Histone H3 binding to the PHD1 domain of histone demethylase KDM5A enables active site remodeling

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Histone demethylase KDM5A removes methyl marks from lysine 4 of histone H3 and is often overexpressed in cancer. The in vitro demethylase activity of KDM5A is allosterically enhanced by binding of its product, unmodified H3 peptides, to its PHD1 reader domain. However, the molecular basis of this allosteric enhancement is unclear. Here we show that saturation of the PHD1 domain by the H3 N-terminal tail peptides stabilizes binding of the substrate to the catalytic domain and improves the catalytic efficiency of demethylation. When present in saturating concentrations, differently modified H3 N-terminal tail peptides have a similar effect on demethylation. However, they vary greatly in their affinity towards the PHD1 domain, suggesting that H3 modifications can tune KDM5A activity. Furthermore, hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) experiments reveal conformational changes in the allosterically enhanced state. Our findings may enable future development of anti-cancer therapies targeting regions involved in allosteric regulation.

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Post-translational modifications of histone proteins are important regulators of chromatin structure and function and are controlled by proteins that write, read and erase these marks. A common and functionally diverse histone modification is lysine methylation, which regulates many cellular processes, including heterochromatin formation, regulation of transcription and DNA repair. Lysine methylation is a reversible modification, and its removal is catalyzed by lysine histone demethylases (KDMs). The KDMs are grouped into several subfamilies depending on their domain composition, substrate specificity and reaction mechanism. The KDM1 family (LSD1 and LSD2) uses a flavin-dependent mechanism, and acts on mono- or di-methylated lysines. A broader range of demethylation is possible by the jumonji C (JmjC) domain-containing family of KDMs (KDM2-9) that utilize a Fe(II)- and α-ketoglutarate (α-KG)-dependent mechanism, and acts on mono- and di-methylated lysines. A broader range of demethylation is possible by the jumonji C (JmjC) domain-containing family of KDMs (KDM2-9) that utilize a Fe(II)- and α-ketoglutarate (α-KG)-dependent mechanism, and acts on mono- and di-methylated lysines. They predominantly act on histone proteins, but in some instances also catalyze demethylation of non-histone substrates. Understanding the role of the chromatin environment in regulating activities of these enzymes is critical to elucidation of context-dependent spatial and temporal regulation of chromatin methylation. Several reports in recent years have pointed out the critical role of chromatin reader domains in regulation of demethylase activities, substrate specificities, and localization.

The human KDM5 subfamily of JmjC demethylases consists of four family members, KDM5A-D, which demethylate H3K4me1/2/3 marks. The proteins in the KDM5 family share common structural features, such as an iron containing active site comprised of the JmjN and JmjC domains, a DNA binding ARID domain, a zinc-finger domain, and either two (for KDM5C and D) or three (for KDM5A and B) plant homeodomain (PHD) chromatin reader domains (Fig. 1a). There has been a considerable amount of interest in the KDM5 family due to their roles in many disorders as all four members have been shown to be involved in various cancers. Specifically, KDM5A is overexpressed in breast cancer and its fragment is known to form a fusion with NUP98 in acute leukemia. Additionally, there is evidence for overexpression of KDM5A in cancer drug resistance in lung cancer models as well as osteoporosis. KDM5B is overexpressed in hepatocellular carcinoma where it promotes metastasis. Additionally, this enzyme is involved in drug resistance in melanoma treatments and regulation of genes involved in stem cell differentiation. KDM5C is highly expressed in neuronal tissues and mutations in this enzyme have been associated with X-linked intellectual disability disorders. KDM5D has been suggested to have a role in spermatogenesis. It is for these reasons that the KDM5 family are of clinical interest, prompting investigations into development of small molecule inhibitors of these enzymes.

KDM5A contains three PHD domains (Fig. 1a), which are commonly recognized as chromatin readers and are often found in chromatin modifying enzymes. PHD domains in demethylases have traditionally been associated with the recruitment of demethylases to chromatin. For example, PHD domain protein BHC80 is a component of LSD1 co-repressor complex that stabilizes the recruitment of LSD1 to chromatin. In addition, PHD domains can also regulate substrate specificity of demethylases, as in the case of PHF8 and KIAA1718. In vitro studies on PHD domains in KDM5A have shown that PHD1 preferentially binds unmodified H3 N-terminal peptides and that PHD3 binds H3K4me3 marks while no function for PHD2 has yet been determined. Binding of the H3K4me3 mark by PHD3 enables the recruitment of the demethylase to its substrate, a role consistent with the prototypical function of reader domains. In contrast to this canonical role, our earlier in vitro studies on the PHD1 domain have uncovered a function of the PHD1 domain in the allosteric regulation of the demethylase activity of KDM5A. Specifically, we found that the engagement of the PHD1 domain by unmodified H3 peptide enhances the activity of the catalytic domain. Since H3 peptide unmodified at Lys4 is the product of demethylation, these findings suggested a positive feedback interaction of demethylases, as previously suggested for the LSD1 family.
regulatory mechanism that may allow for demethylation to be propagated along nucleosomes. Such feedback regulation could be important for removal of broad H3K4me3 domains in early embryo development, a process mediated by KDM5A\textsuperscript{41} and KDM5B\textsuperscript{42,43}.

The molecular basis of allosteric stimulation by the product peptide binding to the PHD1 domain in KDM5A remains poorly understood. In addition, it remains unclear how the allostery is propagated between the PHD1 and JmjC domains. Current crystal structures of KDM5 enzymes are missing the structure of PHD1 and its surrounding regions due to either the PHD1 domain being removed from the construct or being disordered in these structures\textsuperscript{14-16,35}. With limited structural information, here we aim to determine the mechanistic rationale for the catalytic enhancement that results from binding of the peptide ligand to the PHD1 domain. Using purified KDM5A, we demonstrate that this allosteric enhancement is caused by improved substrate binding rather than enhanced catalytic turnover. Differently modified H3 tail peptides bound to the PHD1 domain promote similar effects, indicating that affinity of the modified H3 ligand, which would affect occupancy of the PHD1 domain, drives ligand-induced allosteric stimulation. Hydrogen/Deuterium exchange MS (HDX-MS) was used to probe conformational changes and highlighted regions of the catalytic domain that become less solvent protected in the allosterically enhanced state.

**Results**

Asp292 is important for PHD1 in recognizing H3 peptides. The allosteric regulation of KDM5A by its PHD1 domain highlights a more complex role of this domain rather than simple recognition of chromatin marks. Therefore, it is important to understand the molecular basis of how the PHD1 domain interacts with its ligand. PHD1\textsubscript{KDM5A} binds the N-terminal tail of histone H3 with a preference for unmodified H3, and binding affinity decreases with an increase in H3K4 methylation\textsuperscript{40}. Structural investigations into PHD1\textsubscript{KDM5B} and several other PHD1 domains, which like PHD1\textsubscript{KDM5A} preferentially recognize unmodified H3K4 residues, demonstrate electrostatic interactions between the unmodified H3K4 residue and a conserved carboxylate residue\textsuperscript{39,44-46} (Fig. 1b). However, our initial NMR studies of PHD1\textsubscript{KDM5A} have not detected any interactions between the equivalent conserved carboxylate residue (Asp292) and H3K4 in the histone peptide\textsuperscript{40}, necessitating further investigations into binding of H3K4 by PHD1 of KDM5A.

We suspected that the discrepancy regarding the role of the carboxylate residue in PHD1\textsubscript{KDM5A} and PHD1\textsubscript{KDM5B} could be due to the Asp292 residue in PHD1\textsubscript{KDM5A} being close to the N-terminus of the construct used in our previous studies (V291-K347 used in binding assays, D292-E344 used in NMR experiments). A new construct was generated (S287-K347) (Supplementary Figure 1) and used to measure binding affinity for the H3 peptide via a fluorescence polarization assay (Fig. 1c). A D292A variant was also generated to determine the contribution of the conserved Asp residue to peptide binding. Importantly, this mutation does not impair the overall fold or stability of the PHD1 domain (Supplementary Figure 2).

Compared to the PHD1\textsubscript{V291-K347} construct, the PHD1\textsubscript{S287-K347} construct shows an approximate twofold increase in affinity for the unmodified and mono-methylated, while di- and tri-methylated H3K4 peptides have similar binding affinity to both PHD1 constructs (Table 1). Our observations suggest that the additional N-terminal residues (S287-F290) are contributing to a higher affinity for unmodified and mono-methylated H3K4 peptides. This is further supported by the observation that the longer PHD1\textsubscript{S287-K347} shows a 2.3-fold preference for WT H3 peptide over the H3K4A peptide, whereas the binding affinities of WT H3 and H3K4A peptides to the shorter PHD1\textsubscript{V291-K347} are highly similar (Table 1). Our findings with the extended construct are similar to the 2.5-fold discrimination of WT H3 over H3K4A peptide that has been previously observed for PHD1\textsubscript{KDM5B}\textsuperscript{44}.

We further probed the PHD1-H3 interaction using a PHD1\textsubscript{S287-K347} D292A variant. Compared to the WT PHD1\textsubscript{S287-K347}, the mutant PHD1 domain shows an approximately eightfold decrease in affinity for the unmodified H3 peptide (Fig. 1c, Table 1) and a 10–16-fold decrease in affinity for methylated H3K4 peptides (Table 1) highlighting importance of Asp292 in contributing to PHD1-H3 tail interaction. These large fold reductions in affinity suggest that contribution of D292 extends beyond its likely electrostatic interaction with Lys4 in H3 and into structural organization of the N-terminus of the PHD1 for histone binding. This conclusion is consistent with structural analysis of the interaction of the highly homologous PHD1\textsubscript{KDM5A} with H3, where the equivalent carboxylate residue facilitates recognition of H3T6\textsuperscript{44}. Together, our findings suggest that the proper orientation of the Asp292 residue in the longer PHD1 construct significantly contributes to binding of the H3 peptide to the PHD1 domain.

PHD1 occupancy improves the catalytic efficiency of KDM5A. We have previously shown that binding of a H3 peptide to the PHD1 domain enhances the rate of demethylation by KDM5A in vitro\textsuperscript{40}. To dissect contributions of changes in substrate binding and enzymatic turnover to the overall allosteric enhancement, we investigated how the Michaelis–Menten kinetic parameters were affected by ligand binding to the PHD1 domain. A challenge for such an investigation lies in the nature of the ligand for each of the two sites, the PHD1 domain and the catalytic domain. While the PHD1 domain preferentially interacts with unmodified H3, it also binds H3K4me3, the substrate of the catalytic domain, albeit with a decreased affinity (Table 1). This cross-binding between two sites first prompted us to develop a discrete set of peptide ligands for the two sites in KDM5A (Fig. 2a). After studying the available structural information, N-terminal acetylation of the H3 peptide would likely prevent its binding to the PHD1 domain, as is the case in PHD1\textsubscript{UHRF1}\textsuperscript{46}. Indeed this was the case, as an N-terminally acetylated 21mer H3K4me2 peptide (Ala1-Ala21) does not bind to the PHD1 domain at the concentrations used in our assays (Supplementary Figure 3a). Importantly, although activity is reduced relative to corresponding peptide with a free N-terminus (Supplementary Figure 3c), N-terminal acetylation is tolerated by the catalytic domain (Fig. 2b, c). To develop a peptide selective for the PHD1 domain, we relied on the observation that C-terminal truncation of the K4 methylated H3 tail peptide leads to a dramatic increase in $K_m$ for peptide substrates (Supplementary Figure 3c). This increase in $K_m$ was particularly high for 10mer peptides, both in
which an N-terminally acetylated 21mer peptide substrate (H3 A1-A21) and a 10-mer PHD1 ligand peptide (H3 A1-S10) are used.

We measured the Michaelis–Menten kinetic parameters for substrate demethylation in the presence and absence of the PHD1 domain ligand. The $K_m$ and $k_{cat}$ values were determined by measuring the initial rate of demethylation by KDM5A using the formaldehyde release assay at varying concentrations of acetylated substrate. With the Ac-H3K4me3 21mer peptide alone, we observe a $K_m$ value of 95.5 ± 7.6 μM and a $k_{cat}$ of 2.82 ± 0.06 min$^{-1}$ (Fig. 2b, Table 2). However, in the presence of the H3 10mer effector peptide at a concentration that allows for the saturation of the PHD1 domain allosterically promotes binding of peptide substrates in KDM5A.

**Substrate binding is improved upon PHD1 occupation.** A decrease in $K_m$ upon effector peptide binding to the PHD1 domain suggests that there is improved substrate binding to the catalytic domain under these assay conditions. To further investigate this possibility the direct binding of an N-terminally acetylated substrate peptide was determined using fluorescence polarization assay. Binding assays were conducted in the presence and absence of the effector peptide and FP signal monitored over time (Supplementary Figure 5).

We observe that at 1 min the substrate binds with similar affinity to KMD5A, both in the absence ($K_d = 56.9 ± 7.9$ μM) and presence ($K_d = 62.5 ± 5.4$ μM) of H3 10mer peptide (Fig. 3). However, with no effector peptide, present the fluorescence polarization signal decreases over time. In contrast, when the H3 10mer peptide is present the signal is stable for at least 1 h. This striking difference between the two conditions suggests that the substrate peptide likely dissociates from the catalytic domain over time in the absence of the effector peptide, while the effector peptide occupied PHD1 domain stabilizes the interaction of substrate and the catalytic domain. Our data is consistent with a model where the demethylase is in a slow equilibrium between an open, substrate binding-competent state, and a closed state that does not bind the substrate. Binding of the effector peptide to the

**Table 2 Kinetic parameters of KDM5A1-797 reacting with various substrates with and without effector peptide**

| Substrate | Ac-H3K4me3 21mer | H3 10mer peptide | Ac-H3K4me2 21mer | H3 10mer peptide |
|-----------|-----------------|-----------------|-----------------|-----------------|
| $k_{cat}$ (min$^{-1}$) | 2.82 ± 0.06 | 2.45 ± 0.04 | 1.35 ± 0.11 | 1.40 ± 0.06 |
| $K_m$ (μM) | 95.5 ± 7.6 | 16.8 ± 11 | 229 ± 60 | 54.7 ± 9.9 |
| $k_{cat}/K_m$ (mM$^{-1}$min$^{-1}$) | 29.4 ± 3.3 | 146 ± 12 | 5.88 ± 2.04 | 25.6 ± 5.7 |

![Figure 2](image-url) The catalytic efficiency of KDM5A is enhanced upon ligand binding to PHD1. **a** Schematic representation of the discrete occupancy activity assay in which an N-terminally acetylated 21mer peptide substrate (H3 A1-A21) and a 10-mer PHD1 ligand peptide (H3 A1-S10) are used. **b** Michaelis-Menten plot of KDM5A-catalyzed demethylation of Ac-H3K4me3 21mer peptide in the presence and absence of a saturating concentration (38 μM) of unmodified H3 10mer effector peptide. **c** Kinetic parameters of demethylation of the Ac-H3K4me2 21mer substrate as a function of the presence of the effector peptide. Both (b) and (c) are fitted to the Michaelis-Menten equation. Kinetic parameters are shown in Table 2. Errors (n≥3) represent s.e.m.
PHD1 domain could shift the equilibrium between the two states toward the substrate binding-competent state (Supplementary Figure 6). Our observations that the substrate binding is stabilized in the presence of the PHD1 ligand could explain the decrease in $K_m$ that we observe when an effector peptide is present.

**Impact of H3 modifications on allostery.** The impact of post-translational modifications of H3 on binding of reader domains has been investigated thoroughly. However, it is largely unknown how these modifications may regulate processes beyond effector-reader interactions, such as allostery.

To begin to investigate how modifications impact allostery in KMD5A, we performed competition-based FP assays to determine affinities of a panel of modified peptides (Fig. 4a). In analogy to our earlier findings the PHD1V291-K347 construct, we observe that both methylation and mutation of H3R2 reduces binding affinities, emphasizing the importance of unmodified H3R2 in the PHD1 and H3 interaction. Phosphorylation of either the H3T3 or H3T6 residue greatly reduces the affinity, with H3T3 phosphorylation having a more pronounced effect. Methylation of H3R8 or H3K9 as well as acetylation of H3K9 has a modest or no effect on the affinity for the PHD1 domain. This agrees with the previous observation that the PHD1 domain predominantly interacts with the first five residues of the H3 peptide.

We investigated what impact modifications of the effector peptide have on demethylase activity using our discrete occupation activity assay. Specifically we determined the Michaelis–Menten parameters for demethylation of the Ac-H3K4me3 21mer substrate in the presence of saturating concentrations of various PHD1 ligand peptides (Fig. 4a). Similar to unmodified H3 (Fig. 3), we observed a significant decrease in the $K_m$ value of the substrate peptide for all modified effector peptides, with no significant to modest changes in the $k_{cat}$ value (Fig. 4a, b). The reduction in $K_m$ ranged from 3 to 6.5-fold, with the highest reduction in $K_m$ observed with the K9me3 and K9ac effector peptides, in addition to the unmodified H3 10mer peptide. This could be due to the lack of any H3 modifications that could interfere with optimal ligand engagement with the PHD1 domain in these effector peptides.

Together, our investigations of the impact of H3 modifications on PHD1 binding and KMD5A catalysis indicate a similar reduction in the $K_m$ of demethylation when PHD1 is saturated with effector peptide (Fig. 4b). However, modifications have a profound effect on the interaction of the PHD1 with H3 ligands, which would determine the concentrations of the PHD1 ligand required to saturate. Our findings indicate that the chromatin modifications context can impact the catalytic activity of KMD5A through modulation of the degree to which PHD1 is bound by a ligand.

**PHD1 domain deletion affects substrate binding to KDM5A.** The observation that effector peptide binding to the PHD1 domain reduces the $K_m$ for demethylation raises questions about how the liganded PHD1 domain enables enhanced interaction of the demethylase with its substrate. In order to determine the effect of the PHD1 domain deletion on the kinetics of demethylation, a ΔPHD1 KDM5A construct was generated. While deletion constructs of KDM5 enzymes have been generated previously, these constructs lack both the ARID and PHD1 domains. Our ΔPHD1 construct retains both the necessary JmjN and JmjC domains, as well as the ARID and ZF domains. The demethylase activity of the resulting ΔPHD1 construct toward the Ac-H3K4me3 21mer substrate was evaluated, and compared to the WT KDM5A1-797 construct.

Despite similar $k_{cat}$ values of the two constructs, an approximate eightfold increase in $K_m$ is observed for ΔPHD1 KDM5A-catalyzed demethylation of acetylated substrate (Fig. 5, Table 3). These findings suggest that the binding of the substrate to the catalytic domain is impaired in the absence of PHD1. While we cannot exclude the possibility that the introduction of a GS linker may obstruct substrate binding, our observation that $k_{cat}$ is unaffected indicates that the linker does not inhibit catalysis. Our findings suggest that the PHD1 domain could restructure the catalytic domain to enhance substrate binding, or alternatively directly contribute to substrate binding.

**Assessing active site changes in the enhanced state.** Currently available structures of KDM5 enzymes lack the PHD1 domain, which is either disordered or absent in the crystallized construct. Without structural information, it is difficult to determine how effector peptide PHD1 binding-induced allosteric regulation is communicated between the PHD1 and catalytic domains.

In order to investigate the conformational mobility of KDM5A upon effector peptide binding to the PHD1 domain we performed HDX-MS analysis. HDX-MS measures the rate at which hydrogen atoms exchange with deuterium in the solvent and can report on regions of a protein that undergo changes in hydrogen bonding networks upon ligand binding. We applied HDX-MS to KDM5A1-797 under conditions reflecting those of the discrete occupancy assay (Figs. 2 and 4). The conditions assayed were: KDM5A1-797 bound to the PHD1-specific H3 10mer peptide; KDM5A1-797 bound to the catalytic domain-specific Ac-H3K4me3 21mer substrate; and KDM5A1-797 bound to both
Fig. 4 H3 modifications dictate demethylation activity enhancement in KDM5A. a Binding affinities ($K_d$) of various modified H3 10mer N-terminal peptides for PHD1 and the Michaelis–Menten parameters ($k_{cat}$ and $K_m$) for demethylation of Ac-H3K4me3 21mer substrate peptide in the presence of saturating concentrations of modified H3 10mer peptides. $K_d$ values were determined using a competition-based fluorescence polarization assay, and kinetic parameters for demethylation were measured by the formaldehyde release assay. NB no binding, ND not determined. b Graphical representation of the $K_m$ values in the presence of saturating concentrations of differently modified effector peptides. Error bars refer to the standard deviation. Errors ($n \geq 3$) represent s.e.m.
Fig. 5 Deletion of the PHD1 domain impairs substrate binding by KDM5A. Michaelis–Menten plot for demethylation of Ac-H3K4me3 21mer substrate by KDM5A WT (black symbol) and PHD1 deletion (red symbol) determined by the formaldehyde release assay where Ac-H3K4me3 21mer peptide was used as a substrate. Data are fitted to the Michaelis–Menten equation. Kinetic parameters are shown in Table 3. Errors \( (n \geq 3) \) represent s.e.m.

Table 3 Kinetic parameters of KDM5A1-797 WT and ΔPHD1 constructs reacting with Ac-H3K4me3 21mer substrate

|                 | KDM5A1-797 WT | KDM5A1-797 ΔPHD1 |
|-----------------|--------------|-----------------|
| \( k_{cat} \) \( \text{min}^{-1} \) | 2.82 ± 0.08 | 2.83 ± 0.21 |
| \( K_m \) \( \mu M \)          | 95.5 ± 7.6   | 819 ± 133 |
| \( k_{cat}/K_m \) \( \text{mM}^{-1} \text{min}^{-1} \) | 29.4 ± 3.3 | 3.46 ± 0.82 |

the PHD1-specific H3 10mer peptide and catalytic domain-specific Ac-H3K4me3 21mer substrate.

For all conditions tested, >90% coverage of KDM5A1-797 was obtained thereby enabling us to confidently assign changes in HDX kinetics to various protein domains. While the majority of peptides assigned to KDM5A1-797 showed statistically insignificant changes (gray regions) in the levels of deuterium uptake, various regions of KDM5A1-797 undergo differential deuterium uptake upon peptide binding (Figs. 6a, b, S7, S8). When only the PHD1-specific H3 10mer peptide is present, residues Gly300-Ala342 of the PHD1 domain showed between 2 and 8% reduced deuterium uptake (referred to as protection from deuterium exchange) (Fig. 6a, green regions; Supplementary Figures 7a and 8a). The observation that the majority of the PHD1 domain (Fig. 6c), rather than just the previously mapped peptide binding residues, undergoes a change suggests a broad stabilization of the PHD1 domain upon H3 peptide binding. Conversely, when only the catalytic domain-specific Ac-H3K4me3 21mer substrate peptide was tested no perturbations are observed in the protein (Supplementary Figure 7b), despite using saturating concentrations of the substrate (25-fold over protein concentration, \( \sim 5 \times K_m \)). While it may be expected that residues in the catalytic domain would undergo protection from exchange with solvent upon substrate binding, this lack of perturbation may be due to weak binding of the acetylated substrate peptide, consistent with its high \( K_m \) measured (Fig. 2b, Table 2). Alternatively, substrate interactions with the catalytic domain could be predominantly hydrophobic or hydrogen bonds from the peptide to KDMD5A1-797 could be replacing hydrogen bonds in apo KDM5A1-797. Previous HDX studies have also shown no perturbation in hydrogen/deuterium exchange upon ligands binding. However, when both the PHD1-specific H3 10mer peptide and the catalytic domain-specific Ac-H3K4me3 21mer substrate peptide are present, two regions of KDM5A1-797 undergo significant changes (Fig. 6b, Supplementary Figures 7c, 8c). In addition to the increased protection observed in the PHD1 domain (residues Gly300-Ala342), as in the presence of the H3 10mer peptide alone, a protein segment within the catalytic domain (residues Ser528-Leu545) experiences a statistically significant increase in deuterium uptake (3–4%) suggesting decreased protection (Fig. 6b, yellow regions). These observed perturbations map onto a helical loop region between the fourth and the fifth β-strand of the DSBH motif in the JmjC domain at the interface with the ARID domain (Fig. 6d). These findings reveal a conformation alteration in KDM5A1-797, only present in the PHD1 effector bound higher activity state of KDM5A1-797. Furthermore, the observation that helical loop region becomes more exposed only in the presence of both the effector and substrate peptide indicates a high degree of cooperativity between occupancy of these two sites. Such cooperativity helps explain the coupled energetic effects on catalysis seen in the context of substrate and effector binding (Fig. 2).

While currently available structural data does not allow for clear identification of the role of the helical loop region, we hypothesize that this region may be involved in binding of the methylated peptide substrate. This hypothesis comes from an overlay of structures of KDM5A and the structurally related *Arabidopsis thaliana* demethylase JMJ14 (53.2% sequence identity, 73.4% similarity between catalytic domains of KDM5A and JMJ14 with an RMSD of 0.613) in complex with a H3K4me3 peptide substrate. Many residues of JMJ14, shown to interact with the H3K4me3 peptide substrate, are conserved in KDM5A and comparison between the structures suggests a potential substrate binding groove in KDM5A (Supplementary Figure 9). The difference in length between the 21mer peptide substrate used in our experiments and the 7mer peptide substrate co-crystallized with JMJ14 makes it challenging to compare the residues that participate in binding of the C-terminal end of the peptide substrate. However, several residues conserved in between JMJ14 and KDM5A—Gln353, Leu356 and Val357—bind the H3K4me3 peptide in JMJ14 and are part of the helical loop region that we observe to undergo increased deuterium uptake when both PHD1 effector peptide and the substrate peptide are present. This region has reduced solvent protection only in the presence of both the substrate and effector peptides, and could be responsible for the stabilized substrate binding we have observed in direct binding experiments (Fig. 3).

In order to evaluate if any additional structural changes occur within KDM5A upon effector peptide binding to the PHD1 domain, lysine-directed crosslinking experiments were conducted (Supplementary Figure 10). Isotopically coded reagents were used to probe changes in crosslinking between unoccupied KDM5A and KDM5A with effector peptide bound. Lysine-lysine crosslinks were mostly distributed within the ARID domain and the disordered region linking ARID and PHD1 domains. In contrast, few crosslinks were found to JmjN, JmjC, PHD1, and zinc finger domains. Chemical crosslinking mass spectrometry relies on the presence of accessible, un-hydrogen bound lysine residues located within ionizable tryptic peptides, and is known to preferentially sample disordered protein regions while providing sparse coverage of beta sheets. The quantitative comparison of crosslinks showed minor changes in crosslink occupancy and distribution when effector peptide is present (Supplementary Figure 10). Other than crosslinks between H3 effector peptide and KDM5A, few crosslinks were enriched in either condition more than twofold. Interactions between ARID and the PHD1-ARID linker are somewhat enhanced in the presence of effector (red-colored symbols), while ARID-ARID crosslinks and ARID-linker crosslink are enriched in the absence of effector (blue-colored symbols). However, the magnitude of...
of these crosslink peak area changes relative to the variance due to measurement error, combined with a lack of structural information for the PHD1 and PHD1-ARID linker region, makes these data challenging to interpret.

**Discussion**

Here we show that the allosteric stimulation of KDM5A activity, caused by binding of H3 N-terminal tail effector peptides to the PHD1 domain, is due to a reduced $K_m$ value of peptide substrate demethylation. This was determined by using a novel in vitro activity assay that allows us to discretely occupy the two H3 binding sites in KDM5A. Furthermore, upon assessing the direct binding of peptide substrate to the demethylase we attribute this change to stabilization of the KDM5A-substrate complex only when the PHD1 domain is occupied. The enhancement in demethylation catalysis is largely independent of the type of modification present on the effector.
peptide. However, it is strongly dependent on the occupancy of the PHD1 domain which directly correlates to the affinity of the effector peptide to the PHD1 domain. Therefore it is the affinity for the PHD1 domain that will dictate activity enhancement. Using HDX-MS, we identified a region in the catalytic domain that becomes more exposed only when both the PHD1 domain ligand and the substrate peptide are present. This structural change could be responsible for the stabilized substrate binding and the decrease in $K_{\alpha}$ that we observe in demethylation assays when effector peptide is present.

Active regulation of the catalytic activity of KDM5A by H3 tail as an allosteric ligand has important implications. The observation that the allosteric stimulation is preserved with modified peptides, as long as the PHD1 domain is saturated, suggests that both the nature of the modification and the local concentration of the effector peptide can influence catalysis. Despite the low affinity of PHD1 for H3K4me3 marks, the local concentration of H3K4me3 marks at the sites of recruitment, can enable allosteric enhancement. Once generated, products H3K4me2, H3K4me1, and non-methylated H3 peptides would further enhance the catalytic efficiency of KDM5A due to their higher affinities for the PHD1 domain. Our previous evaluation of nucleosome demethylation suggests that the magnitude of stimulation in the context of chromatin substrate can be far greater than what is observed with the peptides in this study. This positive feedback mechanism, where the reaction is stimulated by the product of the reaction, could contribute to rapid spreading of demethylation of H3K4me3-rich regions, which has been shown to occur in development, during maternal to zygotic transition and in regulation of HOX gene expression. However, rapid removal of these marks and the consequent repression of genes must be tightly regulated as dysregulation of boundary regions between active and silenced genes can disrupt cellular functions and lead to disease.

Our findings implicate PHD1 domain as a possible regulator of demethylation spread.

There are additional examples of allosteric stimulation of enzymes involved in epigenetic modifications. For example, propagation of H3K27 methylation by the PRC2 complex is mediated by product binding to an EED domain within this complex. Similarly, positive feedback regulation is observed in the Suv39h class of histone methyl transferases where H3K9 methylation is enhanced by the binding of the product of the reaction to a chromodomain within the methyltransferase. Additional examples of allosteric regulation in epigenetic enzymes include acetyl transferases p300/CPB, Gcn5, DNA methyltransferase DNMT3a, and DNA demethylase Tet5. Our studies have provided mechanistic rationale for allosteric regulatory role of the PHD1 domain in KDM5A, which expands the function of the PHD class of reader domain in regulation of chromatin methylation. PHD domain mediated allostery has also been shown to occur in the RAG-1/RAG-2 complex where H3K4me3 binding by the RAG-2 PHD finger changes the conformation of RAG-1 leading to enhanced substrate binding and cleavage of DNA at recombination signal sequences.

Our investigation of catalysis in PHD1 deletion construct indicated that the PHD1 domain plays an important role either directly or indirectly, in organizing the catalytic domain for optimal substrate binding. We show that KDM5A retains its catalytic activity after deletion of the PHD1 domain, but that its substrate binding is impaired as reflected in a higher $K_{\alpha}$ value. This finding is also mirrored in studies performed on a ΔAP KDM5B construct which shows a large increase in $K_{\alpha}$, although the precise domain responsible could not be pinpointed. Together, these data further highlight the importance of PHD1 in KDM5 enzymes, and parallel in vivo observations that this domain is necessary for activity of KDM5B and its drosophila homolog Lid.

Our HDX-MS experiments have uncovered residue level dynamics information that cannot be deduced from currently available structures, thereby providing insights into the structural changes in KDM5A that are induced by effector peptide binding to the PHD1 domain. The binding of the effector peptide to the PHD1 domain does not only induce changes in the peptide binding region that we previously mapped, but also affects protection of backbone amide protons throughout the PHD1 domain. Additionally, only in the presence of both the substrate and the effector peptide a helical loop region between the fourth and fifth ß-strand of the DSBH motif in the catalytic domain becomes less protected from solvent. Overlaying the structures of KDM5A and plant homolog JMJD14, bound to a H3K4me3 peptide substrate, suggests that this helical loop region is involved in substrate binding (Supplementary Figure 9). These data could therefore suggest that the improved substrate binding is caused by a potential effector peptide binding-induced repositioning of the PHD1 domain that is coupled to the conformation of the helical loop region which orients itself to optimally bind the substrate.

This possibility is yet to be validated, given a lack of a co-crystal structure between any member of KDM5 family and its H3K4me3 substrate. However, our direct binding experiments lend support to this hypothesis and suggest that occupation of the PHD1 favors a conformation that is able to better bind substrate peptides. While our studies have determined regions of conformational change within KDM5A, additional studies are needed in order to further define the interactions of the PHD1 domain and the catalytic domain, as well as their interaction with chromatin. Our data cannot exclude the possibility that portions of the helical loop region, revealed by HDX-MS, may participate in domain communication within the demethylase.

Our findings contribute to the growing knowledge about allosteric regulation of epigenetic enzymes and highlight the complexity of mechanisms that enable context-dependent...
Fluorescence polarization binding assays. The binding of GST-PHD1 to H3 10mer peptides was measured by either direct or competition-based fluorescence polarization (FP) assays. For competition-based FP assay, 10 μM PHD1 was incubated with varying concentrations of GST-PHD1. Data from direct FP measurements were fitted to equation 1:

$$\text{FP}_{\text{obs}} = \frac{\text{FP}_{\text{max}}(\text{PHD1}) + \text{FP}_{\text{max}}(\text{K})}{K_{d} + \text{PHD1}}$$

where \(\text{FP}_{\text{obs}}\) is the observed FP, \(\text{FP}_{\text{max}}\) is the maximum FP value, \(\text{FP}_{\text{min}}\) is the minimum FP value, [PHD1] is concentration of PHD1, and \(K_{d}\) is the inhibition constant.

For competition-based FP assays, 2 μM GST-PHD1 was incubated with 10 nM of C-terminal fluorescently labeled H3 peptide and different concentrations of unlabeled peptides were used as competitors. Data from competition-based FP assays were fitted to equation 2:

$$\text{FP}_{\text{obs}} = \frac{K_{d}(\text{PHD1}) + \text{FP}_{\text{max}}(\text{K})}{K_{d} + \text{PHD1}}$$

where \(\text{FP}_{\text{obs}}\) is the observed FP, \(\text{FP}_{\text{max}}\) is the maximum FP value, \(\text{FP}_{\text{min}}\) is the minimum FP value, [PHD1] is concentration of PHD1, and \(K_{d}\) is the inhibition constant.

The binding of peptide substrates to KDM5A1-797 was measured using the direct FP assay. A Ac-H3K4me3-fluorescein 31mer peptide was used, rather than the 21mer used in kinetic assays. This was in order to reduce interference of the C-terminal tail of PHD1 with PHD1 KDM5A in the cellular context. Changes that occur in the higher affinity state of KDM5A could also be synergistic with other epigenetic regulatory enzymes.

Expression of recombinant WT and ΔPHD1 KDM5A. WT KDM5A1-797 was expressed in Sf21 cells following Invitrogen Bac-to-Bac baculovirus expression system protocol. The bacmid was transfected in Sf21 cells. Approximately 0.8 × 10^6 cells per well of a six-well dish were allowed to attach in 2 ml of SF-900 II SFM containing 50 mM NaCl, 1.8 mM KH2PO4, 0.5 mM TCEP, and 50 µM ZnCl2 and incubated at 27 °C overnight before the pellet was induced with 0.4 mM IPTG and grown at 18 °C for 5 days or until signs of viral infection were observed. After the induction, the supernatant was collected and sterile filtered to obtain the P1 viral stock. To make P2, 20 ml of SF-900 II SFM containing 1.5 mM MgCl2, 10 mM imidazole, aprotinin 2 µg/ml, and 50 U/ml of formaldehyde dehydrogenase (FDH)-coupled Ac-H3K4me3 peptide was used. The complex with substrate and/or effector peptides was further purified by size-exclusion chromatography using a Hiload 16/60 Superdex 75 gel filtration column. The sample was eluted into a mES cell culture media supplemented with 10% fetal bovine serum. Fifty micrograms of cell lysate was used to determine the amount of recombinant protein.

For HDX-MS analysis. For differential HDX experiments, 5 µl of 20 µM KDM5A1-797 was diluted to 25 µl with D2O-containing HDX buffer (40 mM HEPS, 50 mM KCl, pH 7.9) and incubated at 4 °C for 10, 30, 60, 900, and 3600 s. HDX-MS conditions aimed to mimic the demethylation assay conditions as described in Fig. 2. A pET41a vector and expressed in BL21(DE3) E. coli cells. Expression and purification of all mutants followed the same protocol. Specifically, cells were induced with 0.4 mM IPTG and grown at 18 °C overnight before the pellet was collected. The cells were resuspended in lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.5 mM TCEP, 50 µM MnCl2, 1 mM phe-nylmethylsulfonyl fluoride (PMSF), pH 7.3), lyzed by sonication and centrifuged. The supernatant was purified using Glutathione Sepharose 4B resin, washed with high salt buffer (50 mM HEPS, 700 mM KCl, 10% glycerol, 0.5 mM TCEP, 50 mM MnCl2, 1 mM PMSF, pH 8) and low salt buffer (50 mM HEPS, 150 mM KCl, 10% glycerol, 0.5 mM TCEP, 50 mM MnCl2, 1 mM PMSF, pH 8) and recovered by elution using low salt buffer with 30 mM glutathione. The sample was then concentrated and purified by size-exclusion chromatography using a HiLoad 26/60 Superdex 75 gel filtration column. Samples were eluted into 40 mM HEPS, 50 mM KCl, 0.5 mM TCEP, and 50 mM MnCl2 and flash-frozen.

Peptide identification for HDX-MS. Peptides were identified using tandem MS (MS2 or MS3/MS) experiments performed with a LTQ linear ion trap mass spectrometer (LTQ Orbitrap XL with ETD, Thermo Fisher, San Jose, CA) over a 70 min gradient. Product ion spectra were acquired in a data-dependent mode and five most abundant ions were selected for the product ion analysis per scan event. The MS/MS *.raw files were converted to *.mgf files and then submitted to Mascot ver2.3 (Matrix Science, London, UK) for peptide identification. The maximum number of missed cleavage was set at 4 with the mass tolerance for precursor ions ± 0.6 Da and for fragment ions ± 8 ppm. Oxidation of methionine was selected for variable modification. Pepsin was used for digestion and no specific enzyme was selected in the Mascot during the search. Peptides included in the peptide set used for HDX detection had a Mascot score of 20 or greater. The Mascot protein identification (PDI) search was performed against reverse sequence and false positives were ruled out. The MS/MS spectra of all the peptide ions from the Mascot search were further manually inspected and only the unique charged peptide ions were considered for HDX-MS analysis.
exchange is minimized, and the protein is denatured by dilution to 50 µl with 0.1% TFA in 5 M urea and 50 mM TCEP (held at 4 °C, pH 2.5). Samples are then passed across immobilized pMMAA resin (prepared in house) at 50 µl min⁻¹ (0.1% TFA, 15°C), and the resulting peptides are trapped onto a C₈ trap cartridge (Thermo Fisher, HyperSil Gold). Peptides were then gradient eluted (5% CH₂CN to 50% CH₂CN, 0.3% formic acid over 6 min, 4°C) across a 1 mm × 50 mm C₁₈ analytical column (HyperGold, Thermo Fisher) and electrosprayed directly into a high-resolution orbitrap mass spectrometer (LTQ Orbitrap XL with ETD, Thermo Fisher). Percent deuterium exchange values for peptide isotopic envelopes at each time point were calculated and processed using HDX Workbench. Each HDX experiment was carried out in triplicate with a single preparation of each protein-ligand complex. The intensity weighted centroid value of each peptide envelope was calculated and subsequently converted into a percentage of deuterium incorporation. Statistical significance for the differential HDX data is determined by an unpaired t-test for each time point, a procedure that is integrated into the HDX Workbench software. Corrections for back-exchange were made on the basis of an estimated 70% deuterium recovery and accounting for 80% of the heavy peptide response (corrected for the peak area of the heavy peptide with the peak area of the light peptide). The theoretical elemental compositions were generated if the peptide sequence could not be retrieved from the database.

**Lysine crosslinking MS experiments.** Hundred micrograms of KDMA5Δ1-173 was crosslinked with 1 mM BS₃-D12 (Creative Molecules) under the same conditions. After quenching with 50 mM Tris-base, the light and heavy crosslinked reaction mixtures were combined and precipitated with ice cold acetone. Crosslinked proteins were pelleted by spinning at 13,000 × g, the supernatant was removed, and the pellet was washed once with cold acetone. The precipitate was brought up in 10 µl of SEC buffer (70:30 H₂O:ACN with 0.1% TFA) and 100 µl OMIX C₁₈ tip (Agilent), and evaporated to dryness. Crosslinked products were brought up in 10 µl of SEC buffer (70:30 H₂O:ACN with 0.1% TFA) and 100 µl OMIX C₁₈ tip (Agilent), and evaporated to dryness. Crosslinked proteins were pelleted by spinning at 21,000 × g.

**Fisher). Percent deuterium exchange values for peptide isotopic envelopes at each time point were calculated and processed using HDX Workbench. Each HDX experiment was carried out in triplicate with a single preparation of each protein-ligand complex. The intensity weighted centroid value of each peptide envelope was calculated and subsequently converted into a percentage of deuterium incorporation. Statistical significance for the differential HDX data is determined by an unpaired t-test for each time point, a procedure that is integrated into the HDX Workbench software. Corrections for back-exchange were made on the basis of an estimated 70% deuterium recovery and accounting for 80% of the heavy peptide response (corrected for the peak area of the heavy peptide with the peak area of the light peptide). The theoretical elemental compositions were generated if the peptide sequence could not be retrieved from the database.

**Peaklists were generated using Proteome Discoverer 2.2 (Thermo) and searched for crosslinked peptides with Protein Prospector 5.20.23. In total, 85 of the most intense peaks from each product ion spectrum were searched against a database containing the 11500 protein sequence (structurally and functionally characterized) and the protein N-terminus by semi-hydrolyzed heavy and light BS₂, crosslinking reagents were BS₃-H₂O and BS₅. D₃₂ trypsin specificity was used with two missed cleavages and three variable modifications per peptide were allowed. Unique, crosslinked residue pairs were reported at a 0.3% FDR threshold, corresponding to a Score Difference cutoff of 10.**

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**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file.

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**Received:** 5 March 2018 Accepted: 23 November 2018

Published online: 09 January 2019
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Acknowledgements

We would like to thank Christian Freund, John Gross, Mark Kelly, Geeta Narlikar, Bruce Pascal and Fatima Ugar for their comments on the paper. This work is supported by National Institutes of Health (R01 GM114044 and R01 GM114044-03S1 to DGF and 51015016229 to AB) and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AB).

Author contributions

J.E.L. and D.G.F. wrote the manuscript with input from V.D., M.T., A.B., and P.R.G. J.E.L., C.M.C., M.T., and V.D. designed and performed the experiments. I.O.T., D.G., and K. R. assisted in experimental design and initial experiments. All authors contributed to the interpretation and discussion of the results.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-07829-z.

Competing interests: The authors declare no competing interests.

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