Histone demethylase KDM5A is regulated by its reader domain through a positive-feedback mechanism

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The retinoblastoma binding protein KDM5A removes methyl marks from lysine 4 of histone H3 (H3K4). Misregulation of KDM5A contributes to the pathogenesis of lung and gastric cancers. In addition to its catalytic jumonji C domain, KDM5A contains three PHD reader domains, commonly recognized as chromatin recruitment modules. It is unknown whether any of these domains in KDM5A have functions beyond recruitment and whether they regulate the catalytic activity of the demethylase. Here using biochemical and nuclear magnetic resonance (NMR)-based structural studies, we show that the PHD1 preferentially recognizes unmethylated H3K4 histone tail, product of KDM5A-mediated demethylation of tri-methylated H3K4 (H3K4me3). Binding of unmodified H3 peptide to the PHD1 stimulates catalytic domain-mediated removal of methyl marks from H3K4me3 peptide and nucleosome substrates. This positive-feedback mechanism—enabled by the functional coupling between a reader and a catalytic domain in KDM5A—suggests a model for the spread of demethylation on chromatin.
The dynamic interplay of post-translational modifications (PTMs) on histone tails in nucleosomes provides a molecular mechanism for responding to cellular stimuli by regulating chromatin structure and function. Among the numerous PTMs on histone tails, histone lysine methylation plays a crucial role in controlling gene expression. The dynamics of lysine methylation on chromatin is tightly regulated by the coordinated function of enzymes and proteins that 'write', 'read' and 'erase' this mark. Removal of lysine methyl marks from chromatin is carried out by histone demethylases. Flavin-dependent demethylases LSD1 and LSD2 act on a subset of mono- and di-methylated lysine residues, and the broader and more recently discovered Jumonji C (JmjC) domain-containing demethylase family acts on a wide range of mono-, di- and tri-methylated lysine substrates. Contrary to histone lysine methyltransferases (KMTs), where extensive in vitro and in vivo studies have been conducted to characterize their activity on chromatin, there is limited information on the mechanisms that control the activities of histone lysine demethylases (KDMs), and in particular within the JmjC family. Yet, the need to functionally dissect the function of histone KDMs on chromatin rests on the mounting evidence that JmjC domain-containing KDMs are important during development and are misregulated in several cancers and neurological disorders.

KDM5A (RBP2, JARID1A) belongs to the KDM5 subfamily of Jumonji histone demethylases that act on tri-, di- and mono-methylated lysine 4 of histone H3 (H3K4me3/2/1). The KDM5 family also includes PLU-1/KDM5B, SMCCX/KDM5C and SMCY/KDM5D. The KDM5 enzymes share a highly conserved domain architecture that includes a JmjN domain, the catalytic JmjC domain, a Bright/Arid DNA binding domain, a C 5HC2 zinc finger and two or three PHD domains. Originally described as a binding partner of the tumour suppressor retinoblastoma protein, KDM5A is a critical transcriptional regulator in cellular differentiation and development. KDM5A overexpression promotes tumorigenesis and drug tolerance in cancer cells and thus represents a potential therapeutic target. Despite its importance in physiology and disease, the mechanisms by which the demethylase activity of KDM5A, and KDM5 demethylases in general, are regulated on chromatin are unknown.

Plant homeodomain (PHD) fingers contain a Cys4HisCys3 motif that coordinates two zinc ions in a cross-brace manner and have emerged as sequence- and modification-specific histone recognition domains. To date, PHD domains in demethylases and demethylation complexes have been shown to act as binding modules to regulate occupancy and substrate specificity of demethylases. To investigate whether the functions of PHD domains in demethylases extend beyond recruitment and could contribute to the regulation of the catalytic activity of these enzymes, we set out to interrogate their roles in the context of KDM5A. Among the three PHD domains of KDM5A, PHD3 has been studied in the context of its fusion with the JmjC domain-containing demethylase, the PHD1 is positioned between the JmjN and JmjC domains, and has emerged as sequence- and modification-specific demethylase family acts on a wide range of mono-, di- and tri-methylated lysine tails. While the function of the PHD2 domain is not known; qualitative pull-down assays with isolated PHD1 domain of KDM5A suggest that this domain binds unmodified lysine 4 in histone H3 may be an evolutionarily conserved feature of this reader domain in the KDM5 family of demethylases.

**PHD1 occupancy stimulates peptide demethylation.** Our data indicate that the PHD1 domain in KDM5A preferentially recognizes H3 tail peptides that are unmethylated and, to a lesser extent, mono-methylated at K4 over those containing higher methylation states of this residue. As these preferred binding substrates are the products of KDM5A-mediated demethylation, this observation raises the possibility that the demethylase activity of this enzyme is regulated by a positive-feedback mechanism. To investigate this possibility and to isolate the role of PHD1 from PHD2 and PHD3, we used a KDM5A construct lacking the PHD2 and PHD3 domains (Fig. 1a, KDM5A1-797). We first evaluated the catalytic activity of this construct on H3K4me3, 2 and 1 methylated peptides using an enzyme-coupled fluorescent assay. We found that KDM5A1-797 demethylates all the H3K4 methylation states, with a preference for the H3K4 tri-methylated substrate (Fig. 1e and Supplementary Table 1), consistent with previous findings. Compared with previous in vitro studies on KDM5A (refs 13,14), however, we also detected demethylation of H3K4me1 peptides (Supplementary Fig. 2). While this discrepancy could be due to differences in experimental conditions, our results are consistent with the observation that in vivo KDM5A can demethylate all three methylation states of K4.
We then evaluated the binding selectivity of KDM5A_{1-797} towards differentially methylated H3K4 peptides. In analogy to the isolated PHD1, KDM5A_{1-797} preferentially binds unmethylated and mono-methylated H3K4 peptides compared with di- and tri-methylated H3K4 peptides (Fig. 1f), suggesting that the function of the PHD1 is retained in the context of the demethylase. To test whether binding of the ligand peptide to the PHD1 domain impacts the demethylase activity of KDM5, we compared the activity of the KDM5A_{1-797} construct towards a H3K4me3 peptide in the presence of unmodified H3_{1-18} peptide, ligand for the PHD1 or a truncated H3_{5-18} peptide that does not bind to the PHD1 (Fig. 1b,g). Interestingly, we found that the demethylase activity was stimulated in the presence of H3_{1-18} peptide, but no stimulation was observed with the control H3_{5-18} peptide (Fig. 1g). We also observed a decrease in the catalytic rate of the enzyme at high H3_{1-18} peptide concentrations (>$50 \mu M$) and attributed such effect to inhibition of the active site by this peptide. These results suggest a model whereby binding of an effector peptide to the PHD1 domain allosterically stimulates the activity of the catalytic domain.

**NMR structural analysis of the H3 tail-binding site in PHD1.** To gain insight into the mode of recognition of H3 tail peptide by PHD1 and to identify residues that, when mutated, disrupt the PHD1–H3K4 tail complex, we employed nuclear magnetic resonance (NMR). The two-dimensional heteronuclear single quantum coherence (2D^{15}N-HSQC) spectrum of the apo PHD1 revealed a well-dispersed set of cross-peaks that were assigned to residues by triple-resonance backbone experiments (Fig. 2a). The measured chemical shifts (\textbf{H}n, \textbf{15}N, \textbf{H}a, \textbf{13}Ca and \textbf{13}Cb) were used to generate a CS-Rosetta model of the apo PHD1 domain (Fig. 2b and Supplementary Figs 3 and 4) that was filtered by agreement with measured dihedral restraints (Supplementary...
Table 2). While very similar to the structures of canonical PHD finger domains and, in particular, to the recently determined NMR structure of the PHD1 of KDM5B (ref. 34), the CS-Rosetta models reveal a novel ‘open’ conformation of the L2 loop (residues 323–335; Fig. 2b and Supplementary Fig. 5). On titration with the H3K4me0 peptide, we observed large changes in chemical shifts for many cross-peaks in the PHD1 spectra, consistent with direct binding (Fig. 2a,c and Supplementary Fig. 6). Chemical shift changes were mapped onto the model of the apo PHD1 to display an extended peptide binding site (Fig. 2d,e). The largest perturbations were observed for the residues leading into the first β-strand (Glu305-Asp306), the first β-strand itself (β1, Leu308-Leu310) and the region that harbours the second Zn finger (Cys311-Asp315; Fig. 2c–e). Large chemical shift perturbations were also detected close to the C terminus of PHD1 (Val330-Trp335), as well as in the residues located near the N terminus of the PHD1 (Tyr294-Val295; Fig. 2c–e). Similar chemical shift perturbations were observed in the context of H3 peptide binding to the PHD1 of KDM5B (refs 33,34).

By analyzing the chemical shift perturbations of the PHD1 residues on titration of H3 tail peptide, we were able to identify several residues that contribute to recognition of the H3 tail. Similar to other PHD domains that recognize H3, Trp335 (W335), a highly conserved amino acid (aa) involved in recognition of the N-terminal Ala in H3 is essential for peptide binding since a W335A substitution disrupts H3 binding to
PHD1 (Fig. 2c,f,g)\textsuperscript{24,34,36,37}. We further predict that Asp312 and 315 (D312 and D315), residues that recognize the guanidine moiety of R2 in UHRF1, are important for R2 recognition by KDM5A PHD1 domain (Fig. 2c,f and Supplementary Fig. 7)\textsuperscript{38,41}. Consistent with our prediction, mutations D312A and D315A in the PHD1 of KDM5A have a detrimental impact on peptide binding (Fig. 2g). The large energetic contribution of R2 to binding is further substantiated by our finding that mutation of this residue in the H3 tail peptide to Ala decreases binding affinity more than 25-fold (Fig. 1d).

Similar to the role of D312 in R2 recognition, the NMR structure of the PHD1 domain of KDM5B indicates a critical role of the corresponding D328 in forming a salt bridge with R2 of the H3 tail\textsuperscript{44}. The substitution of R2 by alanine in this system of the PHD1 domain of KDM5B indicates a critical role this residue in the H3 tail peptide to Ala decreases binding affinity 30-fold (Fig. 1d).

### Structural comparison of unmethylated H3K4 PHD readers.

Given that the PHD1 domain of KDM5A preferentially recognizes unmethylated H3K4 tail peptide, we compared this domain with other PHD domains that preferentially interact with an unmodified H3K4 tail (for example, PHD1 of KDM5B, BHC80 and AIRE; Fig. 2f)\textsuperscript{24,34,37}. While recognition of unmodified Lys4 by BHC80 and AIRE PHD domains is achieved by hydrogen bonds with two polar residues and the amino group of Lys4 (D297 side chain and N295 backbone in unmodified Lys4 by BHC80 and AIRE PHD domains is indicated by red dotted lines (Fig. 2f and Supplementary Fig. 7)). The substitution of R2 by alanine in this system decreases binding to the H3 tail by \textasciitilde 30-fold (Fig. 1d).

### PHD1 occupancy stimulates nucleosome demethylation.

Taken together, these experiments demonstrate that peptide binding to the PHD1 domain stimulates the demethylation activity of KDM5A. To get an estimate of the extent of stimulation, we wished to perform demethylation reactions under single turnover conditions. Compared with multiple turnover conditions used to assay peptide demethylation (Fig. 1e,g), where both the product generated in each round of demethylation and the excess substrate could occupy the PHD1 domain, using an excess of demethylase and PHD1 ligand peptide over the substrate allows for direct assessment of the impact of PHD1 domain occupancy on catalysis. The demethylation assays were performed with nucleosomes as demethylation substrates under saturating single turnover conditions (See Methods and Supplementary Fig. 9). We generated homogeneous H3K4 tri-methylated nucleosomes using native chemical ligation between an H3(1–14) thioester peptide tri-methylated on K4 and expressed N-terminally truncated H3(A15C 15–135) protein (Fig. 4a). Following desulfurization, the resulting K4 tri-methylated H3 was incorporated into recombinant nucleosomes\textsuperscript{45} (Fig. 4a). Nucleosome demethylation was assayed by quantitative Western blot-based method that monitors disappearance of the H3K4me3 signal normalized to histone H4, in analogy to a similar assay that we previously developed\textsuperscript{44}. It is important to note that, under assay conditions, the antibody was specific for H3K4me3 mark and that no cross-reactivity against H3K4me2 and H3K4me1 modifications was observed (Supplementary Fig. 10).

To test whether occupancy of the PHD1 stimulates KDM5A demethylation activity on nucleosomes, we monitored nucleosome demethylation in the presence of H3\textsubscript{1–18} peptide or truncated H3\textsubscript{5–18} peptide (Fig. 4b,c). The addition of H3\textsubscript{1–18}, but not binding-impaired H3\textsubscript{5–18}, stimulates KDM5A demethylation on nucleosomes by \textasciitilde 30-fold (Fig. 4c).

These results indicate that binding of the unmodified H3 tail to the PHD1 domain stimulates the catalytic activity of KDM5A and further support a model whereby the function of the PHD1 reader domain and the JmjC catalytic domain are energetically coupled (Fig. 4d).

### Discussion

The presence of both reader and catalytic domains in chromatin-modifying enzymes and/or complexes containing these enzymes has important regulatory implications in chromatin biology. Previous results have demonstrated the role of reader domains in the association of histone demethylases to chromatin. For example, BHC80, a PHD domain-containing protein within the LSD1 co-repressor complex, preferentially binds unmethylated K4 and stabilizes the recruitment of LSD1 to chromatin\textsuperscript{24}. Similarly, a double tudor domain of a jumonji histone demethylase KDM4C recruits this demethylase to regions that contain H3K4me3 marks\textsuperscript{35,46}. In addition, reader domains can regulate substrate specificity of demethylases as in the case of KDM7A and KDM7B (ref. 25).

Here we show that the function of reader domains in demethylases expands beyond these roles. Using a combination of biochemical and structural studies, we show that the PHD1 domain preferentially recognizes unmethylated H3K4 histone tails, the product of KDM5A-mediated H3K4me3 demethylation. Binding of unmethylated H3K4 peptide by PHD1 stimulates the
catalytic activity of KDM5A. This effect is particularly pronounced on homogeneous H3K4me3 nucleosomes as demethylation substrates, where we observe a strong stimulation of KDM5A activity in the presence of PHD1 ligand peptide. We further show that the affinity of the PHD1 domain of KDM5A for histone H3 is modulated not only by methylation of K4 but also by methylation of R2, suggesting an additional layer of regulation of the catalytic activity by the PHD1–H3 tail complex.

Our findings are consistent with a model where product recognition by the PHD1 domain allosterically stimulates the catalytic activity of the enzyme on chromatin (Fig. 4d). Following initial demethylation events, likely enabled by a combination of
the basal activity of the catalytic domain and binding of di- and tri-methylated H3K4 nucleosomes to the PHD1 domain, binding to the resulting H3K4me1/me0 nucleosomes enhances the catalytic activity of KDM5A on the remaining H3K4me3 nucleosomes. Concerted recognition of the product and the substrate by two distinct domains within the same enzyme suggests a model by which demethylation could propagate along nucleosomes through a positive-feedback regulatory mechanism. Positive-feedback regulatory mechanisms have previously been described in several histone methylation complexes and implicated in the propagation of methylation on chromatin. For example, a functional cross-talk between EED, a product-binding WD40 reader subunit of the polycomb repressive complex 2 (PRC2), and the catalytic subunit of the complex, the EZH2 methyltransferase, enables the propagation of H3K27 methylation on chromatin.\(^{11}\) Similar mechanisms also exist for the Suv39h class of histone methyltransferases, the enzymes that add di- and tri-methyl marks to H3K9 (refs. 48–50). Our findings that the catalysis of KDM5A is regulated by a positive-feedback mechanism may be particularly relevant in the context of the transcriptional regulation of the HOX gene clusters, known targets of KDM5A-dependent silencing, which contain large Lys 4 methylated regions.\(^{13.51–53}\)

An alternative, but not mutually exclusive, possibility is that occupancy of the PHD1 domain by the histone tail may stabilize the composite active site formed by JmjC and JmjN domains and, in doing so, contribute to enhance the basal activity of the demethylase. While various modified H3 tails could serve as ligands, optimal stimulation is only achieved by an unmethylated H3K4 tail, given that in its unmodified state the H3 tail has the highest affinity for the PHD1 (Fig. 1c,d,f).

Given the high sequence homology of the PHD1 reader domains across members of KDM5 family\(^{30,33}\), we hypothesize that the functional role of the PHD1 in regulating KDM5A catalysis is conserved among KDM5 enzymes. If conserved, our findings can help explain previous in vivo observations that the deletion of the PHD1 in the Drosophila DMD5 homologue Lid abrogates its catalytic activity in cells.\(^{29,30}\) In addition, it was recently shown that abrogation of H3 tail recognition by point mutation in the PHD1 domain of KDM5B decreases H3K4 demethylation in cells, resulting in the repression of tumour suppressor genes by approximately twofold.\(^{44}\) We anticipate that future mechanistic and structural studies aimed at understanding the functional cross-talk between catalytic and ligand binding domains in KDM5 enzymes will help to better elucidate the role of this cross-talk in the regulation of H3K4 methylation in a cellular context.

Finally, given the oncogenic function of KDM5A in several cancers and its role in drug resistance in cancer, the identification of an allosteric regulatory site provides an attractive opportunity for the development of small molecule allosteric modulators of the activity of this enzyme.

**Methods**

**Expression of recombinant H3 C-terminal fragment.** SMT3(1–98) followed by C-terminal H3 fragment H3 (15–136 A15C) was cloned into pET28b (New England Biolabs) and transformed in Rosetta (DE3) pLysS. Cells were grown at 37°C to an OD600 of ~0.6 and induced with a final concentration of 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 3 h. Cells were harvested and lysed by sonication. Pelleted inclusion bodies were washed twice with wash buffer (50 mM Hepes pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol (BME) and 25 mM imidazole) and 2 volumes of cleavage/dilution Buffer (2 mM Urea, 1 M DTT, 150 mM L-Arg, 10 mM L-Cys, 150 mM NaCl and 50 mM Hepes pH 6.8). Protein was eluted in cleavage/dilution Buffer containing 250 mM imidazole. Eluted protein was diluted so that the final concentration was ~0.25 mg ml\(^{-1}\) and imidazole concentration was <150 mM. Approximately 1/10 of 15% PAGE (419–644) protein to be added was dialyzed in water containing 5 mM BME. Cleaved C-terminal H3 fragment was lyophilized and further purified by semi preparative C-18 RP–HPLC using a 0–60% acetonitril (ACN) with a 0.1% trifluoroacetic acid gradient for 1 h.

**Native chemical ligation and desulphurization of H3K4me3.** Native chemical ligation and desulphurization was performed essentially as previously described. Briefly, 11.1 mg (0.804 μM, 1 equiv.) of H3 (aa 15–136 A15C) was dissolved in 518 μl of ligation/desulphurization buffer (200 mM sodium phosphate pH 7.8, 6 M guanidinium HCl). Dissolved histone was reduced with 28 μl of 1 M Tris(2-carboxyethyl) phosphate TCEP (~50 mM) for 1 h at 37°C. Then 9.25 mg (~100 μM) of 4-mercaptohenylacetic acid (MPAA) and (2.2 μmol, 2.8 equiv.) of H3K4me3-S-bromotyrosine were added. Reaction mixture was incubated in methanol washout buffer with Argon and left to react after storing at 37°C overnight. The product was purified by semi preparative C-18 RP–HPLC using a 0–60% ACN with a 0.1% TFA gradient for 1 h.

**Desulfurization of cysteine 15 to the native alanine was performed by a free-radical-based approach.**\(^{54,55}\) In a typical reaction, 1 mg of H3K4me3 A15C was dissolved in 114 μl of the ligation/desulphurization buffer (200 mM sodium phosphate pH 7.8, 6 M guanidinium HCl). About 20 μl of 400 mM reduced glutathione, 50 μl of 1 M TCEP and 6 μl of 0.2 M VA-01 (Wako Chemicals) was added. The reaction mixture was then incubated at 37°C while vigorously stirring at 50°C until the completion of the reaction (typically overnight). The product was purified by semi preparative C-18 RP–HPLC using a 0–60% ACN with a 0.1% TFA gradient for 1 h.

**Nucleosomes assembly.** Except for H3K4me3, full length histones H2A, H2B and H4 were recombinantly expressed and purified under denaturing conditions.\(^{43}\) H3K4me3 nucleosomes were assembled on 147 bp of DNA using the 601 positioning sequence. The DNA was amplified by PCR and gel purified. The DNA fragment was assembled into mononucleosomes with recombinant **Xenopus laevis** histones by salt dialysis.\(^{43}\)

**Expression of recombinant GST-PHD1 (aa 291–347).** GST-tagged PHD1 (aa 291–347) protein was expressed in E. coli BL21-Gold (DE3) in 2× YT broth (2× Yeast extract and Tryptone) via isopropyl β-D-1-thiogalactopyranoside induction overnight at 18°C. Cells were resuspended in 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 5 mM βME, 50 μM ZnCl₂, 1 mM Phenylmethylsulfonyl fluoride PMSF pH 7.3, lysed by sonication and centrifuged. The supernatant was purified using a Glutathione Sepharose 4B resin, washed with high salt buffer (50 mM Hepes pH 8, 700 mM KCl, 10 mM βME, 50 μM ZnCl₂, 1 mM PMSF) and low salt buffer (50 mM Hepes, 150 mM KCl, 10 mM βME, 50 μM ZnCl₂, 1 mM PMSF). The sample was dialyzed overnight in 40 mM Hepes, 50 mM KCl, 0.5 mM TCEP and 50 μM ZnCl₂. Protein was further purified by anion exchange on MonoQ 10/100 with a linear gradient of low salt buffer (40 mM Hepes, 50 mM KCl, 0.5 mM TCEP and 50 μM ZnCl₂) and high salt buffer (40 mM Hepes, 1 M KCl, 0.5 mM TCEP and 50 μM ZnCl₂).

**Thermal shift assay.** The thermal denaturation of WT and mutant proteins was monitored indirectly by a fluorescence-based thermal shift assay using SYPRO orange dye in a buffer containing 50 μl of 1 μM protein with 1/500 SYPRO orange in 50 mM Hepes, 50 mM KCl pH 7.5. The plate was heated from 25 to 95°C with a heating rate of 1°C/min. The fluorescence intensity was measured with excitation/emission of 492/590 nm, respectively.

**Far-ultraviolet circular dichroism spectrophotometry.** Far-ultraviolet circular dichroism measurements were recorded on a Jasco J-715 spectropolarimeter at 11°C using 1-mm path length from 300 to 200 nm with a scan speed of 500 nm min\(^{-1}\), three scans and 3 ml of buffer for both GST-PHD1 291–347 WT, W335A or 145 mM for KDM5A 279 WT, W335A in buffer (20 mM KH₂PO₄, pH 7.5 10 μM Fe(NH₃)₆(SO₄)₂, 100 mM ascorbate). The spectra were corrected for buffer.

**FP studies.** The association of GST-PHD1 291–347 with H3–15, was measured by either direct or competition-based FP. All measurements were obtained in a buffer containing 50 mM Hepes pH 7.5, 50 mM KCl and 0.01% Tween-20 at 25°C. The binding mixture was incubated for 30 min at room temperature and FP was monitored using a Molecular Devices HT Analyst with excitation and emission wavelengths of λex 480 nm and λem 530 nm, respectively. All data were visualized using Graphpad Prism.

For direct FP binding assay, 10 nM of C-terminal fluorescently labelled H3 peptide (GenScript) were incubated with varying concentrations of GST-PHD1 291–347. Data were analyzed by non-linear regression with a 1:1 binding model (GraphPad Prism 6). For competition-based FP assays, 2 μM GST-PHD1 291–347 was incubated with 10 nM of C-terminal fluorescently labelled H3 peptide and different concentrations...
of unlabelled peptides were used as competitors. All data were visualized using Graphpad Prism and analyzed using the following model adapted from Narlikar et al.57:

\[ K_a = \frac{K_{d} [A]}{K_{d} + [A]} \]

Baculoviral expression and purification of KDM5A-Δ797. KDM5A (aa 1–797) was expressed in sf21 cells following Invitrogen Bac-to-Bac Baculovirus expression system protocol. KDM5A-Δ797 was cloned into a pFASTBAC HTA vector following a ligation-independent PCR cloning method58. Purified bacmid was transfected in sf21 cells. Approximately 0.8 × 10^5 cells per well of a six-well dish were allowed to attach in 2 ml of SF-900 II SFM media containing 50 U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin. While cells attached, 8 µl of Cellcetin II reagent (Invitrogen) in 100 µl of unsupplemented Grace’s Medium was mixed with ~2–5 µg of bacmid in 100 µl of unsupplemented Grace’s Medium and incubated for 15–30 min at 25°C. Once cells were attached, media was removed and cells were washed with 2 ml of Grace’s unsupplemented media. The Bacmid DNA:Cellcetin mixture was then diluted to 1 ml with Grace’s unsupplemented media and added to the well. Cells with Bacmid:Cellcetin II mixture were incubated at 5°C for 24 h. After 24 h, all the cells were detached from the well using a pipette and the supernatant was taken for bacmid transfection.

Demethylation assay on H3 peptides. Demethylation reactions on H3 peptides were performed in a demethylation buffer containing 50 mM Hepes pH 7.5, 50 mM KCl, 1 mM α-ketoglutarate, 50 µM Fe(NH₄)₂(SO₄)₂, and 2 mM ascorbic acid. The reaction was performed under subsaturating single turnover conditions where the effector peptide (H3K4me3) is above its Ki to the PHD domain and KDM5A is 10-fold above H3K4me3 nucleosome concentration. Reactions were initiated by the addition of methylated nucleosomes and quenched with 6 × SDS sample loading buffer and 0.5 M EDTA, pH 8.0. The reaction mixture was analyzed by Western blotting using an anti-H3K4me3 (1:1,000, Millipore cat# 05–1339) anti-H4 (1:1,400, Abcam cat# ab131303) and 15% SDS–PAGE. The protein bands were detected using the ECL detection system.

Nuclear magnetic resonance. A minimal PHD1 construct of KDM5A (D292E–E344) was used in the structural studies. This 6 x -HEK-TEV-PHDI was expressed in M9 minimal media containing 15N ammonium chloride and 13C glucose. The protein was purified as described for the binding studies with the additional steps of TEV protease cleavage and anion exchange chromatography via HiTrap Q (GE Healthcare), 3D-triple resonance experiments for backbone assignment (HNCA (pulse programme: hncagpp3d, TopSpin3.1p18) and CBCA(CO)NH (pulse programme: cbcaconhgpwg3d, TopSpin3.1p18)) and 3D quantitative J-H NMR spectra were recorded on an 800 MHz Bruker Avance III spectrometer equipped with a 12 mm Bruker cryoprobe. The 3D spectra were processed in NMRPipe and proton chemical shifts were referenced to a DSS standard and 13C and 15N were referenced indirectly to this value. Resonance assignments and data analysis, including prediction of ψ and φ dihedral angles from chemical shifts using DANGLE and extraction of 1H-15N chemical shifts, were performed with CCPNMR.

The 3D experiments for the assignment of Apo PHDI used a 180 µM PHDI sample; 2D 13C-15N-HSQC titration spectra were collected with a 5 µM PHDI sample (11 to 260 µM) and the experiments for the assignment of H3-bound PHDI were acquired with a 160 µM PHDI sample containing 2 mM Histone. Assignment of the H3-bound PHDI was deemed critical for correct mapping of peaks that broadened (underwent intermediate time scale exchange) during the titration points prior to saturation. See Supplementary Fig. 6 for high resolution HSQC spectra of all titration points.

Calculation of per residue chemical shift perturbation, all recorded chemical shifts were considered and normalized with the following equation:

\[ \Delta \text{Chemical shift} = \Delta H^\omega + \Delta H^\beta + \left( \frac{\Delta \alpha + \Delta \gamma + \Delta \omega}{4} \right) \]

All experiments were carried out at 298 K (calibrated with 4% v/v MeOH in MeOD) in 50 mM Hepes, 150 mM NaCl, 0.5 mM TCEP and 1 mM ZnCl₂ at pH 7.5 in 5% D₂O. Chemical shifts for the apo and H3-bound KDM5A PHDI will be deposited in the Biological Magnetic Resonance Data Bank.

CS-Rosetta model calculations. After assignment in CCPNMR, chemical shifts for 1H, 15N, 1H, 13Cα and 13Cβ of the apo PHDI protein were submitted to the BMRB CS-Rosetta web server for generation of fragment libraries. The chemical shift–fragmented ions were then used in Rosetta ab initio structure prediction (Rosetta v3.3). Simulations included restraints for the metal coordination of the two Zn-binding sites66, and stereochemistry of the chiral zinc centres was inferred by comparison with homologous structures.

For the apo structure predictions, 30,975 starting decoys were generated with the ab initio protocol. These decoys were ranked by energy and the top 5,000 were further optimized using a fast relax protocol, generating an additional 25,000 decoys. These models were then filtered by score using the all-atom Rosetta force field, and only the lowest scoring fast relax models from the 5,000 ab initio models were included with the ab initio set. Rosetta decoys were sorted by score and all residue pairwise Cα RMSDs were calculated for this set of 35,975 Rosetta decoys using fast_protein_cluster57. For a flow chart of CS-Rosetta model generation see Supplementary Fig. 3a. Plotting the Rosetta all-atom energy as a function of RMSD using different regions of the domain showed that the lowest energy model is not the one with the lowest RMSD. The lowest energy model is the one with the lowest RMSD but the model with the lowest RMSD is not the one with the lowest energy. This is typical for successful structural prediction. The converged regions are those of well-defined secondary structure (the core beta-sheet and the C-terminal alpha-helix; Supplementary Fig. 4).

The top 50 models by Rosetta all-atom energy were compared with the measured dihedral restraints (H10–H12 Supplementary Table 2), and only those with violations <15° were included in the structural ensemble (n = 8) used in the comparison with other known PHD finger structures.

Comparison to structurally determined homologous PHD fingers. In superposition of the KDM5A ensemble of decoys and all closely related PDB homologues, the structural core of the domain is well-conserved. However,
the extended/open conformation of the L2 loop appears unique to KDM5A (Supplementary Fig. 5). The L2 loop was defined as spanning residues 322 through 377. The centers of mass for these regions were calculated by taking the mean Cz position of residues within these regions across each individual model. The relative position of the L2 loop was determined by calculating the center of mass of the zinc ligands (Supplementary Fig. 5a-c). This angle (θ) then defines the relative position of the L2 loop with respect to the core of the PHD finger, with small theta representing a compact/open form and large theta giving a more extended/open form. The angle theta was calculated for the CS-Rosetta ensemble and the homologous structures and these distributions are presented in Supplementary Fig. 5d.

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I.O.T. and D.G.F. conceived the study. I.O.T., K.M.K. and C.I.N. performed the experiments. I.O.T., K.M.K., M.J.S.K. and D.F.G. designed the experiments and wrote the manuscript with inputs from C.I.N. and R.J.F. All authors contributed to the interpretation and the discussion of the results.

Additional information

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