H3K9 Histone Methyltransferase, KMT1E/SETDB1, Cooperates with the SMAD2/3 Pathway to Suppress Lung Cancer Metastasis

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Abstract

Aberrant histone methylation is a frequent event during tumor development and progression. KMT1E (also known as SETDB1) is a histone H3K9 methyltransferase that contributes to epigenetic silencing of both oncogenes and tumor suppressor genes in cancer cells. In this report, we demonstrate that KMT1E acts as a metastasis suppressor that is strongly downregulated in highly metastatic lung cancer cells. Restoring KMT1E expression in this setting suppressed filopodia formation, migration, and invasive behavior. Conversely, loss of KMT1E in lung cancer cells with limited metastatic potential promoted migration in vitro and restored metastatic prowess in vivo. Mechanistic investigations indicated that KMT1E cooperates with the TGFβ-regulated complex SMAD2/3 to repress metastasis through ANXA2. Together, our findings defined an essential role for the KMT1E/SMAD2/3 repressor complex in TGFβ-mediated lung cancer metastasis.

Introduction

Covalent histone modifications have been implicated in the development and progression of various cancers, including lung cancer (1). These histone modifications include acetylation (Ac), methylation (Me), phosphorylation, ubiquitination, and sumoylation. Recent studies have indicated that histone methylation is strongly associated with tumorigenesis and poor prognosis. For example, loss of H3K9Me2 has been found in both prostate and kidney cancer and is associated with poor prognosis (2, 3). Importantly, H3K9Me3 serves as a diagnostic marker of both recurrence and distant metastasis patients with in lung and gastric cancer (4, 5). These observations support the hypothesis that aberrant histone methylation leads to the activation or repression of critical genes during tumorigenesis.

In the past decade, many studies have provided clear indications that HMTs promote tumorigenesis. Suv39h1, a histone H3K9 methyltransferase (H3K9MT), is highly expressed and functions as an oncogene in hepatocellular and colorectal cancer metastasis (6, 7). G9a (EHMT2), a H3K9MT, is overexpressed in lung, prostate, and hepatocellular cancers (8). Suppression of G9a and Suv39h1 causes cell growth inhibition in prostate cancer (9) and induces lung epithelial cells transformation (10). Another H3K9MT, KMT1E, interacts with Dnmt3a to silence gene expression in cervical and breast cancer cells (11). Recent publications using zebrafish have indicated that KMT1E is a pro-oncogene for melanoma development (12). More recently, studies reported that KMT1E is required for cell migration and invasion in A549 cells and prostate cancer (13, 14). Together, these recent studies indicate that KMT1E promotes tumorigenesis; however, the mechanism by which KMT1E promotes metastasis remains to be elucidated.

Metastasis is a crucial step in cancer progression. Recent studies have indicated that cancer metastasis is mediated by cytoskeletal rearrangement, which includes modulating the dynamics of actin, tubulin, or intermediate filaments (15). It is known that the calcium-dependent phospholipid-binding protein ANXA2 plays a role in cytoskeletal rearrangement (16) and cancer metastasis (17). More recent studies showed that ANXA2 was highly expressed and positively correlated with a poor prognosis in non–small cell lung cancer (13, 14). These studies suggest that ANXA2 plays a crucial role in lung cancer metastasis. However, the mechanisms that regulate the transcription of ANXA2 in cancer progression remain unknown. Herein, we propose that ANXA2 expression is mediated by epigenetic regulation.
In this study, we have found that KMT1E serves as a crucial repressor of lung cancer metastasis. We demonstrate that KMT1E cooperates with SMAD2/3 and acts as a novel regulator to inhibit ANXA2 transcription, which suppresses cell invasion and metastasis. These results suggest that a novel pathway, which is controlled in part by a KMT1E/SMAD2/3 repressor complex, regulates epigenetic modification of gene expression during lung cancer metastasis.

Materials and Methods

Cell culture
All cell lines were maintained in RPMI-1640 or DMEM medium containing 10% FBS (Gibco BRL) and cultured at 37°C under 5% CO2. See Supplementary Materials and Methods for the details.

Plasmids
Full-length KMT1E, ANXA2, and SMAD3 were amplified by PCR using embryonic cDNA library as templates. See Supplementary Materials and Methods for details.

Filamentous actin staining
The cells were seeded in 4-well chamber slide and fixed in 4% paraformaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100/PBS, and blocked in 10% normal donkey serum (Jackson ImmunoResearch). Next, they were incubated with Alexa Fluor 488-conjugated phalloidin (Molecular Probes) overnight at 4°C and mounted with Mounting Medium with DAPI (VECTOR labs). The images were taken by ZEISS-LSM510 confocal microscope.

In vitro assay of histone methyltransferase activity
Histone methyltransferase assays were performed as previously described (19). The reaction products were analyzed by 15% SDS-PAGE. After Coomassie Blue staining and destaining, the gels were treated with EN3HANCE solution (NEN Life Science). The dried gels were exposed to Kodak MS X-ray film at −80°C.

In vivo xenograft metastasis assay
Fertilized zebrafish eggs of the Tg(li1:EGFP) genotype were incubated at 28°C in Danieau's solution and raised under standard laboratory conditions. Two days after fertilization (dpf), zebrafish embryos were dechorionated and subsequently anesthetized with 0.04 mg/mL of tricaine (MS-222; Sigma). Before injection, 1 × 106 cells were resuspended in 1 mL serum-free culture medium. Cells were labeled in vitro with CM-Dil (red fluorescence; Vybrant; Invitrogen). Approximately 400 tumor cells were resuspended in 5 μL serum-free RPMI and then injected into the yolk of each embryo using a Nanoject II auto-nanoliter injector (Drummond Scientific). After injection, embryos were incubated for 1 hour at 28°C and checked for the presence of cells at 2 hours posttransplantation; thereafter, they were examined every other day by fluorescent microscopy to monitor metastasis. For mouse model, we performed the in vivo metastasis assay, 1 × 106 cells were resuspended in 0.1 mL PBS and injected into the lateral tail vein of 6 weeks old female SCID mice. After 4 weeks, the mice were sacrificed and analyzed for lung colonization. The protocol was approved by the National Yang-Ming University Animal Care and Use Committee (Taiwan, Republic of China).

Immunoprecipitation
Immunoprecipitation assays were performed using a EZview anti-HA affinity gel (Sigma) according to the protocol of the manufacturer. See Supplementary Materials and Methods for details.

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Millipore) according to the protocol of the manufacturer. See Supplementary Materials and Methods for details.

Luciferase reporter assays
Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). See Supplementary Materials and Methods for the details.

Immunohistochemistry and tissue microarray, Immunoblot analysis, and In vitro migration/invasion assays
See Supplementary Materials and Methods for details.

Statistical analysis
Data analysis was performed using GraphPad Prism software (version 4.03). All data are presented as the mean ± SE. Three independent experiments were compared by the Student t test or binomial proportions test. Statistical significance was calculated using a one-way ANOVA followed by the Dunnett test.

Results

KMT1E is downregulated in the metastatic lung cancer cell line
To study the role of H3K9MTs in lung cancer invasiveness, we investigated the protein levels of H3K9MTs in six sublines of the CL1 lung cancer cell line, a well-known lung cancer metastasis model (20). These six isogenic sublines in the CL1 group, CL1-0, CL1-1, CL1-2, CL1-3, CL1-4, and CL1-5, show increasing metastatic potential (e.g., CL1-0 and CL1-1 are lower in metastatic potential as compared with the rest). Immunoblot analysis showed that G9a protein levels were increased in the highly metastatic cell lines CL1-5, but Suv39h1 protein level remained the same. Strikingly, KMT1E protein levels were significantly reduced in the highly metastatic cell lines CL1-5, but Suv39h1 protein level remained the same. Strikingly, KMT1E protein levels were significantly reduced in the highly metastatic cell lines (Fig. 1A).

To elucidate the clinical relevance of the cell line data, we performed IHC staining of a tissue microarray. The result showed a strong nuclear staining signal of KMT1E in the cancer tissues, whereas no significant signal was observed in the adjacent normal tissues. Among the 192 lung cancer tissue sections, 161 showed positive staining (IHC score >1) for KMT1E (83.8%; Fig. 1B and Supplementary Table S1). In addition, we also compared the intensity scores obtained from a range of cancer tissues that were at different clinical stages (Fig. 1B). As shown in Fig. 1B, KMT1E-positive staining was
higher in the early stages (I: 90.5% and II: 92.6%) compared with the late stages of lung cancer (III: 50% and IV: 50%). The patient details and their clinicopathological characteristics are shown in Supplementary Table S2. These findings are consistent with the results presented in Fig. 1A whereby there is a negative correlation of KMT1E expression with metastasis status. These results suggest that KMT1E may inhibit lung cancer cell metastasis.

**KMT1E suppresses lung cancer cells metastatic ability**

To examine whether KMT1E affects cancer cell migration ability, we performed cell migration assay. We overexpressed KMT1E in the highly metastatic subline, CL1-5 and knocked down KMT1E in the lower metastatic subline, CL1-0. The results showed a dramatic reduction in the migration ability of KMT1E-transfected cells and also showed significantly increased migration ability in si-KMT1E-transfected cell (Fig. 2A and B). To rule out the possibility that reduced migration ability may be due to reduced cell viability in KMT1E-transfected cells, we assessed cell viability in transfected cells using 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays. Three independent experiments showed no difference in the cell viability of transfected cells as compared with control cells (Supplementary Fig. S1).

In addition, KMT1E is known to methylate H3K9 and silence the euchromatic genes in cancer cells (11, 21). To investigate the status of silencing marks, H3K9Me2 and H3K27Me3, we determined the global H3K9Me2 and H3K27Me3 status in transfected cells. The results showed that H3K9Me2 correlated with KMT1E expression level, but no correlation was observed between H3K27Me3 and KMT1E expression levels (Fig. 2C). To further test whether the enzymatic activity of KMT1E was required for tumor cell invasion, we mutated histidine 1224, located in the catalytic domain, into alanine (H1224A), creating a mutant with a loss of methyltransferase activity. As seen in Fig. 2F, the H1224A mutant indeed lost H3K9 methylation activity (Fig. 2F, lane 4) and CL1-5 cells transfected with KMT1E (H1224A)-mutant constructs showed no repression of cell invasion; similar results were observed in CL1-3 and CL1-4 sublines (Supplementary Fig. S2). The KMT1E also contains a methyl-DNA–binding domain (MBD). We created another mutant in which arginine 643 was substituted to alanine, in the MBD. This mutation should leave KMT1E enzymatic activity unaffected (21). It is possible to rule out whether the MBD is in fact the reduced cell invasion ability in transfected cells. As expected, the R643A mutant retained H3 methylation activity (Fig. 2F, lane 3) and repressed invasion ability in CL1-5 cells transfected with the R643A mutant (Fig. 2D and E). Taken together, these data indicate that KMT1E suppresses the invasion ability of lung cancer cells and that this suppression is dependent on KMT1E’s histone H3K9 methylation activity.

**KMT1E acts as a crucial repressor for cellular metastasis in vivo**

To evaluate the role of KMT1E during metastasis, we used a zebrafish tumor model in which Tg (flh:EGFP) zebrafish embryos were implanted with cancer cells. As seen in Fig. 3A, lung cancer cells were labeled with CM-Dil dye and then injected into the yolk sac of 48 hours post-fertilization...
embryos. Three days after injection, the proliferation and metastasis of the cancer cells can be monitored by fluorescence microscopy. The results showed that there was a dramatic increase in the metastatic ability of CL1-0 that had been transfected with a KMT1E-specific siRNAs as compared with the control (scramble siRNA) cells (Fig. 3A). To a lesser extent, we also observed the metastases of the CL1-0 knockdown cells into the trunk regions of the fish. In contrast, control cells remained at the place of injection in the yolk and they invaded neither to other tissues nor into the bloodstream. In three independent experiments (48 fishes/per group), the siRNA-KMT1E group showed that 20.8% of fishes have migrating cells compared with only 2.08% in the control group (Fig. 3B).

Furthermore, we also used a SCID mouse model to test the role of KMT1E in metastasis in vivo. The KMT1E knockout cells were injected into the tail veins of SCID mice. Four weeks after injection, we quantified the number of metastatic nodules in the lung of mice injected with either CL1-0 (control) and KMT1E knockout CL1-0 cells (Fig. 3D). As expected, the more metastatic nodules were observed in lung from injected KMT1E knockout CL1-0 cells (Fig. 3E). These findings indicate that KMT1E plays a role in the suppression of lung cancer cell metastasis.

KMT1E suppresses filamentous actin polymerization and filopodia formation in lung cancer cells

Cancer cell migration and invasion are mediated by the modulation of the dynamics of filamentous actin (F-actin) polymerization and filopodia formation (22, 23). How does KMT1E suppress cancer cell migration and invasion? We investigated the possibility that KMT1E might be involved in F-actin polymerization and filopodia formation. To test this...
hypothesis, we performed F-actin staining by FITC-conjugated phalloidin in transfected CL1-5 and parental CL1-0 cells. Interestingly, we found that CL1-5 cells transfected with KMT1E have more than 23% demonstrated filopodia formation; similar results were also observed in another subline cells (Supplementary Fig. S3). In contrast, of the CL1-0 cells that were transfected with KMT1E, only 1% demonstrated filopodia formation (Fig. 4A). Moreover, 61% (40/65) of CL1-0 cell knockdown of siKMT1E demonstrated filopodia formation (Fig. 4B). These findings suggest that KMT1E inhibits lung cancer cell metastasis through downregulation of the F-actin polymerization in cell migration.

ANXA2 is a direct and functional target of KMT1E in lung cancer cell metastasis

Previous studies have shown that the calcium-dependent phospholipid-binding protein ANXA2 plays a role in F-actin polymerization (24) and cancer metastasis (17). To examine whether ANXA2 plays a role in lung cancer, we performed F-actin staining and in vitro invasion assays. Ectopic overexpression of ANXA2 in CL1-0 resulted in a dramatically increased invasive capacity (Fig. 5A) and promoted filopodia formation (Supplementary Fig. S4) as compared with the control cells (empty vector). In contrast, knocking down ANXA2 expression in CL1-5 resulted in decreased cell invasion ability; a similar

Figure 3. Effects of KMT1E level on cancer cell metastasis in vivo. A, noninvasive lung cancer cells transfected with si-sc or si-KMT1E were labeled (CM-Dil, red) and ectopically injected into the yolk sac of two dpf Tg(FLI1:EGFP) zebrafish embryos. White arrows, primary injected site. B, quantification of metastatic cell in the trunk from fish injected with si-sc or si-KMT1E cells (n = 48/group). C, representative images of lung from injected mice 4 weeks later. CL1-0 and KMT1E-knockout (CL1-0 KMT1E−/−) cells were injected into tail vein of SCID mice. D, quantification of metastatic nodules in the lung from mice injected control (CL1-0) or CL1-0 KMT1E−/− (n = 5/group). E, H&E staining of lung sections from mice injected with control (CL1-0) or CL1-0 KMT1E−/−. Black arrow, the metastatic nodule. Immunoblot analysis of injected cells with indicated antibody. The levels of tubulin (Tub) and histone H3 (H3) were used as loading controls. RF, red fluorescent field; GF, green fluorescent field; BF, bright field. Data represent three independent experiments. Scare bar 50 μm. **, P < 0.01; *, P < 0.05.
KMT1E in CL1-0 cells increased the activity of the ANXA2 promoter approximately 1.5-fold (Fig. 5C). Moreover, the KMT1E(H1224A) mutant was ineffective in suppressing ANXA2 promoter activity in CL1-5; similar results were observed in HEK 293T and A549 cells (Supplementary Fig. S6). To investigate whether ectopic expression of KMT1E was sufficient to regulate the amount of ANXA2 in cancer metastasis, we analyzed the effect of KMT1E level on ANXA2 expression in cancer cells. As seen in Fig. 5D, the immunoblotting showed that in the KMT1E RNAi-knockdown CL1-0 cells ANXA2 protein level was increased by more than 3-fold compared with scramble control. In contrast, overexpression of KMT1E reduced ANXA2 protein level in CL1-5 cells (Fig. 5D).

To investigate whether KMT1E reduces cell invasion through inhibition of ANXA2, we performed a rescue experiment. We cotransfected ANXA2 in KMT1E-transfected cells and subjected the cell line to invasion assays. As shown in Supplementary Fig. S7, the capacity to invade was rescued in cotransfected cells. These findings suggested that ANXA2 was a direct and functional target of KMT1E in lung cancer cell metastasis.

KMT1E physically interacts with the SMAD2/3 repressor complex on the ANXA2 promoter

To further address the mechanism of how KMT1E modulates ANXA2 expression in lung cancer cells, we performed an ANXA2-reporter assay. In Fig. 6A, we show that deleted upstream region −449 to −281 in ANXA2 significantly increased promoter activity. This observation led us to examine whether the promoter region between −449 and −281 is critical for the repression of the ANXA2 promoter by KMT1E. To test this possibility, ChIP was applied to mapping the KMT1E-binding locus on ANXA2 promoter regions. As shown in Fig. 6B, the KMT1E-binding site and the H3K9Me2 modification were enriched in the promoter-proximal region (p2, −376 to −100) of the promoter in CL1-0 cells.

On the basis of the above observations, we hypothesized that this region contains a consensus-binding site for transcription factors. Indeed, we found that the ANXA2 promoter contained a CAGA motif (−355 to −362), which is a SMAD3 consensus-binding site. SMAD3 is a transcription factor that mediates the TGFβ signaling pathway and plays a critical role in malignant invasion, inflammation, and fibrosis. Here, we found that RNAi knockdown of SMAD3 also represses F-actin polymerization and filopoid formation in highly metastatic lung cancer cells (Supplementary Fig. S5C). To test the possibility that KMT1E and SMAD3 act cooperatively to repress ANXA2 promoter activity, we conducted an IP experiment in HEK 293T cells. The cells were cotransfected with plasmids expressing HA-tagged SMAD3 and Flag-tagged KMT1E. We then performed IP with HA or Flag antibody followed by immunoblotting with the indicated antibody as shown in Fig. 6C. The results showed that KMT1E physically interacts with SMAD3 (lane 2, Fig. 6C). A series of different deletion mutants of KMT1E were used to map the interaction domain of SMAD3. We cotransfected KMT1E various deletion mutants with pCMV1a-HA-SMAD3 into HEK 293T cells. IP results showed that HA-SMAD3 interacted well with Flag-KMT1E (658-1291) but that this interaction was poor with Flag-KMT1E (709-1291); compare lanes 3

result was also observed in A549 cells (Fig. 5B). Moreover, inhibition of F-actin polymerization and filopodia formation was observed in the highly metastatic CL1-5 cell line with si-ANXA2 transfection (Supplementary Fig. S5B). The results show that ANXA2 plays a crucial role during lung cancer metastatic progression.

Next, to study whether KMT1E modulates ANXA2 expression, we performed a luciferase assay to analyze the effect of KMT1E level on transcription activity of the ANXA2 promoter. The activity of ANXA2 promoter was suppressed by 40% in KMT1E-transfected CL1-5 cells. Conversely, knocking down KMT1E in CL1-0 cells increased the activity of the ANXA2 promoter approximately 1.5-fold (Fig. 5C). Moreover, the KMT1E(H1224A) mutant was ineffective in suppressing ANXA2 promoter activity in CL1-5; similar results were observed in HEK 293T and A549 cells (Supplementary Fig. S6). To investigate whether ectopic expression of KMT1E was sufficient to regulate the amount of ANXA2 in cancer metastasis, we analyzed the effect of KMT1E level on ANXA2 expression in cancer cells. As seen in Fig. 5D, the immunoblotting showed that in the KMT1E RNAi-knockdown CL1-0 cells ANXA2 protein level was increased by more than 3-fold compared with scramble control. In contrast, overexpression of KMT1E reduced ANXA2 protein level in CL1-5 cells (Fig. 5D).

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This implies that residues 568 to 709 of KMT1E are required for its interaction with SMAD3.

To further examine the role of SMAD3 in KMT1E-mediated ANXA2 repression, we performed ChIP assays on CL1-0 cells. The SMAD3 protein level is low in CL1-0 cells (Supplementary Fig. S8); therefore, we have transfected HA-SMAD3 into this cell line. The results showed that HA-SMAD3 is enriched in the proximal region (p2, −100 to −376) of the ANXA2 promoter, which is similar to the KMT1E and H3K9Me2-binding locus (Fig. 6D). We also performed ChIP assays on CL1-5 cells that ectopically express Flag-KMT1E. As expected, KMT1E, H3K9Me2, and SMAD3 were enriched in the p2 region of the ANXA2 promoter. To further examine whether KMT1E and SMAD3 associate with each other and form a repressor complex on the ANXA2 promoter, we performed a re-ChIP experiment. The results showed that KMT1E and SMAD3 both were enriched in the proximal region (p2, −100 to −376) of the ANXA2 gene promoter (Fig. 6B). These findings demonstrated that KMT1E/SMAD3 forms a repressor complex and inhibited ANXA2 transcriptional activity.

Next, to elucidate the potential role of both KMT1E and SMAD3 on ANXA2 suppression, we performed ChIP experiments in SMAD3 siRNA knockdown CL1-0 cells. These experiments showed that depletion of SMAD3 resulted in a diminished/reduced binding by KMT1E protein on the promoter of ANXA2 in CL1-0 cells. This observation led us to examine whether KMT1E also physically interacts with the other receptor SMAD proteins (R-SMADs). Among the R-SMADs, SMAD2 and SMAD3 are structurally highly similar and mediate TGFβ signals. To test this possibility, we performed re-ChIP assays on CL1-0 cells. As expected, re-ChIP experiment results indicated that KMT1E and SMAD2/3 were enriched in the proximal region (p2, −100 to −376) of the ANXA2 gene promoter (Fig. 6E).

These observations led us to test the effect of the KMT1E/SMAD2/3 complex on ANXA2. To this end, we performed ChIP assays in CL1-0 cells that had been transfected with specific siRNA to knock down the expression of SMAD2 or SMAD3. The results indicated that knockdown of SMAD2 expression resulted in decreased KMT1E-binding on ANXA2 promoter. Moreover, knocking down SMAD2 and SMAD3 together resulted in less KMT1E-binding ability on the ANXA2 gene promoter (Fig. 6F). These findings demonstrate that ANXA2 is negatively regulated by KMT1E/SMAD2/3 repressor complex and that KMT1E physically associates with SMAD2/3 in lung cancer cells.
Discussion

Metastasis is a crucial step in cancer progression and development. Many studies have reported that histone methylation is strongly associated with tumorigenesis and poor prognosis (25, 26). Histone methylation status is dynamic, and regulated through various mechanisms. Recent publications have provided evidence showing that HDMTs and JMJD proteins are required for tumorigenesis (27, 28). Here, we showed that KMT1E, a H3K9MT, acts as a crucial repressor in lung cancer metastasis progression by forming a complex with SMAD2/3 and suppressing AXN2, which, in turn, modulates actin polymerization.

Recently, publications have indicated that KMT1E was found to be a bona fide oncogene undergoing gene amplification-associated activation in lung tumorigenesis (29). The same study also showed that ectopic expression of KMT1E in A549 cells significantly promoted cell invasion. Other studies have found that KMT1E is a H3K9MT involved in the transcriptional silencing of euchromatic genes in cervical and breast cancer cells (11, 21). These findings suggest that KMT1E acts as a repressor of invasion-suppressing genes in cancer cells. Strikingly, Rodriguez-Paredes and colleagues reported that 18 genes have been identified that significantly increased upon KMT1E depletion in both H1437 and DMS-273 cells. Among the 18 genes, 11 genes are known to promote cell invasion in a variety of cancers; these include ANXA3, FGFR2, and IL6 (30–32). These observations led us to propose that KMT1E may play different roles in cancer invasion.

In this study, we found that protein levels of KMT1E are significantly decreased in highly metastatic cancer cell lines (Fig. 1A and Supplementary Fig. S8). Furthermore, the expression of KMT1E was high in tissue samples during the early stages of lung cancer. The results suggest that KMT1E is a proto-oncogene during the early stage of lung cancer. Interestingly, we also observed a significant increase in the invasion ability of A549 cells when transfected with KMT1E (data not shown). These findings strongly suggest that KMT1E plays a significant role in lung cancer metastasis. As seen in Fig. 3, in vivo xenograft experiments showed that loss of KMT1E in non-invasive lung cancer cells promotes cellular invasion. We also determined that KMT1E significantly repressed filopodia formation in lung cancer cells (Fig. 4). Taken together, our results suggest that KMT1E plays different roles in cancer invasion in different cell types, acting both as a suppressor or activator.

During cancer progression, the intermediate filament proteins that mediate filopodia formation are important for tumor migration, invasion, and angiogenesis (22, 23). Up to now, many studies have demonstrated that actin rearrangement and filopodia formation are directly regulated and can be affected by a range of proteins, including the small GTPase, cochlin, and LIM kinase, all of which are critical to tumorigenesis and cancer metastasis (33, 34).

Recent studies also indicated that AXN2 is involved in actin cytoskeletal rearrangement, which is mediated via tyrosine phosphorylation and interaction with a small GTPase (16, 35). In cancer cells, abnormal expression of AXN2 has been associated with cancer progression and metastasis (17, 36–38). One study has indicated that AXN2 promotes cancer cell migration and invasion via F-actin polymerization change of the microstructures in cancer cells (39). Recently, dysregulation of AXN2 has been found to be associated with the metastatic cancer cell state, which suggested that the gene could serve as a diagnostic marker for metastasis (40), although in prior studies, the mechanisms regulating AXN2 transcription remained unknown. As seen in Fig. 4, KMT1E negatively regulates intermediate filaments and represses filopodia formation and cancer cell migration. KMT1E is a H3K9MT in regulating H3K9 methylation and transcriptional silencing, as expected, decreased H3K9Me level in KMT1E-knockdown cells and AXN2 level was increased (Fig. 5D). Here, we have shown that AXN2 is epigenetically regulated via histone modification by KMT1E.

The TGFβ signaling pathway regulates a range of cellular processes, including cell proliferation, differentiation, apoptosis, adhesion, and migration. During cancer progression, TGFβ plays a dual role, acting both as a tumor suppressor and promoter (41–43). How TGFβ functions as a tumor suppressor and driver during tumor development remains to be elucidated. Recently, Chou and colleagues proposed a model in ovarian cancer involving TGFβ/SMAD4 signaling-mediated epigenetic silencing that results in changes in various associated histone modification, which, in turn, affects the epithelial-to-mesenchymal transition (44). Another study showed that SMAD3 also interacts with a KMT that is recruited to a target locus and that represses gene expression (45). Here, we determined that KMT1E physically interacts with SMAD2/3 in vivo (Fig. 6C).

Ectopic expression of Flag-KMT1E in highly invasive lung cancer cells and ChIP analysis results in significant enrichment of SMAD3, which is linked to KMT1E-binding and H3K9Me2 modification within the p2 region of AXN2 (Fig. 6D). Furthermore, the re-ChIP experiments also demonstrated that KMT1E and SMAD2/3 bind each other and form a repression complex on the AXN2 promoter (Fig. 6E). These observations together strongly support the hypothesis that SMAD2/3 is the

![Figure 6](#)
cofactor of KMT1E, and the KMT1E/SMAD2/3 repressor complex can be recruited to the ANXA2 promoter during cancer progression. As seen in Supplementary Table S3, we have indicated the KMT1E DNA-binding sequences in CL1-0 cells as determined by ChIP-seq. Among the conserved motif sequences, the SMAD-binding motif ranked first. The SMAD-binding element (CAGAG) also located approximately within 1 kbp upstream of transcription start site in the list of genes (Supplementary Table S4). Genome-wide analyses of KMT1E/SMAD2/3 complex-binding sites should be done to explore the molecular mechanism of cancer metastasis in the future.

In summary, we showed that KMT1E plays a critical role in lung cancer invasiveness. We also indicated that, at the molecular level, epigenetic suppression of ANXA2 by a KMT1E/SMAD2/3 repressor complex leads to the inhibition of lung cancer metastasis. Findings provide important mechanistic insights into how TGFβ promotes lung cancer metastasis, with possible implications for understanding how to target this broadly important pathway in cancer progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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H3K9 Histone Methyltransferase, KMT1E/SETDB1, Cooperates with the SMAD2/3 Pathway to Suppress Lung Cancer Metastasis

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