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Report

# Lung cancer-associated JmjC domain protein mdig suppresses formation of tri-methyl lysine 9 of histone H3

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**Key words:** mdig gene, histone demethylation, lung cancer, mineral dust, H3K9me3

**Lung cancer is the most common cancer worldwide, accounting for 1.3 million cancer deaths annually. Despite extensive studies over the past decade, the detailed mechanism about the initiation and development of the lung cancer is still elusive. In the present report, we showed that overexpression of mdig is a common feature of the non-small cell lung cancer. Gene silencing or overexpression of mdig revealed that mdig is involved in demethylation of tri-methyl lysine 9 on histone H3, leading to an increase in ribosomal RNA expression. The transcriptional regulation of ribosomal RNA gene by mdig is achieved through abrogating tri-methyl lysine 9 on histone H3 and enhancing RNA polymerase I occupancy in the promoter region of the ribosomal RNA gene as demonstrated by chromatin immunoprecipitation. The pronounced expression of mdig in lung cancer tissues but not normal lung tissues, thus, suggests that mdig possesses oncogenic property through antagonizing tri-methyl lysine 9 on histone H3 and promoting ribosomal RNA synthesis.**

## Introduction

Lung cancer is the leading cause of malignant-related deaths worldwide.[1] Owing to the lack of reliable biomarkers and symptoms in the early stages, nearly 60 percent of patients die within a year after diagnosis and 85 percent die within five years. In addition to tobacco smoking, environmental or occupational exposure to mineral dusts and other hazards is considered to be the second most important causative factor for the lung cancer. Most patients are diagnosed with non-small cell lung cancer, including squamous cell carcinoma, adenocarcinoma and large-cell carcinoma. Despite immense progress in understanding mechanisms and exploring treatments during the past decades, an effective therapeutic regimen for this disease remains elusive. A lung cancer-associated mineral dust-induced gene, mdig, has been previously identified and found to be able to foster proliferation of human bronchial epithelial cells.[2] This gene had also been independently identified in human promyelocytic leukemia HL60 cells as the myc-induced nuclear antigen 53 (mina53) and *Xenopus laevis* as nucleolar protein 52 (NO52),[3,4] respectively. However, it remains unclear how mdig gene regulates cell growth or proliferation that contributes to the development of lung cancer.

It has long been established that tumorigenesis is largely resulted from genetic abnormalities, including genomic mutations, chromosomal translocation and re-arrangement.[5] The importance of epigenetic alterations, especially, the post-translational modifications of histone proteins, in cancer development has just been recognized recently.[6] The basic building blocks of the mammalian genome are nucleosomes that are composed of 146 bp of DNA wrapped around a histone octamer with two copies each of H2A, H2B, H3 and H4. The N-terminal tails of histones H3 and H4 are subject to a dynamic methylation and demethylation through both histone methylases and demethylases. The lysine residues in histones H3 and H4 can be modified by mono-, di- or tri-methylation. It has been generally viewed that tri-methylation of lysines 4 (H3K4me3), 36 (H3K36me3) and 79 (H3K79me3) of histone H3 can create euchromatin structure for active gene transcription. In contrast, tri-methylation of lysines 9 (H3K9me3) and 27 (H3K27me3) of histone H3 is refractory for gene expression due to the formation of the highly condensed heterochromatin architecture. Global downregulation of H3K9me3 and H3K27me3 has been observed in several types of human cancers, which arises from either the increased activity of demethylases or deficiency in the corresponding methylases.[7] For instance, genomic amplification of Jmjd2C (GASC1), a H3K9me3 demethylase, has been observed in lung sarcomatoid carcinoma and oesophageal squamous carcinomas.[8,9] The majority of histone demethylases are JmjC domain-containing proteins.[6] The human mdig gene encodes a protein with a conserved JmjC domain also.[2] It is very likely, thus, that regulatory role of mdig protein on cell growth may be achieved through affecting the methylation states of histone proteins. In the present study, we show that lung cancer expresses

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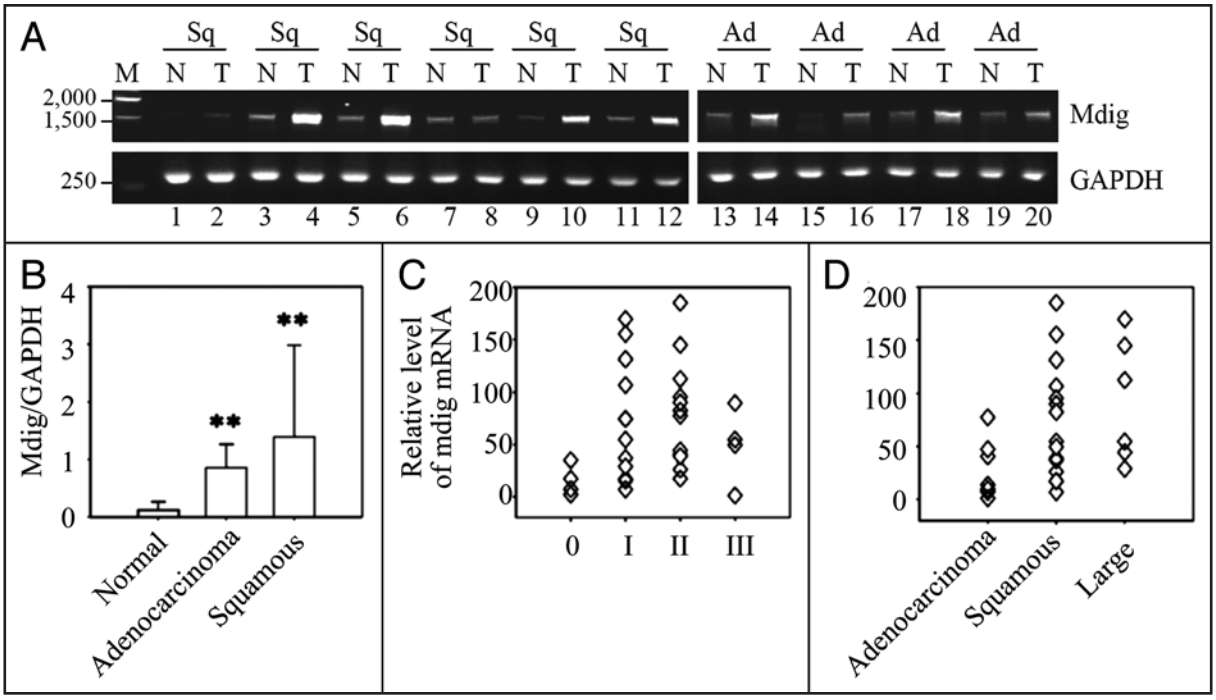


Figure 1. Mdig mRNA is highly expressed in lung cancer. (A) Total RNAs from adenocarcinoma (Ad) and squamous cell carcinoma (Sq) were subjected to RT-PCR using a mdig primer set derived from the exon 1 and exon 10 regions of the mdig gene, respectively, or a primer set for GAPDH. M: DNA marker; N: normal lung tissue; T: lung cancer (tumor). (B) Semi-quantification of the mdig mRNA expressed in normal lung, adenocarcinoma and squamous cell carcinoma. The RT-PCR-amplified cDNA bands from the agarose gel in (A) were quantified by densitometry scan. Data are mean ± standard deviation (SD). The asterisks (**) indicate statistic significance with $p < 0.05$. (C and D) Relative levels of mdig expression in different stages (C, the X axis) and type (D) of the lung cancer. A TissueScan Real-Time panel was used in this PCR reaction, which contains cDNAs from 13 adenocarcinomas, 23 squamous cell carcinomas, six large cell carcinomas, three small cell carcinomas, two neuroendocrine carcinomas and one sarcomatoid carcinoma. Only the data from adenocarcinoma, squamous cell carcinoma and large cell carcinoma were included in (D).

high levels of mdig mRNA and protein, whereas mdig expression is barely detectable in normal lung tissues. Overexpression of mdig in bronchial epithelial cells elevates ribosomal RNA transcription through antagonizing tri-methylation of lysine 9 on histone H3, which associates with an enhanced cell proliferation. Our findings, therefore, may provide a new mechanistic insight into how mdig expression that alters histone H3 methylation contributes to the initiation or development of the human lung cancer.

## Results

**Lung cancer expresses higher levels of mdig mRNA.** In an effort to define the relationship between lung cancer and mdig gene, we compared the expression level of mdig mRNA in lung cancer tissues paired with the adjacent normal lung tissues by RT-PCR. The PCR primer set was derived from the exon 1 and exon 10 regions of mdig gene, which amplifies a 1,510 bp fragment of mdig mRNA encompassing the full length open-reading-frame. An elevated expression of mdig was observed in 11 out of 13 squamous cell lung cancer tissues (lanes 1–12) and six out of eight adenocarcinomas (13–20) relative to the paired normal lung tissues (Fig. 1A and Suppl. Table 1). Semi-quantification of mdig expression showed a more than ten- and six-fold increase of mdig expression in squamous cell carcinoma and adenocarcinoma relative to the normal lung tissues, respectively (Fig. 1B). To further determine the expression of mdig in human lung cancers, we measured mdig mRNA level using lung cancer cDNA panels containing 48 cDNA samples derived from various types and stages of human lung cancers. A higher expression of mdig could be seen in stages 1 and 2 of the lung cancers (Fig. 1C). Both squamous cell carcinoma and large cell lung cancers show increased mdig mRNA expression relative to that of adenocarcinoma lung cancer (Fig. 1D).

**Mdig protein is involved in histone H3 lysine 9 demethylation.** Earlier studies using mdig siRNA indicated an involvement of mdig in the proliferation of bronchial epithelial cells.[2] It is unclear how mdig protein contributes to the regulation of cell growth. The mdig gene encodes a protein with 465 amino acids. Protein sequence analysis predicated the presence of a Jumonji C (JmjC) domain at position 164 to 244 (Fig. 2A). Most recent studies demonstrated that the JmjC domain in several JmjC family proteins functions as a signature motif for histone H3 demethylases.[10-16] Sequence alignment of the mdig JmjC domain with the same domain of the proposed histone H3 demethylases, including FIH,[17] JHDM1,[10] Jmjd2C (GASC1)[15] and Epe1,[17] suggests that mdig contains key elements satisfying cofactor-binding requirements, such as histidine (H) at 179 and lysine (K) at 194 of the mdig protein (Fig. 2A). It has been generally viewed that H3K9me3 and H3K27me3 of histone H3 facilitates formation of heterochromatin and thereby silences gene activation. In contrast, H3K4me3, H3K36me3 and H3K79me3 were associated with active transcription.[18,19] Thus, the methylation status of lysines on histone H3 may determine the

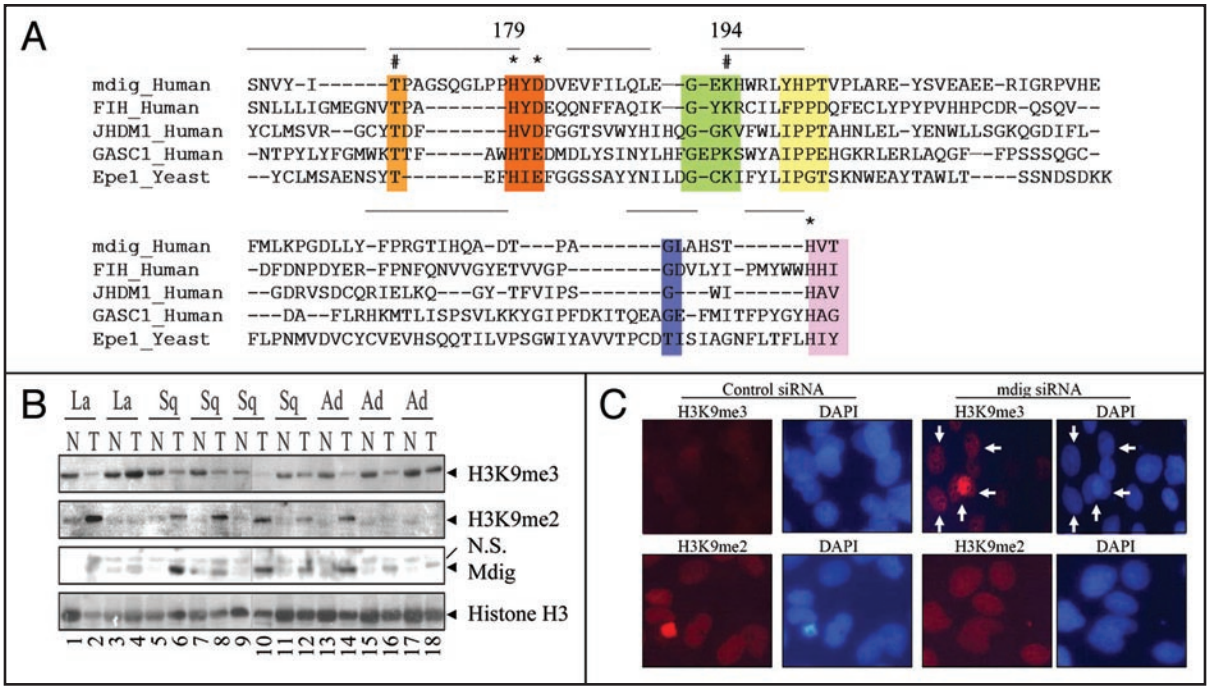


Figure 2. Decrease in the level of H3K9me3 in lung cancers. (A) Alignment of the JmjC domain in human mdig protein with other JmjC family proteins that exhibited demethylase activity or hydroxylase activity. The multiple sequence alignment was made by a Meta Server from BioInfoBank (http://bioinfo.pl/Meta/). Conserved residues are color shaded. The lines on the top indicate the sequences that form β-sheet. The asterisks (*) indicate amino acids that are involved in Fe(II) binding, such as H179. The amino acids for possible α-KG binding were marked with number symbol (#), such as K194. (B) The levels of H3K9me3, H3K9me2 and mdig in lung cancers and the corresponding paired normal tissues were determined by the use of respective antibodies. The same membrane was stripped and re-probed with the antibody against total histone H3 as loading controls. La, large cell lung cancer; Sq, squamous cell carcinoma; Ad, adenocarcinoma; N.S., non-specific band. (C) Silencing mdig by siRNA increases the level of H3K9me3. The nucleus was shown by DAPI staining. White arrows indicate increased level of H3K9me3 in the cells transfected with mdig siRNA.

genomic stability and affect the tumorigenic transformation.[20] In human lung cancers, we noted a decrease in the level of H3K9me3 and an increase of di-methylation of K9 (H3K9me2) relative to the paired normal lung tissues in 8 out of 9 paired samples (Fig. 2B). This notion was confirmed in an additional experimental setting using 5-paired adenocarcinomas, 10-paired squamous cell carcinomas and 4-paired large cell carcinomas (Suppl. Table 2). An immunoblotting analysis using these lung tissue lysates revealed a higher level of mdig protein expression in all of these lung cancers relative to their paired normal lung tissues (Fig. 2B). Such pattern was roughly in agreement with an additional experimental setting using 5-paired adenocarcinomas, 10-paired squamous cell carcinomas and 4-paired large cell carcinomas (Suppl. Table 3).

Given the fact that mdig contains a JmjC domain, the signature motif for histone H3 demethylases, we speculated that the higher levels of mdig mRNA and protein in the majority of lung cancer samples might be responsible for the decrease of H3K9me3. To determine whether mdig affects lysine methylations of histone H3, we next assessed the levels of H3K9me3 and H3K9me2 in the BEAS-2B cells where the expression of mdig was silenced by siRNA. Because of the higher basal level of H3K9me3 in these cells, we stained the control siRNA- and mdig siRNA-transfected cells with the indicated antibodies at a higher dilution (1:300) for a shorter period (30 min). As indicated in Figure 2C, silencing mdig enhanced H3K9me3 (Fig. 2C, the cells marked with white arrows). A marginal decrease in the level of H3K9me2 was observed in the cells transfected with mdig siRNA relative to the cells transfected with the control siRNA (Fig. 2C). These data suggest that mdig contributes to the demethylation of H3K9me3. Repression of mdig expression by siRNA, therefore, increases the level of H3K9me3.

**JmjC domain in mdig protein is important in demethylation of H3K9me3.** To provide direct evidence indicating the involvement of mdig in the demethylation of H3K9me3, we constructed expression vectors for GFP-tagged mdig and transfected these vectors to the BEAS-2B cells. The levels of H3K9me3, H3K9me2 and H3K9me1 in these transfected cells were then determined. In the cells without mdig overexpression, the staining pattern of H3K9me3 is overlapping with the condensed chromosome DNA, whereas such pattern is less obvious for the H3K9me2 and H3K9me1 (Fig. S1). Expression of mdig diminished H3K9me3 (Fig. 3A, the top row) but not H3K9me2 or H3K9me1 (Fig. 3A, the third and bottom rows). A normal level of H3K9me3 was observed in the cells without overexpression of mdig (Fig. 3A, the second row). Expression of the JmjC-deleted mdig showed no effect on H3K9me3 (Fig. 3B), indicating that JmjC domain plays a key role on the function of mdig. It was noted that deletion of JmjC domain resulted in both cytoplasmic and nuclear staining of the expressed protein in some of the transfected cells (Fig. 3B). To further address the role of JmjC domain of mdig protein, we

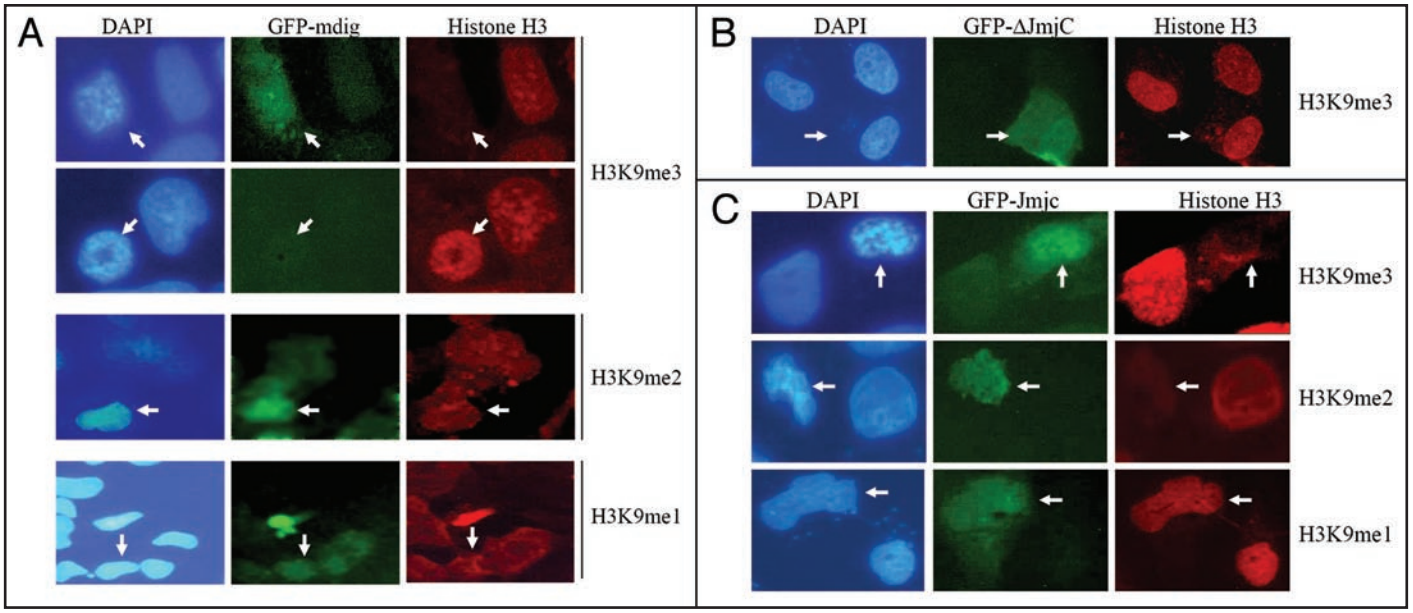


Figure 3. Overexpressing mdig diminishes H3K9me3. (A) The methylation states of H3K9 were determined in the cells expressing GFP-full-length mdig protein by antibodies against H3K9me3, H3K9me2 and H3K9me1. White arrows indicate the cells either expressing GFP-mdig (top, third and bottom rows) or not (the second row). (B) JmjC domain-deleted mdig (GFP-ΔJmjC) has no effect on the level of H3K9me3. (C) Expression of the JmjC domain only construct of mdig decreases the levels of H3K9me3 and H3K9me2 without affecting the level of H3K9me1.

next transfected the cells with a JmjC domain only construct. The expression of this JmjC domain only protein decreased the signals of both H3K9me3 and H3K9me2 without effect on H3K9me1 (Fig. 3C). Together, these results demonstrate that mdig protein is indeed involved in the process of demethylation of H3K9me3, and that the JmjC domain in this protein is critical for such activity. A fluctuation of mdig expression on the level of H3K9me3 was observed among the cells in the different mitotic phases (Fig. S2). Although the prophase cells expressed higher level of mdig, the signal of H3K9me3 appears to be normal, possibly because of the compartmentalizing of the expressed mdig in the nucleolus (see insert of the first panel on the second row of Fig. S2). Following the disassembly of nucleolar structure in mitosis,[21,22] mdig protein appeared to be co-localized with the condensed chromosome (Fig. S2). The H3K9me3 was nearly diminished in the cells in prometaphase, metaphase and early anaphase where the cells expressing the transfected GFP-mdig. In later anaphase and telephase, expression of GFP-mdig failed to affect H3K9me3 (Fig. S2).

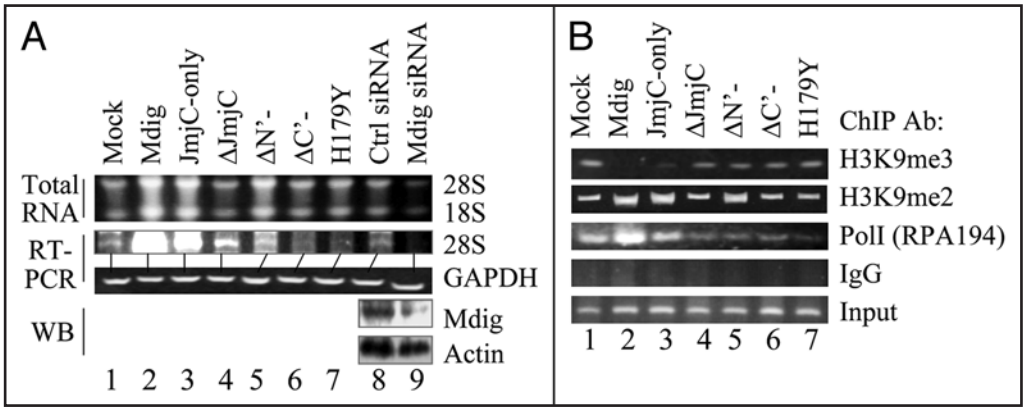


Figure 4. Mdig enhances cell proliferation and expression of ribosomal RNA. (A) Expression of 28S and 18S rRNA in the indicated cells was determined by analyzing the total RNA (top) and RT-PCR using primers corresponding to 28S rRNA (second) and GAPDH (third). The silencing effect of mdig siRNA was verified by immunoblotting using mdig antibody (fourth) and actin antibody (bottom). (B) ChIP analysis for the association of H3K9me3 and H3K9me2 with and the recruitment of poll to the rRNA gene promoter.

**Mdig enhances rRNA synthesis through H3K9me3 demethylation.** Although mdig appears to be involved in the demethylation of H3K9me3, it is unclear how or whether this effect of mdig is directly linked to the cell growth regulation as we reported previously.[2] During the overexpression experiments, we frequently noted that cells transfected with the full-length mdig or the JmjC domain only construct form multiple foci under microscopy and an enhanced proliferation (Fig. S3A and B). We also noted that mdig-transfected cells exhibited elevated levels of 28S and 18S rRNAs (Fig. 4A, top), indicative of active proliferation of the cells. To determine whether mdig or its deletion/mutation constructs affects the expression of rRNAs, we analyzed the level of 28S RNA by RT-PCR using the same number of cells either mock-transfected or transfected with the different mdig constructs. As indicated in Figure 4A, both full-length mdig and the JmjC

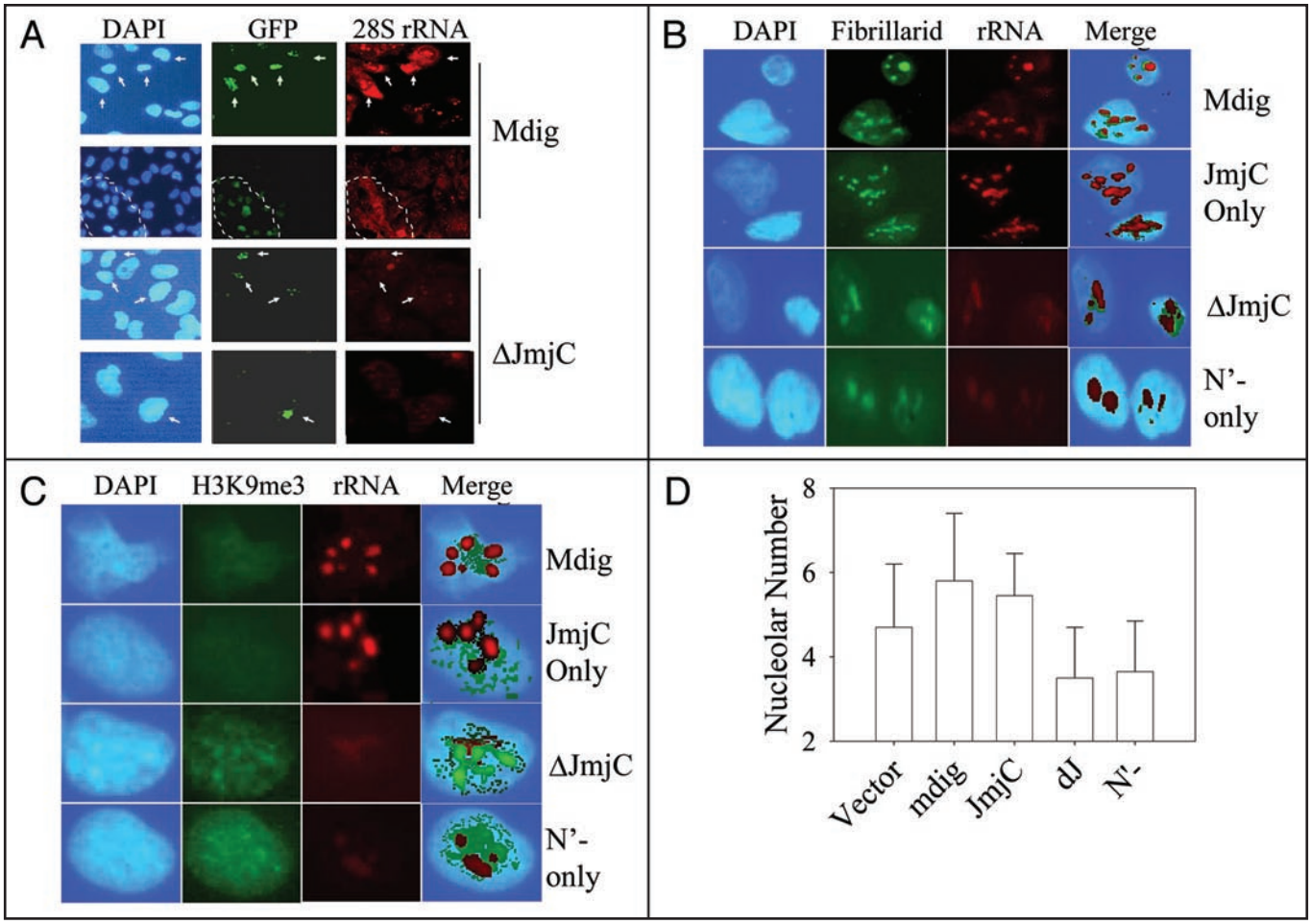


Figure 5. Overexpression of mdig increases rRNA in both nucleoli and cytoplasm. (A) RNA FISH shows increased 28S rRNA synthesis in both nucleoli and cytoplasm of the cells transfected with mdig-GFP but not ΔJmjC-GFP (pointed by white arrows, top row, and encircled by white dashed line, the second row). (B and C) The number of nucleoli in the cells stably transfected with the indicated vectors was determined by staining the cells with anti-fibrillarin, a nucleolar protein. The cells were co-stained with antibody against fibrillarin and 28S rRNA FISH. (D) The level of 28S rRNA is inversely correlated to the level of H3K9me3. The stably transfected cells were co-stained with 28S rRNA FISH probe and anti-H3K9me3 antibody followed by FITC-conjugated second antibody. For the illustration purpose of figures (B and D), the contrast of fibrillarin, H3K9me3 and 28S rRNA images in the "Merge" panels was artificially enhanced and processed as transparency before merging to the DAPI panels.

domain only constructs are able to increase the expression of 28S rRNA. Deletion of the JmjC domain, N-terminal or C-terminal region of mdig dampened the inducibility of mdig on 28S rRNA. Site-directed mutation of H179Y (179H to 179Y mutation) or K194A also compromised the effect of mdig on the expression of 28S rRNA (Fig. 4A, lane 7 and data not shown). As expected, silencing the endogenous mdig attenuated 28S rRNA expression remarkably (Fig. 4A, top and middle). The silencing effect of mdig siRNA on mdig expression was verified by immunoblotting (the fourth and bottom of Fig. 4A). To investigate whether mdig elevates ribosomal RNA synthesis through modulating the association of H3K9me3 and RNA polymerase I (polI) with the rRNA gene promoter, chromatin immunoprecipitation (ChIP) was performed. The antibodies used in this ChIP experiment include anti-H3K9me3, anti-H3K9me2 and anti-RPA194 subunit of polI. Both full-length mdig and JmjC domain only constructs are capable of diminishing the level of H3K9me3 on and enhancing the recruitment of polI to the rRNA gene promoter (Fig. 4B). Again, mutation of the key histidine (H179Y) or deletion of the JmjC domain of the mdig protein prevented the elimination of H3K9me3 on the rRNA promoter (Fig. 4B, lanes 4 and 7).

**Overexpression of mdig increases both nucleolar and cytoplasmic rRNA.** The enhancement of mdig on rRNA synthesis was further confirmed by RNA FISH experiments using fluorescence-labeled anti-sense probe against the 28S rRNA. Comparing to the cells transfected with a JmjC-domain deletion construct (ΔJmjC), the cells transfected with the full-length mdig exhibited higher staining signal for 28S rRNA by RNA FISH (Fig. 5A, circled by white dash line or pointed by white arrows). In the same field of the transiently transfected cells, the cells expressing exogenous mdig-GFP, that is localized in both nucleoplasm and nucleoli, showed an elevated level of 28S rRNA in both cytoplasm and nucleoli, the initial transcription sites of rRNA (Figs. 5A and S4A, pointed by white arrows). A similar level of 28S rRNA was seen between the non-transfected cells and the cells transfected with a ΔJmjC construct (marked by white arrows), suggesting the

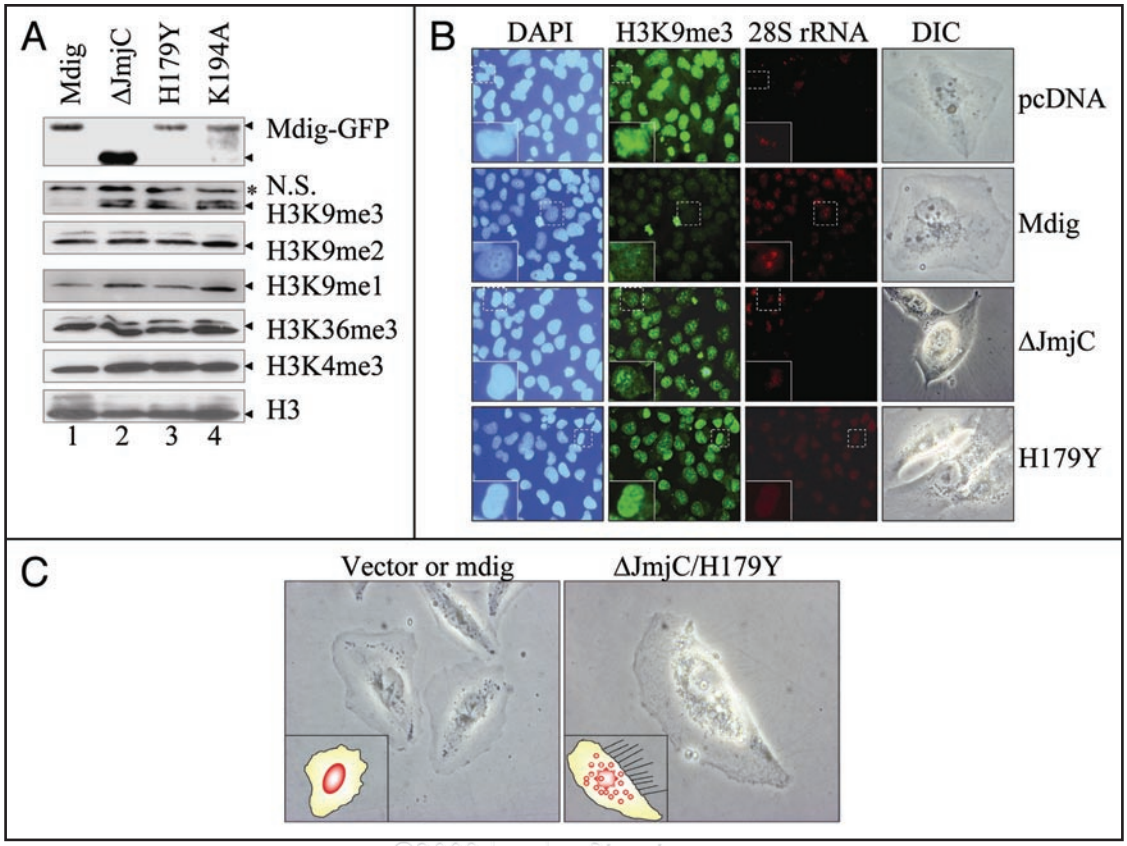


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Figure 6. JmjC domain deletion or the mutations of the key residues of this domain inactivates the demethylase activity of mdig. (A) In vitro demethylase activity assay of mdig and its deletion/mutation constructs. (B) The levels of H3K9me3 and 28S rRNA in these stably transfected cells expressing the indicated vectors were determined by immunofluorescence staining and RNA FISH, respectively. The panels on far right side shows cell morphology of these transfected cells. The area bounded by the dash-lined rectangle is enlarged on the bottom left of each panel showing the details of cell nucleus, H3K9me3 and 28S rRNA staining. (C) Magnified image of cell morphology for the cells stably expressing vector/mdig (left) or ΔJmjC/H179Y.

importance of JmjC domain in mdig protein in the regulation of 28S rRNA (Fig. 5A, the panels in the third and fourth rows). Although some interphase cells transfected with mdig or the JmjC domain construct exhibited dispersion of nucleoli as indicated by fibrillarin staining (Fig. 5B), the difference in the nucleolar numbers among the cells transfected with the indicated vectors is statistically insignificant (Fig. 5B, panels in the second column, and C). Overexpression of mdig appears to be able to enhance the synthesis of the 28S rRNA among the cells in interphase, prophase and metaphase (Fig. S4B). The intensity of the 28S rRNA FISH signal is inversely correlated to the level of H3K9me3. The cells stably transfected with mdig or the JmjC only construct showed a weaker H3K9me3 and a brighter staining of 28S rRNA relative to the cells stably express ΔJmjC or the N'-terminal of mdig (Fig. 5D). Thus, these data strongly suggest that the proliferative effect of mdig is largely resulted from its regulation on the H3K9me3 on the rRNA gene promoter and the consequent expression of ribosomal RNAs.

**JmjC domain deletion or H179Y/K194A mutation inactivates the demethylase activity of mdig.** Above data provides strong evidence suggesting an involvement of mdig in H3K9me3 demethylation. To further validate this, we next measured the demethylase activity of mdig along with other deletion or mutation constructs. As depicted in Figure 6A, wild-type mdig reduced H3K9me3, but not H3K9me2, H3K9me1, H3K4me3 and H3K36me3 in an in vitro histone demethylase activity assay. The demethylase activity was not seen in the assays using ΔJmjC, H179Y or K194A mdig protein (Fig. 6A, lanes 2–4). In the stably transfected cells, these deletion/mutation constructs of mdig are not only unable to reduce the level of H3K9me3, but also failed to maintain the normal morphology of the cells (Fig. 6B and C). Cells stably expressing wild-type mdig or control vector showed a healthy morphology (Fig. 6C). In contrast, those cells stably expressing ΔJmjC, H179Y or K194A showed cytoplasmic disorganization with the presence of poorly defined organelles and ER, swollen organelles, deformations of nuclear envelop, little lipid droplets, glycogen accumulations and irregular cell membrane protrusions. We assumed that the highly bright organelles around nuclei are very likely those swollen mitochondria. These morphological changes complementarily supported our earlier observations indicating that mdig is important for cell growth.

## Discussion

We have described increased expression of mdig mRNA and protein in human lung cancers and the involvement of mdig protein in the demethylation of H3K9me3. The conserved JmjC

domain in mdig protein appears to be essential for its functioning, since deletion of the JmjC domain abolished the demethylation activity of the mdig protein. Overexpression of mdig in human bronchial epithelial cells fosters a fast growth of the cells, which was achieved largely through antagonizing H3K9me3 in the promoter region of rRNA gene. An elevation of the cellular rRNA levels was evidenced by 28S rRAN FISH within the nuclei of the cells ectopically expressing mdig. As the fact that rRNAs are the basic supporting molecules essential for the cellular protein synthesis and other functional activities, aberrant increase in rRNA levels provides the cells with advantage of proliferation and/or tumorigenic transformation.

In recent decades, lung cancer has become the top leading cause of cancer death in both industrialized and developing countries.[23,24] The most common form of lung cancer is the non-small-cell lung cancer that is subdivided into three subtypes: squamous cell carcinoma, large-cell carcinoma and adenocarcinoma. Despite histological distinctions, all forms of lung cancer are highly lethal. A variety of genetic alterations have been attributed to the initiation and progression of the human lung cancer associated with either tobacco smoking or exposure to other environmental hazards. These alterations include chromosomal deletion and/or amplification, genetic polymorphisms of the oncogenes and loss of function of tumor suppressors.[25-27] Because of the nature of multiple abnormalities in cancer genetics, it is unlikely that lung cancer can be determined by malfunction of a single or few genes. Thus, it is intuitively plausible to consider regulatory steps that affect the general stability of the genome.

One of the most important factors that maintain genomic stability is the epigenetic modification of the histone proteins that form basic building blocks for the chromatin fibers containing genomic DNA. The N-termini of the histones H3 and H4 can be modified by methylation, ubiquitination, acetylation or phosphorylation.[6] Aberrations in histone methylation have been linked to the initiation, maintenance and outcome of many human cancers.[28,29] The methylation status at the key lysine residues on histone H3 can determine the formation of heterochromatin and euchromatin structures, and consequently affect genomic stability and the transcription or inactivation of tumor suppressors and oncogenes. The lysine side chains may be mono-, di- or tri-methylated. Tri-methylation of K9 on histone H3 appears to be an essential step for the recruitment of heterochromatin protein (HP1) that facilitates the formation of heterochromatin, which was largely catalyzed by histone methyltransferase, suppressor of variegation 3–9 (SUV39).[30] Abrogation of tri-methylation of K9 on histone H3 causes genomic instability that predisposes to malignant transformation in mice.[31] Gene amplification of Jmjd2C (GASC1), a recently identified demethylase of H3K9me2 and H3K9me3,[15] has been frequently observed in human oesophageal squamous cell carcinoma.[32-34] Interestingly, the Jmjd2C protein also contains a conserved JmjC domain. Similar to the increased expression of Jmjd2C in oesophageal squamous cell carcinoma, the mdig appears to be preferentially expressed in squamous cell lung cancer (Fig. 1).

The heterochromatin structures featured by tri-methylation of H3K9 in human genome are important in controlling genomic stability, cell linage development or differentiation, and the checkpoint mechanisms for cell growth.[35] There are at least three types of heterochromatin structures in human genome: constitutive heterochromatin, facultative heterochromatin and the focal heterochromatin. The constitutive heterochromatin primarily encompasses the regions containing a high density of repetitive DNA elements such as clusters of satellite sequences and transposable elements at centromeres, pericentric foci and telomeres. The transposable elements, which are abundant in human genome, are highly mutagenic by targeting protein-coding genes for insertion, causing chromosome breakage and promoting illegitimate genome rearrangement.[35] Thus, the constitutive heterochromatin maintained by H3K9me3 is pivotal for genomic integrity by preventing abnormal chromosome segregation, recombination and DNA replication. The facultative heterochromatin, on the other hand, occurred mainly at the developmentally-regulated loci, where the chromatin state can change in response to developmental signals.[36] Through interplay with H3K27me3 and DNA methylation, facultative heterochromatin is the key for normal cell linage development and differentiation by somatic methylation and inactivation of the germline-specific genes. In addition, the facultative heterochromatin appears to be responsible for the allelic exclusion, genomic imprinting, or inactivation of the X chromosome and the gene loci of immunoglobulins and T-cell receptor genes. Unlike constitutive and facultative heterochromatins that usually cover large chromosomal regions, the focal heterochromatin represents a repressive chromatin structure embedded in a short euchromatin regions containing actively transcribed genes. The focal heterochromatin may be able to recruit transcriptional repressor complexes, such as CoREST and H3K4me3 demethylase, to regulate nucleosome occupancy in the active gene loci to repress expression of the cell growth genes.[37,38] This assertion was mainly supported by earlier studies showing that Rb-mediated repression of E2F target genes, including cyclin E, cyclin A2, cyclin D1 and B-Myb, was dependent on the formation of focal heterochromatin induced by SUV39 h1-mediated H3K9me3 and HP1 association.[39-42] Thus, any measures that impede H3K9me3 will change the overall nucleosome dynamics and destabilize the genome, leading to de-differentiation of the cells, aberrant gene expression and tumorigenic transformation. The contribution of mdig to demethylation of H3K9me3 will certainly compromise the formation of heterochromatin and thereby enhance the tendency of cancer development in the lung due to genomic instability, overexpression of ribosomal RNAs and immortalization of the lung cells.

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## Materials and Methods

**RT-PCR and western blotting analyses.** Total RNAs from the lung cancer and the paired adjacent normal lung were either purchased from Cureline, Inc. (San Francisco, CA; Fig. 1A) or extracted from lung cancer tissues provided by the Pathologic Archive of West Virginia University. The TissueScan Real-Time panel containing 48 human lung cancer cDNA samples was provided

by OriGene (Rockville, MD). RT-PCR was performed using the AccessQuick RT-PCR system from Promega (Madison, WI) with a reverse transcription at 45°C for 45 min and then 40 cycles of PCR with temperature scales of 95°C 1 min, 59°C 1 min and 70°C 1.5 min. The DNA sequence for each PCR primer has been described previously.[2] Total tissue lysates of lung cancers paired with the adjacent normal lung tissues were purchased from Protein Biotechnologies (Ramona, CA). Western blotting was performed using the indicated antibodies as reported previously.[43] Antibodies against H3K9me3, H3K9me2, H3K9me1, H3K36me3 and H3K36me1 were purchased from Abcam (Cambridge, MA) or Upstate (Charlottesville, VA). The specificity of these antibodies for the methylated histone H3 has been previously described.[15] Antibody against human mdig (NO52/mina53) was a gift of Dr. Marion Schmidt-Zachmann at the German Cancer Research Center, Division for Cell Biology (Heidelberg, Germany).

**Transfection of siRNA and plasmid DNA.** The BEAS-2B cells were seeded at 1 x $10^5$/ml in 6- or 24-well tissue culture plates in DMEM supplemented with 5% fetal bovine serum (FBS) and cultured for 24 to 48 h. Transfection of mdig- and control-siRNA was performed as reported previously.[2] The full-length, truncated or site-directed mutant mdig cDNA was cloned into the *SacII* and *ClaI* sites of the phrGFP-C mammalian expression vector purchased from Stratagene (La Jolla, CA). The PCR primers used for construction of the mdig expressing vectors are listed in Supplementary Method. The plasmid DNA was transfected into the cells with lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) in the serum-free medium for 24 h. The cells were then incubated with the regular cell culture medium containing 5% FBS for an additional 24 h followed by immunofluorescent staining or reporter gene activity assay.

**Immunostaining and RNA fluorescence in situ hybridization (RNA FISH).** BEAS-2B cells in 24-well tissue plates were fixed with 4% paraformaldehyde at room temperature for 10 min and then permeabilized for 5 min with cold PBS containing 0.1% Triton X-100. After extensive washing with PBS, the cells were sequentially incubated with the indicated antibodies in 5% bovine serum albumin for 30 min to 4 h and then incubated with the Alexa Fluor 680 rabbit anti-goat IgG or Alexa Fluor 635 goat-anti-rabbit IgG purchased from Invitrogen Corporation (Carlsbad, CA) for 1 h. For RNA FISH, the fixed cells were first incubated with ULTRAhyb pre-hybridization solution (Ambion, Austin, TX) for 30 min at 68°C and then incubated with 20 μM Alexa Fluor 594-labeled antisense 28S rRNA probe in 5% bovine serum albumin at 4°C for 16 to 48 h. The sequence of the antisense 28S rRNA probe is: AminoC6 + Alexa Fluor 594-CTT AAC GGT TTC ACG CCC TC-3'. One drop of ProLong Gold antifade reagent with DAPI (Invitrogen) was added into each well before the image analysis with the Zeiss Axiovert100 fluorescence microscope connected to a Pixera Pro150ES digital camera.

**Chromatin immunoprecipitation (ChIP).** ChIP was performed using the ChIP kit following the manufacturer's instruction (Invitrogen) and the indicated antibodies. The immunoprecipitated DNA was amplified using PCR primers corresponding to the promoter region of rRNA gene. The primer sequences are: sense: 5'-CGT TTT TGG GGA CAG GTG-3'; antisense: 5'-CGA CTC GGA GCG AAA GAT A-3'. For mock ChIP, the specific antibodies were replaced with rabbit IgG fraction. In addition, whole DNA (input) was used as a reference to estimate the enrichment of proteins at the promoter region of rRNA gene.

**Histone demethylation assay.** In vitro histone demethylation assay was conducted as described by Frescas et al.[44] Patel et al.[45] and Lee et al.[46] with minor modifications. Briefly, the cells transfected with mdig-GFP vectors were subjected to immunoprecipitation using GFP antibody. The core histones were isolated from non-transfected cells using Histone Purification Kit (Active Motif, Carlsbad, CA). The core histones (2 μg) were incubated with GFP-immunopurified mdig proteins from BEAS-2B cells in demethylation buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-oxoglutarate, 40 mM $FeSO_2$, 2 mM ascorbic acid) in a total volume of 30 μl at 37°C for 2 hours. The reaction mixtures were separated on SDS-PAGE followed by western blotting using the indicated histone methylation antibodies.

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**Note**

Supplementary materials can be found at:
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