LETTERS

The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36

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Post-translational modification of chromatin has profound effects on many biological processes including transcriptional regulation, heterochromatin organization, and X-chromosome inactivation[1,2]. Recent studies indicate that methylation on specific histone lysine (K) residues participates in many of these processes[3]. Lysine methylation occurs in three distinct states, having either one (me1), two (me2) or three (me3) methyl groups attached to the amine group of the lysine side chain. These differences in modification state have an important role in defining how methylated chromatin is recognized and interpreted[4–6]. Until recently, histone lysine methylation was considered a stable modification[7,8], but the identification of histone demethylase enzymes has demonstrated the reversibility of this epigenetic mark[9–11]. So far, all characterized histone demethylases show enzymatic activity towards lysine residues modified in the me1 or me2 state[9–11], leaving open the possibility that me3 constitutes an irreversible modification. Here we demonstrate that JHDM3A (jumonji C (JmjC)-domain-containing histone demethylase 3A; also known as JMJD2A) is capable of removing the me3 group from modified H3 lysine 9 (H3K9) and H3 lysine 36 (H3K36). Overexpression of JHDM3A abrogates recruitment of HP1 (heterochromatin protein 1) to heterochromatin, indicating a role for JHDM3A in antagonizing methylated H3K9 nucleated events. siRNA-mediated knockdown of JHDM3A leads to increased levels of H3K9 methylation and upregulation of a JHDM3A target gene, *ASCL2*, indicating that JHDM3A may function in euchromatin to remove histone methylation marks that are associated with active transcription[12].

An extensive family of genes that encode JmjC-domain-containing proteins have been identified in eukaryotic genomes from yeast to human[13,14]. Using an unbiased biochemical approach, we have previously identified the JmjC domain as a signature motif for enzymes that catalyse histone demethylation[10,11]. In an attempt to identify additional histone lysine demethylases, we compared the JmjC domains of other JmjC family members to the known histone demethylases, JHDM1A (also known as FBXL11), JHDM1B (FBXL10) and JHDM2A (JMJD1A)[10,11]. Of the JmjC-domain-containing proteins analysed, the JMJD2 family of proteins[15] were excellent candidates for demethylase activity because amino acids predicted to be associated with enzymatic activity through their involvement in binding of Fe(II) and α-ketoglutarate (α-KG) are conserved (Supplementary Fig. S1a). As JMJD2A is the only JMJD2 family member characterized[16,17], we have focused our efforts in defining whether this protein is an active histone demethylase.

To determine whether JMJD2A is an active histone demethylase, we incubated recombinant Flag-tagged JMJD2A produced in insect cells (Supplementary Fig. S1b) with histone substrates containing radioactively labelled methyl groups corresponding to several characterized methyl-lysine sites on histones H3 and H4. The results presented in Fig. 1a demonstrate that demethylation reactions containing Dim5-labelled histone substrate showed substantial release of labelled formaldehyde. A small, yet reproducible, release of labelled formaldehyde was also observed in reactions containing SET2-labelled substrate (Fig. 1a). These data suggest that JMJD2A may potentially demethylate modified H3K9 and H3K36 residues. To further refine the substrate and methylation-state specificity of JMJD2A, a demethylase assay was carried out on purified native core histones and the modification state of individual methylation sites was analysed by western blot (Fig. 1b and Supplementary Fig. S2a). This analysis revealed that JMJD2A efficiently demethylates both H3K9me3 and H3K36me3 resulting in an accumulation of H3K9me1 and H3K36me1, whereas the levels of other histone methylation sites remained unchanged (Supplementary Fig. S2a). Furthermore, JMJD2A only functions efficiently to demethylate H3K9 and H3K36 within core histone, but not nucleosomal, substrates (Supplementary Fig. S2b), which explains why SET2-labelled nucleosomal histone is not a good substrate in the radioactive formaldehyde-release assay (Fig. 1a). These data verify that JMJD2A is an active histone demethylase and demonstrate that the trimethyl-lysine state is enzymatically reversible. Because JMJD2A is the third JmjC-domain-containing histone demethylase, we have named the protein JHDM3A (JmjC-domain-containing histone demethylase 3A) to reflect its enzymatic function and conform to our existing naming convention.

To confirm that JHDM3A uses an oxidative demethylation mechanism with Fe(II) and α-KG as cofactors, each cofactor was independently omitted from the demethylation reaction. Full enzymatic activity was observed when the complete cofactor/enzyme complement was present in the reaction (Fig. 1c). Omitting Fe(II) from the reaction buffer did not result in loss of activity, suggesting that Fe(II) remains associated with the enzyme during the purification process—consistent with the fact that addition of the divalent chelator EDTA abrogated the enzymatic activity (data not shown). Ascorbate was required for enzymatic activity, presumably owing to is capacity to reduce Fe(III) to Fe(II). Together, these data demonstrate that JHDM3A is an oxidative histone demethylase with the capacity to directly reverse H3K9me3 and H3K36me3 modifications.

To further define the modification-state specificity of JHDM3A, we used methylated peptide substrates in demethylation reactions

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coupled with mass spectrometry (Fig. 1d–g). In agreement with observations made using core histone substrates, JHDM3A is capable of demethylating H3K9me3 and H3K36me3 peptides (Fig. 1d, f). Notably, the H3K9me3 peptide was more extensively demethylated (~80%) than the H3K9me2 (~30%), H3K36me3 (~35%) or H3K36me2 (~25%) peptides, indicating that this is the preferred modification state targeted by JHDM3A (Fig. 1d–g). Demethylation by JHDM3A is not highly processive, as the extent of demethylation becomes less pronounced with the removal of each consecutive methyl group and does not culminate in the production of a single modification state. Furthermore, mass spectrometric analysis demonstrates that JHDM3A has the capacity to actively demethylate all three methylation states (Fig. 1d–g). In combination, our *in vitro* analysis demonstrates that JHDM3A can directly remove methyl groups from modified H3K9 and H3K36 to produce H3K9/H3K36 having me2, me1 and me0 modifications.

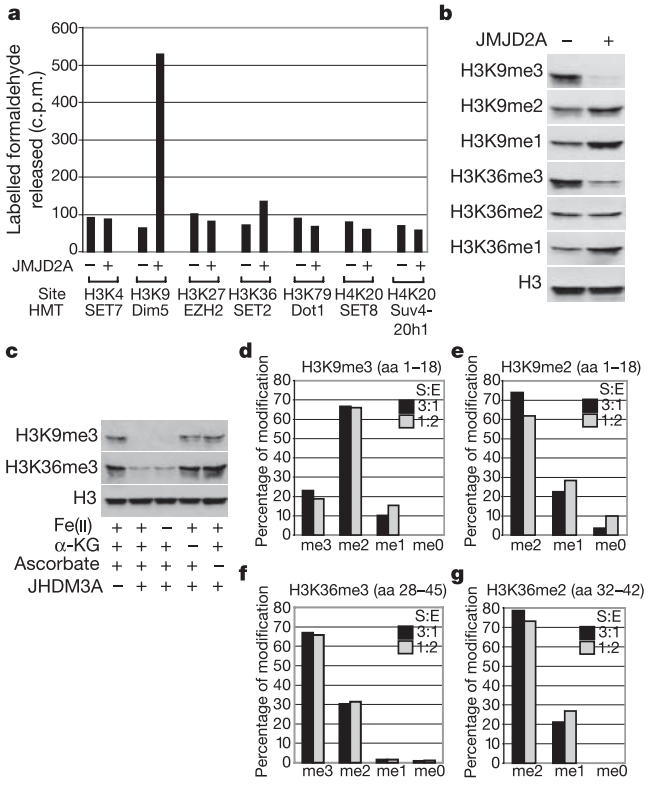

Figure 1 | JMJD2A/JHDM3A is a histone H3K9/H3K36 demethylase capable of removing trimethyl-lysine. a, Histones were labelled using various histone methyltransferase (HMT) enzymes as indicated below the bar graph, and equal counts of the various substrates were incubated with recombinant JMJD2A. The release of labelled methyl groups was measured to assay for histone demethylase activity, in the presence (+) or absence (−) of enzyme. JMJD2A demethylates H3K9 labelled by the histone methyltransferase Dim5, and to a lesser extent H3K36 labelled by SET2. **b**, Hela cell core histones were incubated in the presence (+) or absence(−) of JMJD2A, and following the demethylation reaction histone methylation levels were analysed by western blotting with modification-specific antibodies. JMJD2A demethylates H3K9me3/H3K36me3 resulting in an accumulation of H3K9me1/H3K36me1. For the reasons stated in the text, JMJD2A will hereafter be referred to as JHDM3A. **c**, To demonstrate that JHDM3A carries out demethylation using the predicted oxidative mechanism, the proposed cofactors were omitted from the reaction as indicated (−) below the western blot. Full enzymatic activity relies on the presence of the cofactor α-KG and ascorbate in the reaction. **d–g**, H3K9me3, H3K9me2, H3K36me3 and H3K36me2 peptides were incubated with JHDM3A at substrate to enzyme (S:E) ratios of 3:1 (black bars) and 1:2 (grey bars) in demethylase assays before mass spectrometry. The bar graph represents the percentage of each modification state after the demethylation reaction. The original mass spectrometry data for the S:E ratio of 1:2 is presented in Supplementary Fig. S3. JHDM3A demethylates H3K9me3/2 and H3K36me3/2 peptides, showing the highest activity towards H3K9me3.

Next, we sought to determine whether JHDM3A removes H3K9 and H3K36 modifications *in vivo*. To this end, Flag-tagged JHDM3A was overexpressed in cultured cells and H3K9/H3K36 methylation levels were analysed by indirect immunofluorescence (Fig. 2a–f and Supplementary Figs S4–S6) and western blotting (Fig. 2g). In agreement with the *in vitro* substrate specificity demonstrated for JHDM3A, we observed a near-complete loss of H3K9me3/H3K36me3 methylation in cells expressing JHDM3A (Fig. 2a, d, g, and Supplementary Fig. S5–S6). This activity is dependent on an intact JmjC domain, as an amino-acid substitution (H188A) in the predicted Fe(II)-binding site abrogates these effects (Fig. 2a, d, g, and Supplementary Figs S5–S6). Overexpression of JHDM3A resulted in an increase in H3K9me1-staining intensity in approximately one-third of transfected cells, and caused a subtle increase in H3K9me1 levels as assessed by western blotting (Fig. 2c, g, and Supplementary Fig. S7), but no changes in the level of H3K9me2 were observed (Fig. 2b, g). In addition, H3K36me2 levels were reduced and H3K36me1 levels increased in JHDM3A-expressing cells (Fig. 2e–g). Therefore, overexpression of JHDM3A *in vivo* causes dramatic loss of H3K9me3/H3K36me3 leading to accumulation of H3K9me1/H3K36me1.

Next, we attempted to determine which domains are required for demethylase activity *in vivo*. To achieve this, we generated mutants of JHDM3A harbouring deletions of predicted functional domains (Fig. 2h) and analysed their effect on H3K9me3/H3K36me3 staining in mouse cells. Both the JmjN and JmjC domains are required for demethylase activity (Fig. 2i, j), and deletion of the JmjN domain or the PHD and Tudor domains resulted in both nuclear and cytoplasmic localization of JHDM3A, indicating that these domains contribute to normal nuclear accumulation. Given that the PHD and Tudor domains are dispensable for enzymatic activity, these domains are probably involved in protein localization or protein–protein interaction.

Because little is known about the function of H3K36me modification in mammals[18], we sought to examine in more detail the effects that JHDM3A has on H3K9me-mediated cellular events. Heterochromatin protein 1 (HP1)-alpha and -beta preferentially bind to methylated H3K9 *in vitro*[5,19,20] and localize to pericentric heterochromatin in an H3K9me2/3-dependent manner[21,22]. Therefore, we proposed that JHDM3A activity may influence HP1 localization patterns. Consistent with this prediction, cells expressing high levels of JHDM3A show redistribution of pericentric HP1-alpha/beta (Fig. 3a, b (middle panels) and Supplementary Fig. S8), and this effect is dependent on functional JHDM3A demethylase activity (Figs 3a, b (right panels) and Supplementary Fig. S8). Notably, HP1 localization is retained in a proportion of cells expressing JHDM3A, indicating that factors in addition to H3K9me also contribute to HP1 accumulation at pericentric heterochromatin (Supplementary Fig. S8). These data indicate that elevated levels of JHDM3A can function to antagonize HP1 recruitment to pericentric heterochromatin.

To examine whether JHDM3A has a role in removing histone methylation at a euchromatic gene, we used short interfering RNA (siRNA)-mediated knockdown to manipulate JHDM3A levels and analysed its effects on the only known JHDM3A target gene, *ASCL2* (ref. 17). Consistent with JHDM3A functioning as a negative regulator of transcription and an H3K9/H3K36 demethylase, siRNA-mediated knockdown resulted in *ASCL2* upregulation (Fig. 4b, c), and an increase in the levels of H3K9me3 and H3K36me2 at the *ASCL2* locus as assessed by radioactive polymerase chain reaction (PCR; Fig. 4d). In contrast, no significant changes in methylation were observed at the human chromosome 4 pericentric region (Fig. 4e). To quantify the changes in histone modification, we

used real-time PCR following siRNA-mediated knockdown using two independent siRNA molecules targeting *JHDM3A* (Fig. 4f). This quantitative analysis revealed an approximate fourfold increase in H3K9me3 levels after siRNA treatment, but yielded more subtle changes in H3K36me2 levels than were observed by radioactive PCR, perhaps owing to lower levels of H3K36 methylation at the JAR (for 'JHDM3A-associated region') site. Therefore, occupancy of JHDM3A at a specific target gene is required to maintain reduced levels of H3K9 methylation and, to a lesser extent, H3K36 methylation, in concert with transcriptional repression.

During the revision of our manuscript, another study reported that JHDM3A is a me3-specific H3K9/H3K36 demethylase, with little activity towards lower modification states[23]. In contrast, we clearly observe that JHDM3A demethylates H3K9/H3K36me2/me1. In our hands, JHDM3A shows poor processivity under suboptimal reaction conditions *in vitro*, perhaps providing an explanation for the failure of this previous report to observe significant activity of JHDM3A towards me2/me1 modification states *in vitro*. We are confident that JHDM3A can demethylate lower H3K9/H3K36 methylation states, as several independent *in vitro* and *in vivo* lines of evidence support this conclusion (Fig. 1b, d–g, Supplementary Fig. S2a, Fig. 2a–h and Fig. 4d).

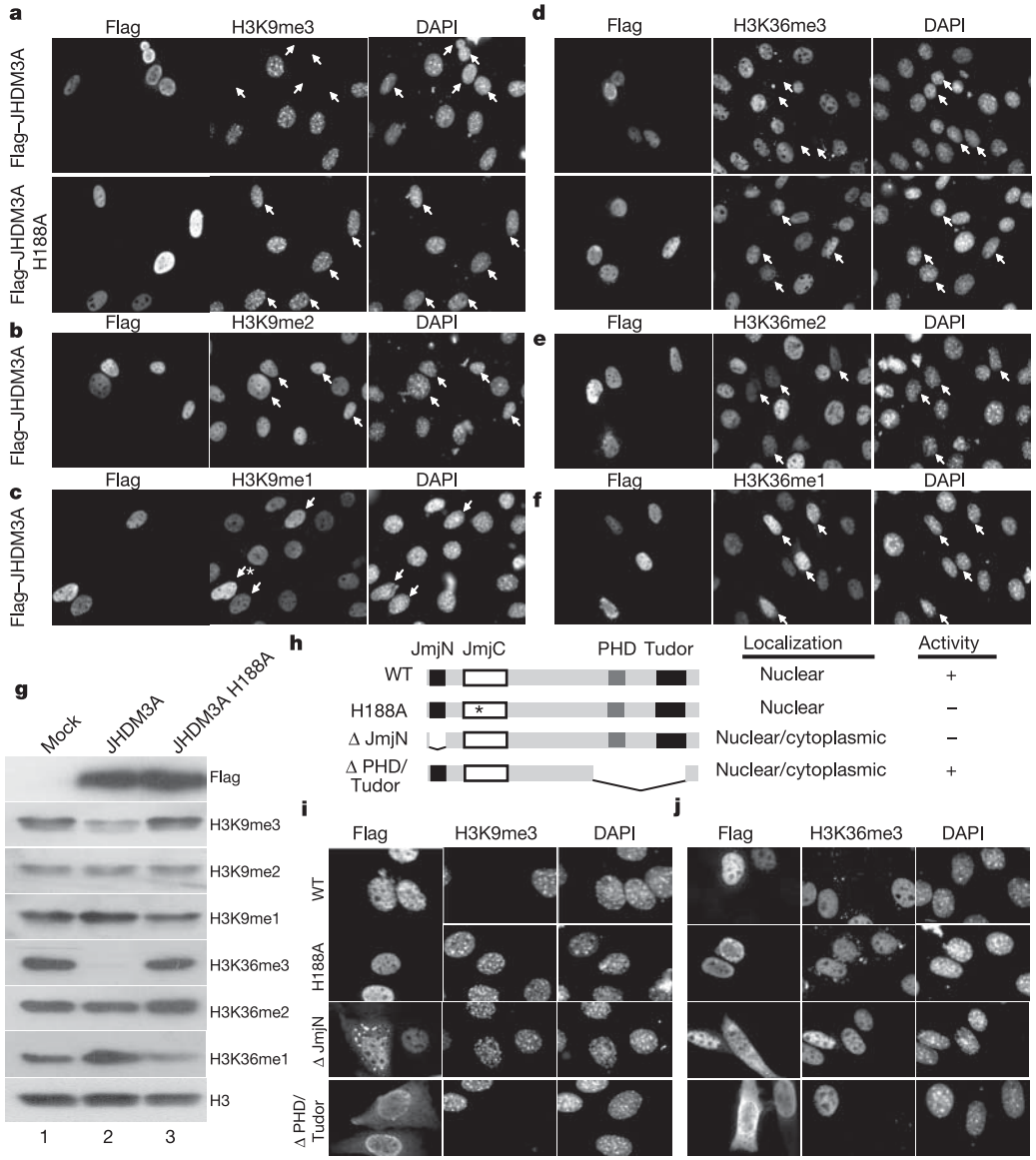

Figure 2 | JHDM3A requires the JmjC and JmjN domains to demethylate H3K9/H3K36 *in vivo*. **a**–**f**, JHDM3A was expressed in mouse NIH3T3 cells as a Flag fusion protein. Indirect immunofluorescence with antibodies against Flag (left panels) or methylated histone (middle panels) were used to analyse the substrate specificity of JHDM3A *in vivo*. DAPI (4,6-diamidino-2-phenylindole) staining (right panels) indicates location of nuclei in each field. Cells transfected with JHDM3A (arrows) showed a pronounced loss of H3K9me3/H3K36me3 staining (**a** and **d**, top panels), and this activity was dependent on an intact JmjC domain as methyl-histone staining was unaffected when a mutation in the predicted Fe(II)-binding site was introduced into JHDM3A (**a** and **d**, bottom panels). JHDM3A-mediated demethylation results in an accumulation of H3K9me1/H3K36me1 signal (the asterisk indicates a cell showing increased levels of H3K9me1) (**c** and **f**). The H3K9me2/H3K36me2 signal is also shown (**b** and **e**). **g**, JHDM3A was expressed in HEK293T cells as a Flag fusion protein, and histones were analysed by western blotting with antibodies against H3K9me/H3K36me modifications. A pronounced loss of H3K9me3/H3K36me3 was observed in JHDM3A-expressing cells (lane 2), and this was dependent on an intact JmjC domain (lane 3). A subtle increase in H3K9me1 and a clear increase in H3K36me1 levels were also evident in JHDM3A-expressing cells. **h**–**j**, JHDM3A wild-type (WT) and deletion proteins (**h**) were expressed in NIH3T3 cells and indirect immunofluorescence was used to analyse H3K9me3/H3K36me3 levels (**i** and **j**). The enzymatic activity of JHDM3A requires both the JmjC and JmjN domains (compare middle panels to top and bottom panels).

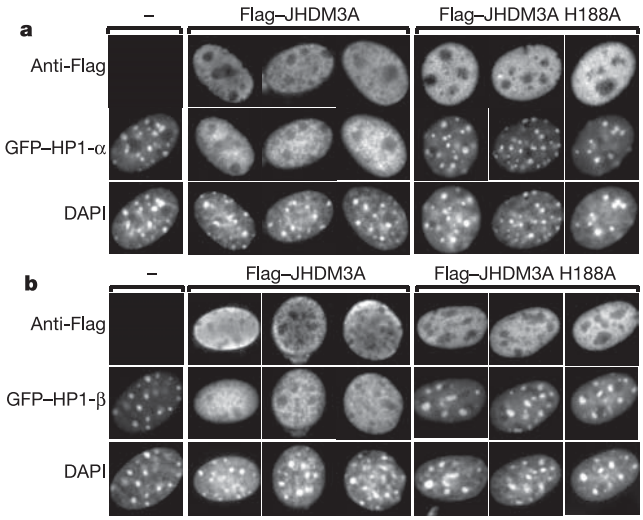

Figure 3 | Overexpression of JHDM3A antagonizes HP1 recruitment to pericentric heterochromatin. a, **b**, Green fluorescent protein (GFP)-tagged HP1-alpha (GFP–HP1-α) and -beta (GFP–HP1-β) show punctate fluorescence corresponding to H3K9me3-containing pericentric heterochromatin in mouse cells (left panels). Overexpression of JHDM3A causes a diffuse re-distribution of GFP–HP1 within the nucleus (middle panels). An intact JmjC domain is essential for antagonizing HP1 recruitment to pericentric heterochromatin, as a mutation in the predicted Fe(II)-binding site abrogated the effect of JHDM3A on GFP–HP1 localization (right panels).

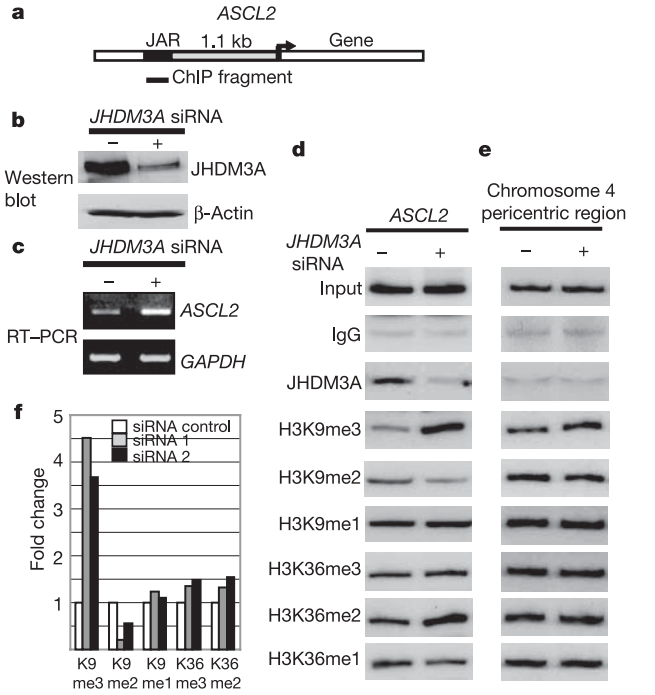

Figure 4 | JHDM3A regulates H3K9/H3K36 methylation at a euchromatic target gene. a, A diagram of the human *ASCL2* gene structure. "JAR" represents the 'JHDM3A-associated region'. **b**, JHDM3A protein levels were efficiently reduced by treatment of cells with *JHDM3A* siRNA **c**, siRNA-mediated knockdown of JHDM3A results in increased expression of the *ASCL2* gene. **d**, **e**, Untreated (−) and *JHDM3A*-siRNA-treated (+) cells were used in ChIP assays to analyse histone modifications at the *ASCL2* gene and the chromosome 4 pericentric region. Reduced levels of JHDM3A caused reduced occupancy of JHDM3A at the *ASCL2* gene and increased levels of H3K9me3 and H3K36me2, as assessed by radioactive PCR. JHDM3A was not specifically enriched at the chromosome 4 pericentric regions, and had no effect on the H3K9/H3K36 methylation profiles at this location. **f**, Quantitative real-time PCR analysis of histone modification levels at the *ASCL2* JAR after treatment with two independent siRNAs (see Methods) revealed a increase in H3K9me3 levels and subtle changes in lower H3K9 and H3K36 modifications.

The recent identification of enzymes that can demethylate histone lysine residues has clearly demonstrated the reversibility of this epigenetic modification[9–11], and our characterization of JHDM3A demonstrates that trimethyl-lysine is also a reversible modification. Little is known about the function of H3K36 methylation in mammals, but this modification is associated with transcribed regions of active genes[18]. A significant amount of H3K9 methylation resides in heterochromatic regions[21,22], but this modification also regulates genes found in euchromatic regions[24,25]. The biological rationale in combining the removal of H3K9 and H3K36 methylation through the activity of one enzyme remains unclear, but suggests that there may be a previously unrealized link between modification of H3K9 and H3K36. Surprisingly, removal of H3K9/H3K36 methylation by JHDM3A is associated with transcriptional repression, indicating that JHDM3A may function to antagonize H3K9/H3K36 methylation, which is associated with gene activity[12,18].

Recent biochemical studies have defined a role for the tandem Tudor domain of JHDM3A in the recognition of methyl-lysine residues in histones H3 and H4 (refs 26, 27). It is tempting to speculate that the enzymatic activity of JHDM3A is recruited by the tandem Tudor domain of JHDM3A to chromatin containing specific histone modifications. Further structural and functional analyses of JHDM3A will be instrumental in defining how this interesting demethylase specifically recognizes, demethylates and contributes to the regulation of the H3K9/H3K36 methylation states *in vivo*.

METHODS

Demethylation assay and mass spectrometry. All histone substrates were radioactively labelled as described previously[10,28]. Equal counts of labelled substrate were used in histone demethylation reactions containing ascorbate. Mass spectrometry was performed also as previously described[10].

Transfection, immunofluorescence microscopy, and western blotting. NIH3T3 and HEK293T cells were grown in DMEM containing 10% FBS and penicillin/streptomycin. For western blot analysis, 10-cm plates were transfected with 20 μg of expression plasmid using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 48 h post-transfection, and histones were acid-extracted and processed for western blot analysis using standard western blotting techniques and enhanced chemiluminescence (ECL) detection. For immunofluorescence, cells grown on coverslips in 6-well plates were transfected with 2–6 μg of Flag–JHDM3A expression plasmid using Fugene 6 transfection reagent (Roche). In experiments using GFP–HP1-alpha or -beta, 250 ng of expression vector was included in the transfection.

***JHDM3A* siRNA, RT–PCR analysis and ChIP.** siRNA-mediated JHDM3A knockdown, RT–PCR analysis of *ASCL2*, and ChIP analysis were carried out as described[17]. Two independent siRNAs targeting *JHDM3A* were used in the experiments (siRNA1: 5′-AAGUUGAGGAUGGUCUUACCU-3′; siRNA2: 5′-AACACAGUUAUUGACCAUACU-3′). A detailed description of this and other relevant methods can be found in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.J.K. carried out most of the experiments in Figs 1–3 and the Supplementary Figures; K.Y. generated recombinant protein; H.E.-B. and P.T. performed mass spectrometric analysis; Y.B., D.Z. and J.W. carried out the experiments in Fig. 4; R.J.K. and Y.Z. wrote the paper.

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