ARTICLES

Histone demethylation by a family of JmjC domain-containing proteins

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Covalent modification of histones has an important role in regulating chromatin dynamics and transcription. Whereas most covalent histone modifications are reversible, until recently it was unknown whether methyl groups could be actively removed from histones. Using a biochemical assay coupled with chromatography, we have purified a novel JmjC domain-containing protein, JHDM1 (JmjC domain-containing histone demethylase 1), that specifically demethylates histone H3 at lysine 36 (H3-K36). In the presence of Fe(II) and α-ketoglutarate, JHDM1 demethylates H3-methyl-K36 and generates formaldehyde and succinate. Overexpression of JHDM1 reduced the level of dimethyl-H3-K36 (H3K36me2) *in vivo*. The demethylase activity of the JmjC domain-containing proteins is conserved, as a JHDM1 homologue in *Saccharomyces cerevisiae* also has H3-K36 demethylase activity. Thus, we identify the JmjC domain as a novel demethylase signature motif and uncover a protein demethylation mechanism that is conserved from yeast to human.

Covalent histone modifications have an important role in regulating chromatin dynamics and function[1]. One such modification, methylation, occurs on both lysine and arginine residues and participates in a diverse range of biological processes including heterochromatin formation, X-chromosome inactivation and transcriptional regulation[2–4]. Unlike acetylation, which generally correlates with transcriptional activation, histone lysine methylation can signal either activation or repression depending on the particular lysine residue that is methylated[4]. Even within the same lysine residue, the biological consequence of methylation can differ depending on whether the lysine residue contains mono-, di-, or trimethylation[5,6].

The steady-state level of a covalent histone modification is controlled by a balance between enzymes that catalyse the addition and removal of a given modification. Although this notion is generally true for many histone modifications, an enzyme capable of removing methyl groups from a methyl-lysine residue has remained elusive, until recently[7]. Using a candidate approach, it has been demonstrated that LSD1/BHC110, a nuclear amine oxidase homologue previously found in several histone deacetylase complexes[8–10], can specifically demethylate monomethyl-H3-K4 (H3K4me1) and H3K4me2 in a FAD (flavin adenine dinucleotide)-dependent oxidative reaction. Although potential LSD1 homologues exist in *Schizosaccharomyces pombe*, they are not found in *S. cerevisiae*, even though at least three lysine residues on H3 can be methylated in this organism. This observation, and that the LSD1 family demethylases are unable to demethylate trimethyl-lysine residues, raises the possibility that additional demethylases that use a different reaction mechanism exist. Using a novel biochemical assay, we have identified a histone demethylase activity from HeLa cells. Here we describe the purification, identification and functional characterization of a family of H3-K36 demethylases. Our results indicate that the JmjC domain, a motif conserved from *S. cerevisiae* to human, is a signature motif for this family of histone demethylases.

Histone demethylase activity in HeLa cell extracts

Recent studies have demonstrated that the methyl groups of 1-methyladenine (1-meA) and 3-methylcytosine (3-meC) in DNA can be removed by the AlkB family of proteins through oxidative demethylation[11,12]. The similarity between the chemistry of removing a methyl group from 1-meA and methyl-lysine (Fig. 1a) prompted us to test whether a similar mechanism is used in histone lysine demethylation. We developed an *in vitro* assay based on detection of formaldehyde, one of the predicted release products. To maximize detection sensitivity, radiolabelled nucleosomal histones were used as substrates. As outlined in Supplementary Fig. S1, labelled substrates were subjected to demethylation reactions in the presence of cofactors Fe(II) and α-ketoglutarate. Released [^{3}H]-formaldehyde was converted to radioactive 3,5-diacethyl-1, 4-dihydrolutidine before being extracted and counted.

Using the assay described above, we analysed the protein fractions derived from HeLa cell nuclear extracts and nuclear pellet[13]. Results shown in Fig. 1b indicate that a demethylase activity is enriched in the nuclear pellet-derived 0.3 M P11 protein fraction. This demethylase activity is dependent on the presence of the protein fraction (Fig. 1c, lane 1), as well as the predicted cofactors (Fig. 1c, lanes 3 and 4). In addition, ascorbate is also required for optimal activity, probably due to its ability to regenerate Fe(II) from Fe(III). These results suggest the presence of a histone demethylase activity in the 0.3 M P11 fraction.

Histone demethylase activity of a JmjC domain-containing protein

To identify the protein(s) responsible for the demethylase activity, we monitored the enzymatic activity through six chromatography columns (Supplementary Fig. S2). After purification of the 0.3 M P11 fraction through DEAE5PW and Phenyl Sepharose columns, we determined the native mass of the enzymatic activity to be about 300 kDa on a Superose 6 column (Fig. 2a). Further purification on a MonoQ column allowed us to correlate two protein bands (marked by an asterisk in the top panel of Fig. 2b) with the enzymatic activity (Fig. 2b, bottom panel). Mass spectrometry analysis identified the larger of the two proteins as F-box and leucine-rich repeat protein 11

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(FBXL11) (Fig. 2c). The identity of the smaller band was inconclusive based on the established criteria for protein identification[14].

FBXL11 is an uncharacterized protein that was originally identified through bioinformatic searches for F-box-containing proteins[15,16]. In addition to an F-box, FBXL11 contains several interesting domains including a JmjC domain, a CxxC zinc finger, a PHD domain and three leucine-rich repeats (Fig. 3a). JmjC domain-containing proteins are predicted to be metalloenzymes that regulate chromatin function[17]. This prediction, and that the demethylase activity requires Fe(II) as a cofactor, are consistent with FBXL11 being the responsible enzyme. We confirmed this possibility by demonstrating that Flag-tagged FBXL11 immunoprecipitated from transfected COS-7 cells exhibits robust histone demethylase activity (Fig. 3b, compare lanes 1 and 2).

To evaluate which domains of FBXL11 are important for its enzymatic activity, we generated a series of expression constructs with deletions of the JmjC domain, CxxC zinc finger, PHD domain, F-box, or leucine-rich repeat, respectively. After transfection and immunoprecipitation, these mutant proteins were subjected to western blot analysis (Fig. 3b, top panel) and demethylase activity assays (Fig. 3b, bottom panel). The results demonstrate that only the JmjC domain is absolutely required, although deletion of the CxxC zinc finger, PHD domain and the leucine-rich repeats also partially impaired the enzymatic activity (compare lanes 4–8 with 2). To demonstrate further the importance of the JmjC domain for enzymatic activity, we generated an H212A mutant. We choose to mutate H212 not only because this histidine is highly conserved in the JmjC domain of FBXL11 orthologues (Supplementary Fig. S3b), but also because the corresponding histidine in FIH (factor-inhibiting hypoxia-inducible factor), a known Fe(II)-dependent oxygenase, directly binds to Fe(II)[18]. Results shown in Fig. 3b indicate that the H212A mutation completely abolishes the enzymatic activity of FBXL11 (compare lanes 2 and 3). On the basis of the above results we conclude that FBXL11 is a novel histone demethylase and that the JmjC domain is critical for its enzymatic activity. Because histone demethylase activity is the first function attributed to FBXL11 and because FBXL11 is the first JmjC domain-containing protein shown to possess histone demethylase activity, we have named the protein JHDM1A (JmjC domain-containing histone demethylase 1A.). The highly related protein FBXL10, which we have named JHDM1B (Supplementary Fig. S3a), is also an active H3-K36 demethylase (data not shown).

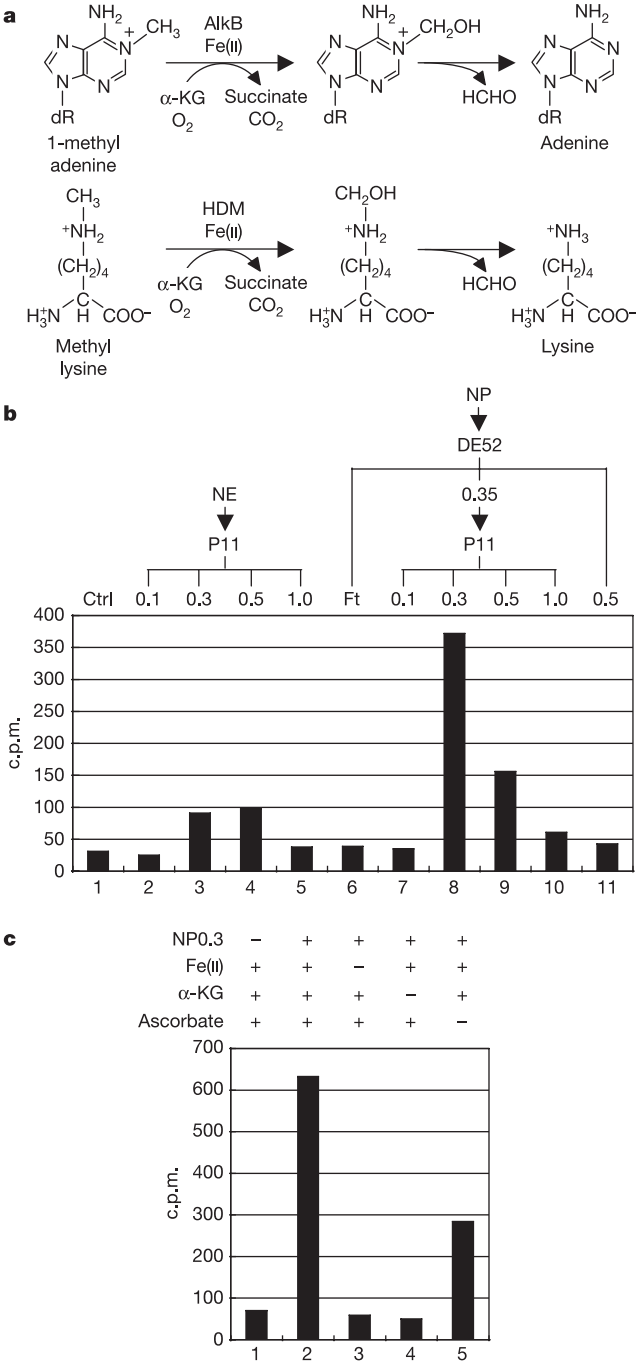

Figure 1 | Identification of a demethylase activity in HeLa cells. **a**, Schematic presentation of the demethylation mechanism of 1-methyladenine by the AlkB family of proteins (top panel). The bottom panel shows the proposed mechanism for histone demethylation. For simplicity, only monomethyl-lysine is illustrated, but the same mechanism can be applied to di-, or trimethylated lysine residues. α-KG, α-ketoglutarate. **b**, Histone demethylase activities in the P11 column fractions derived from HeLa nuclear extracts (NE) and nuclear pellet (NP) fractions. The substrates used are Set2-methylated nucleosomes. The numbers above the panel represent the molar concentration of KCl in the elution buffers. c.p.m., counts per minute. Ft, flow through fraction. **c**, The demethylase activity depends on the presence of Fe(II), α-ketoglutarate and proteins present in the 0.3 M P11 nuclear pellet fraction.

JHDM1A preferentially demethylates H3K36me2

To characterize further JHDM1A, we generated and purified recombinant Flag–JHDM1A from baculovirus-infected Sf9 cells (Supplementary Fig. S4). We analysed its site specificity using histone substrates radiolabelled at all known methylated sites in histones H3 (K4, K9, K27, K36, K79) and H4 (K20, R3). Of the seven substrates, only H3-K36 methylated by Set2 was a substrate for JHDM1A (Fig. 3c). The substrate specificity was also verified by comparing the methylation state of nucleosomal histone substrate after subjecting to JHDM1A-mediated demethylation (Supplementary Fig. S5). Thus, we conclude that JHDM1A is an H3-K36-specific demethylase.

Lysine residues exist in three methylation states (mono-, di- and trimethylation). To determine whether JHDM1A preferentially demethylates a particular methylation state, free histones, mononucleosomes and oligonucleosomes were subjected to a demethylation reaction in the presence or absence of recombinant JHDM1A. Results shown in Fig. 3d indicate that JHDM1A preferentially demethylates K36me2 (second panel, compare lanes 2, 4 and 6 with 1, 3 and 5), although a decrease in K36me1 levels was also observed (first panel). In contrast, no change in trimethyl-K36 (K36me3) levels was detected. Under these assay conditions, JHDM1A was capable of demethylating H3-K36 regardless of whether it was in free, mono-, or oligonucleosome form. To analyse further the K36me2 selectivity of JHDM1A, K36me2 or K36me3 peptides were subjected to demethylation reactions. Mass spectrometry analysis revealed the presence of mono- and unmethylated forms of the peptide in a JHDM1A-dependent manner (Fig. 3e). Demethylation of the peptide occurs in a time- and concentration-dependent manner

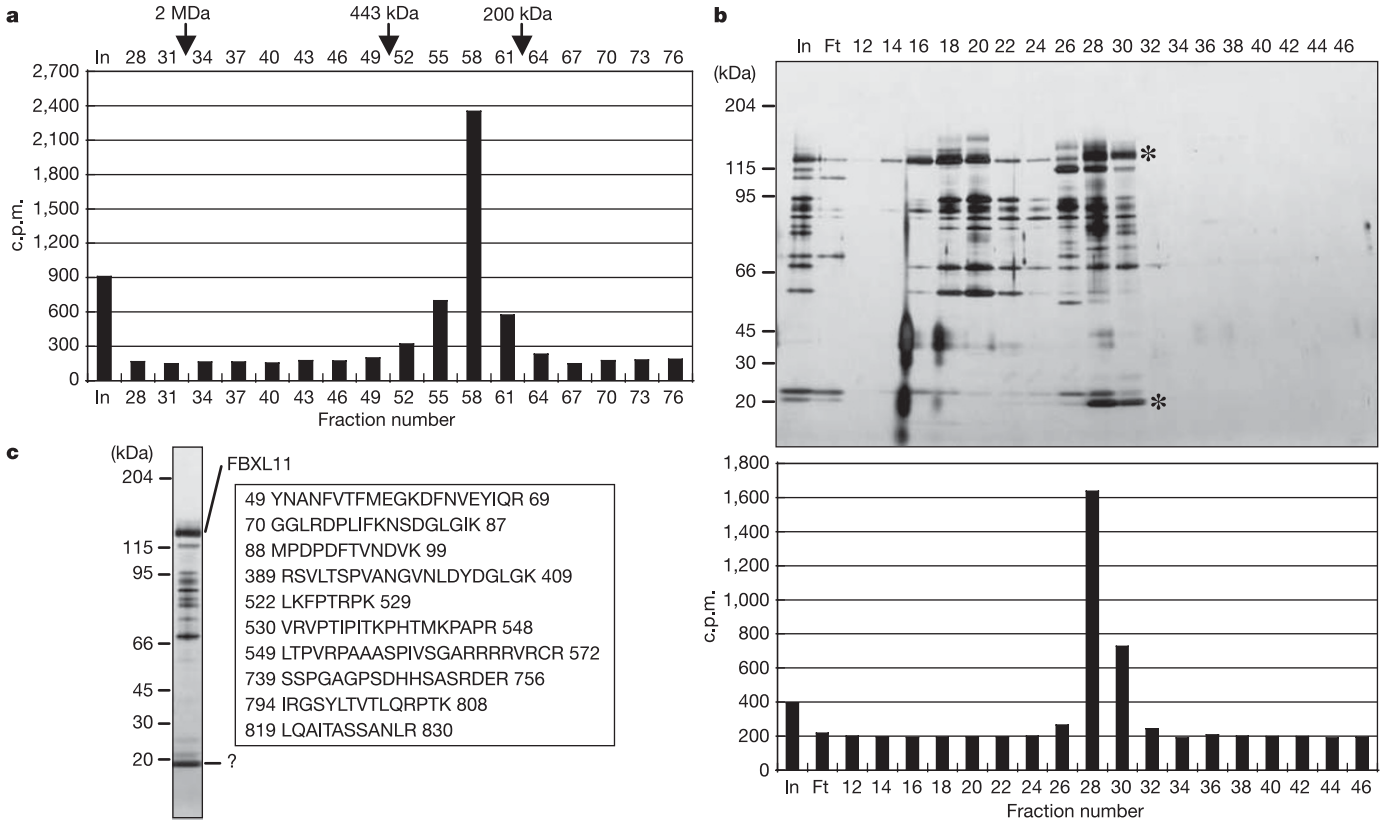

Figure 2 | Purification and identification of a histone demethylase activity. a, Histone demethylase activities of the protein fractions derived from a gel-filtration Superose 6 column. The elution profile of the protein markers is indicated along the top of the panel. **b**, A silver-stained protein gel (top panel) and histone demethylase activities (bottom panel) of the protein fractions derived from a mini MonoQ column. The candidate proteins that co-fractionated with the demethylase activity are indicated by an asterisk. The positions of the protein size markers on SDS–PAGE are indicated to the left of the panel. **c**, A silver-stained protein gel containing the samples for protein identification. A total of 34 tryptic peptides covering 31% of FBXL11 (NP_036440) were identified by mass fingerprinting for the top band, and 10 representative peptides identified are listed. Numbers correspond to the amino acid location within the FBXL11 protein. The question mark represents an unidentified FBXL11-associated protein.

(Supplementary Fig. S6). In contrast, no demethylation was detected when a K36me3 peptide was subjected to a parallel reaction (data not shown). Collectively, the above results indicate that JHDM1A selectively demethylates H3-K36 with a preference for the dimethyl form.

To study the effect of JHDM1A on H3-K36 methylation *in vivo*, we transfected a mammalian Flag–JHDM1A expression vector into 293T cells. Immunofluorescence staining revealed that overexpression of wild-type Flag–JHDM1A, but not the H212A mutant, results in a significant decrease in the level of K36me2 (Fig. 3f). However, overexpression of Flag–JHDM1A does not alter the level of K36me1, K36me3, nor the level of K4me2 (Supplementary Fig. S7). These results indicate that JHDM1A demethylates H3K36me2 *in vivo*.

Demethylation by JHDM1A generates formaldehyde and succinate

The demethylation reaction is predicted to generate formaldehyde and succinate (Fig. 1a). We confirmed this prediction with mass spectrometry. Under the assay conditions, formaldehyde would exist in its protonated form with a mass-to-charge (*m*/*z*) ratio of 31.0184. Consistent with formaldehyde as a reaction product, an ion with an *m*/*z* ratio of 31.0239 was detected when the reaction was carried out in the presence, but not in the absence, of JHDM1A (Fig. 4a). Using a similar approach, we also detected an ion (*m*/*z* 119.0778) that correlated with protonated succinate ($C_4O_4H_7$) in a JHDM1A-dependent manner (Fig. 4b). Because an ion of similar *m*/*z* ratio—119.1215, although lower in abundance—was also detected in the reaction mixture in the absence of JHDM1A (Fig. 4b, bottom panel), we sought to verify that the ion detected in the presence of JHDM1A was indeed succinate. This was achieved by demonstrating that the presumed succinate ion produced in the demethylation reaction generated the identical fragmentation MS/MS spectrum to pure succinate (Fig. 4c). In contrast, no ion was detected in the absence of JHDM1A (data not shown), indicating that the lower abundance ion detected in the control reaction (Fig. 4b, bottom panel) was not succinate. Given that Fe(II)- and α-ketoglutarate-dependent enzymes often catalyse a slow, uncoupled reaction in the absence of substrates, we analysed formation of succinate in the absence of a K36me2 peptide. As expected, JHDM1A catalyses low levels of uncoupled reaction in the absence of the substrate peptide, but production of succinate is stimulated by the presence of the peptide (Supplementary Fig. S8). These results support the hypothesis that JHDM1A-mediated histone demethylation generates formaldehyde and succinate, thus confirming the proposed demethylation mechanism.

Conserved JmjC domain-mediated histone demethylation

Given that JmjC domain-containing proteins exist in organisms from bacteria to human (http://smart.embl-heidelberg.de), we asked whether the demethylase activity of this domain is conserved. In addition to the highly related JHDM1B found in human and mouse, potential JHDM1A homologues were identified in many other eukaryotic organisms including *Xenopus, Drosophila*, *Caenorhabditis elegans*, *S. pombe* and *S. cerevisiae* (Supplementary Fig. S3a). Although the domain structures are not completely conserved among these proteins, their JmjC domains are highly conserved (Supplementary Fig. S3b). Importantly, alignment of their JmjC domains with that of FIH revealed strict conservation—with the exception of the *S. pombe* homologue—of the amino acids, marked

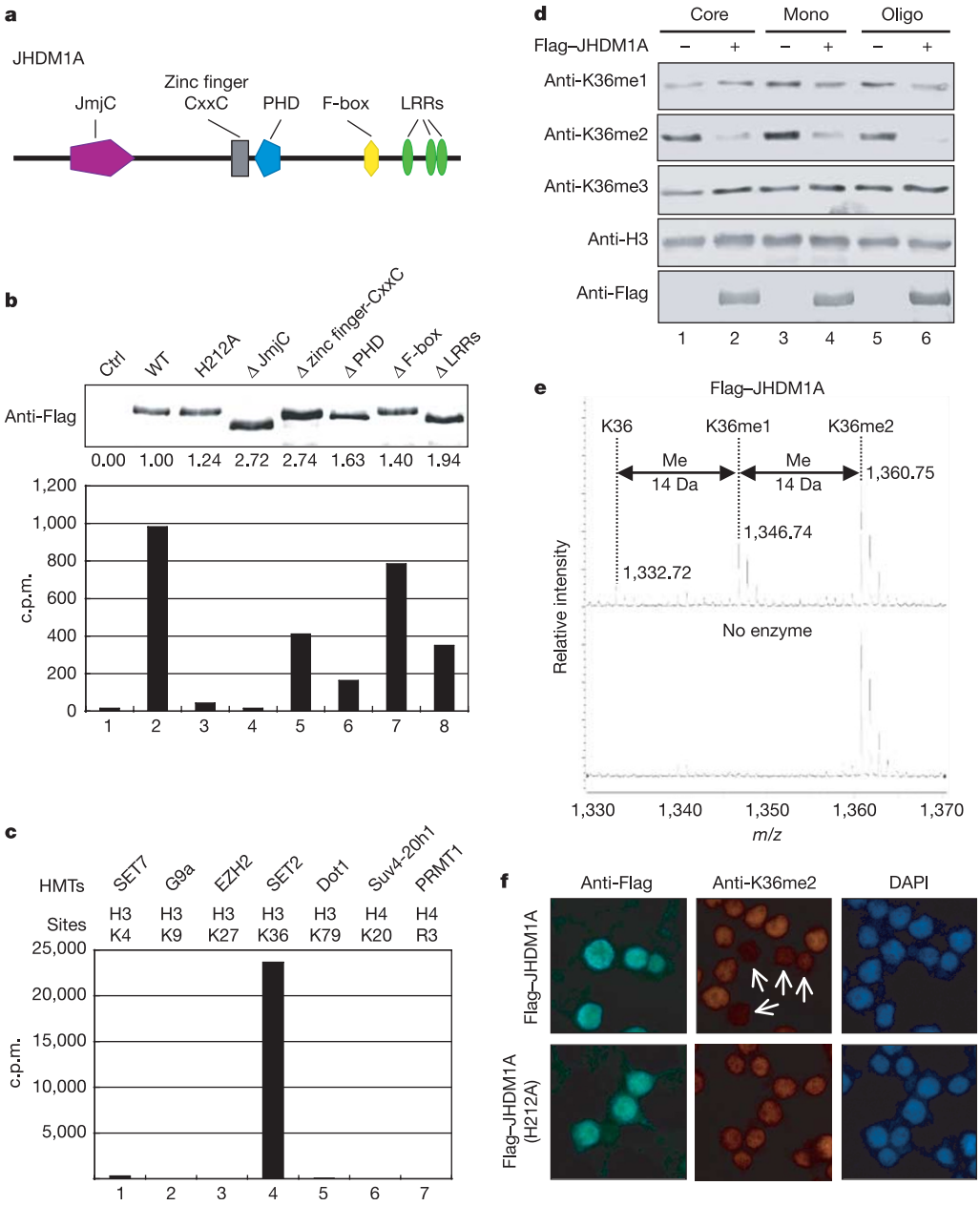

Figure 3 | Characterization of the histone demethylase JHDM1A/FBXL11. **a**, Schematic representation of the JHDM1A protein. Functional domains were identified using the SMART program (http://smart.embl-heidelberg.de). **b**, Wild-type and mutant Flag–JHDM1A proteins were expressed in COS-7 cells, and immunoprecipitated samples were divided and analysed by western blotting (top panel) and demethylase assay (bottom panel). The relative amount of each immunoprecipitated protein, indicated with numbers below the western blot, was quantified with ImageJ (http://rsb.info.nih.gov/ij) and used for normalization of the activities. **c**, Histone demethylase activity of recombinant Flag–JHDM1A, purified from baculovirus-infected Sf9 cells, against various methylated histone substrates. The histone methyltransferases (HMTs) and their sites of methylation are indicated above the panel. The demethylase assays were performed with equal inputs, either by counts or by histone amounts, with similar results. **d**, Western blot analysis of demethylation reactions using various histone substrates. Antibodies used are indicated to the left of the panel. **e**, Mass spectrometry analysis of demethylation of a K36me2 peptide (STGGV2mKKPHRY-C) by purified Flag–JHDM1A. The enzyme/substrate molar ratio of the reaction is 1:40. Numbers represent the masses of the substrate and product peptides. **f**, Overexpression of wild-type (top panels) but not a demethylase-defective mutant (bottom panels) of Flag–JHDM1A in 293T cells resulted in decreased levels of H3K36me2 as marked by arrows.

by an asterisk and a hash symbol, that are involved in Fe(II)- and α-ketoglutarate-binding, respectively[18], indicating that these proteins are likely to be active demethylases. To examine this possibility, we cloned JHDM1B and demonstrated its H3-K36 demethylase activity (data not shown). We also cloned and expressed YER051W, which encodes the *S. cerevisiae* homologue scJHDM1. Recombinant scJHDM1 specifically demethylates methyl-K36, but not methyl-K4 or methyl-K79 (Fig. 5a). The detected demethylase activity is dependent on the JmjC domain, because a point mutation in the JmjC domain (H305A) completely abolished its enzymatic activity (Fig. 5b). These results confirm the conserved function of the JmjC domain in histone demethylation.

Of the JHDM1 family members identified (Supplementary Fig. S3a), only the *S. pombe* homologue Epe1 has been functionally characterized[19]. In the absence of Epe1, centromeres and mating type loci are destabilized, with concomitant changes in methylation patterns of H3-K4 and H3-K9 (ref. 19). Normal function of Epe1 requires an intact JmjC domain, as the JmjC domain mutant Y307A failed to complement a loss-of-function Epe1 mutant. Notably, a tyrosine corresponding to Y307 in Epe1 is conserved in all the

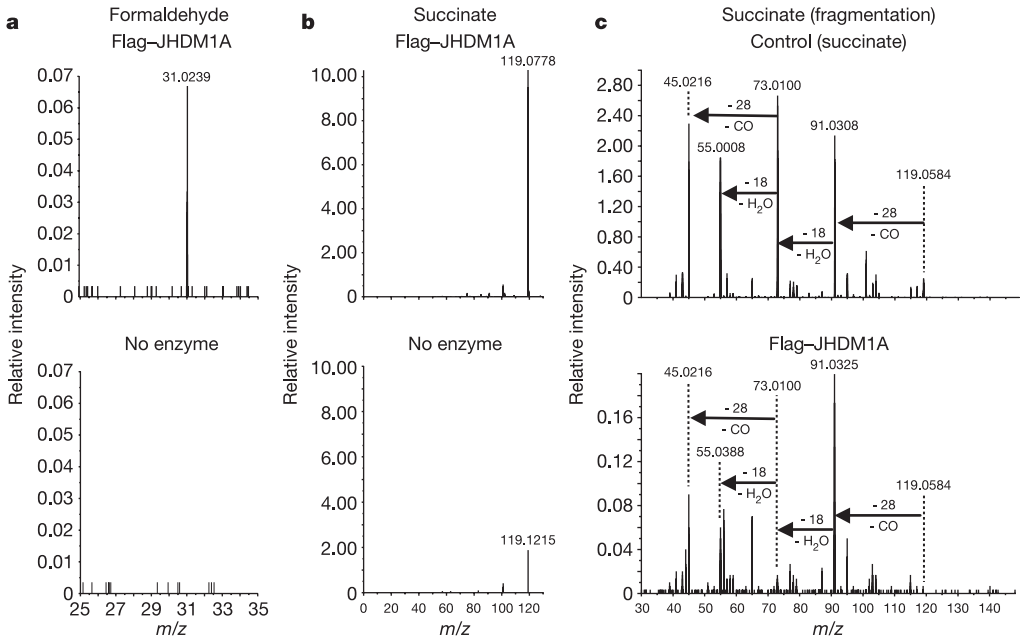

Figure 4 | Flag–JHDM1A-mediated histone demethylation generates formaldehyde and succinate. Demethylation reactions using highly purified Flag–JHDM1A expressed in Sf9 cells and a K36me2 peptide (STGGV2mKKPHRY-C) were subjected to mass spectrometric analysis. **a**, **b**, ESI-MS detection of formaldehyde (**a**; $[M + H]^+_{theoretical} = 31.0184$) and succinate (**b**; $[M + H]^+_{theoretical} = 119.0344$) in the presence (upper panel) and absence (lower panel) of Flag–JHDM1A. **c**, ESI-MS/MS analysis of ions at *m/z* 119 from a standard solution of succinate (600 nM) (top panel), and a Flag–JHDM1A reaction sample (bottom panel). Suggested structures of the succinate fragment ions are shown on the MS/MS spectrum.

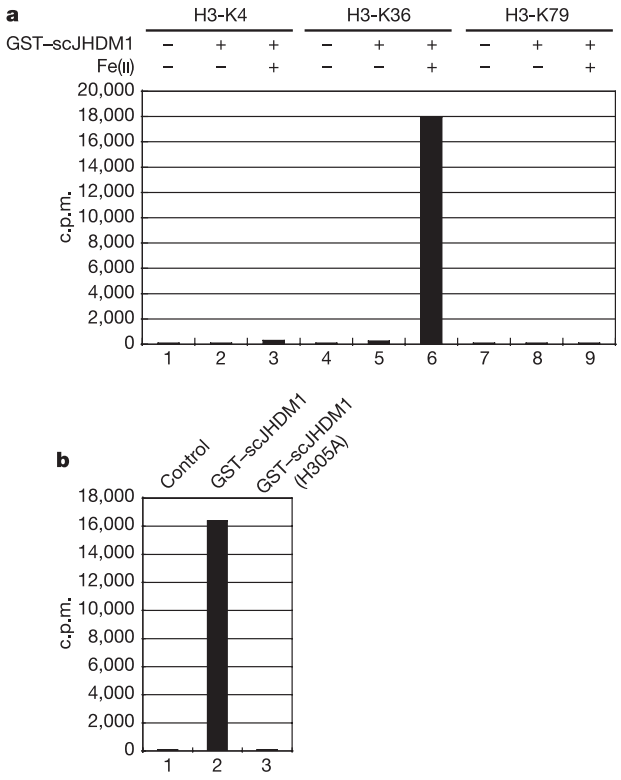

Figure 5 | The *S. cerevisiae* JHDM1 homologue exhibits H3-K36 demethylase activity. **a**, Demethylase activity and site specificity of the *S. cerevisiae* JHDM1 homologue expressed in *Escherichia coli*. **b**, Mutational analysis of the JmjC domain of the scJHDM1 protein. Equal amounts of wild-type and mutant GST–scJHDM1 were used in the demethylase assays. A mutation in the Fe(II)-binding site (H305A) completely abolished the H3-K36 demethylase activity.

JHDM1 family proteins (Supplementary Fig. S3b, marked by '$'), suggesting involvement of this residue in a conserved function, such as histone demethylation. Surprisingly, Epe1 lacks conservation of two residues in the JmjC domain predicted to bind to Fe(II) (Supplementary Fig. S3b). This raises the question of whether Epe1 is an active histone demethylase. Results shown in Supplementary Fig. S9 indicate that recombinant Epe1 purified from Sf9 cells lacks histone demethylase activity towards any of the four lysine residues on *S. pombe* H3 known to be subjected to methylation. Whether native Epe1 is a histone demethylase remains to be determined, but our *in vitro* evidence suggests that the phenotypes associated with Epe1 mutation may not be directly related to histone demethylation. Instead, the phenotypes may result from other affects on histone methyltransferase recruitment or chromatin remodelling.

The JmjC domain is a signature motif for histone demethylases

By monitoring histone demethylase activity, we purified an H3-K36 demethylase and demonstrated that its JmjC domain is critical for its enzymatic activity. This result together with the fact that the JmjC domain is involved in binding to the reaction cofactors Fe(II) and α-ketoglutarate[18], allow us to conclude that the JmjC domain represents a signature motif for a new family of histone demethylases. Our results are consistent with a structure-based prediction that JmjC domain-containing proteins may be histone demethylases[20]. In addition, our study also indicates that histone demethylation catalysed by JmjC domain proteins uses the same oxidative demethylation mechanism used by the AlkB family of DNA demethylases[11,12]. It is worth mentioning that recent studies have demonstrated that methyl-arginine is turned over by a peptidyl arginine deiminase PADI4/PAD4, which converts methyl-arginine to citrulline[21,22]. Because this reaction involves a change in the amino acid residue, it is not a strict demethylation reaction and perhaps a real methyl-arginine demethylase may be found in the JmjC domain family proteins.

The demonstration that JmjC domain proteins function as histone demethylases significantly extends recent findings that LSD1 is an H3-K4 demethylase[7]. First, it reveals a new reaction mechanism that differs from the LSD1 family of proteins in its cofactor requirement

and the reaction products it produces. Second, the LSD1 protein family encompasses only about ten related proteins[23], of which only a few are potential histone demethylases, making it unlikely that these proteins are responsible for modulating the diverse range of histone methylation states[24]. In contrast, JmjC domain proteins form a large protein family that could potentially satisfy the diverse range of enzymatic requirements to regulate and counteract histone methylation. Third, unlike LSD1, which requires a protonated nitrogen for the demethylation reaction, thus limiting the ability of LSD1 to demethylate trimethyl-lysine, there is no such limitation for JmjC domain-mediated demethylation. Given that JmjC domain-mediated demethylation should have the capacity to demethylate the trimethyl state, it was surprising to us that JHDM1A did not carry out this function. One possible explanation is that the methyl-state specificity of JHDM1A might be regulated by its associated proteins. A precedent exists for this possibility, as the H3-K9 methyltransferase ESET alone can only methylate H3-K9 to the dimethyl state, and requires association with mAM for H3-K9 trimethylation[6]. In a more dramatic example, association of androgen receptor with LSD1 can convert LSD1 from a K4 demethylase to a K9 demethylase[25]. A second possibility is that the catalytic pocket of JHDM1A may be too small to accommodate K36me3. Consistent with this possibility, a monomethylase can be converted to a trimethylase by creating a slightly larger catalytic pocket[26]. A third possibility is that an unshared electron pair on the nitrogen of the ε-amino group of lysine is involved in a hydrogen bond within the JHDM1A catalytic site so that the substrate methyl-lysine can be positioned in the catalytic site. In the later two cases, demethylation of trimethyl-lysine may need a JmjC domain with either a larger catalytic pocket or a way of positioning the substrate lysine that does not require the presence of an unshared electron pair on the nitrogen of the ε-amino group. A final possibility is that the trimethyl state is permanent and cannot be reversed. Further characterization of JHDM1A, particularly structural studies, should help to explain why it fails to demethylate K36me3.

METHODS

***In vitro* histone demethylase assay.** To prepare for ^{3}H-labelled methyl-histone octamers or oligonucleosome substrates, histone methyltransferase (HMT) assays were performed as previously described[13]. The HMTs used include GST–SET7, GST–G9a, CBP–Set2–Flag, GST–hDOT1L, GST–PRMT1, GST–Suv4-20h1 and the EZH2 complex. After the HMT reaction, the reaction mixtures were dialysed into histone storage buffer (10 mM HEPES-KOH (pH 7.5), 10 mM KCl, 0.2 mM PMSF and 10% glycerol) before being used in histone demethylase assays.

For histone demethylase assays, core histones, nucleosomes (either ^{3}H-labelled or not) or H3-K36 methylated peptide substrates were incubated with protein fractions or purified Flag–JHDM1A in histone demethylation buffer (50 mM HEPES-KOH (pH 8.0), 7–700 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-ketoglutarate, 2 mM ascorbate) at 37 °C for 1–3 h. Demethylation was analysed by the NASH method, western blotting and mass spectrometry. For detection of ^{3}H-labelled formaldehyde, a modified NASH method[27] was used. After TCA precipitation, equal volume of NASH reagent (3.89 M ammonium acetate, 0.1 M acetic acid, 0.2% 2,4-pentanedione) was added into the supernatant and the mixtures were incubated at 37 °C for 50 min before being extracted with equal volume of 1-pentanol. The extracted radioactivity was measured by scintillation counting. For detection of demethylation with peptide substrates, peptides in the reaction mixture were desalted on an RP micro-tip and analysed by MALDI-TOF as described in Supplementary Methods. For detection of histone demethylation using western blotting, demethylation reactions were subjected to western blotting using methyl-specific antibodies.

Methods for purification of HMTs, JHDM1A, Flag–JHDM1A and immunostaining are described in the Supplementary Methods. Constructs and antibodies are also described in the Supplementary Methods.

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