Regulation of histone methylation by demethylimination and demethylation

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Abstract | Histone methylation has important roles in regulating transcription, genome integrity and epigenetic inheritance. Historically, methylated histone arginine and lysine residues have been considered static modifications because of the low levels of methyl-group turnover in chromatin. The recent identification of enzymes that antagonize or remove histone methylation has changed this view and now the dynamic nature of these modifications is being appreciated. Here, we examine the enzymatic and structural basis for the mechanisms that these enzymes use to counteract histone methylation and provide insights into their substrate specificity and biological function.

SET domain
A sequence motif (named after Su(var)3–9, Enhancer of Zeste, Trithorax) that is found in several chromatin-associated proteins, including members of both the Trithorax group and Polycomb group.

Histone arginine and lysine methylation have attracted much attention in the past six years owing to the identification of enzymes that catalyse these modifications, and owing to their involvement in a wide range of epigenetic processes (reviewed in REFS 1,2). In mammals, histone arginine methylation is typically found on residues 2, 8, 17 and 26 of histone H3 (H3R2, H3R8, H3R17 and H3R26) and residue 3 of histone H4 (H4R3) (reviewed in REFS 3,4). Arginine methylation is catalysed by the PRMT class of histone methyltransferases and contributes to both active and repressive effects on chromatin function[5–8]. Arginine methylation can occur in the mono-methyl, symmetrical di-methyl or asymmetrical di-methyl state (FIG. 1a). Symmetrical di-methylation of arginine refers to the addition of one methyl group to each nitrogen of the guanidinium group, whereas asymmetrical di-methylation refers to the addition of both methyl groups to one nitrogen of the guanidinium group (FIG. 1a). The functional relevance of these defined modification states remains poorly understood.

Histone lysine methylation is catalysed by a family of proteins that contain a SET domain and by yeast Dot1 and its mammalian homologue, DOT1L, which use a novel enzymatic domain (reviewed in REF. 1). Similar to arginine methylation, histone lysine methylation contributes to both active and repressive chromatin functions. In particular, methylation of histone H3K4, H3K36 and H3K79 is associated with active regions of chromatin, whereas H3K9, H3K27 and H4K20 methylation are generally found in silenced regions. Methylation of lysine groups does not affect the overall charge of the histone molecule, which indicates that these modifications function as information storage marks as opposed to disruptors of histone–DNA contacts. In support of this hypothesis, effector proteins can specifically recognize methylated lysine residues and regulate chromatin function[9–15]. Within each histone lysine residue there is an expanded potential to encode additional information, as each lysine amine group can be modified by the addition of one (me1), two (me2) or three (me3) methyl groups (FIG. 1b). The defined methylation state of a lysine residue can lead to differing functional consequences, as effector proteins might recognize one modification state while having comparatively little affinity for other modification states on the same residue[14–16]. This specificity is mirrored by the fact that enzymes that catalyse histone methylation are often capable of producing only defined modification states[17]. Therefore, the modification state of a single methyl-lysine residue can lead to different functional outcomes.

Historically, histone methylation has been considered a static modification, owing to several studies that demonstrated that global turnover of histone methyl groups occurs at a similar rate to histone turnover[18,19]. Therefore, mechanisms to remove methylated histones, including histone replacement or histone tail cleavage, have been proposed as functional alternatives to active enzymatic removal of methyl groups[20,21]. There is no doubt that these processes occur *in vivo*, as actively transcribed regions of chromatin undergo a dynamic replacement of histone H3.1 with the histone variant H3.3 (REF. 22), and histone tail clipping occurs in micronuclei of *Tetrahymena* species[23]. However, it seems unlikely that these processes are specifically designed to counteract histone methylation, as both eventually rely on disassembly of the nucleosome and replacement of the histone molecule to regenerate an intact unmodified nucleosome. This process causes not only loss of the histone methylation marks,

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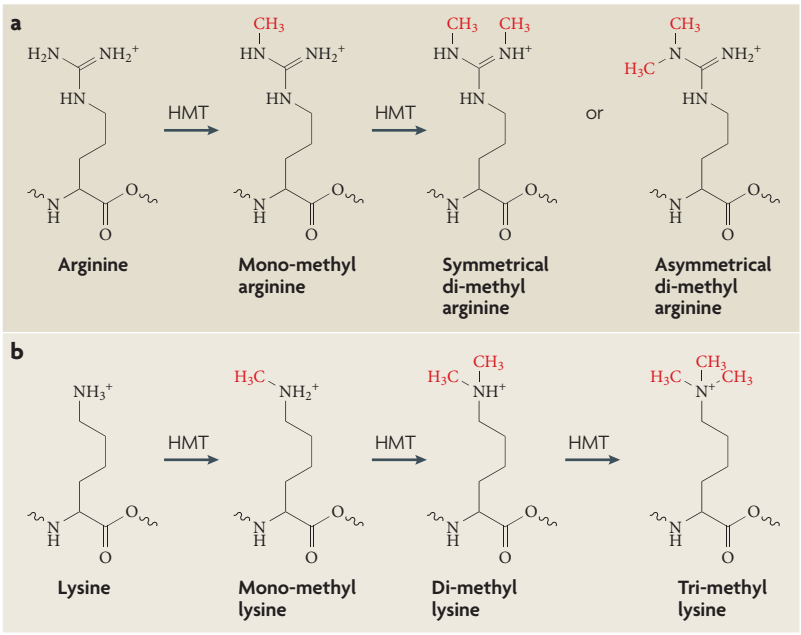


Figure 1 | **Methylation states of arginine and lysine residues in histones. a** | Arginine can be methylated to form mono-methyl, symmetrical di-methyl and asymmetrical di-methylarginine. **b** | Lysine can be methylated to form mono-methyl, di-methyl and tri-methyl lysine. Red lettering highlights the methyl groups. HMT, histone methyltransferase.

but also other important histone modifications found on the same molecule. Despite the lack of evidence for global histone methyl-group turnover, specific instances of active histone methyl-group removal have been observed *in vivo*, supporting the existence of enzymes that actively remove histone methylation *in situ*[24,25].

Over the past couple of years, a tremendous amount of effort has been put into identifying enzymes that actively antagonize or remove histone methylation without the requirement for histone replacement. These novel enzymes include a deiminase enzyme that antagonizes histone arginine methylation, and amine oxidase (AO) and hydroxylase enzymes that directly remove histone lysine methylation (TABLE 1). Here, we examine the enzymatic and structural basis for the mechanisms that these enzymes use to counteract histone methylation and provide insight into their substrate specificity and biological function.

Histone demethylimination by PADI4

Unmodified histone arginine residues can be covalently modified by deiminase enzymes to produce citrulline[26,27] (FIG. 2a, left reaction). This observation led to the hypothesis that methylated histone arginine residues might also be targeted by this class of enzyme to produce citrulline through a demethylimination reaction[28,29] (FIG. 2a, right reaction). Humans and mice encode four peptidylarginine deiminase (PADI) enzymes. Because of its nuclear localization, PADI4 was analysed for potential methylarginine deiminase activity[26]. By incubating recombinant PADI4 with histone substrates it was shown that methylarginine could be readily converted to citrulline, thereby removing the methyl group from modified arginine residues[28,29]. Because PADI4 converts methylarginine to citrulline, rather than to unmodified arginine, it fails to meet the requirements of a true histone demethylase. Furthermore, *in vitro* kinetic analysis of PADI4 enzymatic activity indicates that the enzyme works much less efficiently on methylated arginine substrates than on the same non-methylated substrate[30]. More in-depth analysis is clearly required to understand the relevance of demethylimination by PADI4. Nevertheless, *in vivo*, PADI4 seems to have a role in antagonizing histone arginine methylation in certain instances[28,29].

PADI4 carries out demethylimination of methylarginine by a hydrolase reaction that produces equal amounts of citrulline and methylamine[28,29] (FIG. 2a). PADI4 is dimeric with individual molecules aligning head to tail, and forming contacts between the N-terminal domain of one PADI4 molecule and the C-terminal domain of the second molecule in a structure that resembles a rubber boot[31] (FIG. 2c). The N-terminal domain of PADI4 (amino acids 1–300) forms the active-site cleft that is responsible for deimination (FIG. 2b). Two Ca^{2+} ions are closely associated with the active site and cause a conformational change following binding, which stabilizes this region, resulting in a functional enzyme. The substrate specificity of PADI4 is relatively broad as the active enzyme can deiminate multiple arginines on histones H3 (R2, R8, R17 and R26) and H4R3 (REF. 28), as well as target non-histone substrates[27,32]. When associated with peptides that correspond to the N-terminal tails of histones H3 and H4, PADI4 directs the side chain of the target arginine residue deep into the active-site cleft, and five normally unstructured amino acids of the histone peptide form an ordered β-turn-like conformation[31]. PADI4 does not recognize a defined amino-acid sequence, but instead seems to require an unstructured sequence surrounding the target arginine residue. These structural features explain, in part, the inherent promiscuity of this enzyme with respect to substrate recognition. Of the three arginine methylation states (mono-methyl, symmetrical di-methyl and asymmetrical di-methyl), PADI4 specifically targets deimination of the mono-methyl modification state *in vitro*. To identify the molecular determinants for this specificity, further analysis of enzyme–substrate co-crystal structures is required.

Induction of PADI4 activity in cultured cells results in a global increase in citrullinated histones and reduced levels of arginine methylation, supporting a role for this enzyme in antagonizing histone arginine methylation *in vivo*[28,29]. The recruitment of histone methyltransferase enzymes PRMT1 and CARM1 to promoter regions of hormone-induced genes leads to rapid increases in histone arginine methylation and transcriptional activation. Following the initial hormone-induced transcriptional activation phase, PADI4 is recruited to the promoter region where its presence correlates with the loss of arginine methylation, acquisition of citrulline and disengagement of RNA polymerase II (Pol II) from the gene[28,29]. Therefore, methylation of arginine residues followed by demethylimination seems to contribute to the normal cyclic on and off gene-expression programme

Table 1 | **Enzymes that antagonize or reverse histone methylation**

Enzyme family	Enzyme or enzyme group*	Budding yeast	Fly	Human	Substrate specificity
PADI	PADI4	Absent	Absent	Present	H3R2, H3R8, H3R17, H3R26 and H4R3
LSD	LSD1	Absent	Present	Present	H3K4me2 and H3K4me1
	LSD2	Absent	Absent	Present	ND
JMJC	JHDM1	Present	Present	Present	H3K36me2 and H3K36me1
	JHDM2	Absent	Present	Present	H3K9me2 and H3K9me1
	JHDM3/JMJD2	Present	Present	Present	H3K9me3, H3K9me2, K36me3 and K36me2
	JARID	Present	Present	Present	H3K4me3 and H3K4me2
	PHF8/PHF2	Absent	Absent	Present	ND
	UTX/UTY	Absent	Present	Present	ND
	JmjC only	Absent	Present	Present	Asn hydroxylation and ND

JHDM, JmjC-domain-containing histone demethylase; JMJC, Jumonji-C; ND, not determined; PADI, peptidylarginine deiminase.
*Taken from REF. 60.

of hormone-induced genes. The broad substrate specificity of PADI4 and promiscuity towards both methylated and non-methylated arginine leaves many questions regarding the specific roles of this enzyme in antagonizing histone arginine methylation, but it is clear that demethylimination contributes to transcription regulation and histone modification.

Histone demethylation by LSD1

Over four decades ago, an enzymatic activity was identified in tissue extracts that could demethylate modified lysine by an AO reaction that was proposed to use FAD as a cofactor and produce formaldehyde and unmethylated lysine as reaction products[33]. Later, an enzymatic activity with similar properties was demonstrated to catalyse demethylation of histones[34,35]. However, the identity of this histone demethylase remained elusive. BHC110 (later renamed LSD1) contains an AO domain and was isolated as a stable component of several histone deacetylase (HDAC) protein complexes[36–42]. Based on the presence of LSD1 in chromatin-modifying complexes and its predicted FAD-dependent AO function, Shi and colleagues proposed that LSD1 is the long-sought-after FAD-dependent histone demethylase enzyme. Incubation of recombinant LSD1 with methylated histone substrates resulted in a robust demethylase activity with specificity towards histone H3 methylated on lysine 4 (H3K4)[43]. Enzymatic characterization of the demethylation reaction showed that FAD was required as a cofactor during the removal of a methyl group in a reaction that produced hydrogen peroxide and formaldehyde as products[43] (FIG. 3a). Despite the fact that tri-methylated lysine is a common modification state in histones, the reaction mechanism used by LSD1 requires a protonated nitrogen to initiate demethylation, limiting this enzyme to di-methylated and mono-methylated lysine residues as substrates.

Co-REST alters LSD1 substrate specificity. *In vitro* analysis of recombinant LSD1 revealed that LSD1 alone only demethylates H3K4 in histone substrates that have been stripped of associated DNA[43]. This property was surprising given that nucleosomes are the physiological substrate for LSD1 *in vivo*. Isolation and characterization of LSD1 demethylase complexes from mammalian cells revealed that LSD1 requires Co-REST, a chromatin-associated transcriptional repressor, to demethylate nucleosomal substrates[39,44]. Reconstitution experiments using purified recombinant factors demonstrated that a LSD1–Co-REST association was sufficient to allow nucleosomal demethylation by LSD1 (REFS 39,44). Interestingly, LSD1 is also stimulated by HDAC1-dependent deacetylase activity of the LSD1 complex, revealing a tightly coupled relationship between demethylase and deacetylase activities (BOX 1).

The crystal structure of LSD1 alone[45,46] and in association with Co-REST[47] was recently solved. LSD1 alone forms a structure that contains three domains: the SWIRM domain, the AO domain and the Tower domain (FIG. 3b). The N-terminal SWIRM domain contains a six-helix bundle that packs against the AO domain. The AO domain adopts a two-lobed AO fold that is present in other flavoenzymes. Within the AO domain, one of the lobes functions in substrate recognition and binding, and the other lobe associates with the cofactor FAD. The active site is located within a large cavity between the substrate-binding and FAD-binding domains. A unique feature of the AO domain of LSD1 is an ~105-amino-acid insertion that protrudes from the globular core of the enzyme and forms a third structural domain, the Tower domain. The Tower domain consists of an anti-parallel coiled coil with two extended α-helices that form a left-handed superhelix. The elegant structure of LSD1 in association with Co-REST shows that the Tower domain facilitates interaction with Co-REST[47] (FIG. 3b) by acting as a docking site for the linker region of Co-REST.

Surprisingly, the binding of Co-REST to LSD1 does not cause any significant structural alteration within the catalytic domain to allow nucleosomal demethylation. Instead, Co-REST seems to contribute additional chromatin recognition properties through the function of the SANT domain. Although the SANT domain of

SANT domain
The SANT domain (named after 'switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor ((TF)IIIB)) is a 50-amino-acid motif that is present in nuclear receptor co-repressors and many chromatin-remodelling complexes.

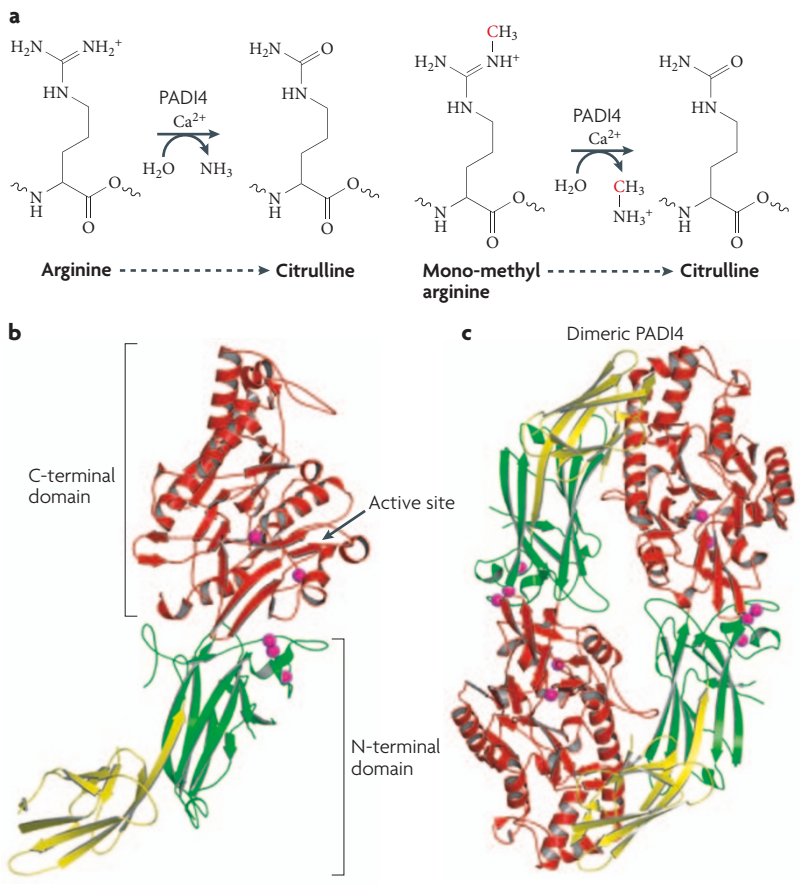


Figure 2 | **PADI4 is a methylarginine deiminase. a** | PADI4 is a Ca^{2+}-dependent peptidylarginine deiminase enzyme that can function to antagonize arginine methylation levels by demethylimination. Deimination of argine and demethylimination of mono-methylarginine is detailed, with the location of the methyl group carbon indicated in red and the reaction product citrulline. **b** | A polypeptide backbone cartoon structure of PADI4. The N-terminal immunoglobulin-like domains (green and yellow) are shown below the C-terminal α/β propeller structure (red) that houses the catalytic domain associated with Ca^{+} (pink spheres). **c** | A polypeptide backbone cartoon depicting the head-to-tail dimeric structure of PADI4. The N-terminal domain of one PADI4 molecule (yellow) interacts with the C-terminal domain of the second PADI4 molecule (red) to form a stable dimer. Parts **b** and **c** were modified, with permission, from REF. 31 © (2004) Macmillan Publishers Ltd.

other proteins has been reported to associate with histone tails, the Co-REST SANT domain binds without apparent sequence specificity to DNA. Point mutations in the SANT domain that inhibit DNA binding also inhibit nucleosomal demethylation[47], suggesting that DNA binding is essential for stimulating LSD1-mediated nucleosomal demethylation. Furthermore, structural modelling suggests that the LSD1–Co-REST complex might recognize a single nucleosome, allowing the methylated H3K4 residue to associate with the LSD1 catalytic site, whereas the SANT domain binds major-groove DNA within the same nucleosome[47] (FIG. 3c). It remains unclear what specific structural role the association between LSD1 and Co-REST has in allowing the catalytic domain to target nucleosomal H3K4, but DNA binding might allow enhanced access of the LSD1 catalytic domain to the H3 tail.

Recognition of H3K4me2 and H3K4me1 by LSD1. Unfortunately, LSD1–H3K4me2 peptide co-crystals revealed no interpretable electron density corresponding to the histone peptide, leaving the substrate recognition properties of LSD1 undefined[47]. Nevertheless, the cavity that contains the active site has an acidic pocket, which probably associates with the basic regions of the histone H3 tail and positions the H3K4 side chain in the vicinity of FAD[45–47]. Because LSD1 catalysis requires a peptide substrate containing at least 21 amino acids[48], it is thought that additional substrate recognition is contributed to by a groove formed between the SWIRM and AO domains of LSD1[47]. As mentioned above, LSD1 is catalytically limited to H3K4me2 and H3K4me1 substrates due to the reaction mechanism used to initiate demethylation. Interestingly, the catalytic cavity of LSD1 is large enough to accommodate an H3K4me3 substrate[46], but competition analysis suggests that H3K4me3 is only a weak competitive inhibitor[48]. This observation indicates that the modification state does in part dictate substrate recognition by LSD1. Specific recognition of H3K4me2 and H3K4me1 *in vivo* could be important for efficient LSD1 function as inappropriate binding of LSD1 to H3K4me3 substrate might lead to a situation in which enzyme is bound to substrate in a catalytically non-productive state.

LSD1 functions as both an activator and a repressor. Previously, the Co-REST complex was shown to repress transcription of neuronal genes in non-neuronal cell lineages[49], suggesting that LSD1 might also contribute to this function. Chromatin immunoprecipitation experiments revealed that LSD1 associates with Co-REST target genes where it demethylates H3K4 and contributes to transcriptional repression[43] (FIG. 3d). In addition to the role of LSD1 in transcriptional repression, an association between LSD1 and the androgen receptor (AR) converts LSD1 to an H3K9 demethylase, allowing it to function as a transcriptional activator[50]. During hormone-induced transcriptional activation, LSD1 is partially required for H3K9 demethylation and AR transactivation[50,51] (FIG. 3e). The structural mechanism by which the AR alters LSD1 specificity remains unknown.

LSD1 homologues have been found in fission yeast (but not budding yeast), worm, fly and mammals. Like their mammalian counterparts, the worm and fly Co-REST and LSD1 homologues physically interact, suggesting that this complex is evolutionarily conserved[52–54]. The fly LSD1–Co-REST complex might have analogous functions to their mammalian counterparts through physical association with the tramtrack 88 transcription factor, which regulates neuronal gene expression in this organism[54], but no link to neuron-specific gene expression has been identified for worm homologues. Given that fission yeast is a single-cell organism that lacks the requirement for diversified cell identity, it was unclear what role the two LSD1 homologues, SWIRM1 and SWIRM2, might have in this organism. Recently, SWIRM1 and SWIRM2 protein complexes were isolated from fission yeast, but no *in vitro* histone demethylase activity has been identified

for either protein[55]. SWIRM1 and SWIRM2 binding patterns throughout the fission yeast genome are remarkably similar, and both tend to associate with the 5′-end of genes. Gene-expression analysis in SWIRM1 deletion strains revealed reduced target gene expression and increased levels of antisense transcripts. These observations indicate that LSD1 homologues in fission yeast might contribute to the transcriptional activation of target genes in a manner that is similar to mammalian LSD1 when bound to the AR[50,51].

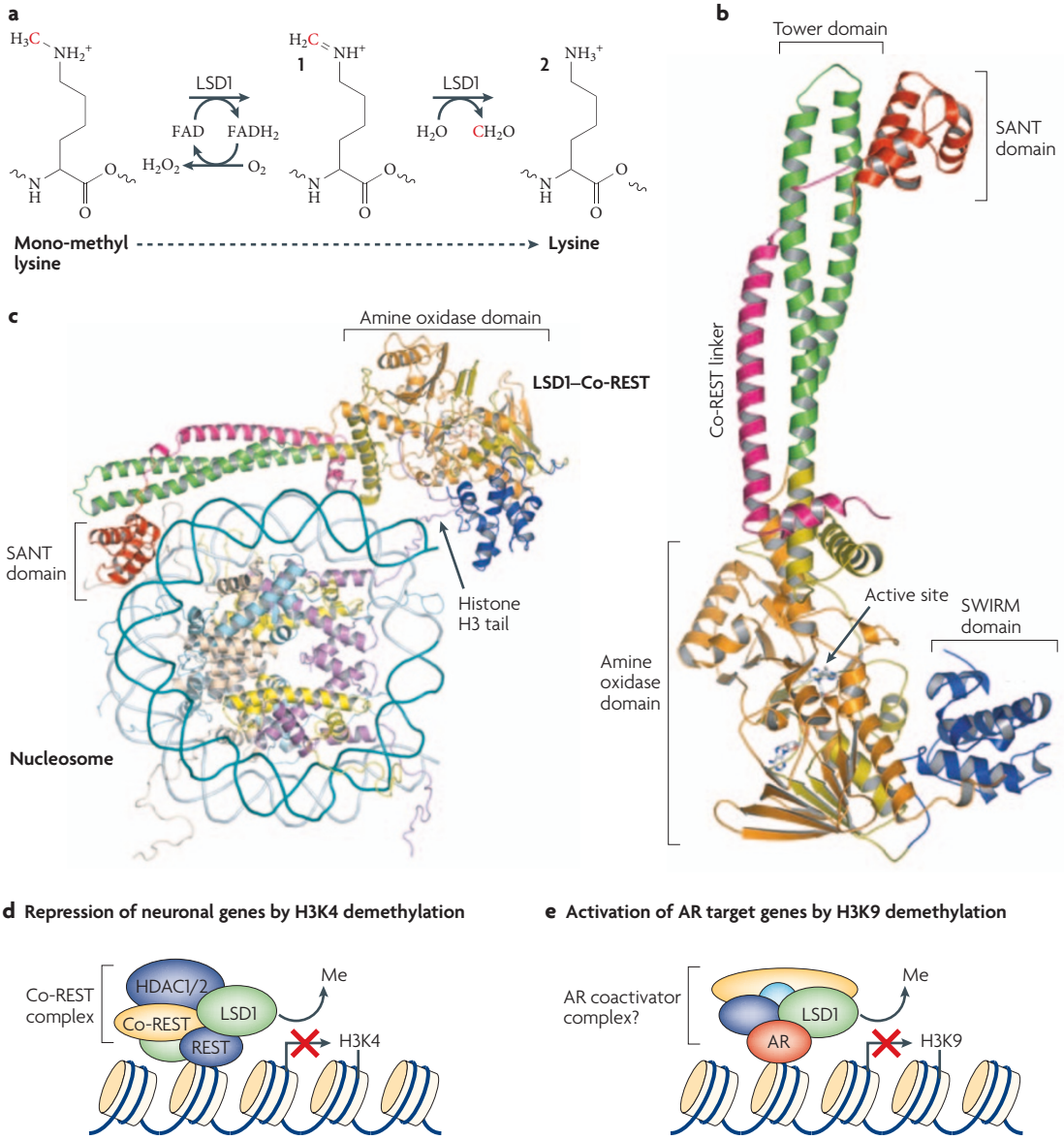


Figure 3 | **LSD1 is an H3K4 and H3K9 demethylase. a** | The LSD1 reaction mechanism detailing the removal of a mono-methyl group. LSD1 is proposed to mediate demethylation of mono- and di-methylated lysine residues through an amine oxidation reaction using FAD as a cofactor. Loss of the methyl group from mono-methyl lysine occurs through an imine intermediate (1), which is hydrolysed to form formaldehyde by a non-enzymatic process (2). **b** | A polypeptide backbone cartoon structure of LSD1 bound to Co-REST and the cofactor FAD. The two-lobed amine oxidase (AO) domain is shown in orange and yellow. The Tower domain is in green and the SWIRM domain in blue. The Co-REST linker region (pink) associates with the LSD1 Tower domain and the SANT domain (red) situated at the top of the Tower domain. **c** | Depiction of the potential association of LSD1–Co-REST with nucleosomal DNA. The bottom half shows a nucleosome with the core histone octamer in the centre and the associated DNA double helix in blue. The LSD1–Co-REST complex modelled onto a nucleosome indicates that the SANT domain of Co-REST (red) could interact with nucleosomal DNA, whereas LSD1 targets the histone H3 tail where it protrudes from the DNA gyres (shown by the arrow). **d** | LSD1 as part of the Co-REST complexes contributes to repression of neuronal genes in non-neuronal cells. LSD1 contributes to repression by removing H3K4 methylation. **e** | When bound to the androgen receptor (AR), LSD1 is converted from a transcriptional repressor to an activator by changing the substrate specificity of LSD1 so that it catalyses the removal of H3K9 methylation. Parts **b** and **c** were modified, with permission, from REF. 47 © (2006) Cell Press.

Box 1 | **LSD1-mediated histone demethylation and deacetylation**

Within cellular chromatin, methylation on histone tails occurs in *cis* with various other covalent histone modifications[1]. The observation that the histone demethylase LSD1 is a component of several histone deacetylase (HDAC) complexes and that it recognizes an extended portion of the histone H3 tail[129] led to the suggestion that recognition of H3K4 methylation might be regulated by histone acetylation at other sites on the same histone tail. In support of this hypothesis, the LSD1–Co-REST complex works less efficiently on hyperacetylated nucleosomes[38,44], and LSD1-mediated demethylation of H3K4 peptides is completely abolished when it also contains acetyl groups on K9, K14 and K18 (REF. 129). Furthermore, reconstitution experiments using purified factors *in vitro* show that demethylation of nucleosomal substrates is enhanced by the LSD1–Co-REST complex in the presence of HDAC1 (REF. 38). The link between demethylation and deacetylation within LSD1-containing HDAC complexes is even more intertwined as the catalytic activity of LSD1 enhances nucleosomal deacetylation[38]. Therefore, H3K4 demethylation and histone deacetylation by LSD1-containing complexes seems to be tightly coupled with both activities, contributing to the overall repressive functions of these complexes.

JmjC proteins are histone demethylases

There are only two LSD1 homologues in the mammalian genome, and the enzymatic mechanism by which these proteins initiate demethylation precludes catalysis of tri-methylated substrates. Given the large number of characterized histone lysine methylation sites and the prevalence of the tri-methyl-modification state, it seemed likely that additional enzymes would be used to catalyse histone lysine demethylation. The identification of the bacterial AlkB protein showed that an iron-dependent and α-ketoglutarate-dependent oxidation reaction mechanism was capable of demethylating DNA, producing formaldehyde as the reaction product[56,57]. Similarities between the AlkB catalytic domain and the JmjC (Jumonji-C) domain in eukaryotes indicated that JmjC-domain-containing proteins might constitute hydroxylase enzymes that function as demethylases[58,59]. Furthermore, many eukaryotic JmjC-domain-containing proteins have characterized roles in transcriptional regulation and contain other domains associated with chromatin function, making this class of proteins attractive histone demethylase candidates[58,60].

Identification of JHDM1. Based on the reaction mechanism used by AlkB to demethylate damaged DNA, a novel histone demethylase assay was designed to isolate potential histone demethylase activities[59]. Using a biochemical purification approach, a robust demethylase activity with specificity towards histone H3K36 was purified from mammalian cells[59]. JmjC-domain-containing histone demethylase 1A (JHDM1A; also known as Fbx11) was shown to be responsible for the demethylase activity, and domain-mapping studies using recombinant protein demonstrated that the JmjC domain was the catalytic core of the enzyme. The JHDM1 reaction mechanism relies on iron and α-ketoglutarate as cofactors to catalyse direct hydroxylation of the lysine methylamine group, producing succinate and carbon dioxide as reaction products (FIG. 4a). The hydroxymethyl group is spontaneously lost as formaldehyde to liberate one methyl group from modified lysine[59] (FIG. 4a). Interestingly, characterization of JHDM1 substrate specificity revealed that JHDM1A is only capable of removing the H3K36me2- and H3K36me1-modification states. This observation was surprising given that the reaction mechanism used by the JmjC domain, unlike LSD1, does not require a protonated nitrogen and therefore is compatible with removal of all three modification states. Structural studies are required to determine the specific molecular explanation for the inability of JHDM1 to demethylate the tri-methyl-modification state, but it seems plausible that, like the histone methyltransferase enzyme SET7/9, the dimensions of the JHDM1 catalytic site mighty dictate substrate recognition[17].

JHDM1 acts as a barrier to H3K36 methylation. Little is known about the function of JHDM1A in mammals. Recently, the closely related mammalian JHDM1B (also known as Fbx110) protein was isolated as a component of the BCOR transcriptional repressor complex, but its contribution to histone H3K36 demethylation was not analysed[61]. The yeast JHDM1 homologue, Jhd1, is also an H3K36me2 and H3K36me1 demethylase, indicating that this demethylation system is evolutionarily conserved[59]. In eukaryotes, H3K36 methylation is targeted to transcribed regions of genes through an association between H3K36 methyltransferase enzymes and the elongating form of Pol II[62–67]. H3K36 methylation within the body of active genes recruits transcriptional repressors to suppress the initiation of intragenic transcription[68–70]. So far, nothing is known about how the mammalian JHDM1 enzymes are recruited to defined chromatin regions, but JHDM1 proteins contain several potential chromatin-targeting domains. Of particular interest is the PHD domain, which is conserved in both yeast and humans[60]. In several other chromatin-associated proteins the PHD domain functions as a methyl-lysine-binding module[14,15,71,72], which indicates that H3K36 demethylase activity could be targeted by the recognition of modified chromatin domains. An *in vitro* proteomic screen of yeast PHD-domain-containing proteins has recently demonstrated that the PHD domain of Jhd1 specifically interacts with histone H3K4me3 (REF. 16). Interestingly, H3K4me3 methylation is found at the 5′-ends of active genes[73] and shows a reciprocal pattern compared with H3K36 methylation, which predominates towards the 3′-ends of genes[74,75]. This demarcation indicates a potential regulatory system by which Jhd1 could function to recognize H3K4me3 chromatin and block the repressive effects of H3K36 methylation at the 5′-ends of genes. In support of this hypothesis, genome-wide location analysis in strains that lack Jhd1 has revealed a global increase in the levels of H3K36me2 methylation near start sites of transcription (J. Fang and Y.Z., unpublished observations). Therefore, in yeast, the removal of H3K36 methylation surrounding promoter elements might be dictated by pre-existing H3K4 methylation marks, providing an intriguing interplay between systems that place and remove histone methylation.

JHDM2 is a H3K9 demethylase. Exploiting the same *in vitro* demethylase assay as was used to isolate JHDM1, a novel H3K9-specific demethylase was biochemically purified from mammalian cells and named JHDM2A[51].

PHD domain
(Plant homeodomain). A zinc-binding domain found in many chromatin-associated proteins. Some PHD-domain-containing proteins have been shown to recognize methylated lysine residues in chromatin.

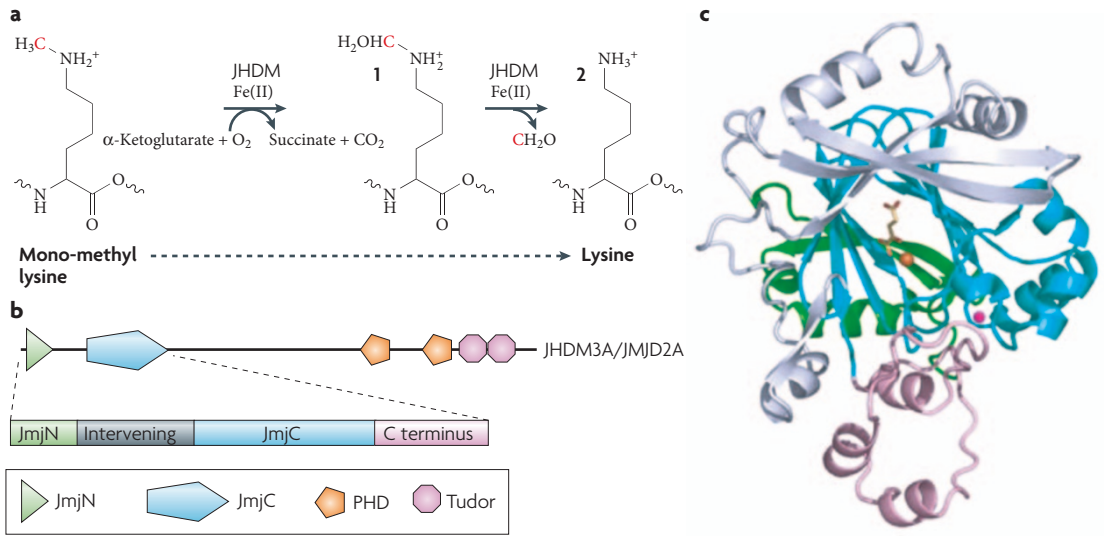


Figure 4 | **JmjC-domain-containing proteins encode histone lysine demethylases. a** | The reaction mechanism shows the removal of a methyl group from a mono-methylated lysine residue catalysed by a JmjC (Jumonji-C)-domain-containing protein. The JmjC domain uses iron (Fe(II)) and α-ketoglutarate as cofactors in an oxidative demethylation reaction that produces hydroxymethyl-lysine, succinate and CO_2 as reactions products (1). The hydroxymethyl group is then spontaneously lost as formaldehyde (CH_2O) to liberate a methyl group (2). Red lettering indicates the methyl group carbon. **b** | The N-terminal portion of JHDM3A/JMJD2A was recently crystallized. The full-length JHDM3A/JMJD2A protein (top) and the crystallized fragment (bottom) are shown. The crystallized fragment contains the JmjN domain (green), intervening region (grey), JmjC domain (blue) and the C-terminal region (pink). The colours used to illustrate each region of the crystallized fragment correspond to structural domains in part **c**. **c** | A polypeptide backbone cartoon structure of JHDM3A/JMJD2A bound to Fe(II) (orange ball), α-ketoglutarate (yellow and red sticks) and zinc (red ball). The catalytic site composed of eight β-strands (blue) forms the core of the JmjC domain and houses the α-ketoglutarate and Fe(II) cofactors. The JmjN domain (green) associates with the JmjC domain on the opposite face to the catalytic site. The C-terminal region (pink) and part of the JmjC domain coordinate the zinc ion. Part **c** was modified, with permission, from REF. 92 © (2006) Cell Press.

Like JHDM1A, JHDM2A demethylase activity can be recapitulated using purified recombinant protein and specifically demethylates H3K9me2- and H3K9me1-modified substrates. There are three members of the JHMD2 family in mammals, JHDM2A–C. JHDM2A and JHDM2B have similar enzymatic properties towards H3K9me2 and H3K9me1, but no H3K9 demethylase activity has been observed for JHDM2C (K. Yamane and Y.Z., unpublished observations)

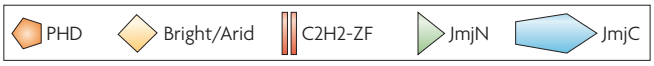
It has previously been demonstrated that human JHDM2C can interact with the thyroid receptor, suggesting that mammalian JHDM2 proteins might contribute to transcriptional regulation by nuclear hormone receptors[76]. To examine this possibility, nuclear receptor proteins were analysed for interaction with JHMD2A *in vitro*, and the AR was shown to specifically bind JHDM2A in a ligand-dependent manner[51]. In cells that are treated with the AR ligand, JHDM2A is recruited to AR target genes where it contributes to transcriptional activation and reduces the levels of promoter-associated H3K9 methylation[51]. Furthermore, small interfering (si)RNA-mediated knockdown of JHDM2A inhibits transactivation of AR target genes concomitant with increased levels of promoter-associated H3K9 methylation. JHDM2 homologues are only found in flies and other higher eukaryotes, indicating that these enzymes might have evolved in multicellular organisms to mediate hormone-dependent transcriptional events[60].

JHDM3/JMJD2 proteins are tri-methyl demethylases. Surprisingly, JHDM1 and JHDM2 fail to catalyse demethylation of their target residues when modified in the tri-methyl state[51,59]. Consistent with the theoretical capacity of JmjC-domain-containing proteins to remove the tri-methyl-modification state, JHDM3A/JMJD2A protein can catalyse demethylation of the tri-methyl H3K9 or H3K36 (REFS 77–80). JHDM3/JMJD2 enzymes preferentially demethylate H3K9me3, H3K9me2, H3K36me3 or H3K36me2, leading to an accumulation of the me1-modification state, but fail to initiate demethylation on me1-modified substrates[77]. In contrast to JHDM1 and JHDM2, the JHDM3/JMJD2 enzymes require both JmjC and JmjN domains to efficiently catalyse demethylation[77,80], and the C-terminal Tudor domain might function as a chromatin-targeting module as it binds methylated histone H3K4, H3K9 and H3K20 (REFS 12,13; FIG. 4b).

Initially, JHDM3A/JMJD2A was isolated as a transcriptional repressor that binds the *ASCL2* gene and represses transcription[81]. This observation is surprising given that JHDM3A/JMJD2A removes histone modifications that are generally associated with transcriptional repression[1]. The recent observations that both H3K9 and H3K36 methylation are associated with the body of actively transcribed genes in mammals[82,83] suggests that JHDM3/JMJD2 proteins might have roles in removing these modifications during the establishment of the fully

Box 2 | **Histone lysine demethylases in budding yeast**

Protein	Substrate
Jhd1	H3K36me2 and -me1
Jhd2	H3K4me3 and -me2
Rph1	H3K9me3 and -me2 H3K36me3 and -me2
Gis1	–
Ecm5	–



Chromatin in the budding yeast *Saccharomyces cerevisiae* contains histone lysine methylation predominantly on histone H3K4, H3K36 and H3K79. In *S. cerevisiae*, there is no LSD1 homologue, but there are five JmjC-domain-containing proteins: Jhd1, Jhd2, Ecm5, Rph1 and Gis1. Using combined bioinformatics and candidate approaches, we have analysed the function of these potential histone demethylase enzymes. Jhd1 is the yeast orthologue of the mammalian JHDM1 proteins and demethylates H3K36me2 and H3K36me1 (REF. 59) (see figure). Jhd2 is the orthologue of the mammalian JARID1 proteins and catalyses demethylation of H3K4me3 and H3K4me2 (REF. 87). Ecm5 is phylogenetically related to Jhd2, but has a highly divergent JmjC domain that lacks cofactor-binding residues that are required for enzymatic activity. Rph1 and Gis1 are orthologues of the JHDM3/JMJD2 proteins in mammals. Rph1 is an H3K36 demethylase that targets the me3 and me2 modification states[130], but Gis1 has a substitution in one of the cofactor binding sites that abrogates demethylase activity.

Interestingly, Rph1, similar to its mammalian counterparts, demethylates H3K9 despite the lack of this modification state in budding yeast. This observation suggests that budding yeast might have once encoded an H3K9 methylation system and that Rph1 is a functional vestige of this modification system[130]. Interestingly, both H3K4 and H3K36 are readily reversible in budding yeast, but there is no JmjC-domain-containing protein that demethylates H3K79. This observation suggests either that H3K79 is not enzymatically reversible in budding yeast, or that demethylation of this residue is catalysed by members of a novel protein family. JHDM, JmjC-domain-containing histone demethylase; JmjC, Jumonji-C.

Tudor domain
A repeated domain first identified in the *Drosophila melanogaster* Tudor protein, which has subsequently been identified in other proteins as a domain capable of mediating protein–nucleotide and protein–protein interactions. Recently, some Tudor domains have been shown to specifically associate with methylated lysine residues.

X-linked mental retardation
A term broadly used in reference to a group of inherited mental retardations with primary genetic defects mapping to the X chromosome.

Trithorax
Antagonists of Polycomb-group (PcG) proteins that maintain the active state of gene expression, whereas PcG proteins counteract this activation by repressing gene expression.

silenced state following phases of active transcription. The function of JHDM3/JMJD2 proteins seem to be relevant to cancer biology, as the JHDM3C/JMJD2C protein is overexpressed in oesophageal cancer cell lines[84] and RNA interference (RNAi)-mediated knockdown of JHDM3C/JMJD2C reduces proliferation of these cell lines[78]. In addition, RNAi-mediated knockdown of the *C. elegans* JHDM3/JMJD2 orthologue results in CEP-1/p53-dependent germ-cell apoptosis and causes defects in the progression of meiotic double-stranded DNA break repair[79]. It will be interesting to determine whether the functional effects of JHDM3/JMJD2 proteins in cancer-cell proliferation and genome integrity rely on JHDM3/JMJD2 demethylase activity.

JARID1 proteins remove tri-methyl H3K4. The primary amino-acid sequence within the JmjC domain of the JARID1 family is highly similar to that of JHDM3/JMJD2 proteins[60], yet JARID1 proteins specifically demethylate H3K4 (REFS 85–87; K. Yamane and Y.Z., unpublished observations). Like the JHDM3/JMJD2 demethylases, JARID1 enzymes target the me3- and me2-modification states *in vitro*, but fail to initiate demethylation on the me1-modification state. In mammals, there are four JARID1 family members: JARID1A (also known as RBP2), JARID1B (also known as PLU-1), JARID1C (also known as SMCX) and JARID1D (also known as SMCY). JARID1-family members generally function as transcriptional regulators, and a reduction of JARID1 protein levels leads to reactivation of target genes and increased levels of H3K4me3 (REF. 85). JARID1A knockout mice are viable but show mild defects in the haematopoietic systems[85]. JARID1B is overexpressed in breast cancer cell lines in which it represses tumour suppressor genes through its H3K4 demethylase activity[88] (K. Yamane and Y.Z., unpublished observations).

Little is known about the function of JARID1C or JARID1D, with the exception that mutations in JARID1C cause X-linked mental retardation[89]. The function of the JARID1 family is evolutionarily conserved as both the fly homologue and the budding yeast homologue are H3K4me3 demethylases[87]. Interestingly, the fly JARID1 protein, Lid, is a Trithorax group protein, indicating that demethylase activity might contribute to epigenetic regulation of gene expression in this organism[86,90]. The budding yeast JARID1 homologue, Jhd2, functions to antagonize Set1-mediated H3K4 methylation and is required for normal telomeric chromatin function, indicating that this demethylation system is evolutionarily conserved[87] (BOX 2).

Structure of the JmjC domain

The primary amino-acid sequence of the JmjC domain is similar to other α-ketoglutarate-dependent oxygenases[91]. Recently, the N-terminal region of JHDM3A/JMJD2A in association with iron and α-ketoglutarate was crystallized, and the core JmjC domain was shown to form a double-stranded β-helix core fold (also known as a jelly-roll fold or double Greek key motif)[92] (FIG. 4). Eight β-strands within the JmjC domain form a two-sided β-helix core fold (DSBH) that is typical of this class of metalloenzymes. The core structure of the JmjC domain provides a rigid scaffold that coordinates an iron (Fe^{2+}) molecule through a typical arrangement of histidine and glutamic acid residues (HxE/Dx_nH) with additional stabilizing interactions provided by the associated α-ketoglutarate cofactor. In addition to the core structure of the JmjC domain, the JmjN domain and C-terminal region contribute to the overall structure of JHDM3A/JMJD2A. The JmjN domain, which is only present in a subset of JmjC-domain-containing proteins[60], is formed by three helices positioned between two β-strands. The JmjN domain associates with the JmjC domain at a position opposite to the catalytic site with its two β-stands integrating into the core of the JmjC domain. Deletion of the JmjN domain renders JHDM3A/JHDM2A inactive[77,80,92], but the structure provides little direct evidence to support a role for the JmjN domain in catalysis given its distance from the active site. The C-terminal portion of the JHMD3A/JMJD2A structure is a mixture of coils and α-helices. Interestingly, this region associates with the JmjC domain and forms a novel zinc-finger motif. This structural motif is achieved by the coordination of zinc by two cysteine residues of the JmjC domain and a cysteine and histidine residue

of the C-terminal region. Attempts to disrupt the zinc-finger motif through substitution mutations caused the JHDM3A/JMJD2A protein to become unstable in solution, suggesting that this feature is required for the structural integrity of the protein.

The lack of a JHDM3A/JMJD2A enzyme–substrate co-crystal structure has made the features required for substrate recognition difficult to interpret. From the existing JHDM3A/JMJD2A structure it is clear that the catalytic core is situated far from the surface of the enzyme and surrounded by a pocket created from protein elements external to the core DSBH[92] (FIG. 4c). It seems probable that the structure of the methylated histone tail, or an actual conformational change in the enzyme, must allow the methylamine group access to the active site[92]. Interestingly, JHDM1A and JHDM3A/JMJD2A both recognize H3K36 methylation, yet there seems to be no primary or secondary structure similarities outside the DSBH. Furthermore, there are few primary amino-acid similarities between JHDM2 and the JHDM3A/JMJD2A demethylases that would provide clues as to the molecular determinants that allow H3K9 demethylation[77]. To further complicate this matter, the JmjC domain of JHMD3/JMJD2 enzymes is most similar at the primary amino-acid level to the JARID1 family of enzymes, which demethylate histone H3K4. Clearly, this suggests that JmjC-domain-containing proteins use unique primary sequences to specify which modification site within histone H3 is recognized. Interestingly, the sequence surrounding histone H3K27 is identical to H3K9 (ARKS), but neither of the H3K9 demethylases can demethylate modified H3K27 (REFS 51,77–80). This further indicates that the sequence immediately surrounding the methyl-lysine residue is not sufficient for enzyme recognition, and suggests a requirement for extended substrate sequence recognition. The characterization of enzyme–substrate co-crystals will be crucial to advancing our understanding of how JmjC-domain-containing proteins recognize methylated residues.

Removal of other methylation marks?

Histone demethylase enzymes of the LSD1 and JmjC class have been shown to remove H3K4, H3K9 and H3K36 methylation. In mammals, H3K27, H3K79 and H4K20 are three other well-characterized methylation sites for which no demethylase enzymes have been identified. Given that the JmjC-domain-containing family of demethylases contains members with potentially uncharacterized enzymatic activity, this family might contain novel demethylases for these sites[60].

H3K27. Although H3K27 methylation is usually associated with stable Polycomb group (PcG)-mediated transcriptional repression[93–96], the H3K27 methylation levels at specific genes have been shown to rapidly decrease during differentiation of mouse stem cells into neuronal lineages[97]. Recently, the human UTX protein was purified as a component of a H3K4 methyltransferase complex[85,98]. Given the opposing roles of H3K4 and H3K27 in gene regulation[99–101], it is tempting to speculate that UTX, or the related UTY and JMJD3 proteins, might constitute functional H3K27 demethylases. Interestingly, mouse JMJD3 is upregulated during embryonic stem-cell differentiation when H3K27 marks seem to be dynamically regulated[97]. Therefore, the UTX/UTY family of proteins are strong candidates for potential H3K27 demethylases.

H3K79. H3K79 methylation might be a histone modification that is not actively reversed. In budding yeast, histone lysine methylation occurs exclusively on histone H3K4, H3K36 and H3K79 (REF. 1). This organism has five JmjC-domain-containing proteins and no LSD1 homologues[60] (BOX2). Three of the JmjC-domain-containing enzymes in yeast are active demethylases that target H3K4 and K36 methylation[59], and the remaining two are inactive due to mutations in the JmjC domain[60] (BOX2). Based on our current enzymatic understanding of histone demethylation, it seems that none of the identified enzyme families can enzymatically remove H3K79 methylation. This is further supported by the observation that H3K4 and H3K36 methylation are dynamically regulated in yeast, whereas changes in H3K79 methylation seem to occur at rates comparable to the dilution effect of unmodified histones during cell division[102–104]. Furthermore, H3K79 is found on ~90% of yeast histone H3 molecules and only seems to be excluded from heterochromatic regions[105,106], supporting the possibility that an enzymatic mechanism to reverse this modification is absent. Therefore, it seems plausible that H3K79 methylation is a more static mark, which demarcates regions of chromatin that are conducive to active transcription and inhibitory to heterochromatin formation.

H4K20. H4K20 methylation is generally considered a repressive chromatin mark. Defined H4K20 methylation states demarcate different genomic regions, with H4K40me3 found in constitutive heterochromatin[107,108] and H4K20me2 or H4K20me1 occurring in a non-overlapping fashion throughout euchromatic regions[109]. Given that H4K20me3 is associated with regions of permanently silenced heterochromatin, it remains possible that this modification state is irreversible. Interestingly, in mammals H4K20me1 is found associated with the body of actively transcribed genes where it has been proposed that this modification might contribute to the silencing of intragenic transcription[82,83]. The global levels of H4K20me1 are dynamically regulated during the cell cycle, but whether this mark is actively removed from the body of genes following transcriptional silencing has not been examined. It will be interesting to determine whether H4K20me1 is actively removed as a consequence of transcriptional silencing and whether any of the currently uncharacterized JmjC-domain-containing proteins contribute to this process.

Novel demethylase enzymes? In addition to the JmjC-domain-containing enzymes, an extensive family of oxygenase-domain-containing proteins are encoded within eukaryotic genomes[91,110,111]. Unlike the JmjC-domain-containing family, which contains conserved chromatin-associated domains, other oxygenases

Polycomb group
(PcG). A class of proteins, originally described in *Drosophila melanogaster*, that maintain the stable and heritable repression of several genes, including the homeotic genes.

S-adenosylmethionine (SAM). A biological compound that is involved in methyl-group transfer in living cells.

seem to lack this feature, making it difficult to exploit bioinformatic approaches to predict which oxygenase sub-families might encode enzymes with activity towards chromatin substrates. Nevertheless, these enzymes might encode additional hydroxylase enzymes that function to demethylate histones. Prior to the identification of the LSD1 and JmjC-domain-containing histone demethylases, Chinenov noted similarities between Elp3, a histone acetyltransferase component of the Pol II elongator complex, and enzymes that use *S*-adenosylmethionine (SAM) in radical reactions[112]. Chinenov proposed that Elp3 might mediate histone demethylation by catalysing scission of SAM to produce a radical species that could drive subsequent reaction steps, leading to the demethylation of arginine or lysine residues[112]. Although no direct evidence has emerged to demonstrate that Elp3 carries out this function, it was recently shown that Elp3 contains a Fe4S4 cluster, binds SAM and can cleave SAM *in vitro*[113]. These observations suggest that Elp3 and other SAM radical enzymes might regulate chromatin function by demethylating histones[114].

Concluding remarks

The identification of a histone deiminase and several histone demethylases has clearly demonstrated that histone methylation is a reversible modification, similar to histone acetylation and phosphorylation[28,29,43,59]. Nevertheless, in contrast to acetylation and phosphorylation, histone methylation is a relatively stable epigenetic mark and enzymes that antagonize these modifications are presumably tightly regulated and targeted to defined loci *in vivo*. The involvement of histone lysine demethylases in transcriptional regulation and cancer-cell proliferation indicate that these factors might have important roles in maintaining cellular homeostasis and that their misregulation could contribute to cancer. Furthermore, the involvement of JmjC-domain-containing proteins in human mental retardation[89,115] indicates that the function of histone demethylases could have an important role in normal neuronal function, perhaps through the regulation of gene expression. An in-depth functional analysis of these interesting enzymes is clearly required to fully appreciate their biological significance.

Understanding how the activity of histone demethylase enzymes is regulated remains an important challenge for future exploration. Overexpression of demethylase enzymes results in global histone demethylation, suggesting that enzymatic activity needs to be tightly controlled to ensure regulation of histone methylation at relevant loci. We think that demethylase activity could be controlled by protein–protein interactions, cofactor availability and post-translational modifications. First, physical association of LSD1 with Co-REST profoundly affects substrate specificity. It will be important to isolate and characterize JmjC-domain-containing protein complexes to examine whether additional factors also regulate the enzymatic properties of this class of enzyme. Second, cofactor availability could be an important means by which histone demethylation is regulated[116]. This mechanism is exemplified by the Sir2 histone deacetylase, which relies heavily on the availability of its cofactor NAD^+ to regulate histone acetylation[117–119]. Because LSD1 and JmjC-domain-containing proteins require cofactors that are also regulated by cellular redox conditions and metabolism, it seems probable that multiple cellular signalling pathways can regulate the potential for histone demethylation. Finally, direct post-translational modification of these enzymes by methylation, acetylation and phosphorylation could also contribute to the regulation of catalysis. Biochemical characterization of the post-translational modifications found on histone demethylase enzymes will be an important aspect of understanding how histone demethylation is regulated.

The identification of histone demethylase enzymes has opened a new frontier in the study of dynamic epigenetic regulation. Recently, it has become clear that histone methylation contributes to maintaining the undifferentiated state of the embryonic stem cells and to the epigenetic landscape during early development[97,120–124]. Understanding how histone demethylation contributes to these processes will be important in advancing our understanding of the basic mechanisms that underpin cell fate and differentiation. The involvement of histone demethylase enzymes in disease and cancer also provides a unique opportunity for pharmacological intervention by designing small-molecule inhibitors that exploit the structure and enzymatic reaction mechanisms of these newly discovered enzymes to counteract their function. Indeed, small-molecule inhibitors have already been identified that inhibit the PADI4 and LSD1 (REFS 125–128). The next few years will certainly be an exciting time to explore how dynamic histone methylation contributes to normal biological functions and disease.

Note added in proof

While this article was in press, a flurry of papers (REFS 85–87, 131–135) appeared online, which demonstrated the H3K4me3 and H3K4me2 demethylase activity of JARID1-family members, including *S. cerevisiae* and *D. melanogaster* homologues.

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Competing interests statement
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