

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

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

Results: Transcriptional inhibition of *HDAC3* expression by KDM2A releases cell cycle and proinvasive 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.

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.

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 the lysine residues involved, among which are histone H3 lysine 4 (H3K4),³ 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 (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-, and 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 epidermal growth factor receptor gene, whereas mutations in tumor suppressor genes, such as *LKB1*, are also

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³ The abbreviations used are: H3K4, histone H3 lysine 4; HDAC, histone deacetylase; NSCLC, non-small cell lung cancer; KDM, histone lysine demethylase; H3K36me2, dimethylated H3K36; DUSP3, dual specificity phosphatase 3; ac, acetylation; qPCR, quantitative PCR.

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detected (9, 10). Thus, much research focus for NSCLC has been put on understanding the 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 up-regulated 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 *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 gene *NANOS1* in two KDM2A-overexpressing NSCLC cell lines. Furthermore, our results suggest that epigenetic repression of *HDAC3* expression by KDM2A is needed for the 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 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; and 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 Technologies, Inc. (IDT). The siRNA sequences are listed in Table 1. As controls, siRNA against luciferase GL3 RNA (siLuc) and siControl were used. Cells (5×10^4) in a 6-well plate were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine RNAiMAX (Invitrogen). Following 72–96 h 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 with that of individual knockdown. In brief, cells ($2\text{--}5 \times 10^4$) were seeded in a 6-well plate 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), and 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 24 h of incubation, cells were retransfected with the 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 with the levels in control cells.

Cell Proliferation and Invasion Assays—To assess cell proliferation, 1×10^4 cells/well in 24-well plates were seeded in triplicate, and cell number were counted at the indicated time points. For the cell invasion assay, the Boyden chamber assay with a modification was performed. In brief, cells (1×10^5) were seeded on the Matrigel-coated membrane in the inserts. After 18 h 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 μ g/ml propidium iodine, 5 mM MgCl₂, 10 mM Tris-HCl (pH 7.0), and 25 μ g/ml RNase A. DNA contents were analyzed using flow cytometry (BD FACSCanto II, BD Biosciences).

For BrdU incorporation assays, $1\text{--}2 \times 10^5$ 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 μ g) were transfected

TABLE 1
PCR primers and siRNAs

F, forward; R, reverse.

Gene	Application	Sequence (5'–3')
CDK6 F	RT-qPCR	TCG ATG AAC TAG GCA AAG ACC
CDK6 R	RT-qPCR	AGG TGG GAA TCC AGG TTT TC
NEK7 F	RT-qPCR	ACT TGG AGA TCT TGG GCT TG
NEK7 R	RT-qPCR	GAC AGC CAA GAG ACC AGA TG
NANOS1 F	RT-qPCR	GGT CGG CTC GAC ATG GGA CG
NANOS1 R	RT-qPCR	CAC ACC CAG CCT TCG CCG TT
RAPH1 F	RT-qPCR	GCC AAC TTT TCT TAC CGC TTC
RAPH1 R	RT-qPCR	GAT TTG ACG CTT ACT GTT TCC TG
β -Actin F	RT-qPCR	GCA CTC TTC CAG CCT TCC
β -Actin R	RT-qPCR	TGT CCA CGT CAC ACT TCA TG
CDK6 F	ChIP-qPCR	CGG AGA GAG TGC TGG TAA CTC CTT
CDK6 R	ChIP-qPCR	TGC GAG TGT CAG TCG GCT CT
NEK7 F	ChIP-qPCR	TTC GGC TCC AGT AGG GAA AC
NEK7 R	ChIP-qPCR	CTG CCC GAT GGA GGC TT
NANOS1 F	ChIP-qPCR	GGA GGA GTG GGC CCG ATA AA
NANOS1 R	ChIP-qPCR	AAA GCC TCC ATG GGC GGG
RAPH1 F	ChIP-qPCR	CAG TCA GTC AGT CAG TCA GTC AGT
RAPH1 R	ChIP-qPCR	AGG GCG AGG CTA ACC ACT CA
siLuc	siRNA (Dharmacon)	Sense, 5'-AACTTACGCTGAGTACTTCGA-dTdT-3' Antisense, 5'-AAUCGAAGUACUCACGGUAAAGUU-3'
siKDM2A-3	siRNA (Dharmacon)	Sense, 5'-AACAAAGGAGAGUGUGGUGUUU-dTdT-3' Antisense, 5'-AAACACCACACUCUCCUUGTT-3'
siKDM2A-4	siRNA (Dharmacon)	Sense, 5'-AAUUACGAAGCCUCACACUAU-dTdT-3' Antisense, 5'-AUAGUGUGAGGCUUCGUAATT-3'
siControl	siRNA (IDT)	Sense, 5'-UCGAAGUACUCACGGUAAAGTT-3' Antisense, 5'-CUUACGUGAGUACUUCGATT-3'
siHDAC3-3	siRNA (IDT)	Sense, 5'-CCCAGUACGAGAAUCAGAACUCACG-3' Antisense, 5'-CGUGAGUUCUGAUUCUCGAGUCCGGUG-3'
siHDAC3-9	siRNA (IDT)	Sense, 5'-GUCAACGACAAUUGUGAUUGGCAUC-3' Antisense, 5'-GGAUGCCAAUCACAAUUGUCGUUGACAU-3'
siCDK6-1	siRNA (IDT)	Sense, 5'-AGAGUGCAAUUUAGAAUAGAAAGT-3' Antisense, 5'-ACUUUCUAGUUCUAAUUUGCACUCUAC-3'
siCDK6-3	siRNA (IDT)	Sense, 5'-GGUGUUGUCAGUACUAAAUGCTT-3' Antisense, 5'-AAGCAUUUAGUACUGACAAACCCUA-3'
siNANOS1-1	siRNA (IDT)	Sense, 5'-GGAGUAGACAGAGUUGUAGUCCGTTG-3' Antisense, 5'-CAGGCCAUACAACUCUGUCUACUCCCA-3'
siNANOS1-3	siRNA (IDT)	Sense, 5'-GGACCACUAUGAACAGAUACAGCGCA-3' Antisense, 5'-UGCGCUGAUCUGUUCUAGUGUCCUU-3'
siScramble	siRNA (IDT)	Sense, 5'-GUGUAUGUAGUGCGGUAGU-3' Antisense, 5'-AUACUACCGCACUACAUCAAC-3'

into either siControl- or siKDM2A-treated cells ($2-5 \times 10^5$ cells) in 60-mm dishes using 10 μ l of Lipofectamine 2000 (Invitrogen). These cells were harvested after 72 h of incubation for further analysis, such as quantitative RT-PCR.

Quantitative ChIP Assay—A ChIP assay was performed as described previously (20). After chromatin immunoprecipitation using specific antibodies, DNA was purified using the Qiagen PCR purification kit. Purified DNAs 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 Study—To determine whether the effect of KDM2A 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 described previously (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 24-h incubation, all three groups of cells were retransfected 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 (1.5×10^6) were subcutaneously injected into the dorsal flanks of male nude mice (8 weeks old). At least five mice were injected for each group and observed for 10 weeks for tumor formation. The ellipsoid volume formula ($1/2 \times L \times W \times H$) was used to calculate the tumor volume. Ten weeks after the injection, the mice were killed, and their tumors and lungs were collected. For pathological analysis, hematoxylin and eosin (H&E) and immunohistochemical staining was performed.

Statistical Analysis—For statistical analysis, each experiment was performed in triplicate and repeated at least three times. Data are presented as the mean \pm S.E. (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.

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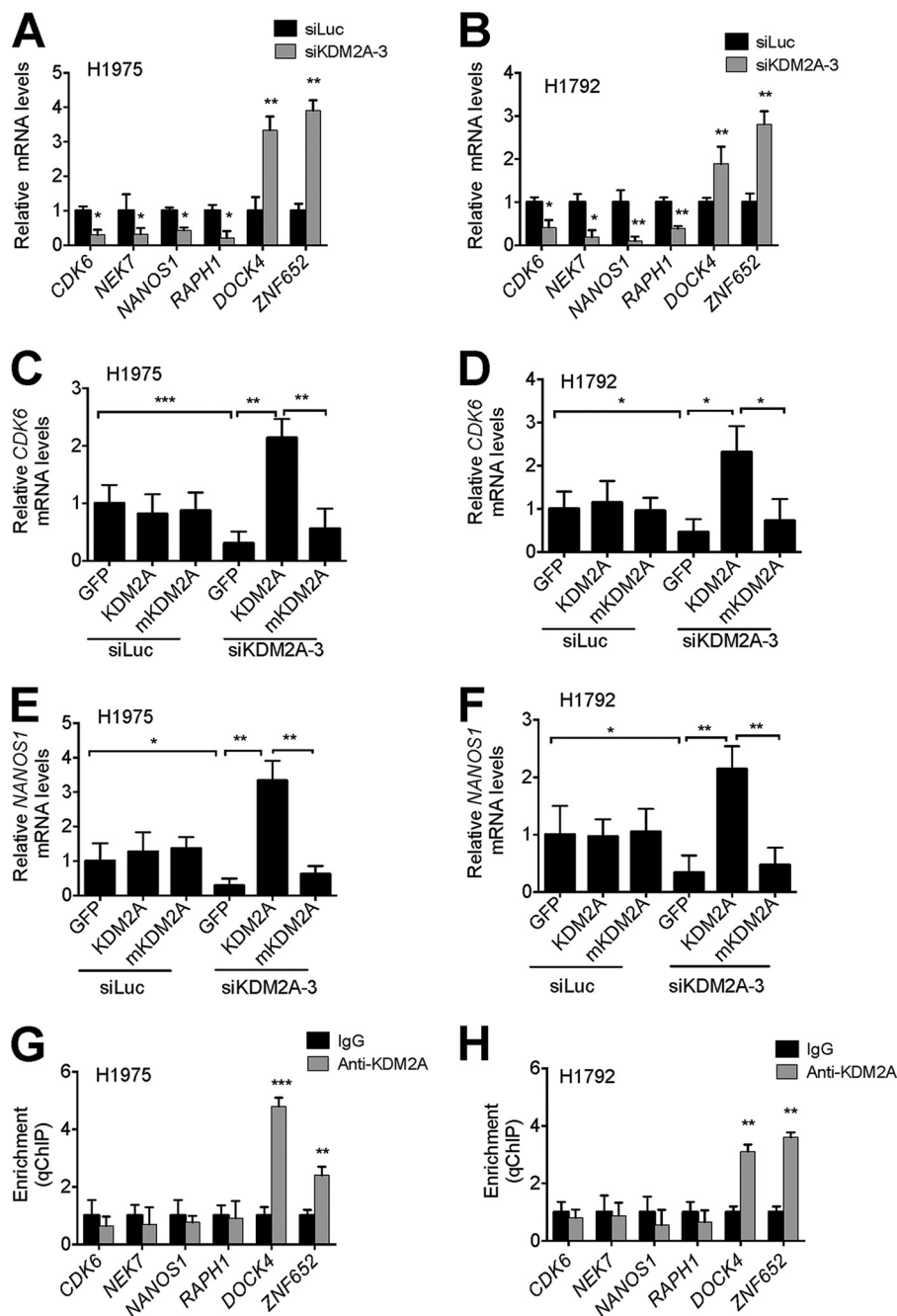


FIGURE 1. Transcriptional expression of cell cycle-associated genes and invasion-related genes is positively regulated by KDM2A in the KDM2A-overexpressing NSCLC cell lines H1975 and H1792. A and B, analysis of *CDK6*, *NEK7*, *NANOS1*, *RAPH1*, *DOCK4*, and *ZNF652* mRNA levels in H1975 (A) and H1792 (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 (*qChIP*). Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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 down-regulated 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 prereplication 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).

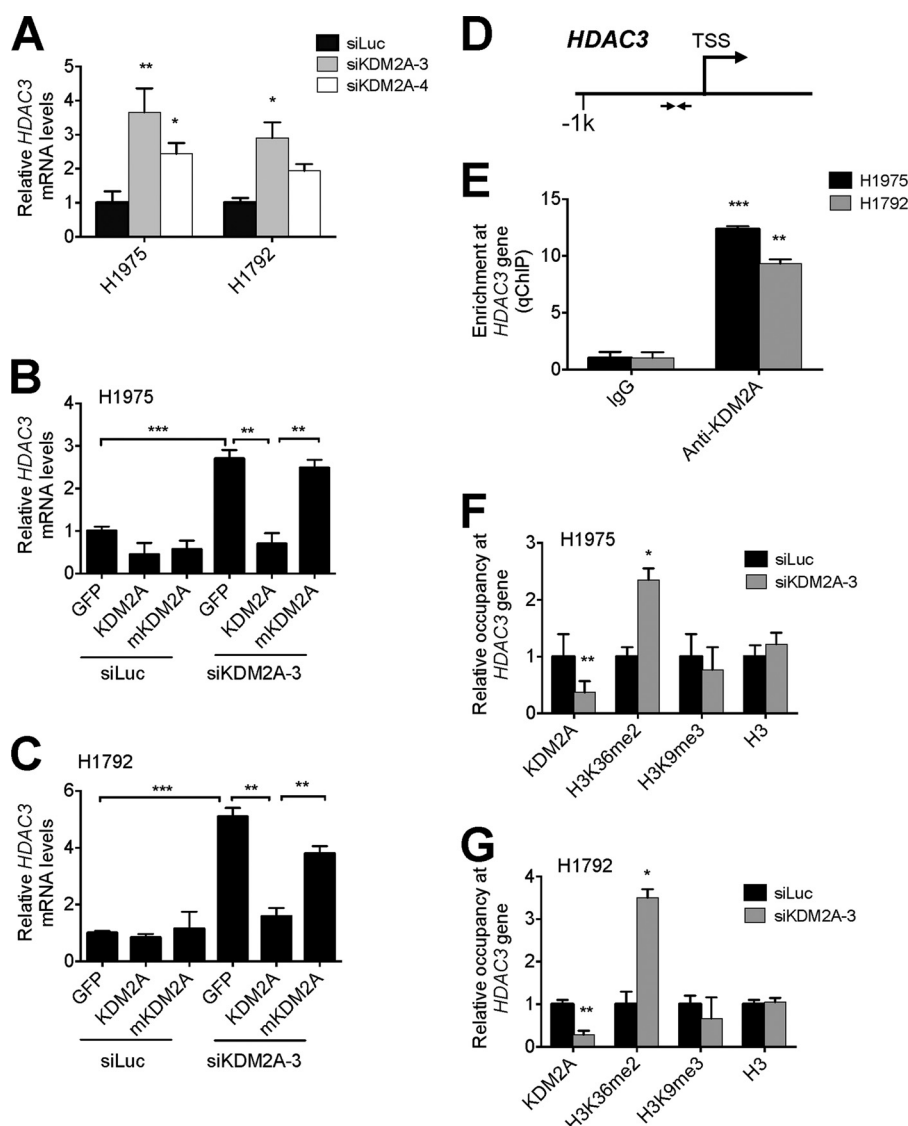


FIGURE 2. KDM2A represses HDAC3 expression by demethylating H3K36me2 at the HDAC3 promoter. *A*, the effect of KDM2A knockdown on HDAC3 mRNA levels in H1975 and H1792 cells. Cells were transfected with two different siKDM2As (siKDM2A-3 and -4). *B* and *C*, analysis of HDAC3 mRNA levels in KDM2A-depleted 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 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 (*qChIP*). *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. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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 using quantitative RT-PCR. Two KDM2A-overexpressing NSCLC cell lines H1975 and H1792 were examined. Our results ensured that these genes were down-regulated by KDM2A knockdown (Fig. 1, *A* 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 described previously (16). Exogenous expression of wild-type KDM2A, but not mKDM2A, significantly rescued expression of these genes in H1975 and H1792 cells (Fig. 1, *C–F*, showing 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. Thus, we determined whether these genes are indirectly or directly regulated by KDM2A using quantitative ChIP assays. Because it has been demonstrated that KDM2A is located 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. 1, *G* and *H*). In line with its role in repressing

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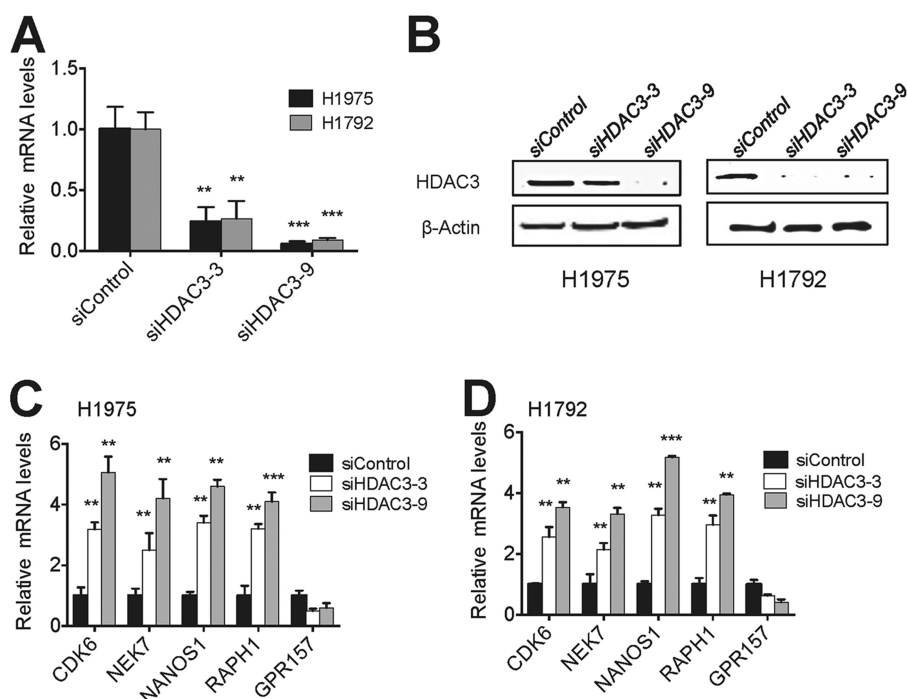


FIGURE 3. HDAC3 represses expression of the cell cycle-associated genes *CDK6* and *NEK7* and the invasion-related genes *NANOS1* and *RAPH1*. *A* and *B*, analysis of HDAC3 knockdown efficacy in H1975 and H1792 cells. Cells were treated with two siRNAs against HDAC3 (siHDAC3-3 and siHDAC3-9). HDAC3 mRNA and protein levels were measured by quantitative RT-PCR (*A*) and Western blot analysis (*B*), respectively. *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. Data are presented as the mean \pm S.E. (error bars). **, $p < 0.01$; ***, $p < 0.001$.

transcription, these results indicate that KDM2A indirectly up-regulates expression of the *CDK6*, *NEK7*, *NANOS1*, and *RAPH1* genes while directly down-regulating expression of the *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. 2*A*). 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. 2, *B* 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. 2*D*). 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. 2*E*). 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 in H1975 and H1792 cells (Fig. 2, *F* 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. 3, *A* and *B*). Quantitative RT-PCR results showed that HDAC3 depletion by two independent siHDAC3s increased expression levels of these genes (Fig. 3, *C* 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. 4, *A–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 support the idea that KDM2A up-regulates expression of these genes by repressing *HDAC3* expression at 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

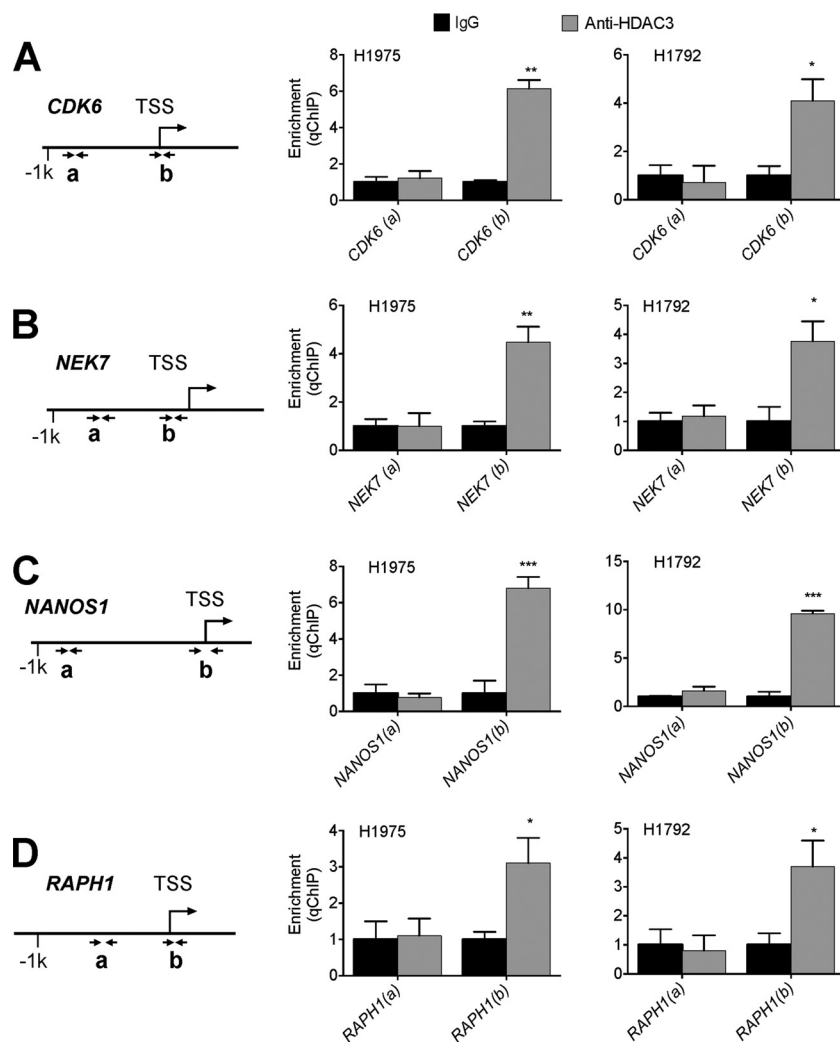


FIGURE 4. HDAC3 occupies the regions spanning the transcription start sites in the *CDK6*, *NEK7*, *NANOS1*, and *RAPH1* genes. A–D, analysis of chromatin occupancy of HDAC3 at the *CDK6* (A), *NEK7* (B), *NANOS1* (C), and *RAPH1* (D) genes in H1975 and H1792 cells by quantitative ChIP (qChIP). A diagrammatic representation of individual genes is also shown. Arrows indicate the PCR-amplified regions (a and b). TSS, transcription start site. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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. 5, A–D). Similar to no obvious effect of KDM2A knockdown on cellular levels of H3K36me₂, 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), whereas NANOS1 modulates cellular invasion in the breast cancer cell lines Hs578T and 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. 6, A–D). On the contrary, NANOS1 knockdown only marginally inhibited cell proliferation and BrdU incorporation but markedly abrogated cellular invasiveness (Fig. 6, E–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 the *CDK6* and *NANOS1* genes in an HDAC3-dependent manner, we reduced HDAC3 in KDM2A-depleted H1975 and H1792 cells using siHDAC3 (Fig. 7, A and B). HDAC3 knockdown significantly restored *CDK6* and *NANOS1* mRNA levels in KDM2A-depleted H1975 and H1792 cells (Fig. 7, C–F). In addition, HDAC3 depletion significantly rescued deficient proliferation of KDM2A-depleted cells by restoring the S phase population (Fig. 8, A–C). Consistent with this, HDAC3 knockdown increased BrdU incorporation in KDM2A-depleted cells (Fig. 8D). Interestingly, HDAC3 depletion did not affect the

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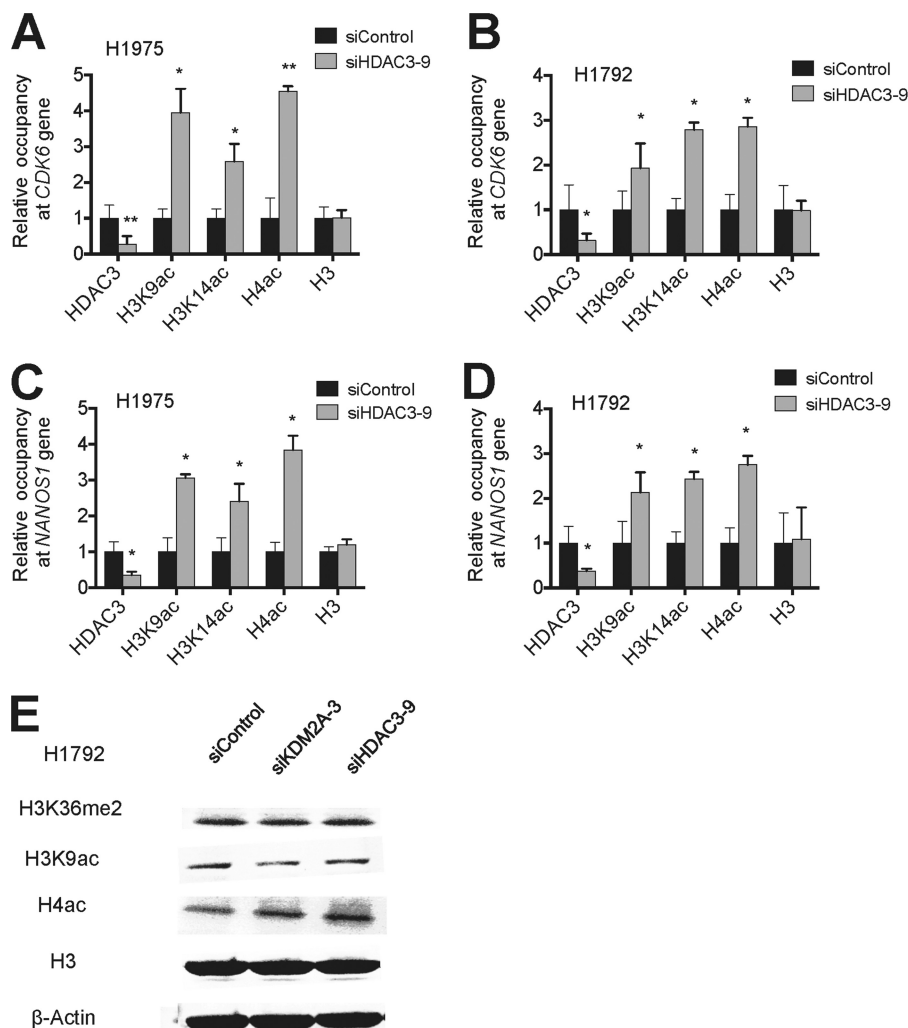


FIGURE 5. HDAC3 knockdown increases acetylation levels in histones H3 and H4 at the promoters of the *CDK6* and *NANOS1* genes. A–D, analysis of chromatin levels of HDAC3, H3K9ac, H3K14ac, H4ac, and H3 at the promoter regions of the *CDK6* (A and B) and *NANOS1* genes (C and D) in H1975 (A and C) and 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 and β -actin were used as internal loading controls. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$.

G_2/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. 9, A and B). These results indicate that transcriptional repression of *HDAC3* by KDM2A may contribute to cell proliferation and invasiveness by releasing cell cycle-associated genes 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. 10, A and B). In agreement with our previous report (16), most mice ($n = 4$ of 5) that were injected with shLuciferase-treated H1792 cells had tumors within 3 months (Fig. 10C and Table 2). In sharp contrast, no mice injected with KDM2A-depleted cells had detectable tumors ($n = 0$ of 5) (Fig. 10C and Table 2). Interestingly, HDAC3 depletion greatly restored defective tumorigenic abil-

ities of KDM2A-depleted H1792 cells (Fig. 10, C and D, and Table 2). Tumors were confirmed by H&E staining, and CDK6 and NANOS1 levels in a control tumor were comparable with those in a tumor that was originated from double knockdown cells (Fig. 10, E 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

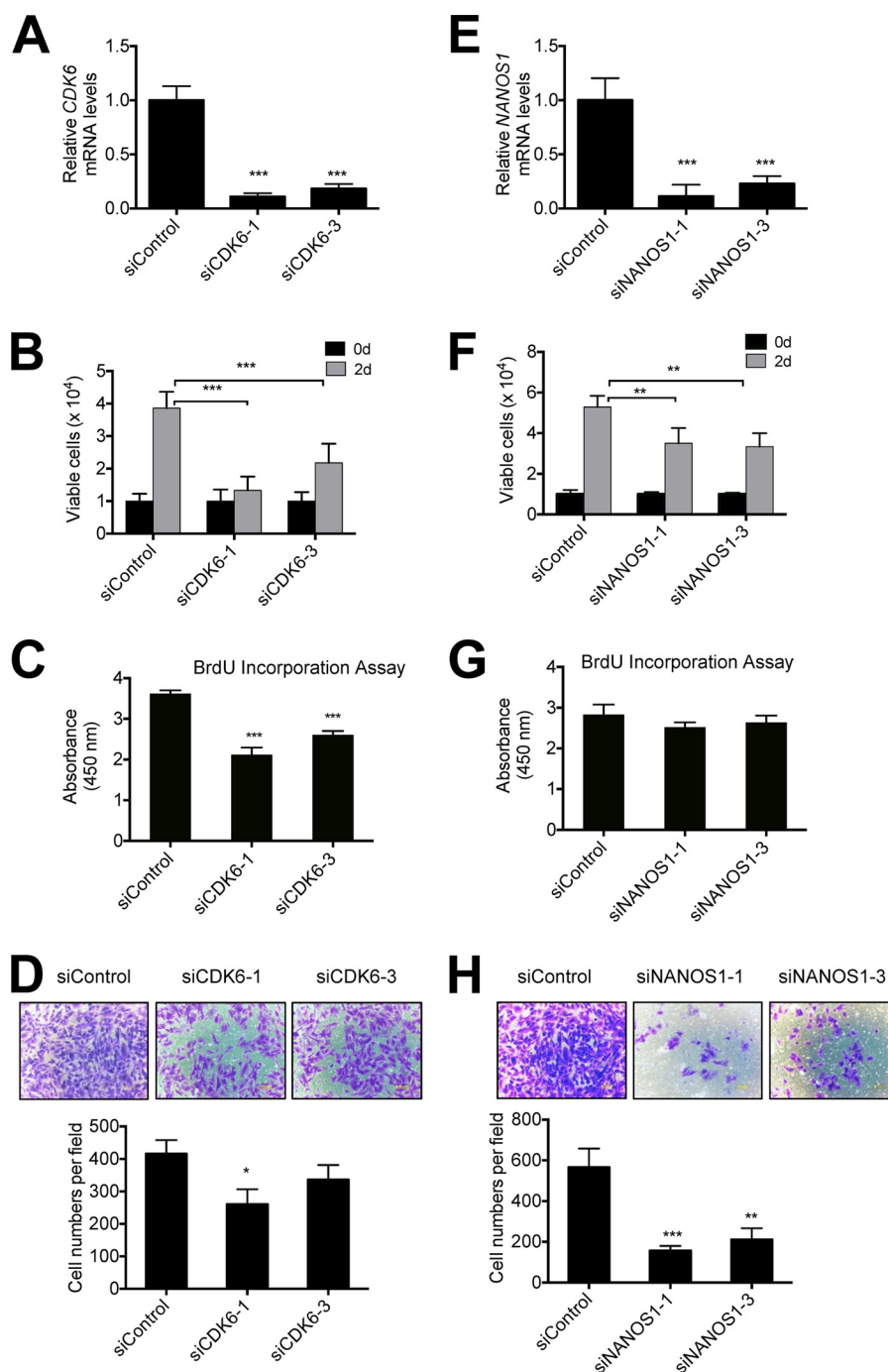


FIGURE 6. CDK6 knockdown inhibits mainly cell proliferation, whereas NANOS1 knockdown impedes largely cell invasiveness. A–D, the effect of CDK6 knockdown on the proliferation and invasiveness of H1792 cells. CDK6 mRNA levels were analyzed in siControl-treated and CDK6-depleted (siCDK6-1 or -3) cells 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. d, days. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

HDAC3. Therefore, our current study uncovered 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 + HDAC3 + cell cycle/invasiveness genes (*e.g.* CDK6 and NANOS1)) (Fig. 10G). In addition, KDM2A-mediated regulation of HDAC3 expression may be largely dependent on the

catalytic activity of KDM2A because the KDM2A catalytic mutant did not repress HDAC3 expression.

It is known that KDM2A acts as a positive regulator for somatic cell reprogramming and cellular antisense (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

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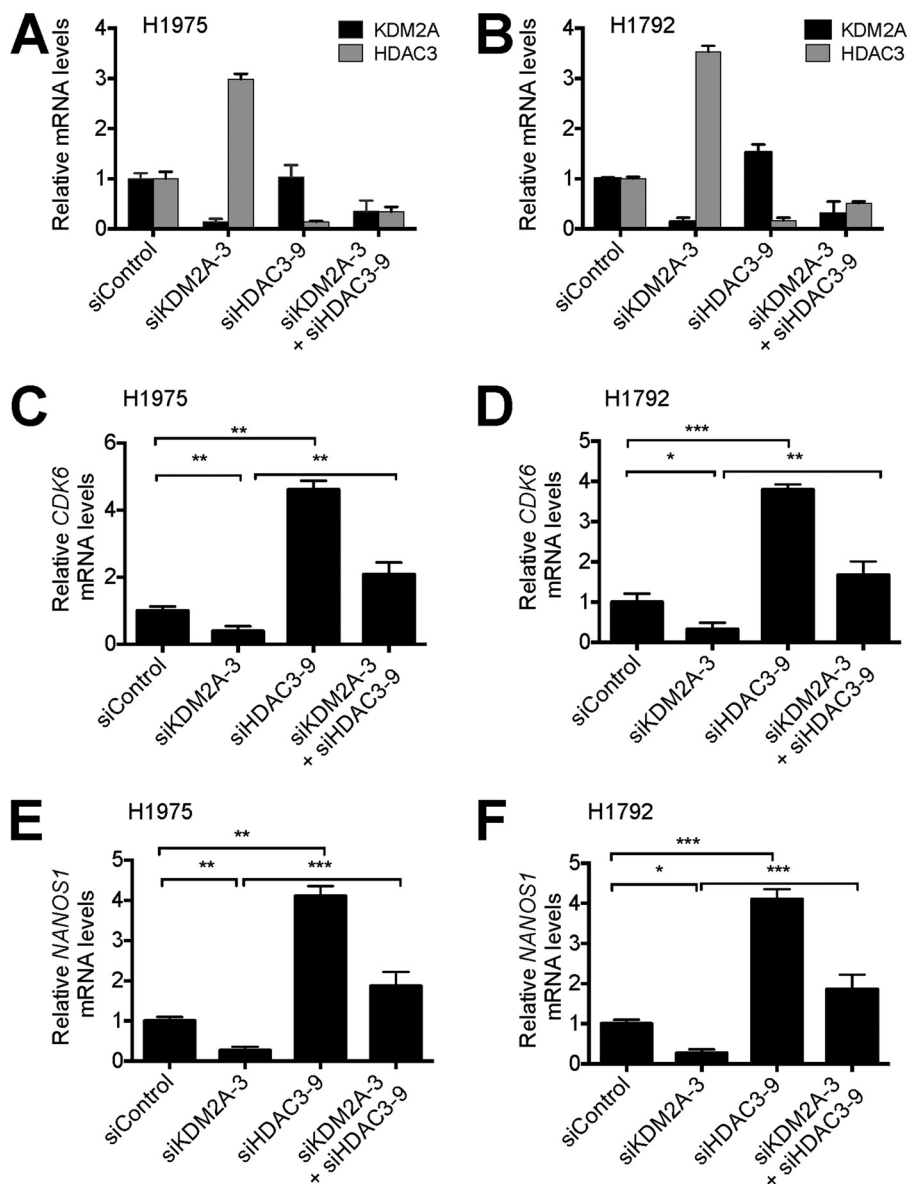


FIGURE 7. HDAC3 knockdown significantly increases expression of the *CDK6* and *NANOS1* genes in KDM2A-depleted NSCLC cells. A and B, analysis of *HDAC3* and *KDM2A* mRNA levels in siControl-treated, KDM2A-depleted (siKDM2A-3), and KDM2A/*HDAC3*-depleted (siKDM2A-3 + siHDAC3-9) cells by quantitative 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. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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 + *DUSP3* + ERK1/2 \rightarrow cell cycle/invasiveness and 2) KDM2A + *HDAC3* + cell cycle/invasiveness genes. Interestingly, treatment of KDM2A-overexpressing cells with the MEK1/2 inhibitor U0126 (10 μ M) 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 *HDAC1* 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 the function of others 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 neu-

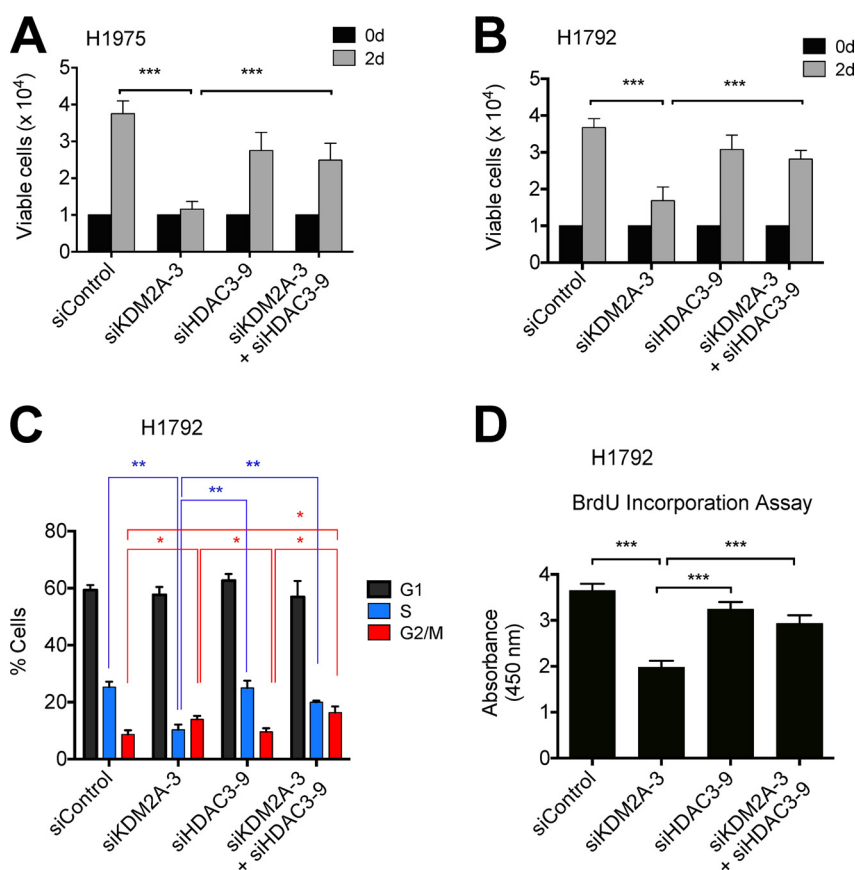


FIGURE 8. HDAC3 knockdown rescues proliferation defect of KDM2A-depleted NSCLC cells. *A* and *B*, the effect of HDAC3 knockdown on the proliferation of KDM2A-depleted H1975 (*A*) and H1792 (*B*) cells. *C*, the effect of single and double knockdown of KDM2A and HDAC3 on S phase cell percentages. The percentages of sub-G₁, G₁, S, and G₂/M phase cells were analyzed. *D*, BrdU incorporation assay. *d*, days. Data are presented as the mean \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

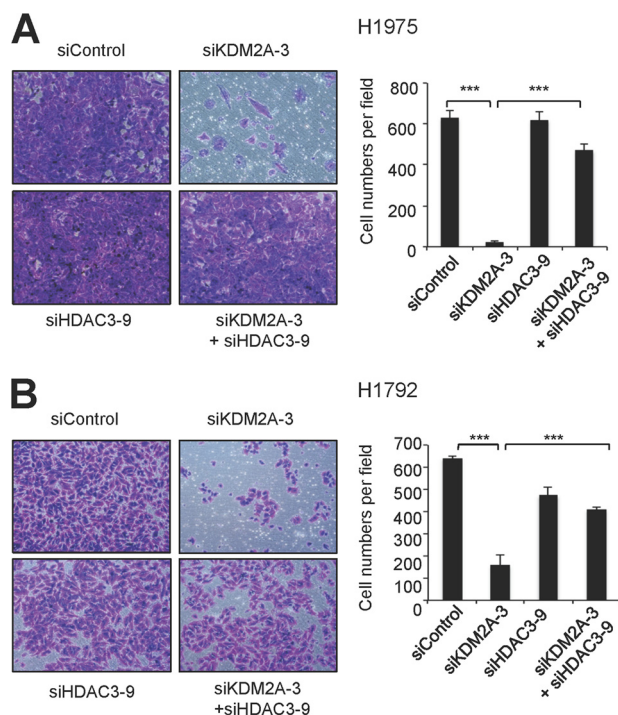


FIGURE 9. HDAC3 knockdown restores deficient invasiveness of KDM2A-depleted NSCLC cells. *A* and *B*, the effect of HDAC3 knockdown on the invasiveness of KDM2A-depleted H1975 (*A*) and H1792 (*B*) cells. Representative images of invaded cells are shown (left panels), and cells were counted (right panels). Data are presented as the mean \pm S.E. (error bars). ***, $p < 0.001$.

tralize 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-associated 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.* (17) reported that via its CXXC domain KDM2A interacts with many transcription start site-associated CpG islands. 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 their levels in a range of NSCLC cell lines (data not shown). These results indicate that KDM2A is a key control factor for *HDAC3* expression

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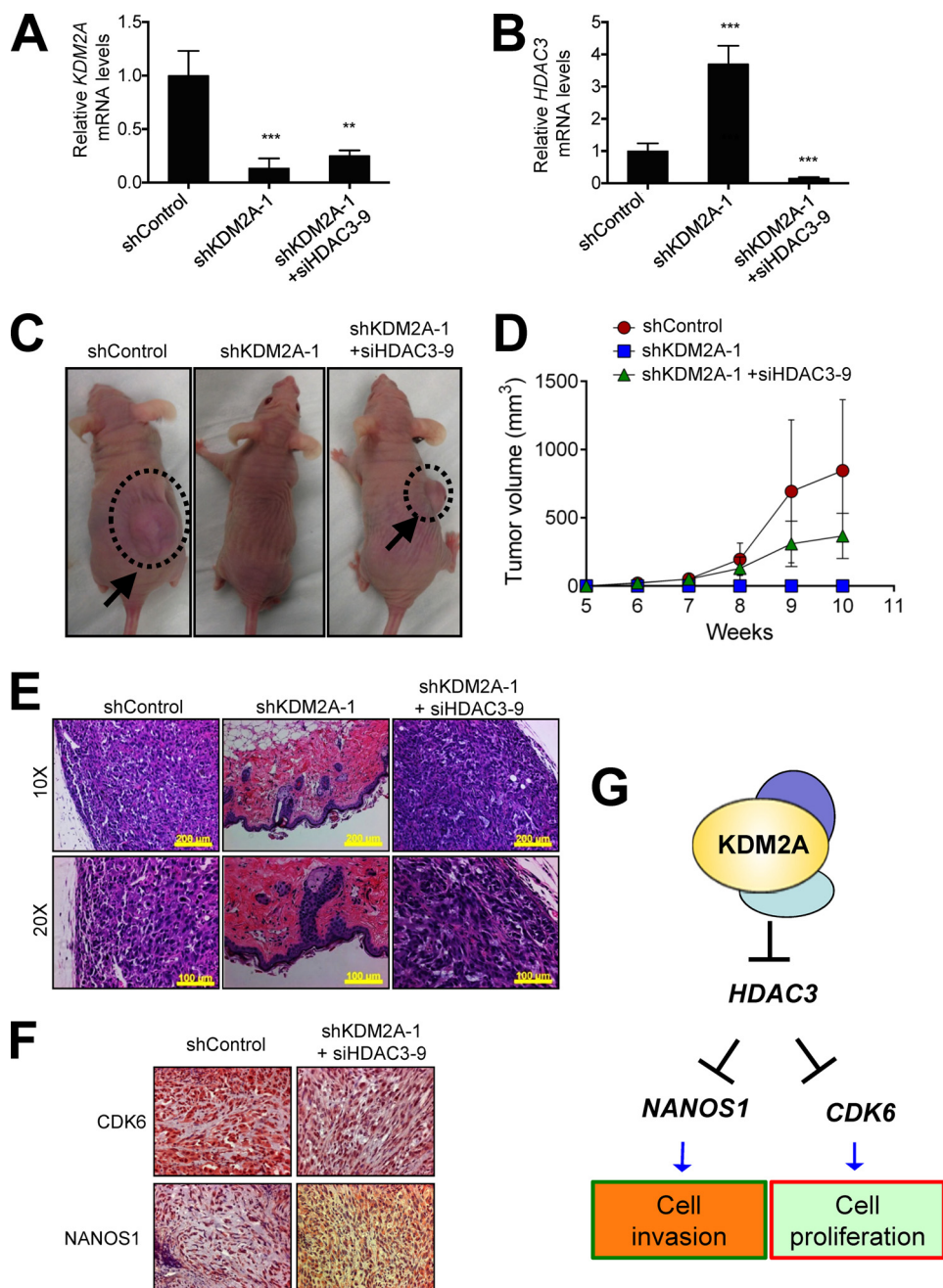


FIGURE 10. HDAC3 knockdown greatly recovers impaired tumorigenicity of KDM2A-depleted NSCLC cells in a mouse xenograft model. *A* and *B*, analysis of *KDM2A* (*A*) and *HDAC3* (*B*) mRNA levels in control (shLuciferase), stably *KDM2A*-depleted (shKDM2A-1), and *KDM2A*/*HDAC3*-depleted H1792 cells. 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 five 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 panels, 200 μ m; lower panels, 100 μ m). *F*, representative images of immunohistochemical staining of NANOS1 and CDK6 in xenograft tumor samples. Immunohistochemical staining was performed only using tumor tissues from the shControl group and shKDM2A-1 + siHDAC3-9 group. Normal mouse skin tissues from the shKDM2A-1 group were not used for staining because they were not a proper control. CDK6 and NANOS1 levels were quantified using the Chromavision Automated Cellular Imaging System (ACIS-III) from Dako and compared between the shControl group and shKDM2A-1 + siHDAC3-9 group: CDK6, 1 versus 0.73; NANOS1, 1 versus 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. Data are presented as the mean \pm S.E. (error bars). **, $p < 0.01$; ***, $p < 0.001$.

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 anticancer agents. Multiple mechanisms underlying the mode of action of *HDAC* inhibitors have been described, including induced expression of tumor suppressor p21 and the proapoptotic

TABLE 2

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

Treatment ^a	Mice treated (n)	Mice with lung tumors (n)
shControl	5	4
shKDM2A-1	5	0
shKDM2A-1 + siHDAC3	5	4

^a 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.

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 proinvasive 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 knock-out 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 antitumor 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 antitumor function of HDAC3 by KDM2A at transcriptional levels may be requisite for the growth and invasion of KDM2A-overexpressing NSCLC tumors.

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