Transcriptional Repression of Histone Deacetylase 3 by the Histone Demethylase KDM2A Is Coupled to Tumorigenicity of Lung Cancer Cells*

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*Running title: Repression of HDAC3 by KDM2A in lung tumorigenesis

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Keywords: histone modification; transcriptional repression; gene regulation; cell cycle; invasion; lung cancer

CAPSULE

Background: Overexpression of the epigenetic repressor KDM2A promotes lung tumorigenesis.

Results: Transcriptional inhibition of HDAC3 expression by KDM2A releases cell cycle and pro-invasive genes from HDAC3-mediated repression and positively regulates cell proliferation and invasiveness.

Conclusion: KDM2A-mediated repression of HDAC3 is linked to KDM2A-promoted tumorigenicity.

Significance: Our findings provide a novel epigenetic insight into how KDM2A promotes lung tumorigenesis and have implications for therapeutic intervention.

ABSTRACT

Dysregulated expression of histone methyltransferases and demethylases is an emerging epigenetic mechanism underlying cancer development and metastasis. We recently showed that the histone H3 lysine 36 (H3K36) demethylase KDM2A (also called FBXL11 and JHDM1A) is necessary for tumorigenic and metastatic capabilities of KDM2A-overexpressing non-small cell lung cancer (NSCLC) cells. Here, we report that KDM2A transcriptionally represses the histone deacetylase 3 (HDAC3) gene by removing methyl groups from dimethylated H3K36 at the HDAC3 promoter in KDM2A-overexpressing NSCLC cells. KDM2A depletion reduced expression levels of cell cycle-associated genes (e.g., CDK6) and cell invasion-related genes (e.g., NANOS1); these levels were rescued by ectopic expression of KDM2A but not its
catalytic mutant. These genes were occupied and down-regulated by HDAC3. HDAC3 knockdown significantly recovered the proliferation and invasiveness of KDM2A-depleted NSCLC cells as well as the levels of CDK6 and NANOS1 expression in these cells. Similar to their previously reported functions in other cell types, CDK6 and NANOS1 were required for the proliferation and invasion, respectively, of KDM2A-overexpressing NSCLC cells. In a mouse xenograft model, HDAC3 depletion substantially restored the tumorigenic ability of KDM2A knockdown cells. These findings reveal a novel cancer-epigenetic pathway in which the antagonistic effect of KDM2A on HDAC3 expression releases cell cycle-associated genes and cell invasion-related genes from HDAC3 repression and indicate the importance of this pathway for tumorigenicity and invasiveness of KDM2A-overexpressing NSCLC cells.

INTRODUCTION

Histone lysine methylation is considered a key chromatin mark that mediates epigenetic and transcriptional regulation of gene expression (1,2). Unlike histone acetylation, which is exclusively associated with gene activation, this modification is linked to activation or silencing of gene expression (3,4). The methylation effect depends on lysine residues involved, among which are histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79, and H4K20 (5). For example, methylated H3K36 is connected to gene activation, whereas methylated H3K27 is related to gene silencing. Like histone acetylation, which can be removed by histone deacetylases (HDACs), histone lysine methylation is reversibly regulated: it is catalyzed by histone lysine methyltransferases (KMTs) (1,2) and can be removed by histone lysine demethylases (KDMs) (6,7). Notably, this modification exists at three different states (i.e., mono-, di- or trimethylation).

Lung cancer is the most prevalent cause of cancer-related mortality in the United States and worldwide (8). Non-small cell lung cancer (NSCLC) is responsible for up to 85% of lung cancer and includes adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. In NSCLC, genetic mutations and abnormalities in kinase signaling pathway members have been well documented (9). For instance, in lung adenocarcinomas, activating mutations for oncogenes frequently occur in K-RAS and EGFR, whereas mutations in tumor suppressor genes, such as LKB1, are also detected (9,10). Thus, much research focus for NSCLC has been put on understanding kinase signaling pathway.

Recently, it has been increasingly evident that dysregulation of histone methylation and its modifiers may be an important factor in lung tumorigenesis and metastasis (11,12). Alterations in histone lysine methylation are associated with clinical prognosis of lung cancer (13,14). For example, global levels of trimethylated H4K20, a gene-repressive mark, are decreased in squamous cell carcinomas, and its low levels correlate with poor prognosis (14). In addition to methylation marks, some histone methylation modifiers are known to have altered expression levels in lung cancer. For instance, the H3K27 methyltransferase EZH2 and the H3K36 methyltransferase MMSET (also called WHSC1 and NSD2) are significantly overexpressed in lung tumors (15). Recently, we showed that the H3K36 demethylase KDM2A (also known as FBXL11 and JHDM1A) is frequently upregulated in NSCLC tumor samples and promotes the tumor growth and invasive abilities of NSCLC cells (16). Consistent with the notion that KDM2A is associated with transcriptional repression by erasing methyl groups from the gene activation mark dimethylated H3K36 (H3K36me2) (17-19), we also reported that KDM2A increases cellular levels of phospho-ERK1/2, a type of oncogenic signal, by down-regulating the expression of dual specificity phosphatase 3 (DUSP3) whose protein dephosphorylates ERK1/2 (16).

To gain greater insights into how KDM2A regulates cell proliferation and invasion, we sought to identify more KDM2A target genes. Here, we provide evidence that histone deacetylase 3 (HDAC3) is an important target gene of KDM2A. Transcriptional repression of the HDAC3 gene by KDM2A-catalyzed H3K36 demethylation up-regulates HDAC3 target genes, including the cell cycle-associated gene CDK6 and the cell invasion-related genes NANOS1, in two
KDM2A-overexpressing NSCLC cell lines. Furthermore, our results suggest that epigenetic repression of HDAC3 expression by KDM2A is needed for tumorigenic and invasive abilities of KDM2A-overexpressing NSCLC cells.

EXPERIMENTAL PROCEDURES

Samples, Reagents, Antibodies, and Animals – H1975 and H1792 NSCLC cell lines were purchased from ATCC. Cell culture reagents were purchased from Life Technologies/Invitrogen; all other chemicals were from Sigma-Aldrich. The KDM2A-specific antibodies (NB100-74602) were purchased from Novus Biologicals. Additional antibodies were purchased as follows: anti-HDAC3 (40968), anti-H3K36me2 (39256), anti-H3K9ac (39138), anti-H3K14ac (39616) and anti-H4Ac (39227) from Active Motif; anti-H3K9me3 (07-442) from Millipore; anti-H3 (ab1971) from Abcam; anti-β-Actin (A5441) from Sigma-Aldrich. Anti-CDK6 (14052-1-AP, Proteintech) and anti-NANOS1 (LS-C164739, Life Span Biosciences) were used for immunohistochemical staining. HRP-conjugated anti-mouse-IgG and HRP-conjugated anti-rabbit-IgG were from Santa Cruz Biotechnology. The nude mice were purchased from MD Anderson Cancer Center, and their care and use were approved by MD Anderson’s Institutional Animal Care and Use Committee.

In Vitro Gene Silencing Using siRNA – For knockdown experiments, siRNAs against KDM2A, HDAC3, CDK6, and NANOS1 were purchased from Dharmacon or Integrated DNA Technology (IDT). The siRNA sequences are listed in Table 1. As controls, siRNA against luciferase GL3 RNA (siLuc) and siControl were used. Cells (5 x 10⁴) in a six-well plate were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine RNAiMAX (Life Technologies/Invitrogen). Following 72 to 96h of incubation, cells were harvested for mRNA and protein analysis or used for cell proliferation and invasion assays.

Double knockdown of KDM2A and HDAC3 were performed, and its effect was compared to that of individual knockdown. In brief, cells (2–5 x 10⁴) were seeded in 60-mm dishes and transfected with one of four different combinations of siRNAs: 1) siControl (70 nM); 2) siKDM2A-3 (35 nM) + siControl (35 nM); 3) siHDAC3-9 (35 nM) + siControl (35 nM); 4) siKDM2A-3 (35 nM) + siHDAC3-9 (35 nM). It should be noted that siControl was added to obtain a final concentration of siRNAs of 70 nM. For transfection, Lipofectamine RNAiMAX was used. After 24h of incubation, cells were re-transfected with same amount of siRNAs. An additional 72 h later, cells were harvested for mRNA analysis or used for cell proliferation and invasion assays.

Quantitative RT-PCR – Total RNA was isolated using RNeasy kits (Qiagen) according to the manufacturer’s instructions. Then, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Quantitative PCR was performed in triplicate using SYBR-green and gene specific primers (Table 1). Messenger RNA levels were quantified using CFX Manager software (Bio-Rad) and were normalized to β-actin mRNA levels. The relative mRNA levels represent the fold change compared to the levels in Control cells.

Cell Proliferation and Invasion Assays – To assess cell proliferation, 1 x 10⁴ cells per well in 24-well plates were seeded in triplicate, and cell number were counted in the indicated time points. For the cell invasion assay, the Boyden chamber assay with a modification was performed. In brief, cells (1 x 10⁵) were seeded on the Matrigel-coated membrane in the inserts. After 18h of incubation, cells that had invaded the Matrigel and migrated to the other side of the membrane were stained and counted.

Cell Cycle Analysis and BrdU Incorporation Assays – For cell cycle analysis, cells were fixed in cold 75% ethanol for 30 min at 4°C, and washed twice in PBS. Then, cells were incubated at 37°C for 30 min in a buffer containing 50 µg/ml propidium iodine, 5 mM MgCl₂, 10 mM TRIS-HCl (pH 7.0) and 25 µg/ml RNaseA. DNA contents were analyzed using flow cytometry (BD FACSCanto II, BD Science).

For BrdU incorporation assays, 1–2 x 10⁵ cells were seeded in 96 well plates in triplicate. BrdU reagent was added to each well, and the
plate was incubated for 24 h. BrdU incorporation was measured at 450 nm absorbance according to the manufacturer’s protocol (Millipore).

Rescue Experiments by Ectopic Expression – To test the effects of ectopic expression of KDM2A in H1975 and H1792 cells, the cDNAs encoding KDM2A, its catalytic mutant mKDM2A and GFP were individually cloned into the mammalian expression vector pFLAG-CMV2 as described previously (16). The resulting expression plasmids (2 µg) were transfected into either siControl- or siKDM2A-treated cells (2–5 x 10^5 cells) in 60 mm dishes using 10 µl of Lipofectamine 2000 (Life Technologies/Invitrogen). These cells were harvested after 72h of incubation for further analysis, such as quantitative RT-PCR.

Quantitative ChIP Assay – ChIP assay was performed as previously described (20). After chromatin immunoprecipitation by specific antibodies, DNA was purified using the Qiagen PCR purification kit. Chromatin immunoprecipitates for KDM2A, HDAC3 and the histone marks of interest were amplified by quantitative PCR (qPCR) and normalized to input. Relative occupancy indicates the change over the control value. Enrichment represents PCR values from specific antibody relative to those from IgG (e.g., anti-KDM2A/IgG and anti-HDAC3/IgG).

Mouse Xenograft Studies – To determine whether the effect of KMD2A knockdown on tumorigenesis is dependent on HDAC3, three groups of cells (shControl-treated, KDM2A-depleted, and KDM2A/HDAC3-depleted H1792 cells) were compared for their tumorigenicity in a subcutaneous xenograft model. KDM2A-depleted cells were generated using shRNA against KDM2A as previously described (21). KDM2A/HDAC3-depleted cells were generated by treating KDM2A-depleted cells with 50 nM siHDAC3-9 using Lipofectamine RNAiMAX. For comparison, the other two groups of cells were also transfected with 50 nM siScramble (a control siRNA). After a 24h incubation, all three groups of cells were re-transfected with the same amounts of siRNAs at the same concentrations. An additional 72 h later, cells were harvested and suspended in RPMI-1640 medium without serum. Cells (1.5 x 10^6) were subcutaneously injected into the dorsal flanks of male nude mice (8 weeks old). At least 5 mice were injected for each group and observed for 10 weeks for tumor formation. The ellipsoid volume formula (1/2 x L x W x H) was used to calculate the tumor volume. Ten weeks after the injection, the mice were killed, and their tumor and lungs were collected. For pathological analysis, hematoxylin and eosin (H & E) and immunohistochemical staining were performed.

Statistical Analysis – For statistical analysis, each experiment was performed in triplicate and repeated at least three times. Data are presented as the mean ± SEM (error bars). Statistical significance was tested by the two-tailed Student’s t-test. * (p <0.05), **(p <0.01) and *** (p <0.001) indicate statistically significant differences. GraphPad Prism software was used for all statistical analyses.

RESULTS

KDM2A Indirectly Up-regulates Expression of Cell Cycle-associated Genes and Cell Invasion-related Genes in KDM2A-overexpressing NSCLC Cells – In our effort to better understand the mechanisms by which KDM2A may regulate the proliferation and invasion of NSCLC cells, we revisited our recent whole genome mRNA expression data in which a number of genes were commonly modulated by two different siKDM2As in two KDM2A-overexpressing NSCLC cell lines H1975 and H1792. Because our recent study showed that KDM2A knockdown decreased the S phase percentages and invasive abilities of H1975 and H1792 cells (16), we paid our attention to cell cycle-associated genes and cell invasion-related genes. In particular, the cell cycle-associated genes CDK6 and NEK7 and the invasion-related genes NANOS1 and RAPH1 were of interest because these genes were downregulated by KDM2A knockdown (16). CDK6 was demonstrated to be often overexpressed in lung tumors (22). CDK6 activity promotes expression of cell cycle regulators and assembly of the pre-replication complex (23), and pharmacologic inhibition of CDK6 induces cell cycle arrest (24). NEK7, a centrosomal kinase, regulates proper spindle assembly and mitotic progression (25). NANOS1
is overexpressed in some human invasive lung carcinomas and increases the production of MT1-matrix metalloproteinase to induce matrix metalloproteinase-dependent invasion of carcinoma cells (26). RAPH1 is known to promote cell proliferation and to play an important role in cell motility (27).

To confirm our previous microarray results, we compared expression levels of the CDK6, NEK7, NANOS1, and RAPH1 genes between KDM2A-depleted cells and control siRNA-treated cells in two KDM2A-overexpressing NSCLC cell lines, H1975 and H1792, using quantitative RT-PCR. Our results ensured that these genes were down-regulated by KDM2A knockdown (Fig. 1A and B). Next, we examined whether ectopic expression of KDM2A restores expression of the KDM2A-regulated genes CDK6, NEK7, NANOS1, and RAPH1 in KDM2A-depleted cells. Wild-type KDM2A and its catalytic mutant mKDM2A were transiently expressed as previously described (16). Exogenous expression of wild-type KDM2A, but not mKDM2A, significantly rescued expression of these genes in H1975 and H1792 cells (Fig. 1C–F show data for CDK6 and NANOS1; similar data are not shown for NEK7 and RAPH1). These results indicate that KDM2A and its demethylase activity may be requisite for expression of the CDK6, NEK7, NANOS1, and RAPH1 genes.

KDM2A is linked to transcriptional repression of its target genes (17-19). Therefore, KDM2A may indirectly up-regulate the CDK6, NEK7, NANOS1, and RAPH1 genes whose expression levels were decreased by KDM2A knockdown. We determined whether these genes are directly regulated by KDM2A using quantitative ChiP assays. Because it has been demonstrated that KDM2A is localized at regions spanning transcription start sites (16,17), we analyzed KDM2A occupancy near those sites in these genes. ChiP results showed that the regions near the transcription start sites in the CDK6, NEK7, NANOS1, and RAPH1 genes were not occupied by KDM2A. In contrast, DOCK7 and ZNF652, whose expression levels were increased by KDM2A knockdown, were KDM2A target genes (Fig. 1G and H). In line with its role in repressing transcription, these results indicate that KDM2A indirectly up-regulates expression of CDK6, NEK7, NANOS1, and RAPH1 genes while directly down-regulating expression of DOCK7 and ZNF652 genes.

**Expression of the HDAC3 Gene Is Repressed by KDM2A-catalyzed H3K36 Demethylation** – Because CDK6, NEK7, NANOS1, and RAPH1 were not direct KDM2A target genes, we hypothesized that these genes may be down-regulated by a transcriptional co-repressor encoded by a KDM2A-repressed gene. Specifically, we reasoned that HDAC3 might be involved in the repression of these genes, as our previous microarray results indicated that HDAC3 is a KDM2A-repressed gene (16). HDAC3 is a well-known transcriptional co-repressor (28-30). Similar to the microarray results, our quantitative RT-PCR data showed that HDAC3 expression levels were up-regulated in KDM2A knockdown cells (Fig. 2A). In addition, our ectopic experiments demonstrated that expression of wild-type KDM2A, but not its catalytic mutant mKDM2A, significantly repressed expression levels of HDAC3 in KDM2A-depleted cells (Fig. 2B and C). These results indicated that KDM2A and its enzymatic activity are critical for KDM2A-mediated repression of the HDAC3 gene.

To determine whether KDM2A is recruited to the HDAC3 gene, we examined enrichment levels of KDM2A over control IgG at a proximal promoter region of the HDAC3 gene using quantitative ChiP assays (Fig. 2D). Our results showed that KDM2A occupied the proximal promoter near the transcription start site in the HDAC3 gene, indicating that HDAC3 is a KDM2A target gene (Fig. 2E). These results are in agreement with our previous study showing that KDM2A is localized near the transcription start site of the DUSP3 gene (16). Because KDM2A removes methyl groups from H3K36me2, we determined the effect of KDM2A knockdown on H3K36me2 levels at the proximal promoter of the HDAC3 gene. Quantitative ChiP results demonstrated that KDM2A knockdown resulted in increased H3K36me2 levels at the proximal promoter of the HDAC3 gene in H1975 and H1792 cells (Fig. 2F and G). These results indicate that KDM2A-catalyzed demethylation of H3K36me2 at the HDAC3 promoter plays an
important role in KDM2A-mediated repression of the HDAC3 gene.

**HDAC3 Directly Represses Expression of the CDK6, NEK7, NANOS1, and RAPH1 Genes** – To examine the possibility that HDAC3 may down-regulate expression levels of CDK6, NEK7, NANOS1, and RAPH1 genes, we depleted HDAC3 in H1792 and H1975 cells using two different siRNAs against HDAC3 (Fig. 3A and B). Quantitative RT-PCR results showed that HDAC3 depletion by two independent siHDAC3s increased expression levels of these genes (Fig. 3C and D). Next, we examined whether HDAC3 is recruited to CDK6, NEK7, NANOS1, and RAPH1 genes using quantitative ChIP. Our results showed that HDAC3 occupied the regions spanning the transcription start sites at these genes (Fig. 4A−D), consistent with the previous report showing that chromatin peaks of HDAC3 cover the transcription start sites (31). These results indicate that HDAC3 directly represses CDK6, NEK7, NANOS1, and RAPH1 genes and also support the idea that KDM2A up-regulates expression of these genes by repressing HDAC3 expression at the transcriptional levels.

To assess whether HDAC3 represses its target genes by deacetylation, we examined the effect of HDAC3 knockdown on histone acetylation levels at the HDAC3 target genes, such as CDK6 and NANOS1. Consistent with the deacetylase activity of HDAC3, our ChIP results demonstrated that HDAC3 depletion increased acetylation levels in histones H3 and H4 at these genes in H1792 and H1975 cells (Fig. 5A−D). Similar to no obvious effect of KDM2A knockdown on H3K36me2’s cellular levels, HDAC3 depletion did not affect total cellular levels of H3K9 acetylation and H4 acetylation, suggesting that HDAC3 erases histone acetylation in a gene-specific manner (Fig. 5E).

**In KDM2A-overexpressing NSCLC Cells, CDK6 Positively Regulates Cell Proliferation, Whereas NANOS1 Is Required for Cellular Invasiveness** – As mentioned earlier, it has been shown that CDK6 regulates cell cycle progression in non-lung cancer cell lines (e.g., U2OS), while NANOS1 modulates cellular invasion in the breast cancer cell lines Hs578T, BT549 and the transformed lung epithelial cell line BZR. To assess whether CDK6 and NANOS1 play similar roles in KDM2A-overexpressing NSCLC cells, we assessed the effect of CDK6 or NANOS1 knockdown on the proliferation and invasiveness of H1792 cells. CDK6 depletion strongly inhibited cell proliferation and BrdU incorporation into the replicated DNA of S phase cells but only weakly impeded cellular invasiveness (Fig. 6A−D). On the contrary, NANOS1 knockdown only marginally inhibited cell proliferation and BrdU incorporation but markedly abrogated cellular invasiveness (Fig. 6E−H). These results suggest that in KDM2A-overexpressing NSCLC cells, CDK6 is associated largely with cell proliferation, whereas NANOS1 is related mainly to cellular invasiveness.

**KDM2A-mediated Repression of HDAC3 Expression Is Important for In Vitro Cell Proliferation and Invasion as well as Xenograft Tumor Formation** – To determine whether KDM2A regulates CDK6 and NANOS1 genes in an HDAC3-dependent manner, we reduced HDAC3 in KDM2A-depleted H1975 and H1792 cells using siHDAC3 (Fig. 7A and B). HDAC3 knockdown significantly restored CDK6 and NANOS1 mRNA levels in KDM2A-depleted H1975 and H1792 cells (Fig. 7C−F). In addition, HDAC3 depletion significantly rescued deficient proliferation of KDM2A-depleted cells by restoring the S phase population (Fig. 8A−C). Consistent with this, HDAC3 knockdown increased BrdU incorporation in KDM2A-depleted cells (Fig. 8D). Interestingly, HDAC3 depletion did not affect G2/M phase population that was increased by KDM2A knockdown (Fig. 8C). Similar to its effects on cell proliferation, HDAC3 depletion significantly recovered cellular invasiveness (Fig. 9A and B). These results indicate that transcriptional repression of HDAC3 by KDM2A may contribute to cell proliferation and invasiveness by releasing cell cycle-associated and invasiveness-related genes from HDAC3 repression.

To determine the effect of KDM2A-mediated repression of HDAC3 on the tumorigenic ability of NSCLC cells in vivo, we depleted HDAC3 in stably KDM2A-depleted H1792 cells using siHDAC3, subcutaneously injected double knockdown cells into mice, and monitored tumor...
growth. Similar to siRNA-based transient knockdown of KDM2A, stable knockdown of KDM2A increased HDAC3 expression (Fig. 10A and B). In agreement with our previous report (16), most mice (n = 4/5) that were injected with shLuciferase-treated H1792 cells had tumors within 3 months (Fig. 10C and Table 2). Interestingly, HDAC3 depletion greatly restored defective tumorigenic abilities of KDM2A-depleted H1792 cells (Fig. 10C and D). Tumors were confirmed by H&E staining, and CDK6 and NANOS1 levels in a control tumor were comparable to those in a tumor that was originated from double knockdown cells (Fig. 10E and F). Results obtained from this subcutaneous xenograft experiment indicate that in vivo formation of tumors by KDM2A-overexpressing NSCLC cells may require KDM2A-mediated repression of HDAC3 expression.

DISCUSSION

In this study, our results provided evidence that KDM2A represses expression of the HDAC3 gene by demethylating H3K36me2 at the HDAC3 promoter. Consistent with our previous study showing that KDM2A positively regulates the S phase cell population and cellular invasiveness (16), the results presented here indicate that KDM2A enhances expression of the cell cycle-associated genes CDK6 and NEK7 and the cell invasion-related genes NANOS1 and RAPH1 in KDM2A-overexpressing NSCLC cells. Interestingly, expression of these genes was directly repressed by the transcriptional co-repressor HDAC3. Therefore, our current study uncover the molecular mechanism in which KDM2A-mediated repression of HDAC3 expression may antagonize the transcriptional repression of cell cycle-associated genes and invasiveness-related genes by HDAC3 in KDM2A-overexpressing lung cancer cells (i.e., KDM2A $\rightarrow$ HDAC3 $\rightarrow$ cell cycle/invasiveness genes [e.g., CDK6 and NANOS1]) (Fig. 10G). In addition, KDM2A-mediated regulation of HDAC3 expression may be largely dependent on KDM2A’s catalytic activity, because KDM2A’s catalytic mutant did not repress HDAC3 expression.

It is known that KDM2A acts as a positive regulator for somatic cell reprogramming and cellular anti-senescence (32,33). Our previous data suggest that KDM2A overexpression promotes NSCLC tumorigenesis and metastasis by increasing ERK1/2 signaling (16). Data reported here show that HDAC3 depletion significantly revived the tumorigenic and invasive abilities of KDM2A-depleted cells and restored expression of the cell cycle-associated gene CDK6 and the invasion-related gene NANOS1 in KDM2A-depleted cells. Thus, this work supports the notion that KDM2A-mediated repression of HDAC3 expression contributes to NSCLC tumorigenesis and invasion, at least in part, by up-regulating certain cell cycle-associated and invasion-related genes. On the basis of our previous and current studies, it is possible that KDM2A promotes tumorigenicity and invasiveness of KDM2A-overexpressing NSCLC cells by controlling two pathways for cell cycle and cell invasion: 1) KDM2A $\rightarrow$ DUSP3 $\rightarrow$ ERK1/2 $\rightarrow$ cell cycle/invasiveness and 2) KDM2A $\rightarrow$ HDAC3 $\rightarrow$ cell cycle/invasiveness genes. Interestingly, the treatment of the MEK1/2 inhibitor U0126 (10 µM) that consequently impedes ERK1/2 phosphorylation did not show any obvious effect on HDAC3 levels, suggesting that these two pathways may be independent (data not shown).

Certain histone modifiers regulate gene expression in a cooperative mode. The H3K9 methyltransferase G9a and the H3K4 demethylase JARID1a co-repress gene expression (34), and the H3K4 demethylase LSD1 and HDAC 1 and 2 cooperatively repress their co-target genes (35). The H3K27 demethylase UTX and H3K4 methyltransferase MLL4 (also known as ALR and KMT2D) may co-activate their co-target genes (36). In contrast, some histone modifiers antagonize others’ function on the same genes. For example, polycomb repressive complex 2, containing the H3K27 methyltransferase EZH2, may enable cancer cells to resist cellular senescence by inhibiting expression of the cellular senescence genes p16^INK4A and p14^ARF (37), whereas the H3K27 demethylase JMJD3 may
 neutralize EZH2 function by activating the same genes (38). Interestingly, the results reported here indicate that the transcriptional co-repressor KDM2A counteracts the gene-repressive effects of HDAC3 on cell cycle-regulatory genes and cell invasion-associate genes by transcriptionally down-regulating the HDAC3 gene in a catalytic activity-dependent manner. To our knowledge, the epigenetic repression of HDAC3 function by KDM2A is a distinct mode of antagonistic regulation in which a histone lysine demethylase opposes the function of a histone deacetylase via transcriptional repression.

As many as 70% of eukaryotic genes contain CpG islands, which represent genomic regions that have higher-than-average genome-wide levels of CpG dinucleotides (39). Blackledge et al. reported that via its CXXC domain, KDM2A interacts with many transcription start site-associated CpG islands (17). Interestingly, the HDAC3 gene has a CpG island spanning the predicted transcription start site (data not shown). Thus, it is possible that KDM2A is recruited to the HDAC3 promoter through its association with the CpG dense region. However, the transcriptional regulation of HDAC3 expression seems more complex. Although our analysis of the tumor database The Cancer Genome Atlas showed a trend for an inverse correlation between KDM2A and HDAC3 mRNA levels in NSCLC tumors, there was no significant correlation between KDM2A and HDAC3 in a range of NSCLC cell lines (data not shown). These results indicate that KDM2A is a key control factor for HDAC3 expression in certain types of lung cancer cell lines and lung tumors, but that other gene-regulatory factors may also be involved in modulating the HDAC3 promoter. Further studies are required for a better understanding of how HDAC3 expression is regulated.

HDAC inhibitors have been recently developed as anti-cancer agents. Multiple mechanisms underlying the mode of action of HDAC inhibitors have been described, including induced expression of tumor suppressor p21 and the pro-apoptotic ligand TRAIL (40-42). Recently, Chen et al. (43) reported that the HDAC inhibitor trichostatin A may increase the expression of the potential tumor suppressor cluster Dleu2/miR-15a/16-1 via HDAC3 inhibition in two NSCLC cell lines A549 and H1299. In contrast, our results suggest that HDAC3 is involved in tumor suppression in the NSCLC cell lines H1975 and H1792. These seemingly contradictory results might result from a cell line-dependent function of HDAC3. Thus, it is possible that HDAC3 may act as an oncogenic factor by repressing tumor suppressor genes in A549 and H1299, whereas it may play a tumor-suppressive role in H1975 and H1792 by down-regulating expression of cell proliferation-related genes and pro-invasive genes.

In addition, the pathologic role of HDAC3 in cancer development might be tissue-dependent. In gastric, prostatic and colorectal cancer samples, HDAC3 overexpression was significantly associated with poor prognosis (44-46). In agreement with these reports, it has been shown that HDAC3 appeared to be up-regulated and to repress the tumor suppressor gene p21 in colorectal cancer cells (47). In contrast, other studies reported that liver-specific HDAC3 knockout mice develop hepatoma (48) and that HDAC3 maintains genomic stability (48) and potentiates apoptosis by down-regulating the proto-oncogene c-Jun (28), indicating an anti-tumor function for HDAC3. In support of the tumor suppressive role of HDAC3, the results reported here showed that HDAC3 knockdown restored defective proliferation and invasiveness of KDM2A-depleted cells. Our additional results indicate that HDAC3 represses cell cycle-associated genes and invasiveness-related genes. Thus, our findings suggest that the inhibition of anti-tumor function of HDAC3 by KDM2A at the transcriptional levels may be requisite for the growth and invasion of KDM2A-overexpressing NSCLC tumors.
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FOOTNOTES:

Acknowledgments – We are thankful to Luanne Jorewicz for manuscript editing. This work was supported by NIH grants CA157919 and GM095659 (to M.G.L.), Cancer Prevention & Research Institute of Texas grant RP110183 (to M.G.L.), a fund from the Center for Cancer Epigenetics at the University of Texas MD Anderson Cancer Center (to M.G.L.), a scholar fellowship from the Center for Cancer Epigenetics at MD Anderson Cancer Center (to S.S.D.), and a fellowship from the Odyssey Program and the Estate of C. G. Johnson, Jr., at MD Anderson Cancer Center (to H.A.).

The abbreviations used are: HDAC3, histone deacetylase 3; NSCLC, non-small cell lung cancer; H3K4, histone H3 lysine 4; KDMs, histone lysine demethylases
FIGURE LEGENDS:

FIGURE 1. Transcriptional expression of cell cycle-associated genes and invasion-related genes is positively regulated by KDM2A in the KDM2A-overexpressing NSCLC cell lines H1975 and H1792. (A and B) Analysis of CDK6, NEK7, NANOS1, RAPH1, DOCK4, and ZNF652 mRNA levels in H1975 (A) and H1792 (B) cells after KDM2A knockdown. Expression levels were analyzed by quantitative RT-PCR. (C–F) Rescue experiments for CDK6 (C and D) and NANOS1 (E and F) expression in KDM2A-depleted H1975 (C and E) and H1792 (D and F) cells. After ectopic expression of GFP, wild type KDM2A, and its catalytic mutant mKDM2A in KDM2A-depleted H1975 and H1792 cells, expression levels were measured by quantitative RT-PCR. The siControl-treated cells were used as controls. (G and H) Analysis of KDM2A occupancy at CDK6, NEK7, NANOS1, RAPH1, DOCK4, and ZNF652 genes in H1975 (G) and H1792 (H) cells by quantitative ChIP.

FIGURE 2. KDM2A represses HDAC3 expression by demethylating H3K36me2. (A) The effect of KDM2A knockdown on HDAC3 mRNA levels in H1975 and H1792 cells. Cells were transfected with two different siKDM2As (siKDM2A-3 and -4). (B and C) Analysis of HDAC3 mRNA levels in KDM2A-depleted H1975 (B) and H1792 (C) cells after ectopic expression of GFP, wild-type KDM2A, and the catalytic mutant mKDM2A. HDAC3 mRNA levels were measured by quantitative RT-PCR. (D) Schematic representation of the promoter region (a) of the HDAC3 gene. Arrows indicate the PCR-amplified region. TSS, transcription start site. (E) Analysis of KDM2A occupancy at HDAC3 gene in H1975 and H1792 cells by quantitative ChIP. (F and G) Analysis of occupied levels of KDM2A, H3K36me2, H3K9me3, and H3 at the HDAC3 promoter region in H1975 (F) and H1792 (G) cells by quantitative ChIP.

FIGURE 3. HDAC3 represses expression of the cell cycle-associated genes CDK6 and NEK7 and the invasion-related genes NANOS1 and RAPH1. (A and B) Analysis of HDAC3 knockdown efficacy in H1975 and H1792 cells. Cells were treated with two different siRNAs against HDAC3 (siHDAC3-3 and siHDAC3-9). HDAC3 mRNA and protein levels were respectively measured by quantitative RT-PCR (A) and Western blot analysis (B). (C and D) The effect of HDAC3 knockdown on CDK6, NEK7, NANOS1, RAPH1, and GPR157 mRNA levels in H1975 (C) and H1792 (D) cells. Expression levels of individual genes were analyzed by quantitative RT-PCR.

FIGURE 4. HDAC3 occupies the regions spanning the transcription start sites in the CDK6, NEK7, NANOS1, and RAPH1 genes. (A–D) Analysis of chromatin occupancy of HDAC3 at the CDK6 (A), NEK7 (B), NANOS1 (C), and RAPH1 (D) genes in H1975 and H1792 cells by quantitative ChIP. Diagrammatic representation of individual genes is also shown. Arrows indicate the PCR-amplified regions. TSS, transcription start site.

FIGURE 5. HDAC3 knockdown increases acetylation levels in histones H3 and H4 at the promoters of the CDK6 and NANOS1 genes. (A–D) Analysis of chromatin levels of HDAC3, H3K9ac, H3K14ac, H4ac, and H3 at the promoter regions of the CDK6 (A and B) and NANOS1 genes (C and D) in H1975 (A and C) and H1792 (B and D) cells. Chromatin levels of proteins and histone marks were measured by quantitative ChIP. Anti-H3 was used as a ChIP control. (E) The effect of KDM2A or HDAC3 knockdown on total cellular levels of H3K36me2, H3K9 acetylation and H4 acetylation. Histone marks were examined by Western blot analysis. H3 was used as an internal loading control.

FIGURE 6. CDK6 knockdown inhibits mainly cell proliferation, whereas NANOS1 knockdown impedes largely cell invasiveness. (A–D) The effect of CDK6 knockdown on the
proliferation and invasiveness of H1792 cells. CDK6 mRNA levels were analyzed in siControl-treated and CDK6-depleted (siCDK6-1 or -3) cells using quantitative RT-PCR (A). Cell proliferation (B), BrdU incorporation (C), and invasion (D) assays were performed. (E–H) The effect of NANOS1 knockdown on the proliferation and invasiveness of H1792 cells. NANOS1 mRNA levels were analyzed in siControl-treated and NANOS1-depleted (siNANOS1-1 or -3) cells using quantitative RT-PCR (E). Cell proliferation (F), BrdU incorporation (G), and invasion (H) assays were performed.

FIGURE 7. HDAC3 knockdown significantly increases expression of the CDK6 and NANOS1 genes in KDM2A-depleted NSCLC cells. (A and B) Analysis of HDAC3 and KDM2A mRNA levels in siControl-treated, KDM2A-depleted (siKDM2A-3), and KDM2A/HDAC3-depleted (siKDM2A-3 + siHDAC3-9) cells by quantitative RT-PCR. H1975 (A) and H1792 (B) cells were treated with siControl, siKDM2A-3, or a mixture of siKDM2A-3 and siHDAC3-9. (C–F) The effect of HDAC3 knockdown on CDK6 and NANOS1 mRNA levels in KDM2A-depleted cells. Expression levels of the CDK6 (C and D) and NANOS1 (E and F) genes in H1975 (C and E) and H1792 (D and F) cells were measured by quantitative RT-PCR.

FIGURE 8. HDAC3 knockdown rescues proliferation defect of KDM2A-depleted NSCLC cells. (A and B) The effect of HDAC3 knockdown on the proliferation of KDM2A-depleted H1975 (A) and H1792 (B) cells. (C) The effect of single and double knockdown of KDM2A and HDAC3 on S phase cell percentages. The percentages of sub-G1, G1, S, and G2/M phase cells were analyzed. (D) BrdU incorporation assay.

FIGURE 9. HDAC3 knockdown restores deficient invasiveness of KDM2A-depleted NSCLC cells. (A and B) The effect of HDAC3 knockdown on the invasiveness of KDM2A-depleted H1975 (A) and H1792 (B) cells. Representative images of invaded cells are shown (Left panels), and cells were counted (Right panels).

FIGURE 10. HDAC3 knockdown greatly recovers impaired tumorigenicity of KDM2A-depleted NSCLC cells in a mouse xenograft model. (A and B) Analysis of KDM2A (A) and HDAC3 (B) mRNA levels in control (shLuciferase), stably KDM2A-depleted (shKDM2A#1), and KDM2A/HDAC3-depleted H1792 cells. Stably KDM2A-depleted H1792 cells (shKDM2A#1) were transfected with siHDAC3-9 to generate double (KDM2A/HDAC3) knockdown cells. (C and D) The effect of HDAC3 knockdown on tumor development of KDM2A-depleted cells in a subcutaneous xenograft model. Three different groups of cells (shControl, shKDM2A#1, and shKDM2A#1 + siHDAC3-9) were subcutaneously injected into 5 mice per group. Representative tumors (10 weeks after subcutaneous injection) are shown in dotted circles and indicated by black arrows (C). Tumor volumes were monitored and plotted for 10 weeks (D). (E) Representative images of H&E staining of xenograft tumor samples (shControl group and shKDM2A#1 + siHDAC3-9 group) and normal skin tissues (shKDM2A#1 group) obtained from the injection sites (10 weeks after subcutaneous injection). Scale bars are indicated (upper panel, 200 µm; lower panel, 100 µm). (F) Representative images of immunohistochemical staining of NANOS1 and CDK6 in xenograft tumor samples. Immunohistochemical staining was performed only using tumor tissues from shControl group and shKDM2A#1 + siHDAC3-9 group. Normal mouse skin tissues from shKDM2A#1 group were not used for staining, because they were not a proper control. CDK6 and NANOS1 levels were quantified using Chromavision Automated Cellular Imaging System (ACIS-III) from Dako and compared between shControl group and shKDM2A#1 + siHDAC3-9 group: CDK6 (1 vs. 0.73); NANOS (1 vs. 0.54). (G) In this hypothetical model, KDM2A transcriptionally represses the HDAC3 gene. Subsequently, cell cycle-associated genes (e.g., CDK6) and invasiveness-related genes (e.g., NANOS1) are released from HDAC3-mediated repression.
Table 1: PCR primers and siRNAs

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Table 2: The effect of HDAC3 depletion in stably KDM2A-depleted H1792 cells on tumor formation upon in a murine subcutaneous xenograft model.

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*Stably KDM2A-depleted (shKDM2A#1) cells, double knockdown (shKDM2A#1 + siHDAC3) cells, and shControl-treated cells were subcutaneously implanted into mice. At the 10th week after injection, numbers of tumor-bearing mice were counted.
FIG 2

A, B, C: Relative HDAC3 mRNA levels in H1792 and H1975 cells transfected with GFP, KDM2A, mKDM2A, siLuc, or siKDM2A-3.

D: Enrichment at HDAC3 gene (qChIP) for H1975 and H1792 cells treated with IgG or Anti-KDM2A.

E: Relative occupancy at HDAC3 gene for H1975 and H1792 cells transfected with siLuc or siKDM2A-3.

F: Relative occupancy at HDAC3 gene for H1975 cells transfected with GFP, KDM2A, mKDM2A, or siKDM2A-3.
**FIG 5**

A

Relative occupancy at CDK6 gene

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B

Relative occupancy at CDK6 gene

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C

Relative occupancy at NANOS1 gene

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Relative occupancy at NANOS1 gene

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E

H1792

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A. H1792

B. H1792

C. H1792

D. H1792

FIG 8
A

siControl
siKDM2A-3

siHDAC3-9
siKDM2A-3 + siHDAC3-9

H1975

Cell numbers per field

siControl
siKDM2A-3
siHDAC3-9
siKDM2A-3 + siHDAC3-9

***

B

siControl
siKDM2A-3

siHDAC3-9
siKDM2A-3 + siHDAC3-9

H1792

Cell numbers per field

siControl
siKDM2A-3
siHDAC3-9
siKDM2A-3 + siHDAC3-9

***

FIG 9
**A** Relative KDM2A mRNA levels

*shControl*  
*shKDM2A#1*  
*shKDM2A#1 + siHDAC3-9*

**B** Relative HDAC3 mRNA levels

*shControl*  
*shKDM2A#1*  
*shKDM2A#1 + siHDAC3-9*

**C** shControl  
shKDM2A#1  
shKDM2A#1 + siHDAC3-9

**D** Tumor volume (mm³)

1500  
1000  
500  
0

**E**

10X

shControl  
shKDM2A#1  
shKDM2A#1 + siHDAC3-9

20X

**F**

shControl  
shKDM2A#1 + siHDAC3-9

CDK6  
NANOS1

**G**

KDM2A  
HDAC3  
NANOS1  
CDK6

Cell invasion  
Cell proliferation

**FIG 10**

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Transcriptional Repression of Histone Deacetylase 3 by the Histone Demethylase KDM2A Is Coupled to Tumorigenicity of Lung Cancer Cells

Shilpa S. Dhar, Hunain Alam, Na Li, Klaus W. Wagner, Jimyung Chung, Yeo Won Ahn and Min Gyu Lee

J. Biol. Chem. published online January 30, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M113.521625

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