenges placed on the discovery of lysine methylated proteins, has been limitations in identification technology. However, the development of new *in silico* prediction resources hold the promise of aiding in the initial annotation of methyllysine on a proteome-wide scale. Although several affinity strategies that utilize commercial antibodies and natural MBDs (see above) have been remarkably successful in the identification of new lysine methylation events when coupled with mass spectrometry, these approaches are inherently biased towards the binding specificity of the protein used for enrichment. *In silico* prediction methods help to overcome this issue by predicting methylation events based on general underlying characteristics of known modified proteins.

During the past decade several attempts for developing methyllysine and methylarginine predictors have appeared in the scientific literature (Chen et al. 2006a, b; Hu et al. 2011; Qiu et al. 2014; Shao et al. 2009; Shi et al. 2012, 2015; Shien et al. 2009). These studies developed their models from the information of methylated sites extracted, mostly, from databases such as UniProtKB, PhosphoSite-Plus, and PubMed, gathering in total few hundreds of methylated sites. Regrettably, a certain number of deficiencies in the preparation of these datasets have been identified (Qiu et al. 2014; Shi et al. 2015), limiting the reliability in some of the currently available predictors. Unfortunately, in almost all cases these predictors omit the effect of the existing imbalance between known methylation sites and those that are assumed not to be subject to methylation during the evaluation of the models. Such an approach leads to optimistic estimations of the errors in the larger class (not-methylated sites) consequently increasing the precision of their outcomes in the validations. Such balanced datasets during evaluation (validation) do not match the challenging imbalanced scenario that these methods have to face when are used in real-life datasets like the entire Human proteome.

In addition to this issue, these predictors have an inherent limitation that undermine their applicability and trust and should be highlighted. Existing predictors have been to an average of only 200 non-redundant methyllysine sites for building and

Fig. 4 (continued) (**b**) Methylated proteins can also be isolated by affinity purification through the use of specific, naturally occurring, modular methyl-binding domains (MBD). Methylated peptide fragments are obtained through protease digest and identified through LC-MS/MS

assessing their models, when the expected diversity of the sequence fragments carrying a methylated site can undoubtedly not be represented with such a few numbers of examples. For reference, as of 2018 the PhosphoSite database reports greater than 2000 Human methyllysine modification sites. The development of reliable *in silico* predictions of methylation does hold significant promise in its ability to annotate an initial enrichment dataset that could to be used to guide targeted mass spectrometry efforts. Future work will help determine how thoroughly MS identification experiments are able to probe the methyllysine proteome. It will also be critical to establish whether these identification technologies, either individually or used in conjunction with each other, will be able to provide a systems-level understanding of how lysine methylation impacts protein signaling, and how dynamic methylation acts to regulate protein, and cellular, function.

6 Summary

Although studies to date have already established that lysine methylation is a prevalent PTM occurring on non-histone substrates with diverse functional roles, it has become clear that we have only scratched the surface when it comes to delineating the complete breadth of the methyllysine proteome and the full spectrum of cellular and developmental processes that it regulates. Just how large is the methyllysine proteome? How is lysine modification dynamically controlled and coordinated in response to cell stimuli? These are critical questions that will be likely addressed in the near future, knowledge of which will provide a greater understanding of protein regulation and of the inner workings of cell biology.

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