

Methylation: lost in hydroxylation?

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Methylation of histone tails is a key determinant in forming active and silent states of chromatin. Histone methylation was regarded as irreversible until the recent identification of a lysine-specific histone demethylase (LSD1), which acts specifically on mono- and dimethylated histone H3 lysine 4. Here, we propose that the fission yeast protein Epe1 is a putative histone demethylase that could act by oxidative demethylation. Epe1 modulates the stability of silent chromatin and contains a JmjC domain. The Epe1 protein can be modelled onto the structure of the 2-oxoglutarate-Fe(II)-dependent dioxygenase, factor inhibiting hypoxia inducible factor (FIH), which is a protein hydroxylase that also contains a JmjC domain. Thus, Epe1 and certain other chromatin-associated JmjC-domain proteins may be protein hydroxylases that catalyse a novel histone modification. Another intriguing possibility is that, by hydroxylating the methyl groups, Epe1 and certain other JmjC-domain proteins may be able to demethylate mono-, di- or trimethylated histones.

Keywords: JmjC; chromatin; Epe1; oxidative demethylation

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Introduction

In eukaryotic cells, DNA is packaged in the form of chromatin. The nucleosome is the basic unit of chromatin and consists of 146 base pairs of DNA wrapped around an octamer of four core histones (H2A, H2B, H3 and H4). The amino-terminal tails of the histone proteins protrude from the nucleosome core and are subject to a diverse array of post-translational modifications that include acetylation, methylation, phosphorylation and ubiquitination. It has been suggested that distinct histone modifications act sequentially or in combination to form a 'histone code' (Strahl & Allis, 2000). The code can be read and interpreted by non-histone proteins that bind chromatin in a modification-sensitive manner, which regulates chromatin organization, transcription,

DNA replication and repair. For example, methylation of individual lysine or arginine residues within histone tails has been causally linked to either transcriptional activation or repression (Kouzarides, 2002; Zhang & Reinberg, 2001). Methylation of histone H3 on lysine 9 allows binding of chromodomain proteins, such as heterochromatin protein 1 (HP1) or Swi6, which contribute to the formation of silent chromatin. In contrast, methylation of histone H3 lysine 4 is linked to transcriptional activation (Lachner *et al*, 2003).

As gene expression is often regulated temporally during the cell cycle or development, particular chromatin modifications must also be dynamic. It is clear that histone acetylation, phosphorylation and ubiquitination are reversible; pairs of antagonistic enzymes catalyse the addition and removal of these modifications (Jenuwein & Allis, 2001). However, for many years, histone methylation was regarded as a stable and irreversible epigenetic mark, which committed the chromatin to a specific transcriptional state (Jenuwein & Allis, 2001; Rice & Allis, 2001). This view was supported by the lack of any identifiable histone demethylase activities and by studies suggesting that histone proteins, and the methylated residues within them, have a similar turnover rate (Byvoet *et al*, 1972; Duerre & Lee, 1974).

Histone methylation is dynamic

The dogma that histone methylation is irreversible has now been disproved. Analysis has shown that certain forms of histone methylation are dynamic. For example, H3 lysine 9 methylation in certain inducible inflammatory genes is erased on activation and restored with post-induction transcriptional repression (Saccani & Natoli, 2002), and histone H3 lysine 4 methylation at a tandem array of mouse mammary tumour virus promoters is rapidly downregulated on glucocorticoid treatment (Ma *et al*, 2001).

Several mechanisms have been proposed for the removal of histone methylation, such as the replacement of methylated histones by histone exchange. Higher eukaryotes have a histone H3 variant, H3.3, which is deposited in a replication-independent fashion at transcriptionally active foci throughout the cell cycle (Ahmad & Henikoff, 2002). In real-time analysis of a single cell, H3 lysine 9 methylation was depleted as histone H3.3 was deposited at active foci (Janicki *et al*, 2004). Methylated histone tails could also be cleaved off, which effectively removes methylation. Evidence for this exists in *Tetrahymena*, in

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which the tails of histones H3 and H4 are subject to proteolytic processing (Allis *et al*, 1980; Lin *et al*, 1991). Both of the above mechanisms ultimately require replacement of the resident histones, but the most direct way of removing histone methylation is by enzymatic demethylation.

Histone demethylases

Arginine is susceptible to enzymatic conversion forming a non-standard amino acid. Human peptidylarginine deiminase 4 (PAD4/PADI4) is capable of converting monomethylated arginines in histones H3 and H4 to citrulline by demethyliminium. However, although this is effectively histone demethylation, it does not represent simple reversal of arginine methylation as the arginine residue is converted to citrulline. The fate of the citrulline residues remains unknown. It is possible that the citrulline is converted back to arginine by an aminotransferase or that the histones containing citrulline are replaced by unmodified versions. However, PAD4/PADI4 also acts on unmethylated arginine to create citrulline, which can no longer be methylated by methyltransferases, suggesting that the role of PAD4/PADI4 may be to deplete the histone substrate for methyltransferases (Cuthbert *et al*, 2004; Wang *et al*, 2004).

The existence of enzymes that directly demethylate histone arginine and lysine residues had been doubted, partly because the amino-methyl bond is refractory to direct cleavage. A histone demethylase activity was described in 1973 (Paik & Kim, 1973), but its molecular identity was never established. However, a direct histone demethylase has recently been found.

Amine oxidases were suggested as potential histone demethylases (Bannister *et al*, 2002), and the elucidation of the function of lysine-specific demethylase 1 (LSD1) confirms this hypothesis. LSD1 is a flavin-dependent amine oxidase that is able to specifically demethylate mono- and dimethylated histone H3 lysine 4 (Shi *et al*, 2004). LSD1 is proposed to catalyse the oxidation of amine via the oxidative cleavage of the α -CH bond of the substrate to form an imine intermediate with the concomitant reduction of the flavin cofactor. The imine intermediate is hydrolysed via a non-enzymatic process to produce a carbinolamine—an unstable group that degrades to release formaldehyde—thereby regenerating the demethylated lysine residue. LSD1 is specific for histone H3 lysine 4 methylation and although LSD1-related proteins could act on other types of histone methylation, only mono- and dimethylated lysine or arginine residues can be demethylated by amine oxidases. This is because formation of the imine intermediate requires a protonated lysine, thereby precluding trimethylated lysines as substrates for amine oxidases. Therefore, other classes of enzymes may also carry out histone demethylation.

It has been hypothesized that direct demethylation of histones could also be catalysed by a subfamily of histone acetyltransferases that are related to elongation protein 3 (Elp3; Chinenov, 2002). In addition to a histone-acetyltransferase domain in the carboxyl terminus, these proteins have an amino-terminal domain similar to that of S-adenosylmethionine (SAM) radical enzymes. These enzymes all catalyse reactions starting with the creation of a reactive 5'deoxyadenosyl radical from SAM. The 5'deoxyadenosyl radical could abstract a proton from the N-methyl group of methyl-lysine or methyl-arginine, which yields an ammonium cation radical. This intermediate is unstable

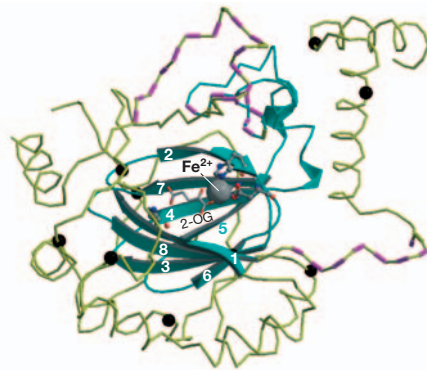


Fig 1 | Predicted structure of Epe1 modelled on the structure of FIH. The three-dimensional-jury algorithm implemented on the meta-server <http://BioInfo.PL/Meta/> was used to predict the fold of the Epe1 protein (Ginalski *et al*, 2003). All 11 algorithms found FIH to be the best hit. The consensus score of 145 is much greater than the cut-off value of 40, at which there is 90% confidence that the prediction has found the correct fold. The JmjC domain including the double-stranded β -helix (DSBH) is shown in cyan and the eight strands of the DSBH are numbered 1–8, corresponding to β -strands 8–15 of FIH, as defined by the PDBsum entry for 1h2k. The fold is viewed roughly in the direction of attack of the substrate, which would be positioned over the Fe^{2+} ion. The black dots represent insertions in Epe1 not present in FIH and the magenta-striped regions are deletions in Epe1 not present in FIH. Insertions or deletions are in surface loops, not within the secondary structure elements or the core of the molecule. Trimethyl-lysine can be modelled in our predicted structure; none of the methyl groups makes a steric clash in the model and all can be accommodated simultaneously.

to hydrolysis and produces a demethylated residue and formaldehyde. Because a protonated nitrogen is not required, this reaction mechanism is compatible with demethylation of trimethylated substrates. Elp3 has been isolated as part of a larger six-subunit complex that is specifically associated with elongating RNA polymerase II (Winkler *et al*, 2001).

Theoretically, methylation could be removed from histones by hydroxylation of the methyl group. This reaction could be catalysed by enzymes such as the 2-oxoglutarate (2-OG)-Fe(II)-dependent dioxygenases, which use Fe(II) in their active site to activate a molecule of dioxygen to form a highly reactive oxoferryl (Fe(IV)=O) species, which can hydroxylate the substrates of the enzyme. Hydroxylation can lead to demethylation; an example of this is the catalysis by the DNA repair demethylase AlkB. AlkB is a 2-OG-Fe(II)-dependent dioxygenase that hydroxylates the methyl groups of certain forms of DNA methylation damage. The oxidized products are thought to be unstable and spontaneously degrade to release formaldehyde, which results in the removal of the methyl group from DNA (Falnes *et al*, 2002; Trewick *et al*, 2002). Recently, a protein, Epe1, has been described in fission yeast, *Schizosaccharomyces pombe*, that affects heterochromatin integrity (Ayoub *et al*, 2003). We predict that Epe1 and other related proteins are good candidates for enzymes that act to remove methyl groups from histones by this hydroxylation mechanism.

Epe1 affects heterochromatin and has a JmjC domain

The Epe1 protein is required for heterochromatin integrity in fission yeast. In the absence of Epe1, silent chromatin at centromeres and the mating type loci are destabilized, which leads to

the disruption of the pattern of histone H3 lysine 4 and lysine 9 methylation within and adjacent to these domains (Ayoub *et al*, 2003). Using yeast two-hybrid assays, we have also identified Epe1 as a protein that interacts with Swi6 (equivalent to metazoan

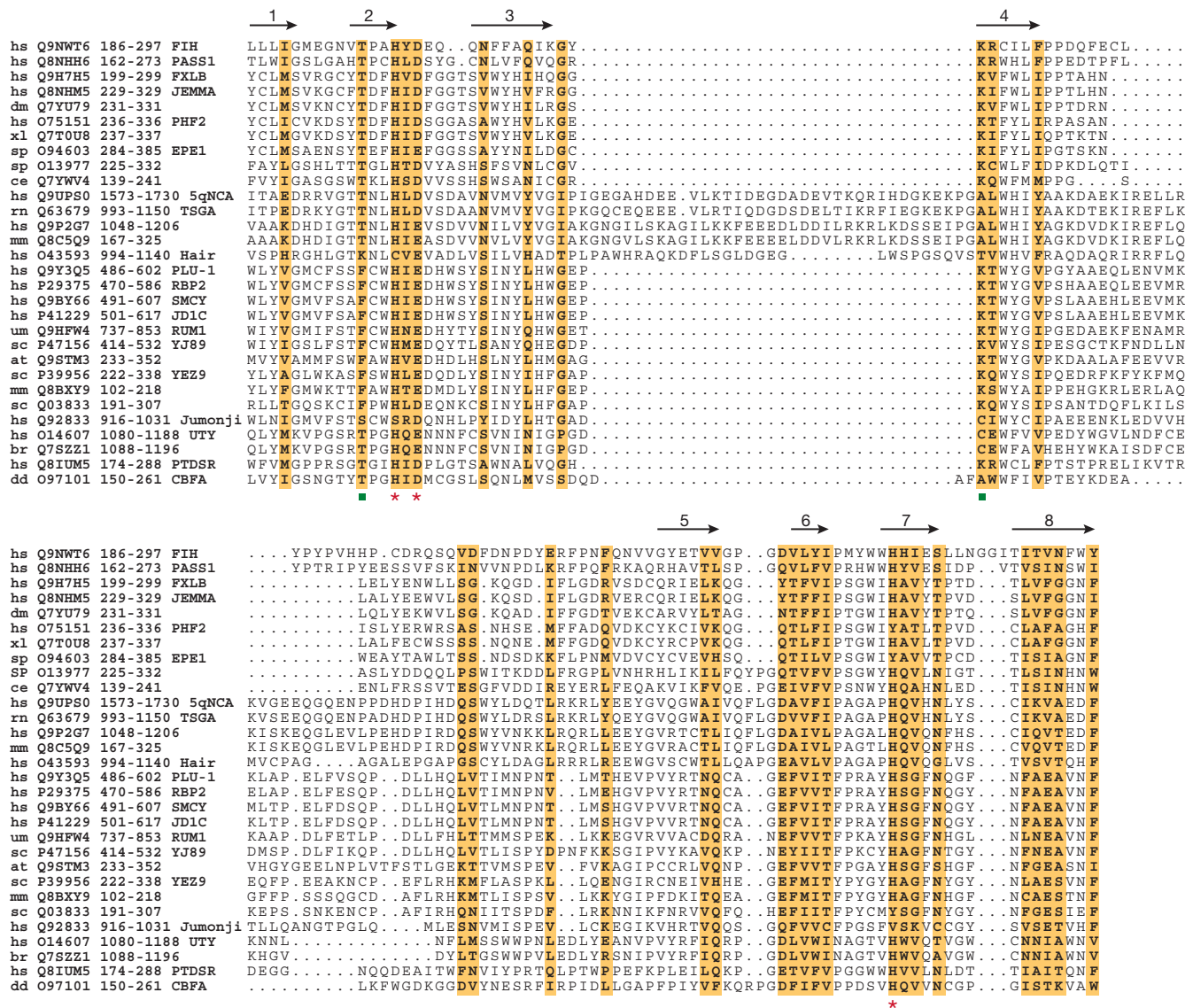


Fig 2 | Multiple sequence alignment of JmjC domains. The JmjC domains of several proteins were aligned using Clustal W. The position of the β -sheets from the crystal structure of Q9NMT6 (*Homo sapiens* FIH) are illustrated above the alignment. Positions with similarity scores greater than 0.5 are highlighted in yellow. The Fe(II) co-ordinating residues (predicted) are marked with a red asterisk. The 2-OG-Fe(II)-dependent dioxygenases bind Fe(II) using the consensus HXD/EXnH. These residues are conserved in most of the JmjC-domain sequences. A subgroup of JmjC domains, including Epe1 and the PHD-finger protein 2, use another Fe(II) co-ordinating residue, tyrosine, in place of the second histidine in this motif, which leads us to predict that Epe1 co-ordinates Fe(II) with His 297, Glu 299 and Tyr 370. The hairless and Jumonji proteins have cysteine and serine residues, respectively, instead of the first conserved histidine of the iron-binding motif. The Jumonji protein also has a valine residue instead of the second histidine so may be catalytically inactive. The 2-OG binding residues (predicted) are shown with green squares. In general, the 2-OG-Fe(II)-dependent dioxygenases use an arginine or lysine residue downstream of the iron-binding motif in strand 8 of the DSBH to interact with the 5-carboxylate of 2-OG. However, structural and sequence analysis of the JmjC-domain protein FIH demonstrates that FIH interacts with 2-OG in a different manner via a threonine (Thr 196) and a lysine (Lys 214) residue in strand 2 and strand 4 of the DSBH, respectively, and by an upstream tyrosine (Tyr 145) residue (Elkins *et al*, 2003). These 2-OG-interacting residues are conserved in many of the JmjC proteins including Epe1. Species abbreviations: at, *Arabidopsis thaliana*; br, *Brachydanio rerio*; ce, *Caenorhabditis elegans*; dd, *Dictyostelium discoideum*; dm, *Drosophila melanogaster*; hs, *Homo sapiens*; mm, *Mus musculus*; rn, *Rattus norvegicus*; sc, *Saccharomyces cerevisiae*; sp, *Schizosaccharomyces pombe*; um, *Ustilago maydis*; xl, *Xenopus laevis*.

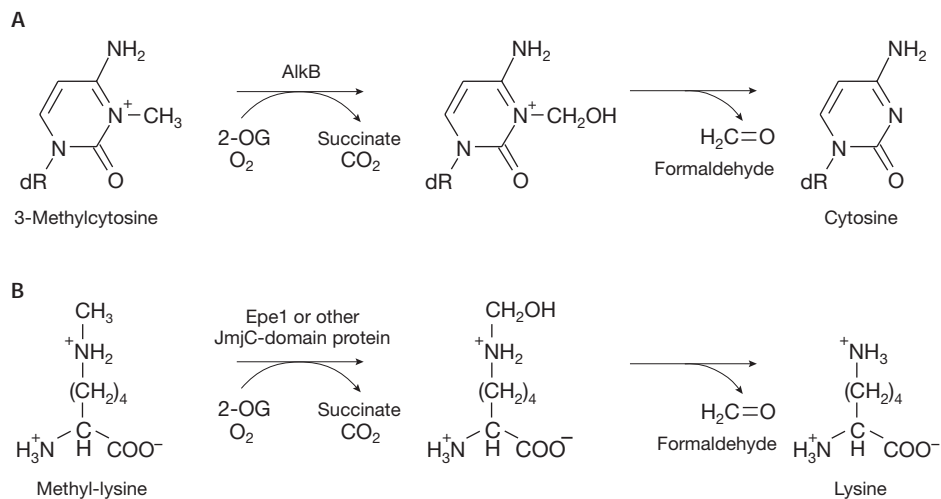


Fig 3 | Demethylation by hydroxylation. (A) Mechanism of demethylation of 3-methylcytosine by AlkB. (B) Proposed mechanism of demethylation of histones by Epe1 and/or other JmjC-domain proteins. The demethylation of monomethyl-lysine is illustrated for simplicity; however, di- or trimethylated residues could also be substrates.

HP1), which in turn uses its chromodomain to bind histone H3 which is methylated at lysine 9 (E. Minc, R. Antonelli, H. Teunissen and R. Allshire, unpublished data). Epe1 contains a JmjC domain, a motif that was first noted in the mammalian jumonji, retinoblastoma (Rb)-binding protein 2, and Smcx proteins (Takeuchi *et al*, 1995). Subsequently, more than 100 JmjC-domain containing proteins have been identified in prokaryotic and eukaryotic organisms (Balciunas & Ronne, 2000; Clissold & Ponting, 2001). The presence of DNA and/or chromatin-binding motifs, such as plant homeodomain (PHD), AT-rich interactive domain (ARID) and zinc fingers, in many JmjC-domain-containing proteins led to the proposal that these proteins regulate the integrity of chromatin structure (Clissold & Ponting, 2001). Several JmjC-domain-containing proteins have been characterized, including the human hairless protein that is mutated in individuals with *alopecia universalis*. The hairless protein is a putative transcription factor and a regulator of apoptosis during hair follicle development (Ahmad *et al*, 1998). Another JmjC protein, Rb-binding protein 2 (RBP2), has been reported to act as a co-activator of nuclear receptors by enhancing receptor-mediated transcription (Chan & Hong, 2001). Msc1, an *S. pombe* JmjC-domain protein, is associated with histone deacetylase activity (Ahmed *et al*, 2004).

Epe1 resembles 2-OG-Fe(II)-dependent dioxygenases

Epe1 can be modelled onto the known structure of FIH (factor inhibiting hypoxia inducible factor), the only JmjC-domain protein with a known three-dimensional structure and biochemical function (Fig 1). FIH is a protein hydroxylase that mediates the hydroxylation of an asparagine residue in the transcription factor hypoxia inducible factor (Hewitson *et al*, 2002). FIH belongs to the 2-OG-Fe(II)-dependent dioxygenase superfamily, a large group of enzymes that catalyse two electron oxidations using an ion of iron in their catalytic core and 2-OG as a co-substrate. Structural analysis of FIH and other 2-OG-dependent

dioxygenases shows that the catalytic core of these dioxygenases contains a motif known as a double-stranded β -helix (DSBH) which consists of two antiparallel β -strands that form a jelly-roll topology (Elkins *et al*, 2003). The predicted structure of Epe1 contains this DSBH motif (Fig 1).

A multiple sequence alignment of JmjC domains from various proteins (Fig 2) shows that Epe1 and many members of the JmjC family show several distinctive features of the 2-OG-Fe(II)-dependent dioxygenases. This suggests that the JmjC-domain proteins may catalyse similar types of reactions. The secondary structure of FIH can be overlaid on the multiple sequence alignment of the JmjC domains and inserts are only seen between the β -strands (Fig 2), which suggests that other JmjC-domain proteins contain the DSBH structural motif. Members of this dioxygenase family co-ordinate Fe(II) at their active site through a 2-His-1-carboxylate triad of amino-acid residues. The carboxylate is normally supplied by the side chain of an aspartate or glutamate residue (Prescott & Lloyd, 2000; Schofield & Zhang, 1999). This Fe(II)-binding motif, which is distinct from the 2-OG-Fe(II)-dependent dioxygenases, has the consensus sequence HXD/EXnH and is found in most of the JmjC-domain sequences, including RBP2, JEMMA (Jumonji domain, EMSY-interactor, methyltransferase motif) and PASS1 (protein associated with small stress; Fig 2). However, the JmjC-domain proteins appear to use different amino acids to interact with their co-substrate, 2-OG. Conserved, positively charged residues appear at distant positions in sequence in the JmjC and 2-OG-Fe(II)-dependent dioxygenases. When the DSBH from representative structures of each class are overlaid, these residues occupy similar positions, which compensates for the buried negative charge on the carboxylate group of the co-substrate 2-OG (supplementary Fig 1 online). In both the FIH asparagine hydroxylase and 2-OG-Fe(II)-dependent dioxygenases family, Fe(II) is liganded in the same way by protein residues and by the proximal carboxylate of 2-OG. The signature residues that differ between families are

involved in liganding the distal region of 2-OG. There are no further signature residues near the reactive centre in either family.

Epe1 is a potential histone demethylase

Epe1 is required for the integrity of heterochromatin in fission yeast and interacts with Swi6 at regions of silent chromatin (Ayoub *et al*, 2003). We suggest that Epe1 ligates Fe(II) at its active site; as the Fe(II) forms a very reactive species with O₂, there is no need to invoke stabilization of a transition state, and so presentation of the substrate to this system may be sufficient for a reaction with molecular oxygen to occur. This leads us to speculate that Epe1 and certain other JmjC proteins may catalyse the addition of hydroxyl groups to histone tails, which is a novel histone modification. However, although Epe1 may be a protein hydroxylase, there is also an intriguing possibility that if Epe1 were to hydroxylate a methyl group on the histone tails, Epe1 might be able to catalyse oxidative demethylation of histones. The hydroxylation of amines that leads to N-dealkylation is widespread in the biochemistry of natural products and occurs in many drugs and xenobiotics (Walsh, 1979). An example of this mechanism is the catalysis by AlkB, which demethylates certain forms of DNA methylation damage by hydroxylation (Falnes *et al*, 2002; Trewick *et al*, 2002). AlkB is also a member of the 2-OG-Fe(II)-dependent dioxygenase family and hydroxylates the methyl group of two lesions in DNA, 1-methyladenine and 3-methylcytosine. The oxidized products spontaneously degrade to release formaldehyde, which results in the removal of the methyl group from DNA (Fig 3A). We propose that Epe1 and certain other JmjC-domain-containing proteins may act as hydroxylases that demethylate lysine or arginine residues in the N-terminal tails of histones. Hydroxylation of the methyl group of mono-, di- or trimethyl-lysine would result in the formation of a carbinoamine, a generally unstable group, which would be expected to release formaldehyde and remove a methyl group from the amino-acid residue (Fig 3B). Unlike the amine oxidase LSD1, which is strictly limited to mono- and dimethylated substrates, the mechanism we propose for Epe1 could allow for the demethylation of mono-, di- or trimethylated histones. This is because demethylation could occur through multiple rounds of hydroxylation, which would result in complete demethylation of even trimethylated lysines. However, Epe1 or other JmjC-domain proteins could be involved in removing the first methyl group from trimethyl-lysine, thereby allowing the action of demethylases such as LSD1.

Many JmjC-domain proteins contain domains that are indicative of chromatin interactions. On the basis of the structural alignments presented here, we predict that some of these proteins may have the ability to directly hydroxylate histones, a novel histone modification, or may represent potential histone demethylases.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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