Trends in Biochemical Sciences

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Review

# Controlling the Controllers: Regulation of Histone Methylation by Phosphosignalling

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Histone methylation is central to the regulation of eukaryotic transcription. Here, we review how the histone methylation system itself is regulated. There is substantial evidence that mammalian histone methyltransferases and demethylases are phosphorylated and regulated by upstream signalling pathways. Functional studies of specific phosphosites are revealing which kinases and pathways signal to the histone methylation system and are discovering the diverse effects of phosphorylation on enzyme function. Nevertheless, the majority of phosphosites have no known kinase or function and our understanding of how histone methylation is regulated is fragmentary. Improved approaches are needed to establish and study the key regulatory phosphorylation sites on histone methyltransferases and demethylases, to avoid focus on constitutive sites which may have little regulatory purpose.

## Highlights

Histone methylation is an important epigenetic modification that controls eukaryotic transcription, and its dysregulation contributes to the aetiology of human disease.

Recent phosphoproteomic studies have revealed that mammalian histone methyltransferase and demethylase enzymes are extensively phosphorylated.

Kinases that phosphorylate histone methyltransferase and demethylase enzymes are from many different signal transduction pathways, however, most phosphosites have no known kinase.

Phosphorylation has diverse effects on histone methyltransferase and demethylase biology, affecting their catalytic activity, chromatin binding, and degradation.

Targeted studies have investigated a tiny proportion of phosphorylation sites, meaning that our understanding of how the intracellular signalling network connects with histone-based gene regulatory systems is rudimentary.

## Histone Methylation Is a Central Regulatory Process in the Eukaryotic Cell

Eukaryotic cells respond to internal and external stimuli via signalling systems. Associated changes in gene expression are mediated by epigenetic modifications (see Glossary) of histone proteins, which affect chromatin compaction and the recruitment of transcriptional coregulators [1]. Histone methylation is a key epigenetic modification that regulates many nuclear processes, including transcription [2], replication [3], and DNA repair [4]. Specific histone methyl marks can have either activating or repressive effects on transcription depending on their position and methylation state [2]. All eukaryotic histone methylation sites demarcate functional sequences in the genome, both throughout a gene's body and within noncoding and regulatory elements, such as promoters and enhancers [5,6]. Importantly, the genomic distribution of histone methylation changes dramatically during growth [7], differentiation [8], and in response to exogenous perturbation [9], to bring about widespread transcriptional reprogramming.

Despite its major regulatory role, little is known about how histone methylation as a system receives information from upstream signalling pathways. As such, it remains unclear how this epigenetic modification is controlled by intracellular and extracellular signals. This review highlights recent efforts to delineate the connection between cellular signalling and histone methylation and discusses the challenges associated with integrating two central regulatory systems of the cell.

## Human Histone Methylation: The Methyltransferases and Demethylases

The mammalian genome encodes 35 histone methyltransferases and 23 demethylases [10–13]. Histone residues modified by these enzymes in the human cell are summarised in Figure 1. Despite much progress in mapping enzymes to their corresponding methylation sites, there is still contention surrounding the specificity of many human methyltransferases and demethylases [14–17]. For example, SMYD3 was initially reported to have H3K4 methyltransferase activity, but was subsequently shown to instead methylate H4K5 and non-histone substrates [16]. Although it was originally classified as a H3K4 methyltransferase, MLL5 does not have methyltransferase

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activity [15]. The overlapping site specificity of histone methyltransferases and demethylases (Figure 1) makes it likely that additional enzymes will be found to methylate or demethylate human histones. Indeed, of the 55 SET domain-containing methyltransferases in the human proteome, approximately half have no known substrates [18].

Given its central role in cellular processes, there is a need to establish how histone methylation is regulated. Underscoring its functional importance, aberrant histone methylation is linked to the aetiology and progression of many diseases [13,19], and histone methyltransferases and demethylases are emerging as anticancer therapeutic targets [11,20]. Consequently, the transcriptional and post-transcriptional regulation of a number of key methylation-related enzymes has been investigated. For example, expression of the oncogenic H3K27-specific methyltransferase, enhancer of zeste homolog 2 (EZH2), is directly controlled by the c-Myc transcription factor [21], and abundance of the resultant transcript is negatively regulated by miRNA-101 [22]. This type of regulation, however, does not fully explain the complexity of the histone methylation system as it only affects the amount of enzyme present. The post-translational modification of the enzymes themselves, being a poorly understood aspect of histone methyltransferase and demethylase biology, is emerging as an important regulatory process; one which connects the intracellular signalling network with histone-based gene regulatory systems, controlling when and where the methylating and demethylating enzymes act.

## Mammalian Histone Methylation Enzymes Are Extensively Phosphorylated

Histone methyltransferase and demethylase enzymes are post-translationally regulated by a number of different modifications, including phosphorylation [23], methylation [24], acetylation [25], ubiquitination [26], and glycosylation [27]. Phosphorylation is of particular interest and high-throughput phosphoproteomic studies are revealing that histone methyltransferase and demethylase enzymes are extensively phosphorylated. Our collation of phosphorylation sites from two phosphoproteomic datasets [23,28] identified 2511 phosphosites on 58 enzymes, of which 1744 are on 35 histone methyltransferases and 767 are on 23 histone demethylases (Figure 2).

### Phosphorylation of Histone Methyltransferase and Demethylase Families Provides Insights into Their Regulation

The phosphorylation profiles of histone methyltransferase and demethylase enzymes in the same family show strong similarity (Figure 2). By contrast, different histone methyltransferase and demethylase families are phosphorylated to markedly different extents, and have different patterns of phosphosites in sequence and structural features. These observations are important and suggest that the cell uses distinct regulatory mechanisms to control specific enzyme families.

A number of histone methyltransferase and demethylase families are extensively phosphorylated and likely to be subject to complex post-translational regulation. For example, all six members of the MLL methyltransferase family harbour at least 60 phosphosites per protein (Figure 2A). Similar trends are observed for the ASH1L methyltransferase family members, which are also extensively phosphorylated. Enzymes with very large numbers of phosphosites are likely to be important integrators of information [29], receiving signals from multiple independent signalling pathways to fine-tune their function.

Several histone methyltransferase and demethylase families receive little information from upstream signalling pathways. For example, arginine methyltransferases (PRMTs) have a smaller number of phosphosites compared with their lysine methyltransferase counterparts (Figure 2A). All five PRMT members have less than 20 phosphosites and show similar phosphorylation

## Glossary

Constitutive phosphorylation site: a phosphorylation site that is always present and is likely essential for protein function and/or structure.
Crosstalk: when multiple post-translational modifications, adjacent on a protein, affect one another or the cognate protein's interactions.
Disordered region: a part of a protein which lacks a defined 3D structure; frequently involved in protein–protein interactions.
Epigenetic modification: heritable alterations in gene expression that are not due to changes in the underlying DNA sequence.
Integrators of information: proteins which receive information from many different inputs to determine downstream functional outcomes. This type of regulation is a hallmark of important regulatory proteins (cf., p53)
Phosphodegron: a short linear motif that, when phosphorylated, is then ubiquitinated by a corresponding E3 ligase, targeting the protein for proteasomal degradation.
Phosphoproteomics: proteome-wide analysis of phosphorylation sites, typically involving affinity-based enrichment and mass spectrometric identification of phosphopeptides and thus phosphoproteins.
Stoichiometry: the proportion of a protein that is modified at a particular site. Calculating stoichiometry requires high-resolution quantitative techniques to determine the amount of modified protein present, compared with the total protein abundance.

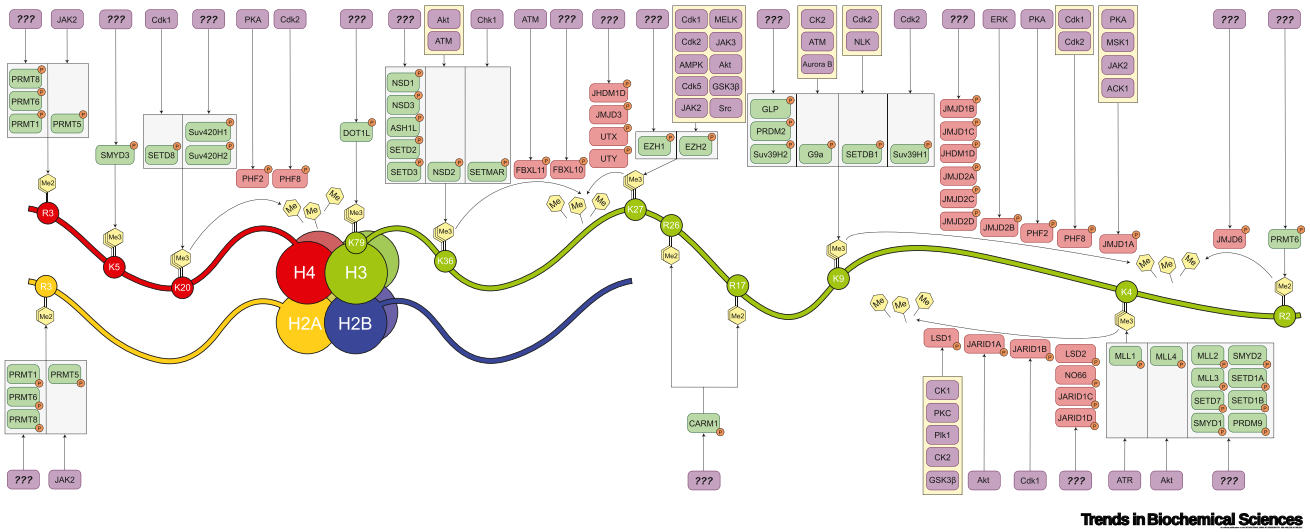



Figure 1. Mammalian Histone Methylation and Its Phosphoregulation. Histone proteins H2A, H2B, H3, and H4 are methylated at lysine (K) and/or arginine (R) residues along their N-terminal tails by methyltransferase enzymes (green). These methyl marks can be subsequently removed by demethylase enzymes (pink). All human histone methyltransferases and demethylases have been shown to be phosphorylated, suggesting regulation from upstream signalling pathways. The kinases responsible for phosphorylating enzymes are shown in mauve, where known. Kinases that are unknown are shown as question marks.

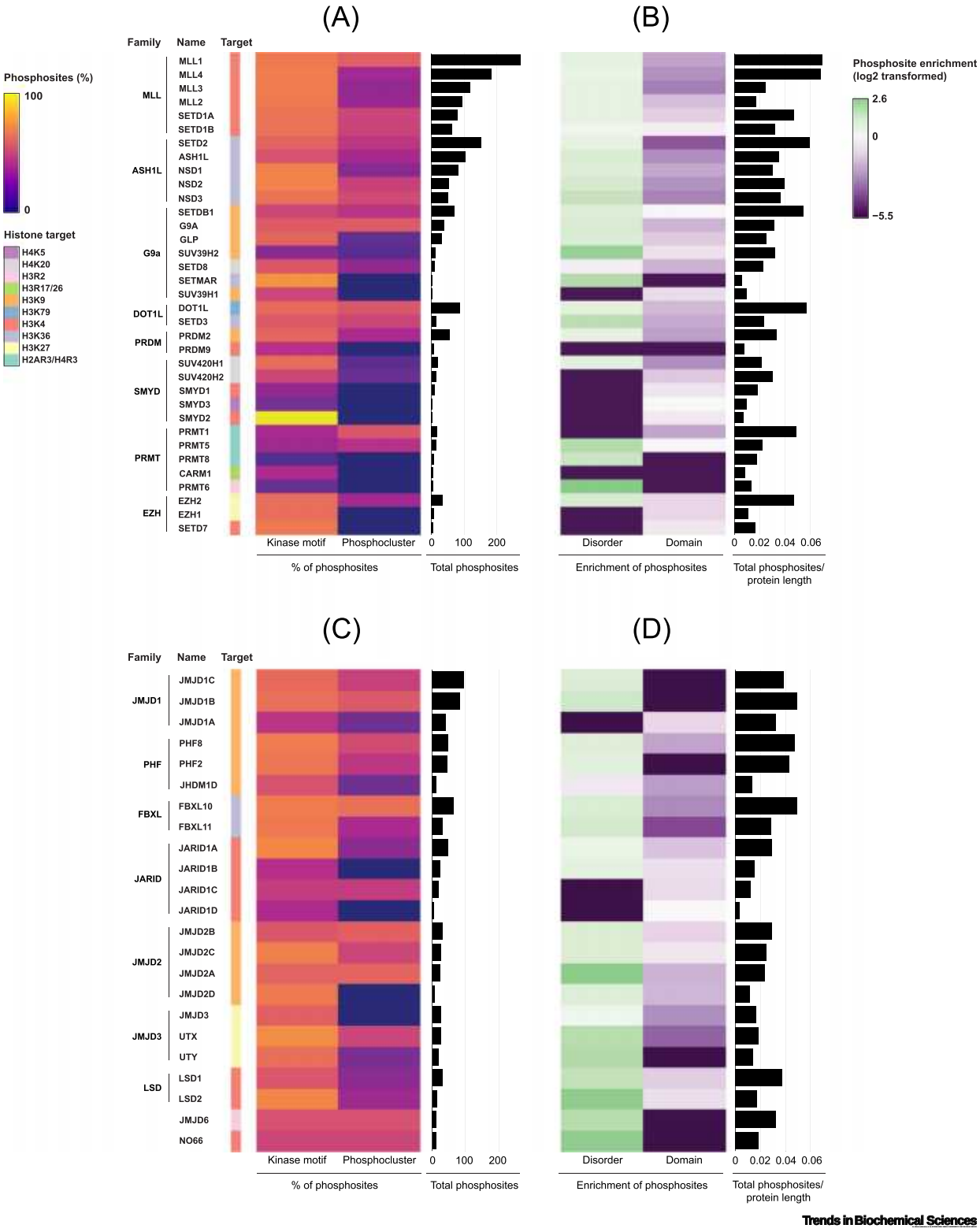
profiles. Enzymes with few phosphosites, such as the PRMTs, might be activated/deactivated on phosphorylation of a specific residue or may predominantly be subject to transcriptional control. Consistent with this is the observation that a single phosphorylation event on tyrosine 291 of PRMT1 alters its substrate specificity and protein–protein interactions [30].

## Phosphorylation Sites in Kinase Motifs Reveal Connections to Known Signalling Pathways

Of the 2511 phosphosites on human histone methyltransferase and demethylase enzymes, 62% are located within a known human kinase recognition motif (Figure 2A,C). These motifs help reveal the signalling pathways that connect to and communicate with the histone methylation system. The most common motifs are for constituents of the MAPK/ERK, mTOR/PI3/Akt, Wnt/β-catenin, cyclin-dependent, and cAMP-dependent signal transduction pathways. This highlights the potential for diverse signalling pathways to transmit cellular information to histone methyltransferase and demethylase enzymes. It also shows that methyltransferase and demethylase enzymes are critical connectors between the intracellular signalling network and the histone methylation system; two major regulatory processes in the cell.

## Phosphorylation Sites in Domains and in Regions of Disorder

Phosphorylation sites within protein domains can regulate catalytic activity and interaction partners [31]. The proportion of phosphorylation sites within domains of histone methylation enzymes is appreciably lower than expected by chance, highlighting a negative enrichment for phosphosites within such domains (Figure 2B,D). Regulation of histone methyltransferase and demethylase function by phosphorylation of domains may thus be less widespread or of lower importance. Of the small number of sites residing in domains, several are located within methyltransferase (SET, DOT1, and PRMT) and demethylase (JmjN and JmjC) domains. Such sites may act as molecular switches, whereby catalytic activity is turned on or off in response to a specific signal, or could target histone methylation enzymes to chromatin features including specific locations in the genome. Consistent with this, several phosphosites are located within chromatin interaction domains (chromodomains, bromodomains, and PHD). Functions of phosphosites are explored in greater detail, as described below.

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Disordered regions in proteins play a key role in signalling as different post-translational modifications alter their conformational fold, binding affinity to specific interaction partners [32], and liquid–liquid phase transitions [33]. In contrast to the small proportion of sites within domains, histone methyltransferase and demethylase enzymes are extensively phosphorylated within regions of disorder. Correspondingly, 74% of mammalian histone methylation enzymes were positively enriched for phosphosites within disordered sequences (Figure 2B,D). While high, this is consistent with the proportion of phosphosites in disorder across the entire phosphoproteome [34]. Notably, however, some enzyme families (e.g., MLL methyltransferases and JMJD3 demethylases) are extensively phosphorylated within disordered regions whereas others are not (e.g., SMYD methyltransferases and JARID demethylases). Sites in regions of disorder can mediate scaffolding and protein–protein interactions of histone methyltransferase and demethylase enzymes, and will be of importance for targeted analysis.

## Phosphorylation Has Diverse Effects on Histone Methyltransferase and Demethylase Function

Phosphorylation of histone methyltransferases and demethylases can have different effects on downstream cellular processes. A review by Treviño et al. in 2015 concisely summarised targeted studies which defined the effect of phosphoregulation for specific human histone methyltransferases and demethylases [35]. Here, we comprehensively review recent studies of methyltransferase and demethylase regulation by phosphorylation. Collectively, these have highlighted the effects that phosphorylation of histone methyltransferases and demethylases can have on enzymatic activity, protein–protein interactions, chromatin binding, and stability (Figure 3).

### Effects on Methyltransferase and Demethylase Activity

A single phosphorylation event can affect the activity of histone methyltransferases or demethylases (Figure 3). This has been shown by in vitro histone methylation assays with wild-type, phosphonull, and phosphomimic versions of enzymes [36,37] or by monitoring changes in cognate histone methylation marks in vivo as a proxy for methyltransferase and demethylase function after mutagenesis of phosphosites [38,39]. Two regulatory mechanisms are evident whereby phosphorylation can directly switch an enzyme's catalytic activity on or off, or influence the formation of catalytically active methyltransferase or demethylase complexes (Table 1).

A comparison of the regulatory effect of phosphorylation between histone methyltransferase and demethylase enzymes reveals a striking trend. For some histone methyltransferases, phosphorylation reduced their capacity to methylate their respective histone residue. This has been shown in five studies to date (Table 1). For example, phosphorylation at serine 363 or threonine 367 of the H3K27-specific methyltransferase, EZH2, by glycogen synthase kinase 3 β (GSK3β), was shown to significantly reduce H3K27 trimethylation levels [38]. Strikingly, and the opposite of what was seen in methyltransferases, three studies into histone demethylase function all revealed that their activity is increased by phosphorylation (Table 1). For example, cyclin E/Cdk2-mediated phosphorylation of PHF8 enhances its demethylase activity, resulting in a global decrease in H3K9 methylation levels [39]. This body of evidence, although as yet only for a small number of enzymes,

Figure 2. Phosphorylation Sites on Human Histone Methyltransferase and Demethylase Enzymes, Their Occurrence, and Enrichment in Sequence and Structural Features. Phosphorylation sites on 35 histone methyltransferases (A and B) and 23 demethylases (C and D) are collated from two phosphoproteomic datasets [23,28]. Enzyme families are grouped and ordered by total number of phosphorylation sites on their constituent members. Phosphosites in kinase motifs, disordered regions, and protein domains are determined using the Eukaryotic Linear Motif [77], MobiDB [78], and InterPro [79] databases, respectively. A phosphocluster is defined as three or more phosphosites in a sliding window of ten amino acid residues. In panels A and C, the percentage of phosphosites occurring within kinase motifs and phosphoclusters are shown as a heatmap, coloured according to the left-hand side scale, while total phosphosite count per protein is displayed as a bar chart. Panels B and D show the proportion of phosphosites within regions of disorder or protein domains, normalised by the total proportion of disorder or domains in each protein, respectively. Enrichment values are shown as a heatmap, coloured according to the right-hand side scale. Phosphosite counts per protein, normalised by total protein length, are displayed as a bar chart.

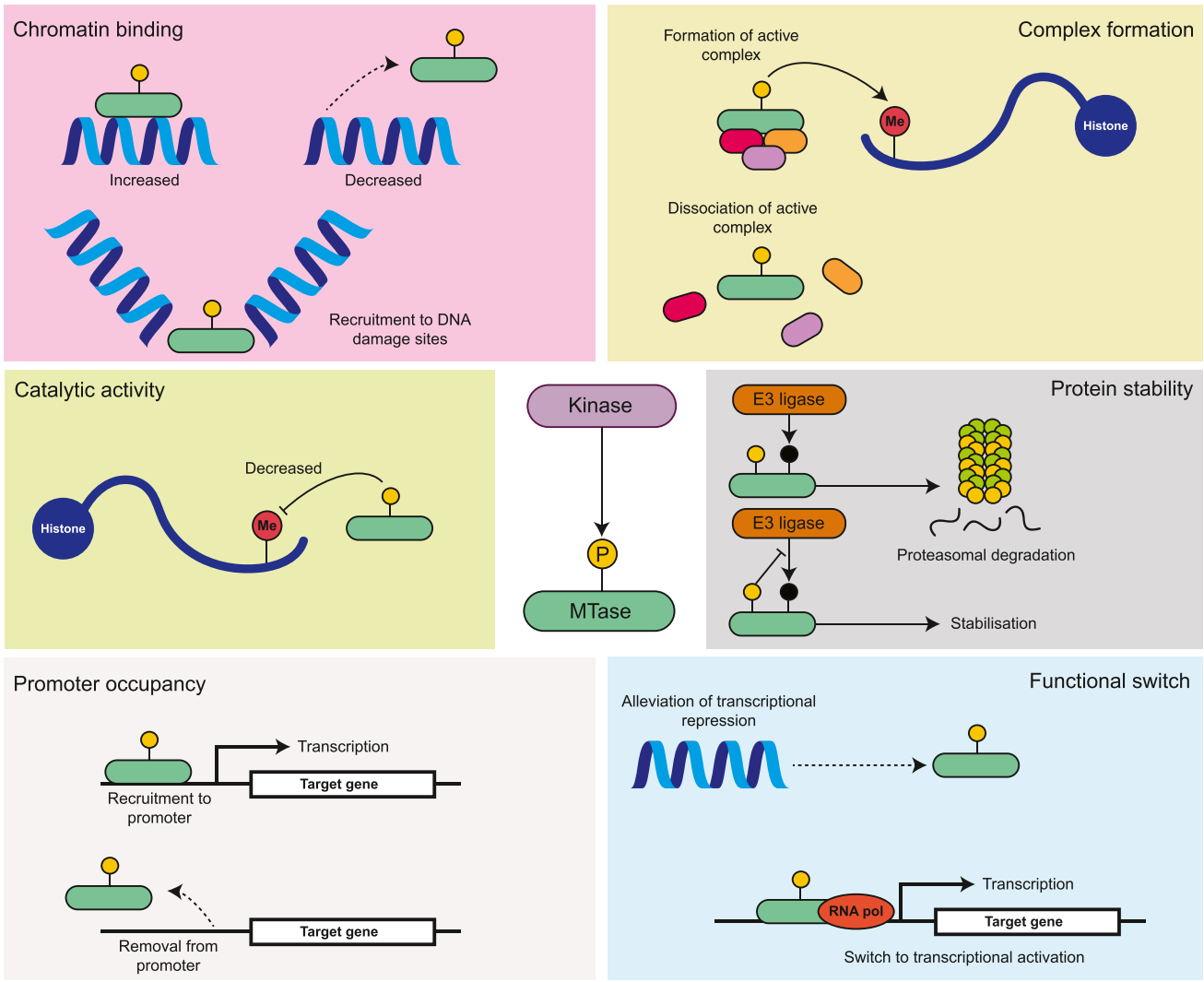


Figure 3. Effects of Phosphorylation on Human Histone Methyltransferase and Demethylase Enzymes. Phosphorylation by an upstream signalling kinase can modulate a histone MTase's catalytic activity, chromatin binding, complex formation, protein stability, and promoter occupancy, or serve as a functional switch. Here, the effects on a histone methyltransferase are shown, but the same processes apply for histone demethylases, with the exception that phosphorylation has only been shown to date to increase catalytic activity, and that a functional switch mechanism for demethylases has not yet been elucidated. Abbreviations: MTase, methyltransferase; RNA pol, RNA polymerase.

raises important questions about how the cell manages methyltransferase/demethylase balance. It is plausible that a single kinase could simultaneously activate a demethylase and inactivate a methyltransferase, leading to widespread removal of a specific histone methyl mark, and increase methyl marks in a converse way. While evidence of the same kinase acting on counteracting histone methyltransferase and demethylase enzymes has not yet been established, it will be fascinating to learn whether this phenomenon applies to the histone methylation system as a whole or in part to coregulate enzyme function in response to a specific signal.

In addition to acting as an on/off switch, phosphorylation can also mediate the formation of catalytically active methyltransferase or demethylase protein complexes (Figure 3 and Table 1). Coimmunoprecipitation assays have revealed that the presence or absence of phosphate groups

Table 1. Phosphorylation Sites on Human Histone Methyltransferases and Demethylases and How They Affect Enzymatic Activity

| Enzyme | Type[a] | Phosphosite | Kinase | Effect | Refs |
|---|---|---|---|---|---|
| Phosphosites which directly affect catalytic activity | | | | | |
| CARM1 | MTase | S217 | ? | Decreased | [37] |
| EZH2 | MTase | S21 | Akt | Decreased | [36] |
| | | S363/T367 | GSK3β | Decreased | [38] |
| MLL4 | MTase | S1331 | Akt | Decreased | [80] |
| PRMT5 | MTase | Y297/Y304/Y307 | JAK2 | Decreased | [81] |
| JMJD1A | DMase | Y1101 | JAK2 | Increased | [82] |
| LSD1 | DMase | S112 | PKC | Increased | [83] |
| PHF8 | DMase | S844 | Cdk2 | Increased | [39] |
| Phosphosites which affect formation of catalytically active protein complexes | | | | | |
| EZH2 | MTase | Y244 | JAK3 | Decreased | [84] |
| | | T311 | AMPK | Decreased | [40] |
| G9a | MTase | T166 | Aurora B | Decreased | [85] |
| NSD2 | MTase | S102 | ATM | Increased | [45] |
| SETDB1 | MTase | T976 | NLK | Increased | [86] |
| LSD1 | DMase | T369 | ? | Decreased | [87] |
| PHF2 | DMase | S1056 | PKA | Increased | [41] |

[a]Methyltransferase (MTase) or demethylase (DMase)

at interfaces can increase or decrease the protein–protein interactions of histone-modifying enzymes. For example, AMPK-mediated phosphorylation of EZH2 at threonine 311 of its N-terminal regulatory region sterically impairs its interactions with SUZ12 and EED [40]. These interactors are both required for the assembly of an active polycomb repressive complex. Conversely, PHF2, which is inactive by itself, forms an active histone demethylase complex with ARID5B upon serine 1056 phosphorylation by protein kinase A [41].

### Effects on Chromatin Binding and Genomic Localisation

Phosphorylation of specific histone methyltransferase and demethylase enzymes can affect their affinity to chromatin (Figure 3). Using pulldown-based approaches, recent studies have observed that specific phosphosites can promote or impede an enzyme's global interaction with histone and nucleosomal substrates (Table 2). For example, methyltransferase G9a is recruited to chromatin upon serine 211 phosphorylation by CK2 [42], while ATM-mediated phosphorylation of the demethylase FBXL11 at threonine 632 considerably abrogates its chromatin binding affinity [43]. These global changes in chromatin association caused by phosphorylation allow eukaryotic cells to broadly regulate histone methylation in response to upstream signals.

Single phosphorylation events have, importantly, also been shown to target a histone methyltransferase or demethylase to a specific location within the mammalian genome (Figure 3 and Table 2). Immunofluorescence techniques have revealed that several methyltransferase and demethylase enzymes, including G9a [44], NSD2 [45], and LSD1 [46], accumulate rapidly at double-strand breaks when phosphorylated in response to DNA damage. This highlights the relationship of histone methylation with DNA damage signalling pathways. At a gene-centric level, chromatin immunoprecipitation assays have shown that phosphorylation of histone-modifying enzymes can influence their occupancy at certain promoters. Indeed, the H3K4-

Table 2. Phosphorylation Sites on Human Histone Methyltransferases and Demethylases and How They Affect Chromatin Binding and Genomic Localisation

| Enzyme | Type[a] | Phosphosite | Kinase | Effect | Location | Refs |
|---|---|---|---|---|---|---|
| EZH2 | MTase | S21 | Akt | Decreased | Global | [36] |
| | | Y696 | Src | Decreased | Global | [53] |
| G9a | MTase | S211 | CK2 | Increased | Global | [42] |
| | | S569 | ATM | Increased | DSBs[b] | [44] |
| LSD1 | MTase | S112 | PKC | Increased | E-cadherin promoter | [48] |
| | | S126 | PLK1 | Decreased | Global | [88] |
| | | S131/S137 | CK2 | Increased | DSBs | [46] |
| NSD2 | MTase | S102 | ATM | Increased | DSBs | [45] |
| SETMAR | MTase | S495 | Chk1 | Increased | DSBs | [60] |
| Suv39H1 | MTase | S391 | Cdk2 | Decreased | Global | [89] |
| FBXL11 | DMase | T632 | ATM | Decreased | Global | [43] |
| JARID1A | DMase | S225/T285/S287/ T1225/S1255 | Akt | Decreased | Cell cycle gene promoters | [90] |
| JARID1B | DMase | S1456 | Cdk1 | Decreased | SOX2 and NANOG promoters | [47] |
| JMJD1A | DMase | S264 | MSK1 | Increased | Global | [91] |
| | | Y1114 | ACK1 | Increased | Global | [92] |
| PHF8 | DMase | S33/S84 | Cdk1 | Decreased | Global | [93] |

[a]Methyltransferase (MTase) or demethylase (DMase).
[b]Abbreviation: DSB, double-strand break.

specific demethylase JARID1B exhibits attenuated occupancy at the promoters of pluripotency related genes (e.g., SOX2 and NANOG) when it is phosphorylated at serine 1456 by Cdk1 [47]. By contrast, LSD1 is recruited to the promoter of E-cadherin upon serine 112 phosphorylation by protein kinase C to induce H3K4 demethylation [48]. The extent to which other histone methyltransferases and demethylases are targeted to specific genomic loci by phosphorylation is unknown and is an avenue for future research.

## Effects on Protein Stability and Proteasomal Degradation

Phosphorylation of histone methyltransferases and demethylases affects their recognition by ubiquitinating and deubiquitinating enzymes (Figure 3 and Table 3). Of eight studies into the effects of phosphorylation on methyltransferase and demethylase stability, five found that phosphorylation sterically hinders ubiquitination of nearby residues and thereby stabilises endogenous protein levels (Table 3). For example, serine 172 phosphorylation of the H3K36-specific methyltransferase NSD2 by Akt kinase disrupts its association with the CRL4 E3 ligase and prevents lysine ubiquitination proximal to the phosphosite [49]. Conversely, three studies which investigated methyltransferase EZH2 showed that phosphorylation encourages ubiquitination and proteasomal degradation (Table 3). EZH2 harbours a Cdc4 phosphodegron motif which, when phosphorylated by Cdk5, is recognised and ubiquitinated by FBW7, triggering EZH2 degradation by the proteasomal machinery [50]. Phosphorylation of methyltransferases and demethylases can also affect their interactions with deubiquitinating enzymes (Table 3). Phosphorylation of LSD1 at serine 683 is a prerequisite for its deubiquitination and subsequent stabilisation by ubiquitin-specific protease 22 [51]. The complex interplay between phosphorylation, ubiquitination, and histone methylation further accentuates the dynamic communication in the cell that exists between major regulatory systems.

Table 3. Phosphorylation Sites on Human Histone Methyltransferases and Demethylases and How They Affect Proteasomal Degradation

| Protein | Type[a] | Phosphosite | Kinase | E3 ligase/ deubiquitinase | Effect | Refs |
|---|---|---|---|---|---|---|
| Phosphosites which affect interactions with E3 ubiquitin ligases | | | | | | |
| EZH2 | MTase | T261 | Cdk5 | FBW7 | Degradation | [50] |
| | | T345/T387 | Cdk1 | ? | Degradation | [94] |
| | | Y641 | JAK2 | β-TrCP | Degradation | [95] |
| MLL1 | MTase | S516 | ATR | SCF/Skp2 | Stabilisation | [61] |
| NSD2 | MTase | S172 | Akt | CRL4 | Stabilisation | [49] |
| SETD8 | MTase | S29 | Cdk1 | APC | Stabilisation | [62] |
| JMJD2B | DMase | T305/S352/ S566/T1065 | ERK | ? | Stabilisation | [96] |
| Phosphosites which affect interactions with deubiquitinating enzymes | | | | | | |
| EZH2 | MTase | S220 | MELK | USP36 | Stabilisation | [97] |
| LSD1 | MTase | S683 | GSK3β | USP22 | Stabilisation | [51] |

[a]Methyltransferase (MTase) or demethylase (DMase).

## Phosphorylation as a Means of Integrating Information on Histone Methyltransferase and Demethylase Enzymes

There is evidence that histone-methylating and -demethylating enzymes function as major integrators of information in the human cell. Given the medical implications, the phosphoregulation of the oncogenic methyltransferase EZH2 [52] has been extensively investigated. A total of 35 phosphorylation sites have been identified on EZH2 (Figure 2), 11 of which have a known kinase and functional consequence (Figure 4A). Phosphorylation sites on EZH2 are transmitted by kinases belonging to independent upstream signalling pathways, including the JAK/STAT, PI3K/Akt/mTOR, and cyclin-dependent signal transduction pathways. These phosphosites have distinct effects on EZH2 function, affecting its H3K27 methyltransferase activity, complex formation with SUZ12 and EED, and proteasomal degradation. Reinforcing its complex post-translational regulation, Src-mediated phosphorylation at tyrosine 696 serves as a functional switch, converting EZH2 from a transcriptionally repressive protein to a transcriptional activator by enhancing its affinity for RNA polymerase II [53]. The convergence of information on EZH2 allows it to respond to multiple incoming signals, in a manner similar to the tumour suppressor protein p53 [54]. It is currently unclear whether these phosphorylation sites display crosstalk and/or work in unison to mediate functional outcomes. Other histone methyltransferase and demethylase enzymes that show extensive phosphorylation may also serve as platforms for complex integration of signals. For example, the H3K4-specific MLL family methyltransferases, H3K36-specific ASH1L family methyltransferases, and H3K9-specific JMJD1 family demethylases all have a large number of phosphorylation sites and some of which group together as phosphoclusters with the propensity to crosstalk (Figure 2).

## New Approaches Are Required to Study the Phosphorylation of Histone Methyltransferase and Demethylase Enzymes

While all 58 mammalian histone methyltransferases and demethylases are phosphorylated, 43 have no known links to an upstream kinase (Figure 1). At the site level, of 2511 phosphosites observed on these enzymes (Figure 2), only 38 (1.5%) have a known upstream kinase and 39 (1.5%) are of known molecular function (Tables 1–3). These numbers are appreciably lower than those for the human phosphoproteome, for which 3.1% of phosphosites have an

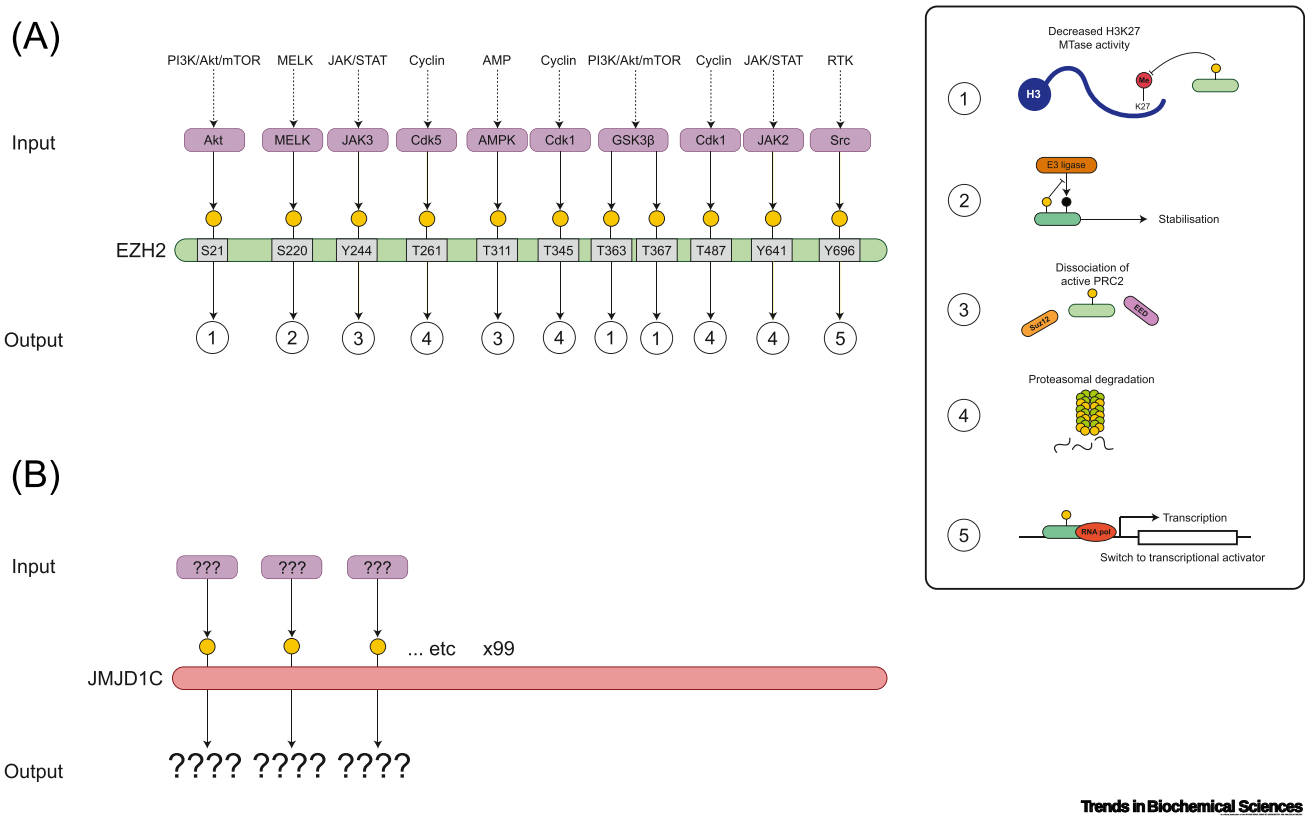


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Figure 4. Human Histone Methyltransferase and Demethylase Enzymes Receive Information from a Range of Upstream Sources. (A) Methyltransferase EZH2 is phosphorylated by a number of kinases from different signalling cascades. The downstream functional effects of these phosphorylation sites have been determined. (B) Demethylase JMJD1C harbours 99 phosphorylation sites, however, none of these sites have had an upstream kinase or biological function determined. Phosphosites on the majority of human histone methyltransferases and demethylases are similarly poorly understood. Abbreviations: EZH2, enhancer of zeste homolog 2; MTase, methyltransferase; PRC2, polycomb repressive complex 2; RNA pol, RNA polymerase; RTK, receptor tyrosine kinase.

associated kinase and 2.8% have an assigned function [55]. Highlighting this gap in our understanding, the histone demethylase JMJD1C has no known kinase or function determined for its 99 phosphosites (Figure 4B).

Recent targeted studies have begun to establish the connections between cellular signalling and histone methylation. However, the studies to date have been fragmentary; almost all investigated the role of a single phosphorylation site on a histone methyltransferase or demethylase, and, in some cases, this site's function or associated kinase could not be fully determined [30,56–59]. Given the large number of enzymes, phosphosites, and the complexities of chromatin, it will be some time before this regulatory system is fully understood. However, two key issues should be addressed to facilitate functional studies: phosphosite selection for targeted analysis and kinase discovery.

## The Challenge of Phosphosite Selection

The selection of phosphosites for detailed analysis is a major challenge as it is unclear which are the most important to study. To date, in cases where reasons have been provided for investigating particular sites, almost all studies used bioinformatic sequence analysis of a methyltransferase or demethylase to identify consensus kinase recognition motifs and thereby focus on phosphosites with candidate kinases [49,60–62]. In other cases, prior knowledge of

established kinase-mediated phosphorylation events informed the selection of individual sites for further study [44,45]. As a result, extensively studied kinases and those with well-defined consensus sequences, including ATM (pS/TQ) [63], Akt (RXRXXpS/T) [64], and Cdk1/2 (pS/TP) [65], have been attributed to many phosphorylation sites on histone methyltransferases and demethylases (Figure 1). By contrast, phosphosites that do not fall within known kinase motifs are often neglected, and kinases without defined recognition sequences are seldom investigated. Unfortunately, these approaches have favoured the continued investigation of multi-substrate kinases rather than the discovery of new kinases that act specifically on histone methyltransferases and demethylases.

A second consideration, in the choice of phosphosites to study, is to ascertain which sites actually have regulatory capacity. There are two challenges to be addressed here: the first is to define all phosphosites on the histone methyltransferases and demethylases, and the second is to understand which actually change in vivo to trigger downstream functions. Few if any studies have fully characterised their enzyme of interest, thus it is likely that constitutive phosphorylation sites, given their high stoichiometry and ease of identification, dominate the focus of targeted studies. This is problematic as while high stoichiometry sites can be essential for function, such sites are not necessarily dynamic or regulatory, and many signal-responsive sites will be of low stoichiometry [66]. Mutating a constitutive phosphorylation site is likely to disrupt the cognate protein's structure and/or function and result in a detectable biological effect. However, such observations do not necessarily show that such sites are of regulatory importance for chromatin dynamics.

Considering the aforementioned limitations of current approaches and the challenges associated with detecting low abundance regulatory phosphosites, improved methods are required to identify sites for targeted analysis. We recommend that future studies initially undertake an in depth characterisation of their enzyme of interest. An example of such an approach was used previously for yeast protein methyltransferases, where comprehensive protein sequence coverage and modification site identification was achieved by using different proteolytic digestions and multiple mass spectrometry fragmentation methods on purified versions of each enzyme [67]. To further address the issue of detection of low stoichiometry regulatory sites, phosphopeptide enrichment techniques, such as immobilised metal affinity chromatography and titanium dioxide chromatography [68], could also be used in sample preparation. During data acquisition, the use of targeted mass spectrometry methods, including data-independent acquisition [69] and selected reaction monitoring [70], can enhance phosphopeptide identification and quantitation, and confidence in site localisation. Collectively, these approaches will improve the identification of low abundance and regulatory phosphosites, including those for downstream analysis.

Functional selection criteria are also needed to shortlist potential regulatory sites for further study. Monitoring changes in the abundance of phosphorylation sites in different cellular contexts and stress conditions will highlight sites that are dynamic, and therefore likely to be regulatory, versus those that are constitutive and static. Moreover, co-monitoring the phosphorylation status of methyltransferase and demethylase enzymes and the methylation status of histone proteins, using either immunoblotting, autoradiography, or mass spectrometry, will help reveal phosphorylation sites that regulate histone methylation. This is because regulatory phosphosites, which typically change in stoichiometry in response to a signal, are likely to be accompanied by concomitant changes in downstream histone methylation levels. Such approaches will shift the focus of targeted studies towards functionally important phosphorylation sites on histone methyltransferase and demethylase enzymes.

### The Challenge of Kinase Discovery

Kinase discovery remains a sizeable challenge and there is currently no 'silver bullet' to identify the kinase responsible for a phosphorylation site. The human genome encodes 538 protein kinases [71]; many of which exhibit functional redundancy and overlapping activity in vivo [72,73]. It is therefore difficult to assign a kinase to a specific phosphorylation site just using single gene knockouts. This issue is further confounded by the challenge of identifying the kinase that acts directly on a protein, rather than a kinase upstream in a signalling pathway which may have secondary effects. Given these challenges, alternate approaches to kinase discovery are required. The use of CRISPR knockout libraries, particularly double knockouts [74], enables high-throughput screening of all nonessential human kinases, while proteome arrays can be used to identify kinase–substrate interactions in vitro [75]. Such approaches can shortlist candidate kinases for targeted validation. To confirm putative kinases, brute-force techniques, which are slow and involved, remain necessary. A recently developed proximity-based crosslinking approach shows promise in establishing kinase-mediated phosphorylation sites in vivo, without the need for overexpression, knockdown, or mutagenesis [76]. Alternatively, in vitro phosphorylation assays or proximity-dependent biotinylation approaches (e.g., APEX and BioID) can be used as confirmatory methods.

## Concluding Remarks

The histone methylation system receives substantial information from the intracellular signalling network. Different histone methyltransferase and demethylase enzyme families are connected to upstream signalling pathways in different ways. Extensively phosphorylated enzymes are emerging as major integrators of information, whereby their function is controlled by multiple independent signals, while enzymes with only a few phosphosites might be regulated by other means (see Outstanding Questions). Considerable work is required to clarify exactly how the histone methylation system, as a whole, is controlled by phosphosignalling.

While targeted studies have begun to highlight the diverse effects of phosphorylation on histone methyltransferase and demethylase biology, the majority of phosphosites have no known upstream kinase or function. This is largely due to the technical and scientific flaws of current approaches that favour the analysis of highly abundant constitutive phosphorylation sites. We have made a series of methodological recommendations, as outlined above, to facilitate the investigation of the most important regulatory sites on histone methyltransferase and demethylase enzymes. Moving forward, these will help establish novel kinase–methyltransferase/demethylase interactions of significant regulatory importance, that may be druggable targets for diseases caused by aberrant histone methylation.

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### Outstanding Questions

How does the eukaryotic cell manage histone methyltransferase/demethylase balance? Can the same kinase simultaneously activate a demethylase and deactivate a methyltransferase to promote widespread removal of a histone methylation mark, and vice versa?

Which signalling pathways are most involved in transmitting information to the histone methylation system? Are these pathways known to regulate epigenetic processes?

There is emerging evidence that certain histone methyltransferase and demethylase enzymes are major integrators of signalling information. Are other less-studied enzymes also subject to complex integration of information from upstream signalling pathways?

Do phosphorylation sites on extensively phosphorylated histone methyltransferase and demethylase enzymes display crosstalk and/or work in unison to mediate functional outcomes?

How is the activity of lesser-phosphorylated histone methyltransferase and demethylase enzymes regulated? Are these enzymes switched on/off in response to a specific phosphorylation event, or are they predominantly regulated at the transcriptional level?

Some phosphorylation sites exist at low site stoichiometry within the cell, while others are constitutive. What is the relative importance of these different types of phosphosites on histone methyltransferase and demethylase enzymes? Do low stoichiometry sites allow for dynamic responses to intracellular and extracellular signals?

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