Science in China Series C: Life Sciences

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Special Topic **Review**

Epigenetic regulation: methylation of histone and non-histone proteins

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Histone methylation is believed to play important roles in epigenetic memory in various biological processes. However, questions like whether the methylation marks themselves are faithfully transmitted into daughter cells and through what mechanisms are currently under active investigation. Previously, methylation was considered to be irreversible, but the recent discovery of histone lysine demethylases revealed a dynamic nature of histone methylation regulation on four of the main sites of methylation on histone H3 and H4 tails (H3K4, H3K9, H3K27 and H3K36). Even so, it is still unclear whether demethylases specific for the remaining two sites, H3K79 and H4K20, exist. Furthermore, besides histone proteins, the lysine methylation and demethylation also occur on non-histone proteins, which are probably subjected to similar regulation as histones. This review discusses recent progresses in protein lysine methylation regulation focusing on the above topics, while referring readers to a number of recent reviews for the biochemistry and biology of these enzymes.

epigenetics, histone, histone modification, histone lysine methylation, histone methylase, histone demethylase, epigenetic inheritance, non-histone methylation

1 Introduction

Epigenetics refers to heritable changes that do not involve alterations of DNA sequences. Chemically, epigenetics refers mainly to DNA CpG methylation and histone modifications, including histone methylation. Methylation occurs on lysine (K) and arginine (R) residues. Thus far, histone lysine methylation has been found to occur at six major sites, including histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20. Interestingly, unlike other modifications, the same lysine residue can be methylated to different degrees to include mono-, di- or trimethyl moieties, which may have different functional consequences. Histone methylation appears to be regulated by a complex network that involves a large number of site-specific methylases, demethylases and methyl recognition proteins ("readers"), which play an important role in controlling the expression of genetic information through transcriptional changes and chromatin structure alterations. In human genome, there are approximately 50 and 25 of known and predicted methylases and demethylases, respectively[1,2]. The number of proteins that are dedicated to the recognition of the different methylation states at these different lysine residues is even greater[3]. This large regulatory network suggests that lysine methyl marks may impart important epigenetic information that has to be precisely regulated in a temporal and spatial manner. For instance, genome-wide ChIP experiments identified what is termed the "bivalent domains", which refer to the co-existence of H3K4me3, an active methyl mark, and H3K27me3, a repressive mark, on developmentally important genes in stem cells. The bivalent domains are believed to protect these genes from premature activation but at the same time poise these genes for future activation. Upon stimulation, the bivalent domains segregate into either H3K4me3- or H3K27me3-

Received March 5, 2009; accepted March 15, 2009
doi: 10.1007/s11427-009-0054-z
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Citation: LAN F, SHI Y. Epigenetic regulation: methylation of histone and non-histone proteins. Sci China Ser C-Life Sci, 2009, 52(4): 311-322, doi: 10.1007/s11427-009-0054-z

marked genes for activation and repression, respectively[4,5]. A fundamental question in the epigenetics field is how the methylation marks can be inherited from mother cells to daughter cells. In *S. pombe*, propagation of the silencing mark, H3K9me3, at the centromeric and Mating type regions requires the RNAi machinery and DNA recognition factors[6,7]. Similarly, in higher eukaryotes, the polycomb group repressive complex 2 (PRC2)-mediated H3K27 tri-methylation (H3K27me3), which plays important roles in gene repression during animal development, also needs specific DNA sequence polycomb response element (PRE) or non-coding RNA, for proper recruitment to specific chromosomal locations[8,9]. Deletion of the components involved in DNA sequence specific recognition results in the loss of the histone H3K27me3 mark, suggesting the inheritance of histone marks requires genetic information encoded by DNA per se and challenging the self-sufficiency of histone methyl marks functioning as carriers of epigenetic information. However, recent studies also provide evidence suggesting that at least some of the methyl marks may be self-sufficient in replicating themselves into daughter cells[10]. Though further studies are clearly required to fully understand this important issue, we discuss the recent progresses that address possible mechanisms for genetic inheritance of histone methylation marks.

While the discovery of histone demethylases highlighted the potential dynamic nature of histone methylation regulation, it also raises interesting questions with respect to how methyl marks are protected for epigenetic inheritance. Are some methyl marks more stable than others and are not reversible by demethylases? Interestingly, demethylases for H3K79 and H4K20 have not been identified, where H4K20 methylation has been suggested to play an epigenetic role[11-13]. This review will also discuss evidence for and against the existence of demethylases for these two methylation sites.

Although lysine methylation was initially identified in histone proteins, recent findings showed that this modification also occurs on non-histone proteins, which is highlighted by the finding of methylation of the tumor suppressor p53[14]. p53 is methylated at multiple lysine residues and can be demethylated by LSD1/KDM1, suggesting that dynamic regulation of p53 methylation may be important for controlling p53 function. In addition to p53, other non-histone proteins have also been found to be subject to methylation regulation[15–17], suggesting that methylation may be a general post-translational modification for regulating protein functions. The fact that the proteins identified to be regulated by methylation are largely transcriptional regulators raises the question whether methylation of non-histone proteins may be a general means to regulate epigenetic information, another subject that will be discussed in this review.

2 Propagation of histone methyl marks through cell cycles

DNA CpG methylation has been shown to play an epigenetic role. The DNA methylation mark was first thought to be propagated via a mechanism that involves DNMT1, a maintenance DNA methylase that catalyzes “new” DNA methylation using hemimethylated DNA as template[18–20]. More recently, UHRF1, which is a DNMT1 interacting protein, has also been implicated in this process by recognizing hemimethylated DNA and recruiting DNMT1 to chromatin[21,22]. Importantly, histone methylation at H3K9 and demethylation at H3K4 have been shown to play a role in the recruitment of DNA methylases[23–25], suggesting an alternative mechanism involving histone methylation for targeting DNA methylases to chromatin for DNA methylation. However, whether and how histone methylation marks can be preserved during DNA replication and mitotic condensation, and be passed on from mother cells to daughter cells remain unclear.

Multiple previous studies suggested that DNA sequence specific recruitment was required for the maintenance of histone methylation[6–9]. However, none of those studies investigated whether histone methylation marks themselves can be faithfully replicated through cell cycles. A recent study focusing on H3K27me3 and its catalyzing machinery, the PRC2 complex, has provided evidence arguing that maintenance of this mark can be accomplished by its own for at least 4 cell divisions[10]. H3K27 tri-methylation was shown to involve two protein complexes, PRC1 and PRC2. While the PRC2 complex contains the enzyme EzH2/KMT6 for H3K27 tri-methylation, the PRC1 complex binds H3K27me3 through PC2 chromodomain and may provide a recruitment function for PRC2[26]. However, in this study, the H3K27 methylase EzH2/KMT6 was found to co-localize with the H3K27me3 mark at G1 phase and the replication sites throughout S phase, but

not CBX8, a subunit of the PRC1 complex, suggesting that PRC2 could bind H3K27me3 on its own. This was further demonstrated by an *in vitro* H3K27me3 peptide pull-down assay, to which an EzH2/KMT6-EED-SUZ12 recombinant trimeric complex was bound. Additionally, EzH2/KMT6 also appeared to associate with mitotic chromosomes across M phase, suggesting that the re-establishment of PRC2-bound chromatin domain after chromosomal de-condensation for the next cell cycle may not require further recruitment event, which would make the DNA sequence specific recognition step dispensable. Importantly, using an inducible GAL4-EED or GAL4-EzH2/KMT6 system, this study further demonstrated that transient recruitment of the PRC2 complex to a GAL4 reporter promoter supported maintenance of the H3K27me3 mark and reporter gene repression for at least four cell divisions after shutting down GAL4-EED or GAL4-EzH2/KMT6 expression. Since there were no DNA elements other than GAL4 binding sites at this artificial promoter for the purpose of PRC2 recruitment, the transmission of the H3K27me3 mark was probably executed by the H3K27me3 mark and PRC2 complex themselves, suggesting that H3K27me3 can be inherited through the action of its own enzymatic machinery.

It is worth noting that on this artificial GAL4 reporter, the maintenance of H3K27me3 mark was only examined for four cell cycles. It is therefore unclear whether maintenance of H3K27me3 can happen beyond four cell cycles. More importantly, future experiments are necessary to determine whether this mode of propagation is applicable to endogenous chromatin locations marked by H3K27me3. Regardless, these findings suggest the possibility that DNA elements (such as PRE) may play an important role in the establishment of H3K27 methylated chromatin, but the maintenance and propagation of H3K27me3 may use a mechanism that is built into the enzymatic complexes which mediate H3K27 methylation.

In addition to H3K27me3, other methyl marks such as H4K20me1 may also be replicated via a similar mechanism. The methylase PR-Set7/KMT5A is responsible for mono-methylation of H4K20, generating H4K20me1, which is further methylated to di- and tri-methylation by the SUV4-20/KMT5B/KMT5C methylases[11,27]. Methylation of H4K20 has been implicated in transcriptional repression and DNA damage response[11–13,28–30]. Many types of cancer have a reduced level of H4K20 methylation globally implicating H4K20 methylation in tumorigenesis[31]. Several recent studies also suggest that H4K20 methylation may have a more general function in chromatin management, which indirectly affects transcription of the affected genomic regions. During cell cycle progression H4K20me1 is found to be tightly regulated; it increases at late-S/G2 phase and peaks at M phase, which corresponds to the change of the protein level of the enzyme, PR-SET7/ KMT5A[32]. PR-SET7/KMT5A inactivation results in a significant reduction of H4K20me1, cell cycle perturbation and de-condensed chromosomes, suggesting the H4K20me1 may play an important role in cell cycle regulation by facilitating chromatin compaction[32]. If H4K20me1 indeed functions in this process, then the mechanism for its inheritance probably occurs at a global level. Consistent with this hypothesis, a recent study found that *in vitro*, H4K20me1/2 and H1bK26me1/2 are recognized by L3MBTL1, leading to chromatin compaction[33]. Importantly, L3MBTL was shown to be associated with DNA in interphase and more intensely with condensed chromosomes during mitosis[34]. These findings suggest that the location information of these methyl marks may be preserved and stored by the chromatin association of L3MBTL1 during mitosis, which may further serve as a docking site for the H4K20me1 methylase in the next round of cell cycle. Supporting this idea, an unpublished result identified a direct interaction between L3MBTL1 and PR-SET7/ KMT5A (unpublished Data, Trojer P. and Reinberg D.), suggesting a possible working model of this system in which the newly synthesized histones are incorporated into chromatin during S phase and L3MBTL1 recruits PR-SET7/KMT5A in late-S/G2 phase to add H4K20me1 mark onto these "new" histones using "old" H4K20me1 as template, thus the epigenetic information is preserved by the binding of L3MBTL1 and H4K20me1 during mitosis and transmitted to daughter cells (Figure 1).

The linker histone H1b has also long been considered to play an important role in chromatin condensation during M phase, and a similar inheritance model may also be applicable to the methylated H1bK26me1/2[33]. In this model, the chromatin associated L3MBTL1 may recruit PRC2 complefx via its interaction with RB1[33,35], and PRC2 catalyzes H1K26 methylation using the parental histones as template[36,37]. A recent study also identified the H3K9 methylase G9A/KMT1C as having methylase activity toward H1bK26, and HP1γ as another "reader" for the H1bK26me1/2 marks[38]. Because of the

ability of HP1γ to interact with G9A/KMT1C and L3MBTL1[16,33], these findings suggest that the H1bK26 methyl marks may also be faithfully replicated into daughter cells using a similar mechanism discussed above.

The findings that lysine methylase complexes also recognize their own reaction products discussed above may not be limited to H3K27me3, H4K20me1 and H1bK26me1/2. Such a mechanism was first reported for the H3K9me3 methylase Suv(Var)3-9/KMT1, which interacts with HP1 that binds H3K9me3[39–41]. More recently, it has been shown that the H3K9me2 methylases G9A/KMT1C and GLP/KMT1D, can also bind to their reaction product H3K9me2 via their ankyrin repeat domains[42]. Is this type of regulatory network being exploited to facilitate the establishment of a particular local chromatin environment or to ensure a faithful inheritance of the methyl marks or both? The answer to this question is likely to be context-dependent. For example, the genomic loci that undergo rapid changes in response to extracellular stimuli or cell cycle related alterations may use this network to ensure the establishment of a local chromatin environment amenable for transcriptional repression or activation. But those large chromosomal domains that play important roles in epigenetic memory, such as the Hox loci, centromere and telomere as well as regions involved in mitotic condensation, may use the enzyme-reader system to replicate the histone marks through successive cell cycles. Further mechanistic studies are still required to address this fascinating puzzle to reveal the nature of epigenetic inheritance of histone methylation marks.

3 Histone demethylation

As discussed earlier, histone methylation is dynamically regulated by a plethora of methylases and demethylases[1,43,44]. What is the role of the demethylases, if any, in regulating epigenetic memory? For those methyl marks that are propagated through cell cycles, they must be protected from the demethylases. It has been reported that the H3K27me3/H3K4me3 bivalent domain is relatively stable and may be important for ES cell self-renewal as resolution of this domain is correlated with stem cell differentiation[4]. Interestingly, the H3K27me3 demethylase UTX/KDM6A is expressed in the ES cells but appears to be prevented from accessing to the bivalent domain via an unknown mechanism[45]. Thus, a precise and balanced regulation of methylase and demethylase function is likely to be important for the maintenance of histone methyl marks.

In addition to the histone methyl marks, can the methyl zero state be inherited through cell division as well? A case in study is the H3K4me2 demethylase LSD1/KDM1 and its associated protein BHC80, which is a PHD domain-containing protein. Upon demethyla-

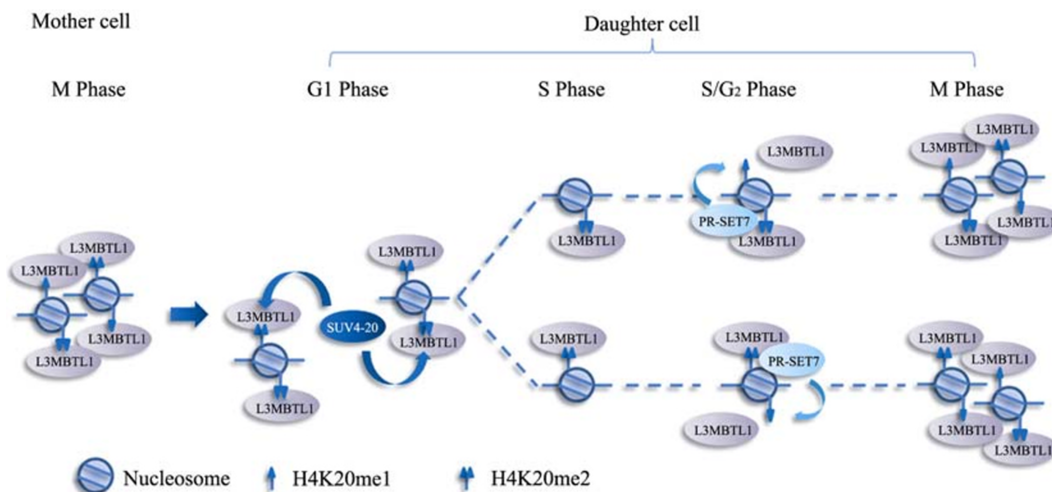

Figure 1 A proposed model for H4K20me1 and H4K20me2 mark transmission. In M phase, L3MBTL1 compacts chromatin via its ability of binding H4K20me1/2 marks, and this reader-mark association is maintained throughout mitosis. At late M/G1 phase, a majority of H4K20me1 marks become methylated to H4K20me2 by Suv4-20/KMT5B-C methylases. After DNA replication in S phase, the fully H4K20me2 methylated nucleosomes become hemimethylated, and then the "new" histones are monomethylated by PR-SET7/KMT5A, which is recruited by L3MBTL1, using the "old" histones as template in late S/G_2 phase. L3MBTL1 recognizes the newly methylated H4K20 marks and compacts the chromatin for next round of mitosis.

tion of H3K4me2 to H3K4me0 by LSD1/KDM1, BHC80 binds the demethylation product H3K4me0 to maintain LSD1/KDM1 at target loci and to prevent re-methylation of H3K4[46]. This is an analogous system to the methylase and methyl "reader" network discussed above, suggesting that epigenetic information may also be stored in the form of the methyl zero state. Indeed, pharmacological inhibition of LSD1/KDM1 results in de-repression of target genes regulated by the maintenance DNA methylase, DNMT1, without changing local DNA methylation, further supporting the idea that H3K4me0 alone also carries epigenetic information[47]. Interestingly, in the germ cells, the histone H3K4me0 state appears to be important for the recruitment of the de novo DNA methylases DNMT3a/b for active DNA methylation[24], highlighting an intimate and complex relationship between H3K4 demethylation and DNA methylation. However, whether H3K4me0 is heritable through the LSD1/KDM1-BHC80 system remains to be determined. Finally, genome wide studies identified the existence of many histone mono-methyl marks, including H3K4me1, H3K9me1, H3K27me1 and H4K20me1[5,48]. How these mono-methyl marks are faithfully replicated through cell cycle? Here again, the demethylases may be proved to play an important role, at least in the generation of the mono-methyl states as multiple demethylases appear to reduce tri- or di-methyl to mono-methyl in vitro[45,49,50]. Whether the mono- methyl states are then recognized by proteins with specific "reader" modules, which is a likely possibility as is the case for H4K20me1, also remains to be investigated.

3.1 Histone H4K20 and H3K79 methylation, are they inherently more stable?

In the past four years or so, a large number of histone demethylases have been identified with activities towards four of the six major histone lysine residues, including H3K4, H3K9, H3K27 and H3K36. Conspicuously missing are demethylases for H3K79 and H4K20, where methylation of the latter has been shown to play a role in chromatin compaction and the methyl marks may be inherited through an enzyme/chromatin-bound "reader" module system discussed earlier. Is this a technical issue or does it imply that these marks are more stable and are not subject to demethylase-mediated demethylation? Here we discuss evidence for and against the existence of H4K20 and H3K79 demethylases.

3.2 H4K20 methylation marks are tightly regulated by cell cycle

As discussed above, H4K20me1/2 marks are recognized by L3MBTL1 and this recognition has been proposed to play an important role in mitotic chromosomal condensation and inheritance of the H4K20 methyl marks[33,51]. Importantly, recent studies revealed a very slow turnover rate of global H4K20 methyl marks[52]. In this study, the authors found that H4K20me2 was the most predominant form of all H4K20 methyl states (including me0); with approximately 98% of newly synthesized histones being di-methylated after 2—3 cell cycles. H4K20me3 only accounts for about 3% of the total H4 in several human cell lines, and its level stays constant[52]. These findings, coupled with the lack of success of finding H4K20 demethylases, raise the question whether the H4K20 methyl marks are not subject to active demethylation by demethylases. However, this does not exclude the possibility that H4K20 demethylases are present and function only within the specific cell cycle windows and/or at specific genomic loci. For example, after mitosis, some of the H4K20me1/2 marks may be demethylated in order to allow the temporary release of a pool of L3MBTL1 to trigger chromatin de-condensation. In addition, the H4K20me2 mark has been found to play an important role in DNA damage response in both fission yeast and mammals[28–30]. During DNA damage, H4K20me2 is bound by the DNA damage response protein Crb2 for yeast and p53BP1 for mammals. Presumably, Crb2/53BP1 is released after DNA repair is completed. How the release is regulated remains an interesting question and can in theory involve a yet-to-be-identified H4K20me2 demethylase. Interestingly, di-methylation of 53BP1 itself at lysine 382 (K382me2) was recently found to be involved in DNA damage response, and the amino acid sequences surrounding p53BP1K382 and H4K20 are very similar, suggesting they might be subject to a similar methylase and demethylase regulation[53](also see next section, and figure 2B). Consistently, PR-SET7/KMT5A was also found to catalyze p53 methylation at K382[54].

Lastly, recent genome-wide mapping studies revealed an interesting correlation between H4K20me1 in the body of the gene and gene expression[5,48]. This observation at the first glance appears to be contradictory to the chromatin compaction function of this mark, which in principle should lead to gene repression. One possibility

is that there are two "types" of H4K20me1. One is bound by L3MBTL1 hence plays chromatin compaction function primarily; while the other type is located in the gene body where it plays a transcriptional role and is subject to dynamic regulation by both methylases and demethylases. To test this model, future studies are required to determine if H4K20 demethylase(s) exists and whether H4K20me1 in the gene is recognized by a "reader" module different from L3MBTL1.

3.3 H3K79 demethylase may exist in fertilized oocytes

Compared with methylation at other lysine residues, H3K79 has two distinct features. First, H3K79 is located on the surface of the globular domain of histone H3, while the other sites are within the flexible N-terminal tail regions[55]. Second, it is catalyzed by a non-SET domain containing methylase, DOT1L/KMT4, while all the other lysine residues are methylated by SET-domain containing methylases[56]. These findings suggest that H3K79 methylation may be regulated by a different pathway.

Similar to H4K20me1, H3K79me2 level also displays a cell cycle related fluctuation in HeLa cells and in an unicellular parasitic protozoan *Trypanosoma brucei*, with the lowest level at late-G2/S phase and the highest level at M phase, which is maintained till the S phase of the next cell cycle[56,57]. Interestingly, loss of H3K79me2 appears to result in the loss of mitosis checkpoint control, and premature cell division before DNA replication was completed[57]. Other studies also identified Dot1/KMT4 function in yeast meiotic checkpoint control[58]. These results suggest that H3K79me2 might play a role in ensuring faithful replication of DNA before cell division, which could be independent of its function in transcription activation. Furthermore, these studies also suggest that if H3K79 demethylases exist, they would have to be tightly regulated, so that the action will be restricted to specific genomic loci and/or limited time window during cell cycle.

Intriguingly, a recent study reported a massive demethylation of H3K79me2 and H3K79me3 within a few hours after oocyte fertilization[59]. In this study, the H3K79me2 mark was found throughout the oocyte genome and H3K79me3 mark at pericentromeric heterochromatin regions. Both marks were dramatically decreased within 4 hours after fertilization, when DNA synthesis had not yet occurred. The DNA synthesis independent H3K79me2/3 demethylation was further confirmed by using aphidicolin to inhibit DNA synthesis in one-cell embryos, which did not affect the global

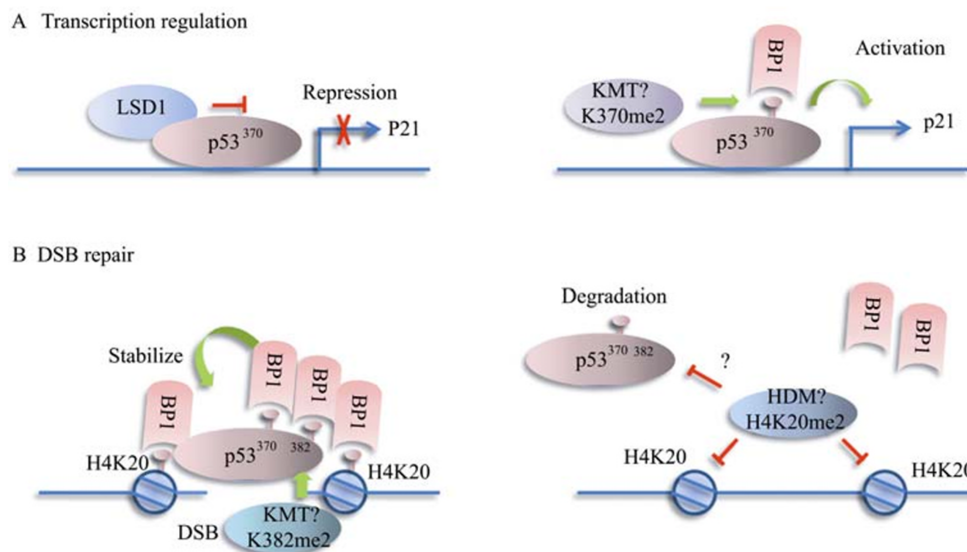

Figure 2 Methyl marks impact p53 function. A, p53K370me2 regulates p53 transcriptional activity through 53BP1. Left, during gene repression, LSD1/KDM1 demethylates p53K370me2 and represses p53 activity. Right, during p53 activation, p53 is di-methylated by a yet-unknown methylase at lysine 370, which is then bound by 53BP1, and this binding facilitates the activator function of p53. B, A proposed model of methyl mark regulation of p53 at DSB site during DNA damage. Left, at DSB site, p53 may be further di-methylated at lysine 382. The two methyl marks on p53 protein, p53K370me2 and p53K382me2, together with histone H4K20me2 mark may trigger a high order protein complex formation and result in p53 stabilization. Right, after DSB is repaired, a yet-unknown demethylase may remove the methyl marks of p53K382me2 and H4K20me2, which results in the release of p53 and 53BP1 complex from the repaired site, and p53 degradation.

H3K79 demethylation. Interestingly, in the same study H3K79me2 demethylation was also observed during somatic nuclei transplanted into enucleated unfertilized oocytes followed by parthenogenetic activation. The hypomethylation at H3K79 site was maintained till the blastocyst stage, except for a transient increase in H3K79me2 at mitosis. These observations together suggest that there might be a pool of inactive H3K79 demethylase(s) in the unfertilized oocytes, which is activated by signals resulting from fertilization, which leads to massive demethylation of H3K79me2/3 marks within a short period of time. This regulation may play an important role in genomic reprogramming following fertilization. However, a cautionary note is that the apparent H3K79 demethylation may also be explainable by histone degradation, a possibility that has not been ruled out just yet.

3.4 Possible ways to identify the demethylases against H4K20 and H3K79

If H3K79 and H4K20 demethylases exist, why have they eluded us thus far? One possible reason is wrong substrates might have been used in the screens for these demethylases. Synthetic methylated peptides and bulk histones are the most commonly used substrates by many groups for demethylase identification, including the efforts to identify H3K79 and H4K20 demethylases. However, it is possible that these demethylases may be inactive or have very low activity towards less physiological substrates such as histone peptides. Such a possibility is not unprecedented. For instance, the methylase activities of PR-SET7/KMT5A and Dot1L/KMT4 are only detectable when nucleosomes were used as substrates[27,56,60]. Furthermore, both H3K79 and H4K20 are located close to the globular domains of histones, thus a natural folding of the nucleosomal substrates) may be needed for the corresponding demethylases to function.

The crosstalks among different histone modifications have recently been explored by several studies, and some interesting links have been discovered. For instance, the PRC2 complex activity is largely stimulated when the native nucleosomal substrates are used, but not the recombinant ones[61,62]. More recently, H2BK120 ubiquitylation was found to dramatically stimulate DOT1L/KMT4 activity toward H3K79 using chemically synthesized nucleosomal substrates, providing a direct evidence of crosstalks between these two modifications[61]. By analogy, a pre-existing (or lack-thereof) histone modification may be important for H3K79 or H4K20 demethylases to function. Since Dot1L/KMT4 activity is coupled with H2BK120 ubiquitination, could the putative H3K79 demethylase be associated with an H2BK120 specific deubiquitinase? A well-designed proteomics approach may provide answer to this interesting possibility.

The third consideration is cofactors and/or posttranslational modifications. Some enzymes acquire significant activities only when associated with other proteins, possibly due to conformational changes in their catalytic domains or substrate recognition sites. For example, EzH2/KMT6 is only active when it is associated with other proteins in the PRC2 complex[62–65]. A recent study found that the H3K36 demethylation activity of the fly dKDM4A is dramatically stimulated upon HP1a association[66]. Another example is LSD1/KDM1, which is active toward nucleosomal substrate only when is associated with its partner protein CoREST[67,68]. The findings suggest that cofactors may be needed for H4K20 and H3K79 demethylases to function, if these enzymes indeed exist. In the case of possible H3K79 demethylases, a fertilized oocyte-specific cofactor and/or post-translational modification might be needed to regulate the demethylation activity after fertilization[59].

4 Methylation and demethylation beyond histones

Although histone methylation has been extensively studied in a variety of organisms as an important epigenetic regulatory modification, exploration of methylation regulation of non-histone proteins is only at the beginning (Table 1). The most well studied example is the tumor suppressor protein p53, whose activity can be either activated or suppressed by methylation depending on the methylation sites and the degrees of methylation. Specifically, several lysine mono- and di-methylation sites have been identified in the C terminus of p53, including K370, K372, K382 and K386. K370me1 and K382me1 which results in p53 repression, while K370me2 and K372me1 leads to p53 activation[69,70]. The SET domain containing protein 9 (SET7/9 /KMT7), which is responsible for p53K372 mono-methylation (p53K372me1), was the first methylase shown to methylate p53[71,72]. This modification prevents SET and MYND domain-containing protein-2 (SMYD2/KMT3C) mediated mono-methylation at p53K370, which re-

presses p53 activity[69]. Interestingly, two recent studies revealed important connections between p53 function and its binding partner p53BP1[53,70]. In one study, p53K370me2 was found to be recognized by the tudor domain of p53BP1 and was critical for the interaction between p53 and p53BP1[70]. Furthermore, LSD1/KDM1 was shown to specifically demethylate p53K372 me2, hence negatively regulating the p53 and p53BP1 interaction. LSD1/KDM1 RNAi significantly increased p53K370me2 level leading to activation of p53 target genes (Figure 2A). The transcriptional activation effect was dependent on p53BP1, since the activation was not observed in p53BP1 RNAi cells. In a second study, p53K382me2 was found to be significantly increased after DNA damage induced by neocarzinostatin, a DNA double strand break inducing drug, and p53K382me2 is also recognizable by p53BP1[53]. However, this interaction seem not to impact p53 transcriptional function, but to stabilize p53 protein level, which also requires p53BP1 binding[53].

How does the same methyl "reader", i.e. 53BP1, bind the two methylation sites on p53 and regulate p53 differently? One explanation could be that 53BP1 binding to these two methylated sites *in vivo* is regulated and with different kinetics, despite the fact that p53BP1 binds both sites to a similar extent *in vitro*[53]. Mutagenesis analyses revealed K370 to be crucial for the interaction of p53 and p53BP1 *in vivo*, however, K382 might only affect a subpopulation of the interaction between p53BP1 and p53[53,70]. These results suggest that K370me2 first recruits p53BP1 to activate gene expression (Figure 2A). A second methylation event on K382 creates an additional binding surface for p53BP1, which may facilitate a high order protein complex formation, which leads to p53 stabilization (Figure 2B). Interestingly, the amino acid sequence surrounding p53K382 (RHKKL) is highly homologous to that of histone H4K20 (RHRKV), and p53BP1 was known to bind H4K20me2[30]. As mention above, H4K20me2 is involved in DNA damage response, we speculate that this stabilization event is likely to occur at the DNA damage sites, and the multiple binding events to p53K370me2, p53K382me2 and H4K20me2 may trigger the formation of a highly stabilized complex containing p53, p53BP1 and chromatin, which is independent of the activator role of p53 (Figure 2B). Although these two studies provided strong evidence of site specific, methylation dependent regulation of p53

Table 1 Non-histone protein lysine methylation and its regulation

Non-histone methylation	Methylase	Demethylase	Methyl reader	Function	*In vivo* evidence
p53K370me1	SMYD2/KMT3C	LSD1/KDM1 *in vitro*	?	p53 repression	Yes
p53K370me2	?	LSD1/KDM1	p53BP1	Transcription activation	Yes
p53K372me1	SET7/9 /KMT7	?	TIP60	p53 activation	Yes
p53K382me1	PR - SET7/KMT5A	?	?	p53 repression	Yes
p53K382me2	?	?	p53BP1	Stabilization	Yes
p53K386me1/2	?	?	?	?	Yes
mDnmt1K1096me1	SET7/9 /KMT7	LSD1/KDM1	?	Destablization	Not definitive
TAF10K189me1	SET7/9 /KMT7	?	?	Stabilize PolII association	Yes
TAF7K5me1	SET7/9 /KMT7	?	?	?	No
ERαK302me1	SET7/9 /KMT7	?	?	Stabilize ERα , increase chromatin association	Yes
Histone H1.4K26me1/2	G9A/KMT1C, EzH2/KMT6	JMJD2D/KDM4D	HP1 γ, L3MBTL1	Chromatin compaction	Yes
G9AK185me2/3 WIZK305me2/3, CDYL1K135me2/3 ACINUSK454me2/3 KruppelK313me? HDAC1K432me? DNMT1K70me2	G9A/KMT1C	?	HP1 β except for DNMT1K70me2	?	No
G9AK239me3	G9A/KMT1C	?	HP1α, β, γ	Colocalization of ectopic G9a and HP1	Not definitive
EuHMT1/GLP K174me? mAM/ATF7IP K16me?	G9A/KMT1C	?	?	?	No

function, the methylases involved remain to be determined and whether demethylases are also involved remain unclear. A complete understanding of the impact and regulation of methylation at these two sites on p53 function will require the identification of the enzymes that are involved in this regulation. Regardless, the C terminus of p53 provides us with an excellent model for studying non-histone protein methylation involving methylases, demethylases and methylation readers.

Another non-histone protein that is regulated by methylation is the DNA methylase DNMT1, an enzyme crucial for the maintenance of cellular DNA CpG methylation[18,19]. In a recent study, mouse Dnmt1 was found to be methylated by Set7/9 at K1096 (K1094 in Human DNMT1), and the methylated K1096 was shown to be a substrate of LSD1-mediated demethylation[15] (Figure 3A). The authors found that although Dnmt1 methylation did not affect its methylase activity, it decreased Dnmt1 protein stability in ES cells. LSD1-mediated DNMT1 demethylation, in contrast, stabilized DNMT1. In the LSD1 knockout ES cells, Dnmt1 protein level was substantially reduced, which resulted in a 43% decrease of global DNA methylation, suggesting that LSD1/KDM1 is important for DNA methylation through regulation of DNMT1 stability. This finding is of particular interest, since both LSD1/KDM1 and DNMT1 play repressive roles in transcription. One might speculate that these epigenetic enzymes collaboratively regulate transcription not only by modifying their substrates in histone and DNA, but also by regulating one another's stability or activity to form a positive feedback loop to achieve an even more stabilized memory of epigenetic information during normal cell proliferation (Figure 3A). It was noted in this study that only the level of Dnmt1 but not those of Dnmt3a and Dnmt3b was reduced in LSD1/KDM1 knockout ES cells, suggesting this feedback loop may specifically apply to the maintenance of DNA methylation, but not *de novo* DNA methylation. In the future studies, it will be interesting to determine if the methylated Dnmt1 is recognized by specific methyl mark "reader(s)", which in turn present the methylated DNMT1 to proteasome for degradation (Figure 3B).

It is also important to note that SET7/9 /KMT7 not only catalyzes p53 and Dnmt1 methylation, but also methylation of TAF7, TAF10, and ERα[71–75]. In all these cases, SET7/9 /KMT7 has been reported to play an activatory role. It is conceivable that SET7/9 on one hand enhances the activity of basic transcriptional machinery through TAF7 and TAF10 methylation, and on the other hand, modifies and attenuates the function of repressor components, such as Dnmt1, and the combined effect is an enhanced transcriptional activity (Figure 3B). If this hypothesis is correct, then one can expect more of such non-histone methylation and demethylation examples to be discovered in the near future.

Although these individual studies are starting to provide important insights into methylation regulation of non-histone proteins, a more systematic proteomics approach is urgently needed to understand the scope and

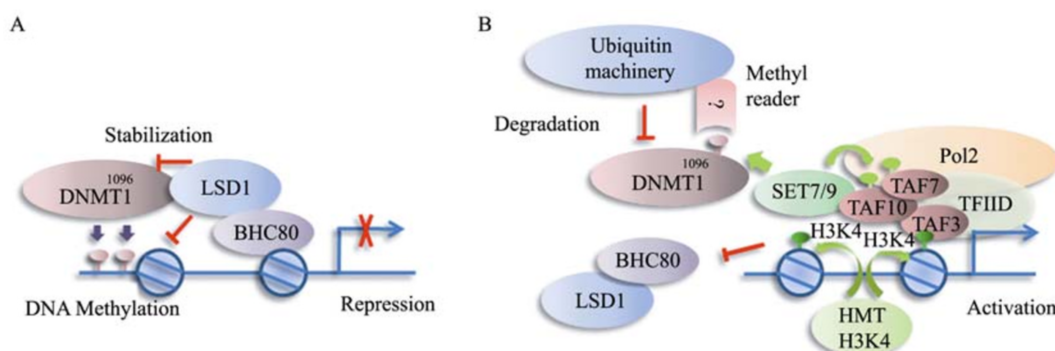

Figure 3 Coordinated models for transcriptional regulation of DNMT1 and LSD1/KDM1target genes. A, LSD1/KDM1 coordinates DNMT1 mediated gene repression. At the repressed gene promoter, LSD1/KDM1 not only maintains histone H3K4me0 status, but also the hypomethylation status of DNMT1K1096, which stabilizes DNMT1 and facilitates DNMT1 mediated DNA methylation. B, A proposed coordination model of DNMT1 and LSD1/KDM1 target gene activation. During gene activation, local histone H3K4 is methylated which prevents BHC80 binding and releases LSD1/KDM1 mediated demethylation on H3K4me2 and DNMT1K1096me2. The TAF3 subunit of TFIID recognizes H3K4me3 and anchors the transcription initiation machinery at promoter region. TAF10 and TAF7 are methylated by SET7/9 /KMT7, which facilitates the association of TFIID and RNA polymerase 2. SET7/9 /KMT7 also methylates DNMT1K1096, which results in DNMT1 destabilization. A yet-unknown DNMT1K1096me reader might be involved to present methylated DNMT1 to an ubiquitin-dependent degradation pathway.

extent of the non-histone methylome. Recently, a histone H3 N terminus sequence permutation based peptide array was used to identify a consensus motif (Arg-Lys) for G9A/KMT1C mediated methylation[17]. Based on this motif, the study further identified several non-histone nuclear substrates of G9A/KMT1C, including CDYL1, WIZ, ACINUS and N-terminal part of G9A/KMT1C itself. Interestingly, the corresponding peptides containing these methylations could be specifically recognized by HP1β, a histone H3K9me3 "reader", further suggesting a potential biological significance of these non-histone methylation marks[17]. Similarly, another study also found G9a methylates itself as well as GLP/KMT1D and mAM at the ARK motifs, and these methylation marks could be recognized by HP1 members[16]. However, whether these sites are methylated *in vivo* and the effect of methylation on G9A/KMT1C mediated gene repression remain to be investigated. In a third example, G9A/KMT1C and EzH2/KMT6 have been found to methylate K26 of histone H1.4[36,38], a putative linker histone although it has not been found within the histone octamers. Notably, the amino acid sequence surrounding H1.4K26 is identical to those surrounding H3K9 and H3K27, respectively. Taken together, looking for methylation consensus sites in non-histone proteins may approve to be a way to identify non-histone methylation events.

The proteomics approach of identifying non-histone protein methylome has certain technical difficulties, such as detection sensitivity and methods of enrichment of methyl peptides. However, one might overcome these difficulties by using recently developed technologies. For instance, one can take the advantage of the SILAC (Stable Isotope Labeling with Amino acids in Cell culture) technology, comparing the differential signals between the proteomes from cell lines carrying wildtype versus catalytic point mutant of any given methylase. Alternatively, one can use methyl "readers" to enrich methylated proteins first before mass spectrometry analysis to increase the odds of finding methylated peptides. Finally, there are many SET domain and several JmjC domain containing putative methylases and demethylases that showed no known activity toward histones or nucleosomes. It is interestingly to speculate that non-histone proteins may be primary substrates for these putative enzymes.

5 Perspective

Over the last decade, a large number of histone methylases, demethylases and methyl mark "readers" have been found, and our understanding of epigenetic regulation of histone methylation has been greatly improved. However, it is clear that more studies are required for the further understanding of the function of methylation in various biological processes. Methylation provides a fascinating means of regulation; the sites and degrees of methylation can carry different functional meanings, and methylation on the same lysine residue could be interpreted differently under different contexts. How methylation is precisely regulated to ensure the storage and delivery of proper epigenetic messages via methylation of histone and non-histone proteins remains an important and exciting question, and will keep many investigators occupied for years to come. Given the complication of this regulatory system, novel system biology approaches appear to be urgently needed to accelerate the discoveries in the field, which we expect to significantly expand our knowledge in the regulation of genomic information by a comprehensive understanding of this tiny chemical modification.

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