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REVIEW

Emerging roles of lysine methylation on non-histone proteins

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Abstract Lysine methylation is a common posttranslational modification (PTM) of histones that is important for the epigenetic regulation of transcription and chromatin in eukaryotes. Increasing evidence demonstrates that in addition to histones, lysine methylation also occurs on various non-histone proteins, especially transcription- and chromatin-regulating proteins. In this review, we will briefly describe the histone lysine methyltransferases (KMTs) that have a broad spectrum of non-histone substrates. We will use p53 and nuclear receptors, especially estrogen receptor alpha, as examples to discuss the dynamic nature of non-histone protein lysine methylation, the writers, erasers, and readers of these modifications, and the crosstalk between lysine methylation and other PTMs in regulating the functions of the modified proteins. Understanding the roles of lysine methylation in normal cells and during development will shed light on the complex biology of diseases associated with the dysregulation of lysine methylation on both histones and non-histone proteins.

Keywords Lysine methylation · SETD7 · G9a · SMYD2 · p53 · ERα

Dynamic posttranslational modifications (PTMs) of histone proteins, especially those that occur on their N-terminal unstructured tails, are key to the regulation of chromatin dynamics [1]. These modifications, which include acetylation, methylation, phosphorylation and ubiquitination, serve as hallmarks of the chromatin state and gene expression status [2]. In general, acetylation is associated with gene activation, whereas methylation can be associated with either gene activation or gene silencing, depending on the residues being methylated. For instance, methylation of histone H3 lysine 4 (H3K4), H3K36 and H3K79 is typically linked to active gene expression, whereas di- and trimethylation on H3K9, H3K27, and H4K20 are usually associated with gene silencing [3–5].

Almost all of the enzymes that deposit and remove methyl groups from lysine residues were originally discovered as histone-modifying enzymes; therefore, they were named histone lysine methyltransferases (HKMTs) and histone lysine demethylases (HKDMs), respectively [5, 6]. As more examples of these enzymes acting on non-histone proteins were uncovered, they were renamed as lysine methyltransferases (KMTs) and lysine demethylases (KDMs) [7], respectively, to reflect their general roles in modifying proteins beyond histones. Like histone lysine methylation, non-histone lysine methylation facilitates interactions with specific readers to execute downstream signaling, and the crosstalk between lysine methylation and other PTMs also impacts the biological functions of the modified proteins. Therefore, the interactions of lysine methylation and other PTMs on non-histone proteins can

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be seen as extending the "histone code" to a more general "PTM code" that is likely to regulate all eukaryotic proteins. This review will describe the current knowledge of the KMTs and KDMs, including SETD7, G9a, and SMYD2, that have more known non-histone substrates than other enzymes, focusing on their role in the methylation of non-histone proteins. In the second part of this article, we will use p53 and nuclear receptors, especially ERα, as examples to discuss the roles and mechanisms of lysine methylation in regulating non-histone proteins.

The enzymes that act on non-histone substrates

Most known KMTs contain a catalytic SET domain, an evolutionarily conserved domain named after its founding members: *Su(var)3-9*, a *Drosophila* position-effect variegation suppressor gene, *Enhancer of zeste*, and *Trithorax* [8]. Based on primary amino acid sequence, domain architecture and histone substrate specificity, KMTs are classified into eight distinct subfamilies [9]. A handful of KMTs have been shown to methylate non-histone proteins, among which SETD7, G9a, SMYD2, SET8, and EZH2 have multiple non-histone protein substrates (Table 1). We will briefly describe these enzymes below.

Setd7

SETD7 (also known as KMT7 or SET7/9) is the only KMT7 family member due to its unique enzymatic activity and protein domain architecture. It was initially identified as a histone H3K4 mono-methyltransferase associated with transcriptional activation [10]. However, later studies revealed that SETD7 knockdown in cells did not alter histone H3K4 methylation, indicating that SETD7 does not play a major role in maintaining histone methylation [11]. Instead, SETD7 preferentially catalyzes the methylation of numerous non-histone proteins [12, 13] (Table 1). Structural and functional analysis revealed the minimal consensus sequence for SETD7 substrate recognition: K/R-S/T-**K** (**K** is the target lysine residue) [14]. The identification of this consensus sequence led to the discovery of over 90 putative non-histone substrates of SETD7 in mammalian cells [15]. To date, more than 30 non-histone proteins involved in diverse cellular processes have been confirmed as SETD7 substrates.

SETD7-mediated methylation of transcription factors and epigenetic regulators can lead to either gene activation or repression via modulating protein stability. For example, methylation of the tumor suppressor protein p53 and estrogen receptor α (ERα) by SETD7 stabilizes these proteins and is required for their activation [16, 17]. In contrast, SETD7-mediated methylation of transcription factors such as the RelA/p65 subunit of NF-κB and E2F1, or the DNA methyltransferase 1 (DNMT1) reduces their protein stability and impairs function [18–21]. The functional outcome of lysine methylation is also site-specific: SETD7-mediated methylation of different lysine residues within the same protein is linked to distinct biological functions. For example, SETD7 methylates the FoxO transcription factor 3 (FoxO3) at two residues, K270 and K271. Methylation at K270 represses its DNA-binding activity and transactivation [22], whereas K271 methylation promotes its transcriptional activity, but leads to decreased protein stability [23].

SETD7 can also modulate its substrates' functions without affecting protein stability. For example, SETD7 methylates androgen receptor (AR) and enhances AR-mediated transactivation. This methylation facilitates the inter-domain communication and the recruitment of AR to chromatin, while the overall AR protein level is not affected [24, 25]. Likewise, SETD7-mediated methylation of farnesoid X receptor (FXR) stimulates its transcriptional activation activity without altering protein stability [26].

Methylation of proteins by SETD7 can also influence protein–protein interaction. SETD7 methylates the tumor suppressor retinoblastoma protein (Rb) and regulates Rb-dependent cellular functions through modulating protein–protein interactions. SETD7-mediated methylation of Rb at K873 promotes its interaction with the heterochromatin protein HP1, whereas methylation of K810 impedes Rb binding to cyclin-dependent kinases [27, 28]. Similarly, SETD7 methylates TAF10, a component of the general transcription factor TFIID complex. This modification enhances the interactions between TAF10 and RNA polymerase II during the formation of the transcription pre-initiation complex [29]. In addition, another component of the TFIID complex TAF7 is also a putative target of SETD7 [14], suggesting that non-histone protein methylation may be a general means of transcriptional control that extends beyond the TAF10–RNAPII interaction. Finally, SETD7-mediated protein methylation may also regulate protein–RNA interaction. SETD7 methylates the HIV transactivator Tat protein and this methylation enhances the interaction between Tat and the HIV trans-activating response RNA element [30, 31].

In addition to the direct modification of transcription factors, SETD7 also modifies epigenetic regulators and transcription cofactors such as histone-modifying enzymes. For instance, SETD7 methylates SUV39H1 at two residues (K105 and K123), and this methylation inhibits the H3K9 methyltransferase activity of SUV39H1 leading to heterochromatin relaxation and genome instability [32]. SETD7 methylates poly-ADP-ribosyltransferase 1 (PARP1) and triggers its recruitment to DNA damage sites [33]. It has also been reported that SETD7 methylates the

Table 1 Lysine methyltransferases and their histone and non-histone substrates in humans

KMTs	Other names	Substrates and modified lysine residues					
		Histones	References	Non-histone proteins	In vitro	In cells	References
SETD7	KMT7, SET7/9	H3K4me1	Nishioka et al. [10] and Dhayalan et al. 15]	p53 K372me1	Protein MS	WB	Chuikov et al.[16]
				ERα K302me1	Peptide MS	WB	Subramanian et al. [17]
				MYPT1K442me1	Protein MS	Auto-rad	Cho et al. [104]
				RelA K37me1, K314me1 and K315me1	K37: peptide MS K314/315me1: protein MS	K37me1: WB; K314/315me1: Pan-Me WB	Link et al. [18] and Yang et al. [19]
				DNMT1 K142me1	Protein Auto-rad	WB	Esteve et al. [20]
				E2F1 K185me1	Protein Auto-rad	WB and Auto-rad	Kontaki and Talianidis [21]
				FoxO3 K270me1, K271me1	Peptide MS	IP and WB	Xie et al. [22] and Calnan DR et al. [23]
				AR K630me1, K632me1	K632: peptide MS K630: Auto-rad	OE, IP and Pan-Me WB	Ko et al. [24] and Gaughan et al. [25]
				STAT3 K140me2	Protein Auto-rad	WB	Yang et al. [106]
				TAF7 K5me	Protein Auto-rad	N/A	Couture et al. [14]
				TAF10 K189me1	Protein Auto-rad	IP and Pan-Me WB	Kouskouti et al. [29]
				Rb K873me1, K810me1	K873me1: Auto-rad K810me1: protein MS	IP and WB	Munro et al. [27] and Carr et al. [28]
				HIV Tat, K51me1	Protein MS and Auto-rad	OE, IP and WB	Pagans et al. [30] and Pagans et al. [31]
				FXR K206me	Peptide Auto-rad	IP and Pan-Me WB	Balasubramaniyan et al. [26]
				PARP1 K508me1	Peptide Auto-rad	OE, IP and WB	Kassner et al. [33]
				Yap K494me1	N/A	IP and Pan-Me WB; MS	Oudhoff et al. [35]
				IFITM3 K88me1	Protein WB	OE and WB	Shan et al. [36]
				PCAF K89me1	Protein Auto-rad	IP and WB	Masatsugu and Yamamoto [34]
				SUV39H1 K105me and K123me	Protein Auto-rad	WB	Wang et al. [32]

Table 1 continued

KMTs	Other names	Substrates and modified lysine residues					
		Histones	References	Non-histone proteins	In vitro	In cells	References
				AKA6, CENPC1, MeCP2, MINTme2, PPARBP, ZDH8me1, Cullin1, IRF1, and TTK	Peptide Auto-rad	OE and MS	Dhayalan et al. [15]
G9a	KMT1C, EHMT2	H3K9me2 H3K27me2 H1.4K26me2 H1.2K187me2 H3K56me1	Tachibana et al. [37], Trojer et al. [43], Weiss et al. [44], Wu et al. [45] and Yu et al. [46]	G9a K165me3 K239me3	Peptide Auto-rad	K165me3: WB K239me3: N/A	Sampath et al. [54] and Chin et al. [150]
				C/EBPβ K39me	Protein Auto-rad	N/A	Pless et al. [55]
				p53 K373me2	Protein Auto-rad	IP and WB	Huang et al. [56]
				MyoD K104me2	Protein MS	IP and WB	Ling et al. [57]
				Reptin K67me1	Protein MS	IP and WB	Lee et al. [58]
				MEF2DK267me2	Protein MS	IP and WB	Choi et al. [59]
				MTA1 K532me2	Protein WB	IP and WB	Nair et al. [60]
				CDYL1 K135me3, WIZ K1162me2; K305me/2/3, ACINUS K654me2/3, DNMT1 K70me2, G9a K185me2/me3, CSB, HDAC1, and KLF12	Peptide Auto-rad; CDYL, WIZ, G9a: protein MS	N/A	Rathert et al. [48]
				DNMT3A K47me2	Protein Auto-rad	IP and WB Protein MS	Chang et al. [145]
				SIRT1 K622me	Protein Auto-rad	N/A	Moore et al. [62]
SMYD2	KMT3C	H3K36me2 H3K4me1	Brown et al. [68]	p53 K370me1	Peptide Auto-rad	IP and WB	Huang et al. [70]
				Rb K860me1, K810me1	Peptide MS	K860: IP and WB K810: OE, IP and WB	Saddic et al. [71] and Cho et al. [75]
				HSP90 K209me1, K615me1	Protein MS	N/A	Abu-Farha et al. [73]
				HSP90 K531me, K574me1	Peptide MS	Auto-rad, K574: OE, IP and WB	Hamamoto et al. [146]
				ERα K266me1	Peptide MS, protein Auto-rad, protein WB	IP and WB	Zhang et al. [72]
				PARP1 K528me1	Peptide MS	OE, IP and WB	Piao et al. [74]
SETD8	KMT5A, SET8, PR-SET7	H4K20me1	Yin et al. [76]	p53 K382me1	Peptide MS, peptide Auto-rad, protein WB	IP and WB	Shi et al. [79]

Table 1 continued

KMTs	Other names	Substrates and modified lysine residues					
		Histones	References	Non-histone proteins	In vitro	In cells	References
				PCNA K248me1	Peptide MS, protein Auto-rad, protein WB	OE and WB	Takawa et al. [80]
				Numb K158me2, K163me2	Peptide MS	OE, IP and Pan-Me WB	Dhami et al. [81]
EZH2	KMT6	H3K27me3	Cao et al. [82]	GATA4 K299me1	Peptide MS and protein WB	IP and WB	He et al. [83]
				RORα K38me1	Peptide MS	OE, IP and WB	Lee et al. [84]
SETD1A	KMT2F, SET1A	H3K4me3	Lee and Skalnik [87]	HSP70 K561m2	N/A	IP and WB, IHC	Cho et al. [88]
SETDB1	KMT1E, ESET	H3K9me3	Schultz et al. [85]	HIV Tat K50me, K51me	Peptide Auto-rad	N/A	Van Duyne et al. [86]
SETD6		H2A.ZK7me1	Binda et al. [94]	RelA K310me1	Peptide MS and protein WB	WB	Levy et al. [95] and Chang et al. [96]
				PAK4	Protein Auto-rad	OE, IP and Pan-Me WB	Levy et al. [151]
				PLK1	Protein Auto-rad	OE, IP and Pan-Me WB	Levy et al. [151]
SMYD3	KMT3E	H3K4me3	Hamamoto et al. [89]	VEGFR1 K831me2	Protein WB	WB	Kunizaki et al. [90]
				MAP3K2 K260me1/2/3	Protein and peptide auto-rad	IP and WB	Mazur et al. [152]
NSD1	KMT3B	H3K36me2	Wang et al. [92]	RelA K218me1, K221me2	N/A	MS and WB	Lu et al. [93]
CaM KMT		N/A		CaM K115me3	Peptide MS	N/A	Magnani et al. [97]
VCP KMT	METTL21D	N/A		VCP K315me3	Peptide Auto-rad	MS and WB	Kernstock et al. [98] and Cloutier et al. [99]
METTL22		N/A		KIN K135me3	Protein Auto-rad	N/A	Cloutier et al. [99] and Cloutier et al. [101]
METTL21A		N/A		HSPA1 K561me3, HSPA8 K561me3, HSPA5 K585me3	Protein Auto-rad	HSPA1: WB; others: N/A	Wang et al. [99] and Jakobsson et al. [102]

Auto-rad autoradiography, *IHC* immunohistochemistry, *IP* immunoprecipitation, *MS* mass spectrometry, *N/A* not available, *OE* overexpression, *Pan-Me WB* Western blotting using Pan-methylation antibodies, *WB* Western blotting using site-specific methylation antibodies

p300/CBP-associated factor (PCAF), but the biological effect of this modification is unknown [34].

So far, we have presented several examples of how SETD7 can directly influence chromatin and transcription, but SETD7 also methylates proteins in the cytoplasm that are important for various cellular responses and pathways. SETD7 methylates Yes-associated protein (YAP), a Hippo pathway transducer to promote its cytoplasmic retention [35], as well as the interferon-induced transmembrane protein 3 (IFITM3) to reduce its antiviral activity [36]. Other non-histone substrates of SETD7 include AKA6, CENPC1, MeCP2, MINT, PPARBP, ZDH8, Cullin 1, IRF1, and TTK, all of which were identified by a peptide array methylation-based analysis [15]; however, the biological functions of these methylation events remain to be determined.

G9a

G9a (also known as euchromatin histone methyltransferase 2 [EHMT2] or KMT1C) and its homolog, G9a-like protein, a.k.a. EHMT1 and KMT1D (GLP), are the main enzymes contributing to H3K9 mono- and dimethylation (H3K9me1/2) at euchromatin for transcriptional repression [37–40]. G9a and GLP, which can form both homodimers and G9a/GLP heterodimers, are each required for the in vivo function of the other: G9a cannot compensate for the loss of GLP function and vice versa [41, 42]. In addition to its main target at H3K9, G9a was shown to also methylate H3K27, H3K56, and residues on certain histone H1 variants, including H1.4K26 and H1.2K187 [43–46]. G9a-mediated H3K56 methylation serves as a chromatin docking site for PCNA prior to its function in DNA replication [46], and H1.4K26 methylation provides a recognition surface for the chromatin-binding proteins HP1 and L3MBTL1, promoting H1 deposition and retention on chromatin [43, 47].

G9a is ubiquitously expressed in all cell types during development and plays an important role in various biological processes, including mouse embryo development, germ cell development, immune response, and brain function [48]. At the molecular level, G9a is essential for transcriptional repression, gene imprinting, provirus silencing, and DNA methylation [49–53]. G9a exerts most of these functions through the methylation of histone H3K9; however, this activity does not seem to be required in the regulation of DNA methylation in embryonic stem cells [42, 49, 51].

Besides methylating histones, G9a can also methylate a number of non-histone proteins (Table 1). The first identified non-histone substrate of G9a was G9a itself [54]. Automethylated G9a recruits HP1 to enhance the negative regulation of gene transcription. The other known non-histone substrates of G9a are mainly transcription factors. Through methylating these transcription factors, G9a suppresses target gene expression in addition to depositing the repressive H3K9me2 mark. G9a was shown to methylate C/EBPβ [55], p53 [56], and the myogenic regulatory factor MyoD [57] to inhibit their transactivation activity. Additionally, G9a also methylates the chromatin-remodeling factor Reptin [58], myocyte enhancer factor 2 (MEF2) [59], and metastatic tumor antigen 1 (MTAl) [60]. The G9a-mediated methylation on MEF2 and MTAl can be removed by LSD1/KMT1A, which is required for the precise control of their transcriptional activity in response to different signals [59, 60].

Given the wide array of G9a substrates, researchers sought to define a G9a methylation consensus sequence using a peptide array. The G9a substrate consensus sequence is composed of an R–K sequence flanked with a hydrophilic amino acid at position -2, a small amino acid at position -1, a hydrophilic amino acid at position +1, and a hydrophobic amino acid at position +2 [48, 61]. The identification of this motif led to the discovery of additional potential non-histone substrates of G9a, such as WIZ, CDYL1, CSB, ACINUS, HDAC1, DNMT1, KLF12 [48], and SIRT1 [62] (Table 1). However, it remains to be determined whether these proteins are bona fide G9a methylation targets in vivo.

G9a was previously found to be a coactivator of nuclear receptors, such as AR, ERα, and the glucocorticoid receptor (GR) as well as the osteoblast-specific transcription factor Runx2 [63–66]. The coactivator role of G9a has been shown to be largely methylation independent [67]. However, unpublished data from our group indicate that G9a-mediated methylation of ERα protein contributes to the positive regulation of ERα target gene expression. Overall, G9a functions as either a transcriptional corepressor or coactivator in a context-dependent manner. Future studies will be needed to determine how G9a makes the switch between histone and non-histone substrates during transcriptional control.

SMYD2

SMYD2 (also known as KMT3C) was initially identified as a member of the SMYD family of KMTs that contains a SET domain and a MYND motif. SMYD2 was shown to methylate both H3K36 and H3K4 and act as a transcriptional coactivator [68, 69]. Like SETD7 and G9a, SMYD2 can methylate diverse non-histone proteins in addition to histones (Table 1). The currently known SMYD2 non-histone substrates include p53, Rb, ERα, PARP1 and the chaperone protein HSP90 [70–74]. As opposed to the SMYD2-mediated methylation of histone proteins that leads to gene activation, SMYD2-mediated methylation of

non-histone proteins is normally associated with an inhibitory effect. For example, SMYD2 represses the transactivation activity of p53 through the methylation of p53K370, which negatively influences the SETD7-mediated p53-activating methylation of p53K372 [70]. SMYD2 inhibits the positive role of Rb in transcription by methylating RbK810 [75] and K860 [71]. In addition, SMYD2 inhibits ERα-mediated transcriptional activation. Recently, our group reported that SMYD2 monomethylates ERα at K266 in the hinge region to prevent ERα from being recruited to chromatin, thus suppressing transcriptional activation of its target genes [72].

Other KMTs that target non-histone substrates

In addition to the three KMTs discussed above, several other KMTs have also been reported to methylate some non-histone substrates (Table 1). The histone H4K20 mono-methyltransferase SETD8 (also known as PR-SET7 or KMT5A) [76–78] methylates p53 [79], PCNA [80] and the p53 associated tumor suppressor protein Numb [81]. Enhancer of Zeste Homolog 2, a.k.a. KMT6 (EZH2), the catalytic subunit of the Polycomb-repressive complex 2 (PRC2) that deposits the repressive H3K27me3 mark [82], can also methylate GATA4 [83] and the orphan nuclear receptor RORα [84]. Other examples include: methylation of the HIV Tat protein by the H3K9 methyltransferase ESET [85, 86], HSP70 methylation by the H3K4 methyltransferase SETD1A [87, 88], VEGFR1 and MAP3K2 methylation by the H3K4 methyltransferase SMYD3 [89–91], and RelA methylation by both the H3K36 methyltransferase NSD1 [92, 93] and SETD6 [94–96].

In addition to the SET domain-containing KMTs, there are other types of methyltransferases that can modulate non-histone proteins (Table 1). These enzymes include the calmodulin lysine N-methyltransferase (CaM KMT) and the methyltransferase-like (METTL) proteins. The calcium-binding messenger CaM is methylated at K115 by a class I, non-SET domain-containing protein methyltransferase, CaM KMT [97]. CaM KMTs and their catalytic activities on CaM are highly conserved across species from human to insect to plant. In addition to the class I methyltransferase signature domain, CaM KMTs possess unique flanking regions at their C- and N-termini that may dock the substrates' methylation sites into the enzymes' active sites for trimethylation. The METTL family contains METTL21D, METTL22, and METTL21A. METTL21D trimethylates K315 of VCP/p97, a type II AAA + ATPase. This methylation negatively affects various cellular functions of VCP/p97 including ubiquitin-dependent protein degradation [98–100]. METTL22 trimethylates KIN/kin17 at K135, which regulates the association of KIN with chromatin and possibly its functions in DNA repair, DNA replication and mRNA processing [99, 101]. METTL21A appears to act on the HSP70 family of chaperone proteins including HSPA1, HSPA8, and HSPA5 [99, 102]. Trimethylation of HSPA8 leads to a decrease in the interaction of this chaperone with α-synuclein, which likely has implications in the etiology of Parkinson's disease [102]. Together, these studies implicate protein methylation in regulating a broad range of cellular functions through direct impacts on non-histone substrates.

KDMs that act on non-histone proteins

There are only four KDMs known to demethylate non-histone proteins: LSD1 (a.k.a. KDM1A or AOF2), JMJD2A (a.k.a. KDM4A), FBXL1 (F-box and leucine-rich repeat protein 11, a.k.a. KDM2A), and PHF2 (Table 2). LSD1 antagonizes SETD7-mediated methylation on both histone H3K4 and various non-histone proteins including MYPT1, DNMT1, E2F1, p53, STAT3, and Tat [20, 21, 103–107]. LSD1 is also involved in the removal of methylation marks on ERα and AR that are mediated by KMTs other than SETD7 [72, 108–111]. Similar to the opposing functions of LSD1 and SETD7, the Jumonji domain-containing H3K9 and H3K36 demethylase JMJD2A [112, 113] appears to pair with G9a in modulating the methylation dynamics of various substrates including WIZ, CDYL1, CSB, and G9a itself [114] (Table 2). Another histone H3K36 KDM, FBXL11, opposes NSD1-mediated methylation on the RelA subunit of NF-κB [93, 115]; and the H3K9 demethylase PHF2 can act on its effector partner ARID5B [116, 117]. With time, it is likely that more non-histone KMT and KDM targets will emerge.

Non-histone proteins regulated by lysine methylation

As we discussed above, transcription factors such as p53, NFκB and several nuclear receptors are modified by lysine methylation. Here, we will describe the roles and mechanisms of these methylation events on non-histone proteins using p53 and ERα as examples. Lysine methylation on non-histone proteins is generally involved in the regulation of protein–protein interaction, protein stability, and in some cases, protein localization. Similar to methylation on histones, lysine methylation on non-histone proteins often occurs in regions that are also enriched in many other PTMs. Orchestration of each modification and crosstalk among them lead to distinct functional outcomes under different biological conditions. Thus, similar to their role in creating the "Histone Code" for epigenetic regulation of chromatin, lysine methylation and other modifications on non-histone proteins constitute a more general "PTM

Table 2 Lysine demethylases and their histone and non-histone substrates in humans

KDMs	Other names	Substrates and modified lysine residues					
		Histones	References	Non-histone proteins	In vitro	In cells	References
LSD1	KDM1A, AOF2	H3K4me2	Shi et al. [103] and Metzger et al. [109]	p53 K370me2	Peptide Auto-rad	WB	Huang et al. [105]
				DNMT1K142me1	Peptide Auto-rad	Auto-rad	Wang et al. [141]
				E2F1 K185me1	Protein Auto-rad	Auto-rad	Kontaki and Talianidis [21]
				MYPT1K442me1	Peptide MS	Auto-rad	Cho et al. [104]
				STAT3 K140me2	Protein Auto-rad	WB	Yang et al. [106]
				MEF2DK267me2	Protein WB	IP and WB	Choi et al. [59]
				MTA1 K532me2	Protein Auto-rad	N/A	Nair et al. [60]
				ERα K266me1	N/A	WB	Zhang et al. [72]
				HSP90 K209me1, K615me1	Protein Auto-rad	N/A	Abu-Farha et al. [73]
				HIV Tat K51me1	Protein WB	WB	Sakane et al. [107]
JMJD2A	KDM4A	H3K9me2/3, H3K36me2/3	Whetstine et al. [112] and Klose et al. [113]	G9a K185me3, WIZ K305me3, CDYL1 K135me3, CSB K170me3, K297me3, K448me3, K1054me3	Peptide MS	N/A	Ponnaluri et al. [114]
FBXL11	KDM2A	H3K36me2	Tsukada et al. [115]	RelA K218me1, K221me2	N/A	WB	Lu et al. [93]
PHF2	JHDM1E	H3K9me2/1	Wen et al. [117]	ARID5BK336me2	Protein WB	IP and WB	Baba et al. [116]

Auto-rad autoradiography, *IP* immunoprecipitation, *MS* mass spectrometry, *N/A* not available, *WB* Western blotting using site-specific methylation antibodies

code" that is likely to regulate most, if not all, eukaryotic proteins in diverse cellular processes.

p53

p53 is a well-known tumor suppressor that binds to specific DNA sequences and transcriptionally activates target genes that regulate several key cellular processes. These include cell cycle control, apoptosis and DNA repair in response to genotoxic stress [118]. p53 is probably the most studied non-histone protein that is regulated by lysine methylation. Interestingly, all known lysine methylation events on p53 occur in the C-terminal unstructured regulatory region of the protein, which mimics the N-terminal unstructured tail of histone H3 (Fig. 1).

Both the positions of the methylated lysine residues and the methylation states (e.g., mono or dimethylation) influence p53 functions (Fig. 1). For example, monomethylation of p53K370 (p53K370me1) by SMYD2 is linked to transcriptional inhibition [70]; whereas its dimethylation, by a yet unknown enzyme, is linked to p53 activation [105]. LSD1 demethylates p53K370me2 to p53K370me1 and thus can switch p53 from an active state to an inactive form [105]. Other KMTs that act on p53 are SETD7 and G9a/GLP. SETD7 monomethylates p53K372 whereas G9a/GLP dimethylates p53K373 [16, 56]. Like their contrasting roles in controlling transcription by methylating histone H3K4 to activate transcription or H3K9 to repress transcription, SETD7- and G9a/GLP-mediated methylation of p53 are associated with target gene activation and repression, respectively [16, 56]. Likewise, the H4K20 methyltransferase SETD8 monomethylates p53 at K382, which suppresses p53′s functions in checkpoint activation and apoptosis [79]. In addition, p53K386 can also be mono- and dimethylated, by enzymes that are yet to be identified [119].

As the methylated lysine residues of p53 are in close proximity, it is not surprising that modification on one residue can influence modifications on other residues. For example, the active mark of p53K372me1 deposited by SETD7 negatively impacts the repressive marks of SMYD2-dependent p53K370me1 [70] and the G9a-mediated p53K373me2 [56] (Fig. 2a, b). In addition to methylation, the lysine residues within the C-terminus of

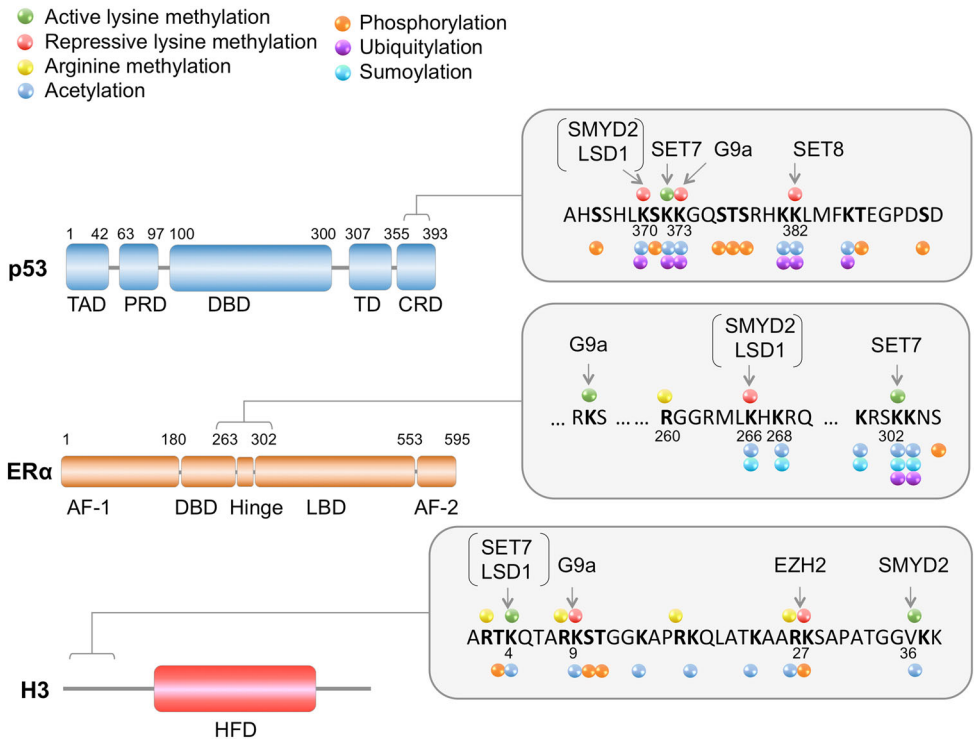

Fig. 1 Schematic representation of protein structure and posttranslational modifications on p53 and ERα. PTMs are represented by *colored dots* that are defined in the figure. Protein domains are abbreviated as follows: p53: *TAD* Transactivation domain, *PRD* proline-rich domain, *DBD* DNA-binding domain, *TD* tetramerization domain, *CRD* C-terminal regulatory domain; ERα, *AF-1* activation function domain 1, *DBD* DNA-binding domain, *LBD* ligand-binding domain, *AF-2* activation function domain 2; H3, *HFD* histone fold domain. The first and last amino acids of selected domains are indicated. The protein primary sequence and PTMs of the p53 C-terminal CRD domain, ERα DBD and hinge regions, as well as the histone H3 N-terminal tail, included for comparison, are shown in the zoomed-in *boxes*. The KMTs catalyzing these lysine methylation events are listed. Residues that are demethylated by LSD1 are also shown

p53 are also modified by acetylation, ubiquitylation and sumoylation [120, 121] (Fig. 1). Crosstalk among these PTMs provides a molecular mechanism that mediates rapid switching of p53 function upon various stimuli such as DNA damage response. The role of p53 in DNA damage response is mediated by modification of p53K382, where methylation and acetylation compete with each other to inhibit or promote p53′s role in DNA damage response. SETD8-mediated p53K382me1 inhibits DNA damage-induced acetylation at the same residue (p53K382ac) thus impairing p53-mediated DNA damage responses [79] (Fig. 2c). In contrast, the dual methylation of p53K370me2 and p53K382me2 promotes p53 DNA damage responses by inhibiting MDM2-mediated p53 protein ubiquitination and degradation [122].

Like the methylation marks on histones, methylation on p53 can be recognized by reader proteins. The chromatin compaction factor L3MBTL1 recognizes p53K382me1 through its triple malignant brain tumor (MBT) repeats [123]. The interaction between L3MBTL1 and p53 brings L3MBTL1 to promoters to repress the transcription of p53 target genes such as p21 and PUMA [123] (Fig. 2d). Upon DNA damage, monomethylated p53K370 and K382 are dimethylated by currently unknown enzymes. These dimethylation marks are recognized by the tandem Tudor domain of p53 Binding Protein 1 (53BP1) that acts as a transcriptional coactivator of p53 to promote p53 target gene expression during the DNA damage response [105, 119, 124] (Fig. 2d). In addition to 53BP1, p53K370me2 and p53K382me2 are also recognized by the second Tudor domain of PHF20. The two Tudor domains of PHF20 dimers each bind p53K370me2 or p53K382me2 simultaneously, and thus stabilizing and activating p53 during DNA damage response [122] (Fig. 2e).

Although a number of PTMs, including lysine methylation, have been identified within the p53 C-terminal regulatory region, these PTMs may only fine tune the functions of p53 and some modifications are likely functionally redundant. This could explain why the loss of p53K372 methylation in SETD7 knockout mice has no effect on p53-dependent cellular functions such as cell cycle arrest and apoptosis in response to radiation, genotoxic agents, and oncogenic agents [125, 126]. Future research should carefully combine in vitro and in vivo

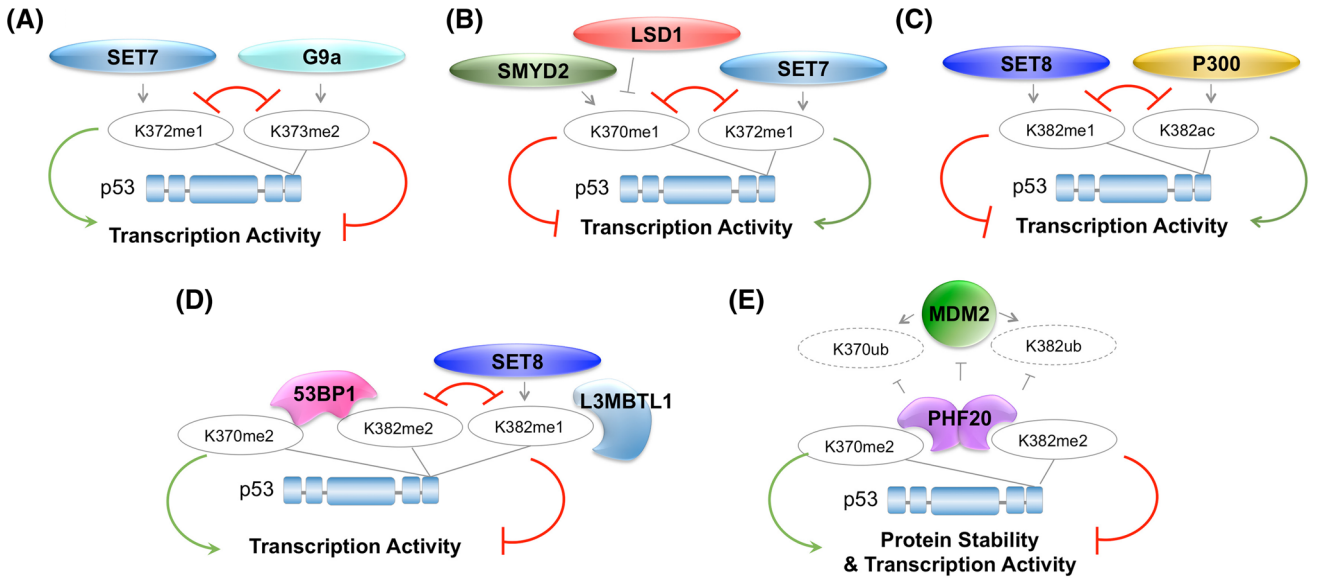

Fig. 2 Crosstalk and functional outcomes of p53 lysine methylation. **a** SET7-catalyzed monomethylation of p53K372 (K372me1) (*activating mark*) competes with G9a-catalyzed dimethylation of K373 (K373me2) (*repressive mark*) to promote the transcriptional activity of p53. **b** SET7-catalyzed K372me1 (*activating mark*) competes with SMYD2-catalyzed K370me1 (*repressive mark*) to promote the transcriptional activity of p53. LSD1 demethylates p53K370me1. **c** SET8-catalyzed K382me1 (*repressive mark*) competes with p300-catalyzed K382 acetylation (K382ac) (*activating mark*) to repress the transcription activity of p53. **d** L3MBTL1 recognizes p53K382me1 to repress p53 target gene expression. Upon DNA damage, 53BP1 recognizes the dual marks of p53K370me2 and p53K382me2 to act as a transcriptional coactivator of p53. **e** Dual methylation of p53 K370me2 and K382me2 recruits the PHF20 dimer to inhibit MDM2-mediated p53 ubiquitylation and degradation, thus promoting p53 protein stability and transcription activity

approaches to investigate not only the methylation events, but also their interplay with other PTMs and regulators of p53.

ERα and other nuclear receptors

ERα is a member of the nuclear hormone receptor family that controls cellular responses to estrogens [127]. ERα shares a conserved protein structure with other NRs, including an N-terminal regulatory domain, known as activation function domain 1; a central DNA-binding domain (DBD); a C-terminal ligand-binding domain, known as activation function 2; and two flexible domains: a hinge region that connects the DBD with the ligand-binding domain, and a regulatory domain at the C-terminus (Fig. 1). The binding of hormone ligands is an essential step in the activation of ERα and other NRs that leads to a sequence of events including conformational changes, protein dimerization, translocation from the cytoplasm to the nucleus, and binding to specific DNA sequences known as estrogen response elements [127]. The ligand-induced allosteric changes of ERα alter its interactions with different coregulators that are essential for ER-mediated transcription. Transcriptional coregulators, namely coactivators and corepressors, regulate the activity of DNA-bound transcription factors [128, 129]. One of the main functions of ERα coregulators is to modulate histone modifications, thus controlling the accessibility of the underlying DNA to the transcriptional machinery [128, 129].

Like histones, the ERα proteins are subjected to a number of PTMs including phosphorylation, methylation, acetylation, ubiquitination, and sumoylation. Except for phosphorylation, these modifications are enriched in the DBD domain (aa 181-263) and hinge regions (aa 264-302) (Fig. 1), and are believed to play important roles in regulating ERα protein stability, estrogen sensitivity, subcellular localization and DNA-binding affinity [130–132]. All five lysine residues (K266, K268, K299, K302, and K303) at the border between the DBD and the hinge region are reported to be acetylated by p300/CBP [133, 134]. Depending on the target sites, lysine acetylation can lead to opposing outcomes: acetylation of K299, K302 and K303 inhibits ERα target gene expression, whereas acetylation of K266 and K268 promotes ERα target gene expression by enhancing its DNA binding.

To date, there are only two reported lysine methylation events of ERα that regulate ERα, each with a distinct outcome. In the first, SET7 monomethylates ERα at K302, which stabilizes ERα and promotes its transactivation

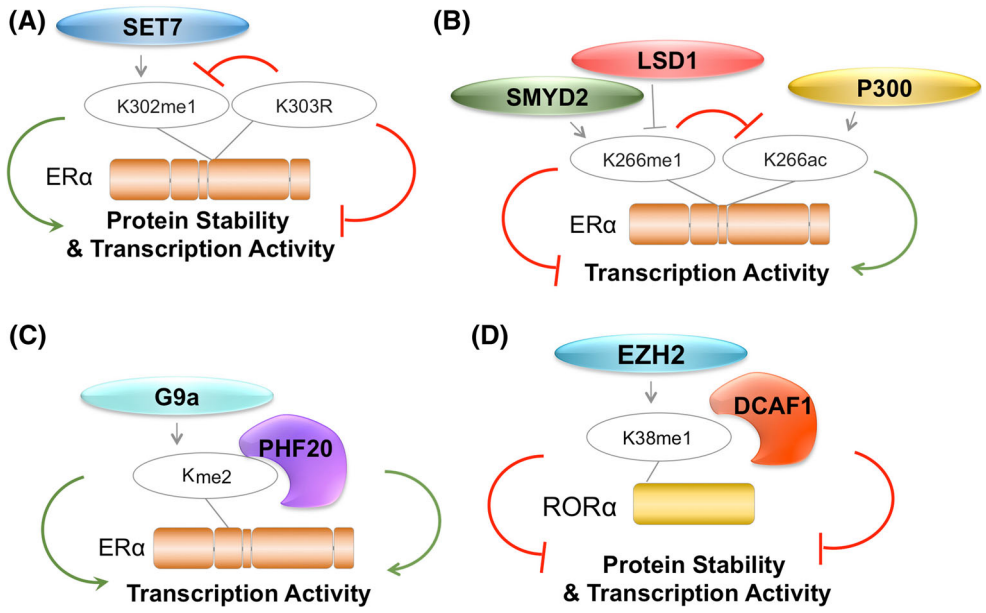

Fig. 3 Crosstalk and functional outcomes of ERα lysine methylation. **a** SET7-catalyzed ERαK302me1 (*activating mark*) promotes ERα protein stability and transcriptional activity. The breast cancer-associated K303R mutation inhibits ERαK302me1. **b** SMYD2-catalyzed K266me1 (*repressive mark*) competes with the p300-catalyzed K266ac (*activating mark*) to repress the transcription activity of ERα. LSD1 demethylates K266me1. **c** G9a-mediated ERα methylation of ERα recruits PHF20 to promote the transcriptional activity of ERα. **d** EZH2-mediated RORα K38 methylation is recognized DCAF1, which facilitates the CUL4–DDB1-dependent ubiquitination and protein degradation, resulting in a loss of transcriptional activation of RORα

activity [17]. This modification is negatively regulated by the K303R mutation [17] (Fig. 3a), which has been associated with breast cancer [135–137]. The second methylation event was recently reported by our group. SMYD2 monomethylates ERα at K266, which prevents acetylation of the same residue, thus keeping ERα in a repressed or inactive status [72]. Upon estrogen activation, the repressive K266me1 mark is removed by LSD1, which then allows p300 to acetylate this residue to activate the ERα transcriptional response (Fig. 3b). In our own research, we have identified a third ERα methylation event. Through in vitro screen, we found that G9a can methylate ERα (unpublished results). The G9a-mediated ERα methylation is recognized by the Tudor domain of PHF20, which recruits the MOF histone acetyltransferase complex to deposit histone acetylation, thus coordinating the in trans crosstalk between ERα and chromatin modifications (Fig. 3c).

In addition to ERα, a few other nuclear receptors, such as AR, retinoic acid receptor (RAR), and RAR-related orphan receptor (ROR), are also regulated by lysine methylation. AR is methylated on two residues, K630 and K632, by SETD7 [24, 25]. As is the case for methylation of ERα by SETD7, these methyl marks on AR enhance its transcriptional activity. RARα is trimethylated at K347, which facilitates the interaction between RARα and its modulators including p300/CBP, receptor-interacting protein 140, and retinoid X receptor (RXR) [138]. Monomethylation of RARα at K109 also modulates its ligand-dependent activation and interaction with coregulators, probably through coordinating the synergy between the receptor DBD and the ligand-binding domain (LBD) [139]. Nevertheless, the enzymes responsible for K347 and K109 methylation have yet to be unveiled. RORα is another nuclear receptor that undergoes lysine methylation. The H3K27-specific methyltransferase EZH2 monomethylates RORα at K38 [84]. This modification is recognized by the DDB1 and CUL4-associated factor 1 (DCAF1) adaptor, resulting in ubiquitination-dependent degradation and a loss of transcriptional activation of RORα [84] (Fig. 3d).

Other non-histone proteins modulated by lysine methylation

In addition to those already discussed, several other proteins are modulated by lysine methylation. These include the NFκB subunit, RelA/p65, Rb, DNMT and HSP proteins (Table 1). The NFκB subunit, RelA/p65, is a component of the canonical NFκB signaling pathway that is important for immune responses. Six lysine residues in RelA have been reported to be methylated by three KMTs: K37, K314, and K315 are monomethylated by SETD7 [18, 19]; K218 and K221 are mono- and dimethylated by NSD1 [93]; and K310 is monomethylated by SETD6 [95, 96]. In response

to either TNFα or IL-1β stimulation, RelA is methylated at K37 by SETD7, which promotes NFκB promoter binding and target gene expression [18]. In contrast, SETD7-mediated methylation of K314 and K315 triggers proteasomal degradation of NFκB [19]. Methylation of K314 and K315 is impaired by the acetylation of K310, leading to prolonged protein stability and enhanced RelA transcriptional activity [140]. Monomethylation of RelA at K310 by SETD6 recruits G9a/GLP, thus attenuating NFκB-induced transcriptional responses by depositing the histone H3K9 methylation mark [95]. In contrast, methylation of RelA at K218 and K221 by NSD1 is associated with NFκB activation [93]. Interestingly, NFκB signaling drives the expression of FBXL1/KDM2B to remove these methylation marks, thus forming a negative regulatory feedback loop [93].

Retinoblastoma protein was the first tumor suppressor found to function in inhibiting abnormal cell cycle progression and excessive cell growth. SETD7 monomethylates Rb at K873, which recruits the heterochromatin protein HP1, to facilitate Rb-dependent transcriptional repression, cell cycle arrest and cellular differentiation [27]. Similarly, the K860 monomethylation mark deposited on Rb by SMYD2 interacts with the transcriptional repressor L3MBTL1 to repress the expression of cell cycle genes in response to anti-proliferative signals [71]. Both SETD7 and SMYD2 monomethylate Rb on another lysine residue, K810 [28, 75]. Methylation of K810 by SETD7 impedes the binding of cyclin-dependent kinase (CDK) thus preventing Rb phosphorylation and maintaining Rb in the hypophosphorylated, growth-suppressing state [28]. In contrast, SMYD2-mediated methylation on Rb K810 enhances S807/S811 phosphorylation to accelerate E2F transcriptional activity and to promote cell cycle progression [75]. It is unclear how a single modification can lead to such divergent outcomes. One possibility is that SETD7 and SMYD2 may also modify other proteins involved in these processes, leading to activation or repression of the Rb-associated kinases. Another possibility is that SETD7 and SMYD2 may recruit other proteins that sterically block or open up one or more phosphorylation sites, which may be independent of their enzymatic activity.

DNA methyltransferase 1 is the only DNA methyltransferase responsible for the maintenance of CpG methylation. The discovery that DNMT1 itself is methylated bridges two important epigenetic regulatory mechanisms: histone methylation and DNA methylation. DNMT1 is monomethylated by SETD7 at K142 and is believed to increase DNMT1 protein turnover [20]. This methyl mark can be removed by LSD1, which protects DNMT1 from proteasomal degradation [141–143]. However, DNMT1K142me1 can also be recognized by the Tudor domain of PHF20L1, which blocks the ubiquitination of DNMT1 and inhibits its degradation [144], thus making it less clear under what conditions K142me1 promotes or inhibits DNMT1 turnover. Regardless, DNMT1 can also be dimethylated by G9a at K70 in vitro, but any biological consequence of this modification has yet to be determined [48]. The de novo DNA methyltransferase DNMT3A is also methylated by G9a. G9a-mediated dimethylation of DNMT3A on K47 is recognized by the chromodomain of the methyl-H3K9-binding protein MPP8, forming a DNMT3A-MPP8-G9a silencing complex on chromatin [145].

HSPs function as molecular chaperones in various cellular processes. So far, HSP70, HSP90, and HSP90AB1 have all been shown to be substrates of KMTs. HSP70 is dimethylated at K561 by SETD1A [87], which promotes cell growth by enhancing HSP70 nuclear translocation and interaction with Aurora kinase B [88]. HSP70 isoforms such as HSPA1, HSPA8, and HSPA5 can be methylated by METTL21A to modulate protein–protein interactions [99, 102]. SMYD2 monomethylates HSP90 on K209 and K615 [73] and HSP90AB1 on K531 and K574 [146]. HSP90 K615 methylation promotes the formation of an HSP90-involved protein complex important for myocyte function [147, 148]. SMYD2-mediated HSP90AB1 methylation facilitates chaperone complex formation [146].

Perspectives

As we have discussed above, the substrates of KMTs and KDMs include not only histones, but also tumor suppressors, nuclear receptors, transcription factors, epigenetic regulators and chaperone proteins. Compared with the plethora of information about histone methylation, our understanding of non-histone protein lysine methylation is very limited. We do know that lysine methylation regulates protein function through two main mechanisms: interplay with other PTMs and effects on protein–protein interactions.

Similar to its role in the epigenetic regulation of histones, lysine methylation is associated with both positive and negative functions of the modified proteins in a site- and state-specific manner. Several groups have attempted to analyze and catalog protein lysine methylation at the proteome-wide level using different approaches: array-based enzymatic assays or mass spectrometry coupled with affinity or chemical enrichment (see review in [149]). With the increasing number of non-histone lysine methylated proteins being identified, it is likely that lysine methylation will be found to be a ubiquitous PTM that modulates proteins involved in all cellular processes. However, challenges still remain. For those interactions that have

been identified in vitro, it is important to validate their occurrence in vivo. Further, it is imperative to determine the biological significance of these modifications. Future studies are needed for better understanding of the writers, erasers and readers of lysine methylation the signaling pathways that control protein lysine methylation and their dysregulation in diseases.

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