The histone H3K4-specific demethylase KDM5B binds to its substrate and product through distinct PHD fingers

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SUMMARY

The histone lysine demethylase KDM5B regulates gene transcription and cell differentiation. It contains three PHD fingers, the biological roles of which remain elusive. Here, we show that the first PHD1 finger of KDM5B binds unmodified histone H3, whereas the third PHD3 finger prefers the trimethylated mark, H3K4me3. RNA-seq analysis indicates that KDM5B functions as a transcriptional repressor for a set of genes. Biochemical analysis reveals that KDM5B associates...
with components of the nucleosome remodeling and deacetylase (NuRD) complex and may cooperate with HDAC1 in gene repression. Compared with the estrogen receptor positive breast cancers, KDM5B is downregulated in the triple-negative breast cancer. Overexpression of KDM5B in the MDA-MB 231 breast cancer cells suppresses cell migration and invasion ability, and the PHD1-H3K4me0 interaction is important for inhibition of migration. These findings highlight tumor-suppressive functions of KDM5B in triple-negative breast cancer cells and suggest a novel multivalent mechanism for KDM5B-mediated transcriptional regulation.

INTRODUCTION

The histone lysine demethylase KDM5B (also known as PLU-1 and JARID1B) regulates gene expression and is implicated in cancer development and proliferation (Klose et al., 2006). KDM5B belongs to the KDM5/JARID1 family that catalyzes the removal of methyl groups from tri-, di- and monomethylated lysine 4 of histone H3 (H3K4me3/2/1) and also includes KDM5A/RBP2, KDM5C/SMCX and KDM5D/SMCY in mammals (Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007; Yamane et al., 2007). Fly and yeast each has a single orthologue of KDM5: the Drosophila Little imaginal disks (Lid) and S. cerevisiae Jhd2p/YJR119Cp (Eissenberg et al., 2007; Lee et al., 2007; Liang et al., 2007; Secombe et al., 2007; Seward et al., 2007). The KDM5 proteins have highly conserved domain architecture. They contain a catalytic JmjN/JmjC domain, a DNA-binding ARID/Bright domain, a C5HC2-zinc-finger, and two or three PHD fingers, with the exception of yeast KDM5, which consists of only the catalytic module and one PHD finger.

The expression of the KDM5B gene is restricted in normal adult tissues except for testes and ovaries, but it is often upregulated in human malignancies, including breast, prostate, bladder, lung and cervical cancers and leukemias (Hayami et al., 2010; Roesch et al., 2010; Xiang et al., 2007). KDM5B interacts with transcription factors PAX9, FOXG1 and FOXC2 (reviewed in (Cloos et al., 2008)) and associates with nuclear receptors, such as estrogen receptor alpha (ERα), androgen receptor and progesterone receptor, to repress or promote activation of target genes (Catchpole et al., 2011; Krishnakumar and Kraus, 2010; Vicent et al., 2013; Xiang et al., 2007). Microarray analyses reveal that KDM5B represses genes of antiproliferative and cell cycle regulators, including the tumor suppressor BRCA1, HOX5A and MTs in mammary epithelial cancer cell line MCF7, while positively regulating E2F1 and E2F2 in A549 and SW789 cells (Hayami et al., 2010; Scibetta et al., 2007; Yamane et al., 2007). Knockdown of KDM5B decreases the growth of MCF7 cells both in vitro and in vivo, suggesting that KDM5B is required for the proliferation in this type of cancer (Catchpole et al., 2011; Li et al., 2011; Yamane et al., 2007).

Recent studies have implicated KDM5B in continuous growth of xenografted melanoma cells and metastatic progression (Roesch et al., 2010), and overexpression of KDM5B in immortalized normal breast cancer MCF10A cells was found to enhance cell invasion (Yoshida et al., 2011). However, in addition to functioning as an oncprotein, KDM5B has been shown to possess tumor-suppressive activities. KDM5B associates with the retinoblastoma (Rb) tumor suppressor, and KDM5B knockdown phenocopies loss of Rb in Rb-dependent senescence models (Chicas et al., 2012; Nijwening et al., 2011; Roesch et al., 2005). Consistent with its tumor suppression function, overexpression of KDM5B in the triple-negative breast cancer cell line MDA-MB 231 inhibits cell migration and invasion potentials (Li et al., 2011). Thus, the growing evidence suggests that KDM5B can act as an oncprotein or a tumor suppressor in a cell type-dependent manner. However, the molecular mechanism by which KDM5B contributes to gene regulation and exerts opposite functions in carcinogenesis remains unclear.
In this work, we show that KDM5B functions as a broad transcriptional repressor for genes involved in inflammatory response, cell proliferation, cell adhesion and migration in the MDA-MB 231 triple-negative breast cancer cells. Mass spectrometry, IP and genomic analyses demonstrate that KDM5B associates with components of the NuRD complex and may cooperate with the HDAC1 deacetylase and the CHD4 ATPase in repression. We found that of the three PHD fingers present in KDM5B, two (PHD1 and PHD3) bind to histone tails. Whereas PHD1 is highly specific for H3K4me0, PHD3 is more promiscuous and recognizes H3K4me3 as well as lower methylation states of H3K4. KDM5B inhibits migration and invasion abilities of the MDA-MB 231 breast cancer cells, and binding of the PHD1 finger to H3K4me0 is required to suppress cell migration. Together, our data highlight tumor-suppressive functions of KDM5B in triple-negative breast cancer cells and suggest a multivalent mechanism underlying KDM5B-dependent repression.

RESULTS AND DISCUSSION

KDM5B is downregulated in ER− breast cancer cells

Breast cancer is a heterogeneous disease. Molecular profiling has identified several distinct subtypes of breast cancer, which correlate with hormone response, patient prognosis and response to therapy (Morris and Carey, 2007). KDM5B has been shown to play a significant role in breast cancer progression and metastatic capacity (Catchpole et al., 2011; Li et al., 2011; Yamane et al., 2007), however it remains unclear whether KDM5B expression and tumorigenic activities are the same in different subtypes of tumors. To clarify this, we examined expression levels of KDM5B in 12 different breast cancer cell lines (Fig. 1a). Cell nuclear extracts were isolated and probed with anti-KDM5B antibodies. Compared with the immortalized “normal” breast cancer cells (76NF2V and MCF-10A), KDM5B was overexpressed in ER-positive (ER+) cells, including MCF7, T47D, MDA-MB 361 and UACC 812 (Fig. 1a). Surprisingly, we found that the expression level of KDM5B is significantly lower in ER-negative (ER−) cells, including HER2+ SKBR3 and AU565 cells and the triple-negative MDA-MB 231, MDA-MB 436 and MDA-MB 468 cells. The triple-negative subtype of breast cancer is particularly associated with worse surviving prognosis in comparison with the ER+ breast cancers. To determine the general expression level of KDM5B in the triple negative subtype, we analyzed KDM5B gene expression in breast cancer patients in the Curtis breast tumor dataset available in Oncomine. We observed lower expression levels of KDM5B in the triple negative breast cancer patients compared with patients with ER+/PR+ subtype (Supplementary Fig. S1 and Supplementary Table S1).

The differential expression levels of KDM5B imply distinct roles of this protein in ER+ and ER− cancer subtypes. Although the function of KDM5B in ER+ MCF7 cells has previously been characterized (Catchpole et al., 2011; Li et al., 2011; Scibetta et al., 2007; Yamane et al., 2007), little is known about KDM5B activities in more aggressive ER− subtypes. To assess the role of KDM5B in triple-negative breast cancer, we used two shRNAs that reduced the KDM5B protein level to different degrees in MDA-MB 231 cells. As shown in Figure 1b, full knockdown of KDM5B led to the increased H3K4me3 level, and this is consistent with the H3K4-specific demethylase activity of KDM5B. It also indicates that the orthologous KDM5 demethylases do not substitute for KDM5B, which has also been observed in ER+ MCF7 cells (Catchpole et al., 2011; Yamane et al., 2007).

KDM5B is required for repression of a set of genes involved in immune response and cell proliferation in MDA-MB 231 cells

To identify KDM5B-regulated genes in MDA-MB 231 cells on a genome-wide scale, we performed RNA-seq gene expression analysis in the cells treated with a KDM5B-target shRNA or a control non-targeting shRNA in duplicates. We identified 423 genes that were
upregulated and 333 genes downregulated in KDM5B knockdown MDA-MB 231 cells (Fig. 1c). These results imply that KDM5B correlates with both gene activation and repression. Gene ontology (GO) analysis revealed that KDM5B represses genes involved in immune response and cell proliferation, as well as regulation of angiogenesis, cell adhesion and migration, whereas downregulated genes in KDM5B knockdown cells did not cluster (Fig. 1c and Supplementary Table S2). The expression of the KDM5B-dependent genes was further validated by quantitative real-time PCR (Fig. 1d). A substantial increase in gene expression was seen in both KDM5B-knockdown cells, and the extent of upregulation inversely correlated with the extent of KDM5B knockdown, suggesting that the observed differences are caused by the elimination of KDM5B. Furthermore, chromatin precipitation (ChIP) assays in KDM5B-overexpressing cells demonstrated that KDM5B binds to the promoters of these genes, substantiating that these genes are likely KDM5B direct targets (Fig. 1e). Together, these results suggest that KDM5B represses a set of target genes in MDA-MB 231 cells.

KDM5B associates with the deacetylase NuRD complex

The mechanism underlying transcriptional repression by KDM5B is not well understood, and the question of whether it relies on the demethylase activity per se or encompasses other epigenetic processes remains open. To examine if KDM5B is capable of associating with other nuclear proteins, we immunoprecipitated KDM5B from HEK 293 cells stably expressing Flag-tagged KDM5B, followed by SDS-PAGE and silver staining (Fig. 2a). Mass spectrometry and western blot analyses of the IP-purification showed that KDM5B co-precipitated with two catalytic subunits of the NuRD complex (reviewed in (Allen et al., 2013)). One subunit is a chromodomain helicase DNA-binding protein 4 (CHD4) ATPase, which hydrolyses ATP necessary for DNA sliding and repositioning of nucleosomes. The second catalytic subunit is histone deacetylase 1 (HDAC1), which deacetylates acetylated lysine residues of histones. Furthermore, two non-catalytic subunits of NuRD, metastasis associated 2 (MTA2) and retinoblastoma-binding protein 4 (RBBP4), were also identified. The direct interaction between KDM5B and HDAC4 has previously been reported (Barrett et al., 2007), therefore we tested whether KDM5B is able to associate with HDAC1. Co-IP experiments in cells transfected with Flag-HDAC1 demonstrated that HDAC1 indeed interacts with endogenous KDM5B (Fig. 2b). These data suggest an intriguing possibility of a cooperative action of the three catalytic components, coupling lysine demethylation, lysine deacetylation and ATPase-mediated chromatin remodeling, all of which are important for transcriptional repression (Fig. 2c).

KDM5B co-localizes with HDAC1 and CHD4 at chromatin

To confirm that KDM5B, HDAC1 and CHD4 co-localize in vivo, we performed analysis of ChIP-seq datasets generated by the ENCODE consortium in K562 cells. KDM5B is recruited to ~140,000 genomic regions, and ~50% (76268 out of 140,000) of these regions overlap with the regions occupied by HDAC1 (Fig. 2d). Furthermore, 99% of the CHD4-bound regions overlap with the KDM5B-HDAC1-bound regions, which represents ~17% (12811 out of 76268) of KDM5B-HDAC1 associations. Together, these results suggest a strong co-occupancy of KDM5B with the NuRD complex on the chromatin.

The genome-wide analysis reveals that ~50% of the KDM5B-HDAC1 binding sites overlap with tri-, di- or monomethylated H3K4 (H3K4me3/2/1), which are substrates for the KDM5B enzymatic activity (Fig. 2e and Supplementary Fig. S2). Notably, ~95% of the KDM5B-HDAC1-CHD4-bound regions correlate with chromatin enriched in H3K4me3, and these regions comprise ~50% of the KDM5B-HDAC1-H3K4me3 overlapping regions (Fig. 2f and Supplementary Fig. S2). Active, weak or poised promoters and enhancers were particularly occupied by KDM5B-HDAC1-CHD4. These results imply that KDM5B co-
localizes with the NuRD components at the sites containing its substrate (H3K4me3), removal of which by KDM5B would yield transcriptionally inactive chromatin.

**PHD1 and PHD3 fingers of KDM5B but not PHD2 have histone-binding activity**

KDM5B contains three PHD fingers, the biological roles of which remain unclear (Fig. 3a). As PHD fingers from other proteins have recently been shown to possess histone-binding activity, we generated individual KDM5B PHD finger constructs (PHD1, PHD2 and PHD3) and tested these proteins in pull-down assays and histone peptide microarrays. GST-fusion PHD1, PHD2 and PHD3 fingers were incubated first with biotinylated histone peptides containing single methylation marks and then with streptavidin Sepharose beads. The histone peptide-bound proteins were detected by western blotting. As shown in Figure 3b, the KDM5B PHD1 finger recognizes the N-terminus of histone H3, either unmodified or methylated at Lys9. The second PHD2 finger was incapable of histone binding, whereas the third, PHD3 finger showed preference for H3K4me3. This high specificity was confirmed using an extended peptide microarray. The PHD1 finger bound to histone H3 (H3K4me0) regardless of posttranslational modifications (PTMs) present on Lys9, Lys14 or Lys18, whereas PHD3 was specific for methylated H3K4 (Supplementary Fig. S3).

To examine the effect of the PTMs commonly found in the first six residues of histone H3 on binding of the KDM5B PHD fingers, we hybridized these proteins to a PTM library microarray (Fig. 3c, d). The GST-tagged PHD fingers were screened against an extensive library of multiply modified histone peptides (Supplementary Table S3). In agreement with the pull-down data, the KDM5B PHD1 finger associated with unmodified histone H3 tail, however, PTMs on the first six residues of H3 blocked this interaction. In particular, dimethylation or citrullination of Arg2, phosphorylation of Thr3, methylation of Lys4 and phosphorylation of Thr6 were not tolerated. On the other hand, binding of the PHD3 finger to H3K4me3 was affected to a lesser extent. PTMs of Arg2 were permissible, while phosphorylation of Thr3 reduced this interaction and phosphorylation of Thr6 further inhibited it.

**Mechanism of the H3K4me0 recognition by PHD1**

To determine the molecular basis for the interaction between KDM5B PHD1 and unmodified histone H3, we purified ¹⁵N-labeled protein and carried out ¹H,¹⁵N heteronuclear single quantum coherence (HSQC) titration experiments (Fig. 4). Addition of the H3K4me0 peptide (aa 1–12 of H3) induced large chemical shift changes in the PHD1 finger, indicating direct binding (Fig. 4a). A number of peaks disappeared at a protein to peptide ratio of 1:1, and many changed their positions, revealing slow-to-intermediate exchange regime on the NMR time scale, indicative of strong interaction. The precise binding affinity of the KDM5B PHD1 finger for H3K4me0 was found to be 1.5 µM, as measured by fluorescence spectroscopy (Fig. 4e).

To identify residues in the histone-binding site of the PHD1 finger, we produced ¹³C/¹⁵N-labeled KDM5B PHD1 finger. We assigned backbone and side chain ¹H, ¹³C, and ¹⁵N chemical shifts using a set of three dimensional triple resonance experiments and analyzed perturbations in the ¹H,¹⁵N HSQC spectra of the protein upon binding to H3K4me0. Plotting chemical shift changes for each backbone amide allowed us to identify the residues of KDM5B PHD1 that are directly or indirectly involved in the interaction (Fig. 4b). The most perturbed residues were clustered in the middle region of the PHD1 finger sequence, encompassing residues E321-C330, and a shorter region around W351 was perturbed to a lesser degree. To obtain mechanistic details of the interaction, we used the assigned ¹³Cα, ¹³Cβ, ¹Hα, ¹⁵N and ¹HN chemical shifts and a CS-Rosetta protocol (Shen et al., 2008) to generate a model of the apo-state. The resulting model showed a typical PHD/
RING finger fold and was superimposed with the structure of the CHD4 PHD2 finger with an rmsd of 2Å. Mapping the KDM5B residues with significantly perturbed amide resonances onto the model diagram revealed that these residues are located in or around the first β-strand of the PHD1 finger (Fig. 4c). This binding site is in good agreement with the binding sites seen in previously determined structures of PHD fingers bound to H3K4me0 (reviewed in (Musselman and Kutateladze, 2011)).

To assess the inhibition effect of Lys4 methylation, we characterized the interaction of the KDM5B PHD1 finger with H3K4me1, H3K4me2 and H3K4me3 peptides by NMR and fluorescence spectroscopy (Fig. 4d, e). We found that the PHD1 finger binds to H3K4me1 and H3K4me2 peptides 5- and 100-fold weaker than it binds to H3K4me0 (Fig. 3c). Trimethylated H3K4me3 peptide caused very small changes in the protein NMR spectra, indicating an almost negligible association in the mM range of affinities.

**The H3K4me0 binding mode is conserved**

The elongated histone-binding site of KDM5B PHD1 and high sequence similarity suggest that the binding mode of this protein is similar to that of the AIRE, BHC80, CHD4 and TRIM24 PHD fingers, and this was supported through mutational analysis (Fig. 4c and Fig. 5a). $^{1}H,^{15}N$ HSQC and pull-down experiments showed that substitution of L326, a conserved hydrophobic residue preceding the third zinc-coordinating Cys, to a tryptophan abolished interaction of KDM5B PHD1 with H3K4me0 (Fig. 5b-d). Likewise, replacement of the corresponding hallmark Cys or Met residues in other H3K4me0-specific PHD fingers with a bulky tryptophan completely disrupts this binding (Lan et al., 2007; Musselman et al., 2009). Another distinguishable feature of the H3K4me0 recognition is the presence of extensive acidic patches on the surface of a PHD finger that are involved in electrostatic and hydrogen bonding contacts with unmodified lysine and arginine residues of H3. Although the KDM5B PHD1 sequence N-terminal to the first zinc-coordinating Cys is less acidic than the sequences of other H3K4me0-binding PHD fingers, KDM5B contains more acidic residues in the loop connecting zinc-coordinating second and third Cys residues, as well as following the fourth zinc-coordinating Cys (Fig. 5a). Whereas replacement of some surface acidic residues with an alanine resulted in insoluble proteins, soluble D320A and D328A mutants showed a decrease in binding to H3K4me0, corroborating the importance of electrostatic contacts with the highly basic H3K4me0 tail (Fig. 5b-d).

Despite the sequences of human KDM5B PHD1, human KDM5D PHD1 and a single PHD finger of yeast KDM5 are very similar, KDM5D and the yeast orthologue bound to H3K4me0 much weaker (Fig. 5a, and Fig. 5e and f, left panels). The respective equilibrium dissociating constants ($K_d$s) were measured through $^{1}H,^{15}N$ HSQC titrations and found to be 3.5 mM and 117 µM, respectively. Such a weak association was not enhanced by methylation of H3K9, as was found for the CHD4 PHD fingers (Fig. 5e, right panel), and the reduction of the surface negative charge further diminished it (Fig. 5f, right panel).

Notably, the key L326 residue in the KDM5B sequence is replaced by a phenylalanine in KDM5D (Fig. 5a). Much like a tryptophan in the L326W mutant of KDM5B, the bulky phenylalanine may cause steric hindrance, impeding this interaction. Another critical residue, an invariable leucine (L324 in KDM5B), is substituted by a threonine in yeast KDM5, most likely accounting for the decrease in binding (Fig. 5a).

**PHD3 finger prefers H3K4me3 but also binds H3K4me0**

The amino acid sequence of the third PHD3 finger of KDM5B has 70% identical residues compared to the sequence of the PHD3 finger of KDM5A, including the two tryptophan residues that have previously been shown to be required for binding to H3K4me3 (Wang et al., 2009) (Fig. 6 and Supplementary Fig. S4). Accordingly, mutation of the W1502 and
W1512 residues in KDM5B PHD3 disrupted interaction with H3K4me3, whereas replacement of acidic D1506 with an arginine reduced it (Fig. 6c-e). These results imply that the mechanism of H3K4me3 recognition by the PHD3 finger of KDM5A is conserved in KDM5B, with W1502 and W1512 of the KDM5B PHD3 finger most likely being involved in caging the trimethylammonium group of H3K4me3 and D1506 contributing to restraining the guanidino group of Arg2 of the histone peptide. Surprisingly, we found that the KDM5B PHD3 finger shows only slight preference for the trimethylated state of H3K4. We compared interactions with histone H3K4me3/2/1/0 peptides using NMR and fluorescence spectroscopy (Fig. 6b, d). An almost identical pattern of chemical shift changes induced by H3K4me3, H3K4me2, H3K4me1 and H3K4me0 in the $^1$H,$^15$N HSQC spectra of PHD3 suggested that all peptides occupy the same binding pocket (Fig. 6b). The slow exchange regime on the NMR time scale for the methylated peptides revealed an equally robust interaction. Even interaction with the unmodified peptide was in the slow to intermediate exchange, which is indicative of strong binding. In agreement, binding affinities of the KDM5B PHD3 finger for all four peptides were in the range of 0.5 to 4 µM, as measured by fluorescence (Fig. 6d). Together, these data demonstrate that the KDM5B PHD3 finger binds strongly to either methylated or unmodified histone H3K4, yet it prefers the trimethylated species.

**PHD1-H3K4me0 interaction is important for suppression of MDA-MB 231 cell migration**

GO analysis of MDA-MB 231 cells suggests that in triple-negative breast cancer KDM5B represses genes involved in the regulation of cell adhesion and migration, the two processes imperative for cancer metastasis. We therefore sought to examine the role of KDM5B and its PHD fingers in cell migration and invasion. Consistent with the GO analysis, knockdown of KDM5B in MDA-MB 231 cells increased cells’ capability to migrate through the Boyden chambers nearly two fold (Fig. 7a), whereas overexpression of KDM5B decreased cell migratory potential by 50% (Fig. 7b). To determine the effect of KDM5B on cell invasion potential, MDA-MB-231 cells were tested in matrigel-coated chambers. The invaded cells were fixed, stained with Crystal Violet and counted using a light microscope (Fig. 7c). We found that overexpression of KDM5B reduced the MDA-MB-231 cell invasion potential. Thus, KDM5B is required to block migration and invasion ability of the ER$^-$ type triple-negative breast cancer cells.

To determine the role of the PHD fingers and their histone binding in MDA-MB 231 cell migration, we generated cell lines stably expressing full-length WT KDM5B and KDM5B mutants, harboring loss-of-function mutations either in the PHD1 finger (L326W and D328A) or in the PHD3 finger (W1502A) (Fig. 7d, right panel). These stable cell lines were then used in cell migration assays. As expected, WT KDM5B decreased cell migration by 50% compared to the control cells, however, the L326W and D328A mutants of PHD1 impaired in binding to H3K4me0 failed to suppress cell migration (Fig. 7d, left panel). These results suggest that the PHD1 finger mutants may act as dominant negative mutants, altering the dynamics and interactions of endogenous KDM5B. In contrast, the W1502A mutant of PHD3 defective in H3K4me3 binding showed the inhibitory effect on cell migration similar to the effect of the wild type protein. These results suggest that recognition of H3K4me0 by the PHD1 finger of KDM5B is important for inhibiting migration of MDA-MB 231 cancer cells, whereas recognition of H3K4me3 by the PHD3 finger of KDM5B is dispensable in suppression of cell migration.

**CONCLUDING REMARKS**

Previous observations and data presented in this study demonstrate that the expression level of KDM5B is highly cancer cell type-dependent. KDM5B is downregulated in ER$^-$ breast cancer cells, including the MDA-MB 231 triple-negative breast cancer cells, whereas in line

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with previous findings (Hayami et al., 2010; Scibetta et al., 2007; Yamane et al., 2007), it is upregulated in ER+ breast cancer cells, such as MCF7. Depending on the type of cancer cells, KDM5B appears to elicit opposite effects, acting much like other major transcriptional regulators p53 and TGF-β; in contrast to its tumor promoting function in the MCF7 cells (Catchpole et al., 2011; Li et al., 2011; Yamane et al., 2007), KDM5B inhibits migration and invasion of the MDA-MB 231 cells.

Our structural and biochemical analyses reveal that the two PHD fingers of KDM5B, PHD1 and PHD3, associate with histone tails. The PHD1 finger specifically binds to H3K4me0, and the PHD3 finger is selective for H3K4me3. Thus, not only does the KDM5B demethylase erase the H3K4me3/2/1 marks, producing unmodified H3K4me0, it also reads the target mark and the product of its own catalytic activity through distinct PHD fingers. A combination of two ‘readers’ capable of recognizing distinctive epigenetic marks is likely to have significant impact on KDM5B activity. Binding of PHD1 to H3K4me0 may provide an anchoring mechanism for KDM5B to sense H3K4me3 through PHD3 and slide along the H3K4me3-enriched promoters, demethylating nearby methylated H3K4 and further spreading the transcriptionally inactive state of chromatin (Fig. 6g). Furthermore, the ability of the PHD3 finger to bind unmodified H3 as well implies that the sliding process may also occur when H3K4me3 level is low.

The findings that KDM5B is co-purified and co-localizes with components of the NuRD complex indicate that KDM5B and NuRD may cooperate in transcriptional repression (Fig. 2c). The NuRD complex contains two catalytic subunits, the deacetylase HDAC1 and the CHD4 ATPase, with both subunits being essential in the regulation of gene expression and chromatin remodeling. A cooperative action of the three catalytic proteins, KDM5B, HDAC1 and CHD4, links H3K4-demethylation, lysine deacetylation and ATPase-mediated chromatin remodeling, providing a powerful mechanism for a rapid shut off of actively transcribed genes (Fig. 2c). Multiple contacts of other NuRD components with chromatin likely add another layer of complexity. Histone-binding activities of two PHD fingers of CHD4 and the WD40 domain of RBBP7/4, as well as DNA binding by the ARID domain of KDM5B and two chromodomains of CHD4 may fine tune the affinity and specificity of this gene repressive machinery (Bouazoune et al., 2002; Murzina et al., 2008; Musselman et al., 2009; Scibetta et al., 2007) 60. Future functional studies are necessary to establish the importance of crosstalk between these epigenetic components in KDM5B-mediated transcriptional repression.

**EXPERIMENTAL PROCEDURES**

**NMR Spectroscopy and sequence specific resonance assignments**

NMR experiments were performed at 298K on a Varian INOVA 600 MHz spectrometer equipped with a cryogenic probe. For backbone assignments, uniformly $^{15}$N- and $^{13}$C-labeled KDM5B PHD1 (1 mM in Tris-HCl buffer pH 6.8, 150 mM NaCl, 15 mM DTT, and 8 % D$_2$O) was used to collect three-dimensional triple-resonance HNCACB, CBCA(CO)NH, C(CO)NH, and HC(CO)NH experiments, as described (Ali et al., 2013). The NMR data were processed with nmrPipe (Delaglio et al., 1995) and analyzed with nmrDraw. Initial sequence specific assignments were obtained in the PINE program and completed using CcpNMR Analysis v1.6 (Vranken et al., 2005).

Chemical shift perturbation experiments were carried out using 0.1 mM uniformly $^{15}$N-labeled wild type or mutated KDM5B PHD1, KDM5B PHD3, KDM5D PHD1 and yeast KDM5 PHD fingers. The $^1$H,$^{15}$N heteronuclear single quantum coherence (HSQC) spectra were recorded in the presence of increasing concentrations of 12-mer histone tail peptides (synthesized by the University of Colorado Denver Peptide Core Facility). The dissociation
constants \((K_d)\) were determined by a nonlinear least-squares analysis in Kaleidagraph using the equation:

\[
\Delta \delta = \Delta \delta_{\text{max}} \left( [L] + [P] + K_d \right) - \sqrt{\left( [L] + [P] + K_d \right)^2 - 4[P][L]} / 2[P]
\]

where \([L]\) is concentration of the peptide, \([P]\) is concentration of the protein, \(\Delta \delta\) is the observed chemical shift change, and \(\Delta \delta_{\text{max}}\) is the normalized chemical shift change at saturation. Normalized (Grzesiek et al., 1996) chemical shift changes were calculated using the equation

\[
\Delta \delta = \sqrt{(\Delta \delta H)^2 + (\Delta \delta N/3)^2},
\]

where \(\Delta \delta\) is the change in chemical shift in parts per million (ppm).

**Fluorescence Spectroscopy**

Spectra were recorded at 25 °C on a Fluoromax-3 spectrofluorometer (HORIBA). The samples containing KDM5B PHD1 and PHD3 fingers in PBS buffer and progressively increasing concentrations of the histone peptide were excited at 295 nm or 280 nm. Emission spectra were recorded over a range of wavelengths between 305 and 405 nm with a 0.5 nm step size and a 1 s integration time. Three scans were averaged and recorded. \(K_d\) values were calculated using a nonlinear least-squares analysis and the equation:

\[
\Delta I = \Delta I_{\text{max}} \left( [L] + [P] + K_d \right) - \sqrt{\left( [L] + [P] + K_d \right)^2 - 4[P][L]} / 2[P]
\]

where \([L]\) is the concentration of the histone peptide, \([P]\) is the concentration of KDM5B PHD1 or KDM5B PHD3, \(\Delta I\) is the observed change of signal intensity, and \(\Delta I_{\text{max}}\) is the difference in signal intensity of the free and bound states of the PHD finger. \(K_d\) represents the average of three separate experiments (two for the PHD1-H3K4me2 interaction), with error computed as the standard deviation between those values.

**Cell migration and invasion assays**

Cell migration and invasion assays were performed as described previously using Boyden chambers (BD Biosciences) and modified Boyden chambers coated with Matrigel matrix (Li et al., 2011). \(1 \times 10^5\) of MDA-MB 231 cells were starved for 16–20 h in DMEM containing 0.1% BSA, and in 300 µl serum free medium and were placed to the upper chamber of the transwell. The chambers were then transferred to 24-well plates containing 500 µl of media supplemented with 10% FBS in each well and were incubated for less than 24 h at 37°C. Non-invading cells were removed by wiping the upper side of the membrane, and the invaded cells were fixed and stained with Crystal Violet. The number of invaded cells was counted under a light microscope from at least five fields and three experiments. The average numbers from the three experiments were calculated between control and the samples using Student’s t-test.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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KDM5B binds to its histone target and the product through distinct PHD fingers. KDM5B may cooperate with the NuRD complex in transcriptional repression. KDM5B suppresses migration and invasion of the triple-negative breast cancer cells.
Figure 1. KDM5B is a broad transcriptional repressor

(a) KDM5B is overexpressed in ER-positive (ER+) but downregulated in ER-negative (ER−) breast cancer cells. Western blot analysis of KDM5B protein levels across a panel of breast cancer cells is shown. (b) Knockdown of KDM5B leads to the increased global H3K4me3 levels. Western blot analysis of KDM5B and H3K4me3 levels in KDM5B knockdown and control cells. (c) KDM5B represses the expression of genes involved in immune response, proliferation and migration. The heatmap of up- and downregulated genes in MDA-MB 231 cells treated with KDM5B-specific shRNA vs. control shRNA. Gene ontology (GO) analysis of upregulated genes is shown on the right. Downregulated genes are not enriched in any specific GO term with FDR<5 (see Supplementary Table S2). (d) Quantitative real-
time PCR analysis of the expression of KDM5B-regulated genes in control and KDM5B knockdown MDA-MB 231 cells. Error bars represent s.e.m. (e) KDM5B ChIP analysis on the promoters of the indicated genes in MDA-MB 231 cells stably expressing Flag-KDM5B. IgG is shown as a negative control. Error bars represent s.e.m. See also Figure S1 and Tables S1 and S2.
Figure 2. KDM5B associates with components of the NuRD complex

(a) Mass spectrometric analysis of the KDM5B protein complex. Silverstaining of the IP-purified complex and number of peptides and scores of KDM5B-associated proteins are shown. (b) Co-IP of KDM5B with HDAC1. (c) A model for the cooperative action of KDM5B and the NuRD complex. (d) Venn diagram showing overlap of the KDM5B-, HDAC1- and CHD4-bound genomic regions. (e) Overlaps of the H3K4me3-, KDM5B-HDAC1- and KDM5B-HDAC1-CHD4-bound regions are shown in black. (f) KDM5, HDAC1 and CHD4 chromatin profiles over a 50 Kb region of human chromosome 7. Peaks are indicated with a black line and a gray line over the profiles for KDM5B-HDAC1-CHD4 and KDM5B-HDAC1, respectively. Chip-seq data for H3K4me3 and RNA-seq in K562

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cells are shown. The two lower tracks correspond to the coding regions containing exons and introns, as well as DNase I accessible regions. See also Figure S2.
Figure 3. The KDM5B PHD1 and PHD3 fingers but not PHD2 show histone-binding activity
(a) Schematic representation of the KDM5 family of proteins. (b) Western blot analysis of
GST-KDM5B PHD fingers bound to the indicated biotinylated peptides. (c, d) GST-
KDM5B PHD1 and PHD3 were analyzed on a PTM library microarray. Relative average
signal intensities are shown in graphs. Error bars represent s.e.m. from pooled averages of
two independent array experiments. Scatter plot of two arrays probed with GST-KDM5B
PHD3 is also shown. Blue and red dots indicate H3K4me3-containing and H3K4me2-
containing peptides, respectively. The correlation coefficient was calculated by linear
regression analysis using GraphPad Prism v5. The list of PTM-containing peptides used in
the assays and complete analysis are shown in Supplementary Table S3 and Supplementary
Table S4. See also Figure S3.
Figure 4. The PHD1 finger of KDM5B is specific for H3K4me0
(a) Superimposed $^1$H,$^{15}$N HSQC spectra of KDM5B PHD1 collected upon titration of H3K4me0 peptide. Spectra are color coded according to the protein:peptide molar ratio. (b) The normalized chemical shift changes observed in the PHD1 finger upon binding to H3K4me0 as a function of residue. Differences greater than the average plus one standard deviation (SD), the average plus one-half SD, and the average are shown in red, orange and yellow, respectively. An asterisk indicates disappearance of a signal. (c) A model of the PHD1 finger fold generated using assigned $^{13}$Cα, $^{13}$Cβ, $^1$Hα, $^{15}$N and $^1$HN chemical shifts and a CS-Rosetta protocol. The most perturbed in (b) residues of the PHD1 finger are colored red and labeled. (d) Superimposed $^1$H,$^{15}$N HSQC spectra of KDM5B PHD1
collected upon titration with H3K4me0, H3K4me1, H3K4me2 and H3K4me0 peptides (residues 1-12 of H3). (e) Binding affinities of wild type (WT) KDM5B PHD1 for the indicated histone peptides measured by tryptophan fluorescence (a) or NMR (b). (f) Representative binding curves used to determine the K_d values by fluorescence and NMR.
Figure 5. The molecular mechanism of the KDM5B PHD1-H3K4me0 interaction
(a) Alignment of the PHD finger sequences: absolutely, moderately and weakly conserved residues are colored pink, yellow and blue, respectively. The regions important for the interaction with H3K4me0 are indicated by red bars. Each tenth residue of KDM5B is labeled. (b) Overlays of $^1$H,$^15$N HSQC spectra of the PHD1 L326W and D320A mutants collected upon titration of the H3K4me0 peptide. (c) Western blot analysis of GST-KDM5B PHD1, WT and mutants, bound to the biotinylated peptides. (d) Binding affinities of human KDM5B, human KDM5D and yeast KDM5 PHD1 fingers for the indicated histone peptides measured by tryptophan fluorescence (a) or NMR (b). NB-no binding by Western blot (c). (e, f) Overlays of $^1$H,$^15$N HSQC spectra of the PHD1 fingers of KDM5D and yeast KDM5
collected upon titration of the indicated peptides. Spectra are color coded according to the protein:peptide molar ratio.
Figure 6. The PHD3 finger of KDM5B is selective for H3K4me3
(a) Alignment of the PHD finger sequences: absolutely, moderately and weakly conserved residues are colored pink, yellow and blue, respectively. The Lys4me3 binding residues are indicated by red ovals. Each tenth residue of KDM5B is labeled. (b, c) Superimposed $^1$H,$^{15}$N HSQC spectra of KDM5B PHD3, WT or D1506R, collected upon titration with the indicated peptides. (d) Binding affinities of the KDM5B PHD3 finger as measured by tryptophan fluorescence (a) or NMR (b). (e) Western blot analysis of GST-KDM5B PHD3, WT and mutants, bound to the indicated biotinylated peptides. (f) Representative binding curves used to determine the $K_d$ values by fluorescence. (g) A model
for KDM5B anchoring at chromatin and spreading the transcriptionally inactive state. See also Figure S4.
Figure 7. KDM5B represses cell migration in a PHD1-dependent manner

(a) Knockdown of KDM5B increases cell migration. Representative views of cells migrated through the Boyden chambers. An averaged migration activity of KDM5B knockdown cells (relative to control cells) from three independent experiments is shown on the right. (b, c) Overexpression of KDM5B represses cell migration and invasion activities of MDA-MB 231 cells. Representative views of cells migrated through the Boyden chambers (b) or extracellular matrix-coated chambers (c) are shown. Averaged migration (b) or invasion activity (c) of KDM5B-expressing cells (relative to control cells) from three independent experiments are shown on the right. Error bars represent s.e.m. (d) Repression of cell migration by KDM5B requires histone binding of PHD1, but not histone binding of PHD3.

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Shown are: averaged cell migration activities of MDA-MB 231 cells expressing WT or mutated PHD1 and PHD3 (left), and expression levels of the KDM5B proteins (right).