A regulatory circuit composed of DNA methyltransferases and receptor tyrosine kinases controls lung cancer cell aggressiveness

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Abstract

Overexpression of DNMT1 and KIT is prevalent in lung cancer, yet the underlying molecular mechanisms are poorly understood. While the deregulated activation of DNMT1 or KIT has been implicated in lung cancer pathogenesis, whether and how DNMT1 and KIT orchestrate lung tumorigenesis are unclear. Here, using human lung cancer tissue microarrays and fresh frozen tissues, we found that the overexpression of DNMT1 is positively correlated with the upregulation of KIT in tumor tissues. We demonstrated that DNMT1 and KIT form a positive regulatory loop, in which ectopic DNMT1 expression increases, whereas targeted DNMT1 depletion abrogates, KIT signaling cascade through Sp1/miR-29b network. Conversely, an increase of KIT levels augments, but a reduction of KIT expression ablates, DNMT1 transcription by STAT3 pathway leading to in-parallel modification of the DNA methylation profiles. We provided evidence that KIT inactivation induces global DNA hypomethylation, restores the expression of tumor suppressor p15INK4B through promoter demethylation; in turn, DNMT1 dysfunction impairs KIT kinase signaling. Functionally, KIT and DNMT1 co-expression promotes, whereas dual inactivation of them suppresses, lung cancer cell proliferation and metastatic growth in vitro and in vivo, in a synergistic manner. These findings demonstrate the regulatory and functional interplay between DNA methylation and tyrosine kinase signaling in propelling tumorigenesis, providing a widely applicable approach for targeting lung cancer.

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Competing Interests: The authors state no conflict of interest.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)
Introduction

Lung cancer, an extremely heterogeneous group of human malignancies, is a multi-step process involving genetic (e.g., tyrosine kinase mutations) and epigenetic (e.g., DNA methylation) alterations. DNA methylation takes place mainly at the C5 position of cytosines within CpG dinucleotides and is catalyzed by DNA methyltransferases (DNMTs). It is typically believed that DNMT3a and DNMT3b are de novo methyltransferases that newly methylate cytosines\(^1\), but DNMT1 is a maintenance DNMT that is responsible for transferring DNA methylation patterning from parental to daughter cells at cell division.\(^2\), \(^3\) However, certain findings reveal that DNMT1 is also an active de novo methyltransferase that cell cycle-independently initiates CpG methylation in cancer cells.\(^2\), \(^4\), \(^5\) In accordance, ectopic \textit{DNMT1} expression leads to genome-wide DNA hypermethylation,\(^6\), \(^7\) whereas genetic or pharmacological inhibition of \textit{DNMT1} expression leads to DNA hypomethylation and the reexpression of epigenetically-silenced tumor suppressor genes (TSGs) coupled by cell cycle arrest, apoptosis and differentiation.\(^8\) - \(^10\)

The prevalent upregulation of \textit{DNMTs}, particularly \textit{DNMT1}, strongly associates with shorter survival and serves as an independent prognostic factor in lung cancer.\(^11\) - \(^13\) Mechanistically, \textit{DNMT1} overexpression silences TSGs through promoter DNA hypermethylation, thus conferring a survival advantage to lung cancer cells. A few studies show that multiple factors controlling \textit{DNMT1} gene are increased during cell division and \textit{DNMT1} levels reach a maximum during S phase, suggesting a cell cycle-dependent \textit{DNMT1} regulation.\(^14\) - \(^16\) However, these findings are insufficient to explain why \textit{DNMT1} was highly expressed in quiescent lung cancer cells and nuclear run-on assays failed to detect obvious change in DNMT1 transcriptional activity throughout the cell cycle. Given that many cells (e.g., cancer stem cells) in lung tumors are quiescent and/or slow-dividing, we propose that cell cycle-independent regulation of \textit{DNMT1} gene is indispensable in lung tumorigenesis.

Constitutively active receptor tyrosine kinases (RTKs) are usually detected in lung cancer patients.\(^17\), \(^18\) The hyperactive KIT, a class III RTK family member, could result from both gene mutations and overamplification, but the relatively low incidence of \textit{KIT} mutations suggests its overamplification to be more essential for lung cancer pathogenesis. In fact, \textit{KIT} overexpression has been documented in the development, progression and drug resistance of lung cancer and survival in some patient subpopulations.\(^17\) - \(^21\) Inhibitors (e.g., PKC412) for aberrant KIT signaling have been tested in various preclinical and clinical models,\(^22\) - \(^26\) but they did not bring long-lasting benefits. Given that inhibitor-associated gene overamplification is a frequent event,\(^19\), \(^27\) - \(^29\) focusing on \textit{KIT} gene regulation could gain better understanding of lung cancer molecular biology and subsequently develop more efficient cancer therapies.

While, as independent entities, the roles of epigenetic dysregulation and hyperactive tyrosine kinases in lung cancer have been studied to a certain extent, the reciprocal gene regulations and functional cooperation between these oncogenic pathways remain elusive. This study aims to test the hypothesis that DNA methylation machinery and KIT kinase signaling may cooperatively regulate lung tumor aggressiveness. That is, KIT kinase may partially
transform cells by fostering epigenetic silencing of TSGs. Likewise, through induction of kinase hyperactivity, DNMT1 may facilitate its own persistent activation in cancer cells. We report here that, in lung cancer cells, DNMT1 and KIT form a regulatory circuit consisting of KIT, Sp1/miR-29b, STAT3 and DNMT1, whose intercommunication promotes lung tumorigenesis. Dual-genetic and dual-pharmacological inhibition of DNMT1 and KIT leads to a more profound inhibition of tumor cell growth in vitro and in vivo. These findings support that the DNMT1-KIT loop is a promising therapeutic target in lung cancer.

Results

DNMT1 upregulation is positively correlated with KIT overexpression in lung cancer cells

To determine whether expression of DNMTs and KIT is upregulated in lung cancer, human NSCLC TMAs were immunostained using anti-DNMTs and anti-KIT, and the target levels were quantified using H-scores. Representative images are shown in Figure 1a and Supplementary Figure S1a. Compared to the normal adjacent tissues, the tumor tissues displayed higher levels of KIT (53%), DNMT1 (69%), DNMT3a (71%) and DNMT3b (71%) (Supplementary Figure S1b), consistent with the previous reports. When correlated the gene levels with disease stages, H-scores for DNMTs and KIT were significantly higher in later stage (Figure 1b and Supplementary Figure S1c). Importantly, higher levels of KIT were accompanied by DNMT overexpression, but lower KIT expression was observed in tumor tissues carrying lower DNMTs (Figure 1c and Supplementary Figure S1d), indicating a positive correlation between these variables. In support of DNMT1 abundance in DNA methylation, DNMT1 and 5mC levels displayed an in-parallel correlation in tumor tissues (Figure 1d). Although KIT is positively related to all three DNMTs, we mainly focused on DNMT1, because DNMT1 carries both maintenance and de novo DNA methylation activity, and its levels serve as an independent prognostic factor in NSCLC. To this end, we assessed DNMT1 and KIT RNA expression in fresh-frozen NSCLC (never smoker) and matched adjacent non-tumor lung tissues (n=30) and in 10 lung cancer cell lines. In agreement with IHC staining results, DNMT1 and KIT was 2.3±0.8 and 5.7±0.5 fold higher, respectively, in tumors than normal tissues (Figure 1e). All tested cell lines displayed higher KIT and DNMT1 expression compared to normal fibroblast MRC-5 (Figure 1f). In line with this, DNMT1 and KIT protein levels were 2.4-fold and 2.7-fold higher, respectively, in fresh-frozen lung tumors than their adjacent normal control (n=7) (Supplementary Figure S1e and 1f). The correlation analysis revealed that DNMT1 and KIT mRNA expression was positively associated in 30 pairs of patient samples (Figure 1g) and 10 cell lines (Figure 1h), but their protein levels exhibited a trend toward positive correlation in patient tissues (n=7, Supplementary Figure S1g). These results support the regulatory and functional interactions between DNMT1 and KIT in lung cancer cells.

DNMT1 upregulates KIT through Sp1/miR-29b network

Given the positive correlations of DNMT1 and KIT expression in lung cancer cells, we proposed the existence of a regulatory loop between DNA methylation and KIT signaling, in which KIT activity controls the expression of DNMT1, whose alteration modifies KIT transcription in parallel. To test this, DNMT1 was knocked down in NSCLC H1299...
(carcinoma) and H1975 (adenocarcinoma) cells by a Smart siRNA pool containing 4 specific siRNAs or the shRNA3 that showed the most DNMT1 reduction (>80%, Supplementary Figure S2a). DNMT1 depletion significantly decreased global DNA methylation (Supplementary Figure S2b) and KIT expression followed by the dephosphorylation of AKT (Figure 2a), a KIT downstream effector.31 In contrast, enforced DNMT1 expression resulted in the increased DNA methylation (Supplementary Figure S2c) and the upregulation of KIT coupled by AKT hyperphosphorylation (Figure 2b). Because p53 function is impaired in H1975 (p53 mutation) and H1299 (p53 null) cells, to address if KIT signaling in the context DNMT1 involves p53 function, we manipulated DNMT1 in NSCLC A549 cells (wild-type p53), and independently verified the findings from H1975 and H1299 cells (Supplementary Figure S2d), supporting a limited role of p53 in DNMT1-related KIT expression. Finally, we tested whether DNMT1 regulates additional kinases, such as KRAS, EGFR and MET that are important for lung tumorigenesis, and found that DNMT1 ablation impaired expression of these three kinases (Supplementary Figure S2e). Collectively, DNMT1 is a positive regulator for tyrosine kinases in lung cancer.

To elucidate how DNMT1 regulates the KIT gene, we focused on the Sp1/miR-29b network, because Sp1/miR-29b has been shown to regulate KIT transcription in leukemia,31 and miR-29b was silenced in lung cancer.11 First, quantification of IHC-stained TMAs revealed Sp1 overexpression in tumor versus normal tissues with the highest levels in stage III disease (Figure 2c and Supplementary Figure S2f). Second, Sp1 and DNMT1 expression in tumors showed a positive association (Supplementary Figure S2g, left). Third, DNMT1 abrogation reduced (Figure 2d, left), whereas its overexpression increased (Figure 2d, right), the levels of Sp1 and KIT, supporting DNMT1 as a positive Sp1 regulator. Moreover, Sp1 depletion impaired expression of KIT and DNMTs (Figure 2e). Given a positive correlation of Sp1 with KIT (Supplementary Figure S2g, right), these findings identified DNMT1-Sp1 axis as KIT gene regulator. Fourth, to understand how DNMT1 regulates Sp1, we initially examined if DNMT1 regulates miR-29b, a Sp1 repressor,8, 31 and found that miR-29b expression was restored upon DNMT1 inactivation (Figure 2f, left). As no CpG islands were identified in miR-29b promoter (not shown), miR-29b upregulation might occur in a DNA methylation-independent manner through DNMT1-associated NFκB or c-Myc, the reported miR-29b suppressors.31, 32 Indeed, similar to overexpression of wild-type DNMT1, enforced expression of catalytically inactive DNMT1 did not rescue miR-29b suppression (Figure 2f, right). Further, DNMT1 knockdown disrupted the physical interaction of DNMT1 with NFκB or DNMT1 with c-Myc (Figure 2g), which is crucial for their transcriptional activities. Finally, miR-29b induction abolished Sp1 and KIT expression (Figure 2h; Supplementary Figure S2h), where DNMT3a and DNMT3b were used as positive controls, and attenuated DNMT1-induced Sp1 upregulation (Figure 2i), supporting that DNMT1 upregulates Sp1 through miR-29b suppression. Based on these, we conclude that KIT gene is governed by a DNMT1-Sp1/miR-29b cascade.

**Tyrosine kinases positively regulate DNA methylation**

To explore the contribution of KIT to DNA methylation, we initially knocked down KIT by siRNA in H1975 and H1299 cells and observed AKT dephosphorylation (Supplementary Figure S3a) coupled by a remarkable DNMT downregulation (Figure 3a). This was verified.
by the results from shRNA-triggered KIT depletion (Supplementary Figure S3b). In contrast, KIT overexpression upregulated DNMT1 gene (Figure 3b). Consistent with the role of Sp1/miR-29b in KIT and DNMT1 regulation,9, 31 enforced KIT expression increased, but KIT knockdown suppressed, Sp1 expression (Supplementary Figure S3c), which was in contrast to the change of miR-29b levels (Supplementary Figure S3d). Consequently, targeted KIT depletion decreased (Figure 3c, left; Supplementary Figure S3e), whereas ectopic KIT expression increased (Figure 3c, right), global DNA methylation, in line with the positive correlation of KIT with 5mC levels in lung tumors (Supplementary Figure S3f). Notably, while DNMT1 is classically believed to be cell cycle-related enzyme,2, 3 we did not see obvious changes in cell cycle in cells with KIT knockdown or overexpression (not shown). This supports the notion that the alterations of DNMT1-dependent DNA methylation via KIT manipulation occur in a cell cycle-independent manner and really reflect the changes of DNMT1 gene abundance or/and enzymatic activity.

Because TSGs are known to be silenced by promoter DNA hypermethylation in NSCLC, we used p15INK4B, an inhibitor of cyclin-dependent kinases (INK) that plays a critical role in growth inhibition and cell differentiation, as readout to determine whether kinases regulate TSG p15INK4B. qPCR revealed that KIT knockdown restored, but KIT overexpression further silenced, p15INK4B expression (Figure 3d). Using bisulfite sequencing, we checked the methylation status of p15INK4B promoter (-4 to +535) in H1975 cells. Because +221 to +535 region was barely methylated (Supplementary Figure S3g), we focused on the region from -4 to +247. KIT depletion reduced p15INK4B promoter DNA methylation from 11% to 4%, but KIT overexpression increased it from 12% to 19% (Figure 3e). Finally, depletion of KRAS, EGFR and MET by their respective shRNAs (Supplementary Figure S3h) led to STAT3 dephosphorylation with a concurrent DNMT1 downregulation and DNA demethylation (Supplementary Figure S3i). Together, the tyrosine kinases serve as unconventional DNA methylation regulators in lung cancer.

**STAT3 activity is responsible for KIT-driven DNMT1 upregulation**

To understand how KIT regulates DNMT1 gene, we decided to target STAT3 signaling, because KIT significantly regulates STAT3 phosphorylation.33 Once phosphorylated, STAT3 binds specific DNA elements regulating target expression.34 Given the existence of STAT3 binding elements in DNMT1 gene promoter, we proposed that DNMT1 expression in lung cancer cells is, at least partially, governed by the KIT-STAT3 axis. Indeed, KIT depletion decreased STAT3 phosphorylation in H1975 and H1299 cells (Supplementary Figure S3j). STAT3 overexpression enhanced (Figure 3F, right), whereas STAT3 dephosphorylation by siRNA (Figure 3F, left), shRNA (Supplementary Figure S3k) or its selective inhibitor NSC74859 (Figure 3G) remarkably suppressed, DNMT1 expression. Mechanistically, 1) ChIP analysis revealed that enforced STAT3 expression enhanced, while STAT3 knockdown diminished, the binding of total- and phospho-STAT3 in DNMT1 promoter (Figure 3H); 2) reporter assays showed that ectopic STAT3 expression increased (Figure 3I, left), whereas NSC74859 treatment decreased (Figure 3I, right), the luciferase activities driven by DNMT1 promoter region containing STAT3 binding elements, supporting that STAT3 binds and transactivates DNMT1 promoter in lung cancer cells. Finally, STAT3 dephosphorylation by NSC74859 and siRNA/shRNA reduced global DNA methylation (Figure 3J, Supplementary

*Oncogene. Author manuscript; available in PMC 2018 March 04.*
Figure S3l), substantiating the role of STAT3 in DNMT1 regulation and suggesting STAT3 inactivation as an alternative approach to achieve DNA demethylation. Collectively, STAT3 signaling bridges the gap between KIT and DNMT1-dependent DNA methylation in lung cancer.

**DNMT1 and KIT cooperatively modulate lung tumor cell proliferation in vitro**

The above results demonstrate that DNMT1 and KIT form a positive regulatory circuit in lung cancer cells. Such positive regulation insinuates a likely biological link and cooperative features in controlling tumor cell growth. To clarify as to whether such biological communication of DNMT1 and KIT impacts cancerous lesions, we first assessed the effects of single gene manipulation on lung cancer cells. We found that depletion of KIT or DNMT1 in H1975 cells remarkably decreased colony number and restrained cell migration (Supplementary Figure S4a). Conversely, enforced expression of KIT or DNMT1 enhanced the clonogenic and wound-healing potential (Supplementary Figure S4b), and, in a time-dependent manner, largely increased the rate of cellular proliferation (Supplementary Figure S4c). To rule out the off-target effects, we transfected H1299 cells with individual shRNA targeting three different region of the DNMT1 or KIT gene. As expected, knockdown of DNMT1 or KIT by individual shRNA led to similar phenotypes, including reduction of colony number and impairment of wound-healing capability (Supplementary Figure S4d and e). Importantly, the rescue experiments revealed that overexpression of DNMT1 cDNA attenuated the inhibition of wound healing mediated by shRNA-triggered DNMT1 knockdown in H1299 and H1975 cells (Supplementary Figure S4f and g). Together, these results support the notion that, as independent events, the hyperactive DNMT1 or KIT signaling pathway functions as lung cancer promoting factor.

To test the effects of DNMT1 and KIT interaction on tumor cell growth, we transfected H1975 cells with KIT or/and DNMT1 siRNA at suboptimal doses (50 nM). The siRNA co-transfection resulted in a synergistic downregulation of KIT and DNMT1 (Figure 4a and Supplementary Figure S5a), leading to a more profound DNA demethylation (Figure 4b). Functionally, DNMT1 and KIT co-knockdown synergistically decreased colony number (Figure 4c) and attenuated wound healing (Figure 4d). In contrast, co-transfection with KIT and DNMT1 expression vectors resulted in the most robust increase of their protein expression (Figure 4e and Supplementary Figure S5b) and global DNA methylation (Figure 4f) coupled by the higher colony number (Figure 4g) and the enhanced wound-healing potential (Figure 4h). These findings support that DNMT1 and KIT are functionally interactive in regulating cancer cell growth.

**The combined expression of KIT and DNMT1 synergistically accelerates lung tumor cell growth and metastasis in vivo**

To determine if DNMT1 and KIT orchestrate tumor growth, we overexpressed DNMT1 or/and KIT in H1975 cells. About 1×10^6 transfected cells were subcutaneously injected into nude mice and the experiments were terminated at day 28 after engraftment. Compared to single gene, DNMT1 and KIT co-expression had a more potent effect on tumorigenesis, as indicated by the enhanced tumor volume (control, 54±7 mm^3; DNMT1, 243±73 mm^3; KIT, 231±84 mm^3; DNMT1 plus KIT, 512±127 mm^3) and the increased tumor weight (control,
56±6 mg; DNMT1, 266±26 mg; KIT, 248±25 mg; DNMT1 plus KIT, 465±51 mg) (Figure 5a–c). Moreover, KIT and DNMT1 co-expression led to the largest lesion area (control, 5±2%; DNMT1, 36±6%; KIT, 42±9%; DNMT1 plus KIT, 85±8%) and the most metastatic foci (control, 1.7±0.5; DNMT1, 9.7±1.5; KIT, 11.0±3.0; DNMT1 plus KIT, 20.0±4.1) in lungs (Figure 5d and 5e). Histological examination of tumor sections using Ki-67 or H&E staining revealed that the co-expression mice displayed higher levels of tumor proliferation than other groups (Figure 5f). Mechanistic investigation showed that tumor cells from DNMT1 and KIT co-transfection group exhibited the highest levels of DNMT1, KIT and 5mC with the least levels of E-cadherin, a known suppressor of tumor cell invasion and migration (Figure 5f). Altogether, these findings support that KIT overexpression communicates with DNMT1 upregulation to induce more aggressive lung cancer.

**Dual inactivation of KIT and DNMT1 leads to synergistic blockage of lung tumorigenesis and pulmonary metastatic growth**

While the synergistic effects of KIT/DNMT1 co-expression in nude mice clearly support their functional interplay in lung cancer, the recipient mice are immunodeficient. To this end, we employed the C57BL/6 mouse with competent immune system and LL/2, which is syngeneic to C57BL/6 with lung metastatic potential. We initially transfected LL/2 cells with mouse DNMT1 or KIT siRNA and found, similar to the results from H1975 cells, DNMT1 depletion impaired KIT expression and phosphorylation; in contrast, KIT knockdown decreased DNMT1 expression (Supplementary Figure S6a). We then knocked down both KIT and DNMT1 in LL/2 cells (Supplementary Figure S6b, left and middle) and evidenced a synergistic reduction of global DNA methylation (Supplementary Figure S6b, right), tumor cell migration (Supplementary Figure S6c) and colony number (Supplementary Figure S6d), compared to single gene depletion.

To examine the in vivo effects of dual knockdown of DNMT1 and KIT, the transfected LL/2 cells (0.5×10^6) were subcutaneously injected into C57BL/6 mice (n=6 mice/group), which were sacrificed at day 20 post- engraftment. Compared to the mice injected with the single agent alone, the mice injected with cells with DNMT1 and KIT co-depletion had the smallest tumor volume (scramble, 585±87 mm^3; DNMT1, 260±39 mm^3; KIT, 247±31 mm^3; DNMT1 plus KIT, 39±6 mm^3) and the least tumor weight (scramble, 643±80 mg; DNMT1, 276±41 mg; KIT, 245±28 mg; DNMT1 plus KIT, 123±16 mg) (Figure 6a, 6b and Supplementary Figure S6e) and also displayed the fewest nodules (scramble, 21.2±5.5; DNMT1, 11.5±5.4; KIT, 10.0±2.6; DNMT1 plus KIT, 1.2±1.2) and the smallest lesion area (scramble, 71±7%; DNMT1, 40±8%; KIT, 35±11%; DNMT1 plus KIT, 8±5%) in lung (Figure 6c and d). Moreover, as shown in Figure 6e, KIT/DNMT1 co-suppression resulted in the lowest levels of tumor cell proliferation (evident by Ki-67 and H&E staining), suggesting that KIT/DNMT1 co-dysfunction has more robust effects on blocking lung tumorigenesis in vivo, which might take place through the synergistic suppression of KIT and DNMT1 oncogenic pathways, as supported by the lowest levels of KIT, DNMT1 and 5mC, and the highest E-cadherin expression in co-knockdown group. In agreement with the dual-depletion results, treatment with the combination of decitabine and PKC412 led to more robust inhibition of cell migration in H1975 cells expressing relatively higher level of DNMT1 and KIT, but no obvious changes in cell migration of H1650 cells expressing relatively lower
level of them (Supplementary Figure S7), supporting an idea that the double-targeted therapy might be more efficient in NSCLC cells highly expressing both DNMT1 and KIT, which merits systematic investigations. Importantly, such combination approach in tumor-bearing mice achieved greater therapeutic outcomes, as demonstrated by the smallest tumor volume (control, 1559±380 mm$^3$; PKC412, 855±223 mm$^3$; decitabine, 894±212 mm$^3$; decitabine plus PKC412, 431±153 mm$^3$), the least tumor weight (control, 1865±203 mg; PKC412, 1069±180 mg; decitabine, 992±276 mg; decitabine plus PKC412, 413±102 mg) and the fewest nodules (control, 25±4; PKC412, 12±3; decitabine, 14±4; decitabine plus PKC412, 6±2) in lung (Supplementary Figure S8). Collectively, these findings further strengthen the idea of DNMT1-KIT crosstalk in regulating the invasive and migratory abilities of lung cancer cells.

**Discussion**

We and others have previously shown that abnormal DNMT or kinase activities are functionally essential for cancer pathogenesis.\(^8\),\(^31\),\(^35\) However, whether and how DNMT1 and KIT genes are reciprocally regulated in cancer cells, and whether and how DNMT1 and KIT deregulation orchestrates cancerous lesions remain intriguing. In the present study, we used lung cancer as readout model and provided a series of evidence underscoring two important concepts. On the one hand, DNMT1 and KIT form a regulatory circuit, in which, KIT positively regulates DNA methylation through STAT3 pathway; in turn, DNMT1 reinforces KIT kinase cascade via Sp1/miR-29b network; on the other hand, DNMT1 and KIT functionally cooperate in provoking lung tumorigenesis, and dual inactivation of them results in more robust cancer cell growth inhibition in vitro and tumor regression in vivo. Thus, DNMT1-KIT vicious circle acts as a key regulator of cell fate decision in cancer pathogenesis representing an attractive target for designing innovative therapeutic strategies.

Our initial motivation in studying aberrant kinase in concert with epigenetic programming in lung cancer came from our previous demonstrations that 1) oncogene abundance, when compared to mutations, could be better therapeutic target;\(^9\),\(^31\) 2) disruption of oncogenic expression abolishes the enzymatic activity, regardless of mutation status; 3) Sp1 and miR-29b are involved in the transcriptional regulation of DNMT1 and KIT, respectively.\(^8\),\(^9\),\(^11\),\(^31\) In the present study, we showed that DNMT1 and KIT levels were much higher in lung tumor tissues and cell lines than in normal counterparts. Importantly, KIT expression was positively correlated with DNMT1 expression at both protein and RNA levels, together, leading to our hypothesis that DNMT1 and KIT have regulatory interactions in controlling their cellular levels and functional cooperation in the regulation of cancer cell growth. In fact, modulation of DNMT1 activity positively changed the functions of multiple kinase signaling; conversely, KIT expression upregulated DNMT1 gene leading to an increase of global DNA methylation and further p15$^{INK4B}$ silencing by promoter DNA hypermethylation. In support of this, KIT inactivation decreased the levels of DNMT1 expression and global DNA methylation with a concurrent p15$^{INK4B}$ re-expression through promoter DNA hypomethylation. Functionally, we demonstrated that, as individual events, overexpression of DNMT1 or KIT increased, whereas inhibition of them impaired, lung cancer cell expansion, consistent with previous reports that upregulation of DNMT1 or KIT predicts poor prognosis.\(^11\)-\(^13\),\(^20\) More importantly, dual-manipulation of DNMT1 and KIT
function displayed more robust effects on tumor cell growth in vitro and tumorigenesis in vivo. Given that EGFR, MET and KRAS positively regulate DNMT1-dependent DNA methylation or vice versa, our findings establish a novel mechanistic model describing epigenome-kinome crosstalk in controlling cancer cell survival and proliferation.

It is postulated that DNMT1 upregulation partially involves the deregulation of miRs (e.g., miR-29b) or the disruption of p53/Sp1 interaction. Although AKT1 kinase has also been found to phosphorylate and stabilize DNMT1 protein, a comprehensive characterization of whether and how tyrosine kinases regulate DNMT1 transcription has not been yet initiated. Our data uncovered KIT functions as additional modulators for, not only specific genomic loci (e.g., \( p15^{INK4B} \)), but also the global DNA methylation, through DNMT1 transactivation in lung cancer. In support of this, ectopic KIT expression increased the levels of DNMT1 and DNA methylation, while KIT ablation diminished DNMT1-dependent DNA methylation and restored \( p15^{INK4B} \) expression. Mechanistically, we demonstrated that KIT regulates DNMT1 gene, at least partially, through the STAT3 pathway, because KIT significantly modulates STAT3 functions, whose changes paralleled DNMT1 levels. Given that DNMT1 can be phosphorylated on multiple sites by kinases, like AKT or PKC isoforms, and as AKT is one of KIT downstream effectors, we can’t exclude the possibility that KIT may indirectly phosphorylate and stabilize DNMT1 protein, another latent mechanism controlling KIT-driven DNA hypermethylation. Collectively, these results define KIT as a non-classical epigenetic regulator in lung cancer.

KIT overamplification strongly associates with an aggressive tumor phenotype in lung cancer, but how KIT gene is upregulated remains elusive. The positive correlation of KIT with DNMT1 expression in lung cancer cells raises the possibility that DNMT1 overexpression could be one cause of KIT upregulation, which has not yet been demonstrated. We provide compelling evidence that DNMT1 positively regulates KIT gene, mechanistically, through Sp1/miR-29b loop. Notably, DNMT1 manipulation positively changed Sp1 levels, but negatively altered miR-29b expression. Given that miR-29b indirectly suppresses DNMT1 gene, the current findings complete an DNMT1/miR-29b negative loop through Sp1 in lung cancer. Additionally, we previously reported that Sp1 is an upstream regulator of DNMT1 gene, here we identified DNMT1 as Sp1 gene activator, thus finalizing Sp1/DNMT1 positive circuit in lung cancer. Although this possibility has not been evaluated, DNMT1 may regulate KIT via the alterations of promoter DNA methylation, because Sp1 binding elements (CG-rich region) on KIT promoter have been shown to be modified by DNA methylation. Given the multiple CpG islands at KIT promoter, it is likely that KIT expression is partially attributed to promoter DNA methylation in lung cancer cells, thus raising a question of how Sp1/miR-29b network and promoter DNA methylation achieve a balance to ensure that KIT levels meet its cellular functions. The answers to this question are new insights into the cooperative features of epigenome and kinome in cancer biology.

In summary, our results demonstrate a vicious circle between DNMT1 and KIT in determining lung cancer cell fate. Such regulatory and functional cooperation is not restricted to a specific pathway, but possibly extended to the kinome and epigenome. Not only do these findings add a new layer to the complexity of molecular processes regulating
cancerous lesions, but also highlight the capability of tyrosine kinases to modulate DNA methylation; in turn, DNA methyltransferases to alter kinase cascades. Given the highly heterogeneous features of lung tumorigenesis, it remains unclear why certain lung cancer subtypes exhibit highly expressed kinases or DNMTs, but not both simultaneously. Further elucidation promises to be informative about the molecular rules governing epigenetic and kinase aberrations.

Materials and Methods

Plasmids and chemicals

\(STAT3\) expression plasmid was constructed by cloning the PCR fragments into the HindIII and XhoI sites of pcDNA4 vector. \(KIT\) expression plasmid was obtained by inserting \(KIT\) gene sequence into pBABE-puro retroviral vector. Construction of \(DNMT1\) luciferase reporter was obtained by subcloning promoter fragment (−1048/+36) containing two STAT3 binding sites (−194/−186: TTTACTCAA and −423/−416: TTAAAAAA) into the KpnI and HindIII sites of pGL3-basic. The catalytically inactive \(DNMT1\) (C1226W, cysteine to tryptophan) plasmid was generated using a QuikChange XL Site-directed mutagenesis Kit (200521, QuikChange, Agilent Technologies, Santa Clara, CA). Primer sequences are listed in Supplemental table. All constructs were verified by DNA sequencing. The shRNA and scramble vectors for \(DNMT1\), \(KIT\) and \(STAT3\) were obtained from BMGC RNAi (University of Minnesota). Synthetic \(miR-29b\) (AM00LRA3) or negative scrambled control (AM17111) were purchased from Ambion. The On-target\textsuperscript{plus} Smart pool siRNAs containing a mixture of 4 oligonucleotides for \(KIT\) (L-003150), \(DNMT1\) (L-004605), \(Sp1\) (L-026959) and their scramble oligos were purchased from GE Healthcare. \(STAT3\) siRNA and scramble (sequences in Supplemental table) were synthesized by Integrated DNA Technologies. PKC412 (P-7600) and nilotinib (N-8207) were purchased from LC Laboratories (Woburn, MA), decitabine (A3656) and procainamide (P9391) from Sigma-Aldrich (St. Louis, MO), and NSC74859 (S3I-201) from SelleckBio (Houston, TX).

Cell lines and transfections

Cell lines (H1975, H1299, A549, H1650, H520, H460, H441, H358, H69, H82, LL/2 and MRC-5) were obtained from American Type Culture Collection (Manassas, Virginia). LL/2 was cultured in DMEM, human normal lung fibroblast MRC-5 in EMEM and others in RPMI1640 containing 10% fetal bovine serum (FBS, 16140-071, Life Technology, Waltham, MA). Cell lines were newly purchased with no further authentication and no further testing for mycoplasma. The transfection of siRNAs (100 nM), \(miR-29b\) (100 nM), and expression and shRNA vectors (2 μg) were performed as previously described.\textsuperscript{29,40,41}

Lung cancer tissue microarray

Lung cancer tissue microarray (TMA) slides were obtained from US Biomax (LC10012, Rockville, MD). The TMAs contain 45 cases of non-small cell lung carcinoma (NSCLC, 26 cases of squamous cell carcinoma, 18 cases of adenocarcinoma, 1 case of large cell carcinoma) at different stages (18 cases of Stage I, 16 cases of Stage II, 10 cases of Stage III and 1 case of Stage IV), and adjacent tissue, 5 normal tissues with duplicate cores per case. The slides were stained with antibodies from Abcam: anti-DNMT1 (ab19905),
anti-DNMT3a (ab2850) and anti-DNMT3b (ab13604); Cell Signaling Technology: anti-KIT (3074); Santa Cruz Biotechnology (Dallas, Texas): anti-Sp1 (sc-14027), and quantified as previously described.29,41

Western blot
After the various treatments, the whole cellular lysates were prepared by harvesting the cells in 1×cell lysis buffer.29,40,42 The antibodies used are from Santa Cruz Biotechnology: anti-Sp1 (sc-59) and anti-β-Actin (sc-1616); Cell Signaling Technology: anti-KIT (3392S), anti-phospho-KIT (Tyr719, 3391L), anti-AKT, anti-phospho-AKT (Ser473, 9272), anti-STAT3 (9139), and anti-phospho-STAT3 (Tyr705, 9131); New England Biolabs: anti-DNMT1 (M0231L, Ipswich, MA); EMD Millipore (Billerica, MA): EGFR (04-281); Proteintech: MET (19971-1-AP, Rosemont, IL) and Abcam: anti-KRAS (ab180772).

Chromatin immunoprecipitations (ChIP)
ChIP was performed in H1975 cells transfected with STAT3 expression, shRNA or control vectors (2 μg) as previously described.31,42 The anti-STAT3 (9139) and anti-phospho-STAT3 (Tyr705, 9131) (Cell Signaling Technology) were used.

RNA isolation, cDNA preparation and qPCR
RNA was isolated using miRNAeasy Kit (217004, Qiagen) and cDNA was synthesized by SuperScript® III First-Strand Synthesis System (18080-051, Invitrogen). qPCR was performed by TaqMan (DNMT1, KIT, Sp1) or SYBR-Green (4309155, p15INK4B) master mix, which was normalized by 18S levels. The primer sequences are listed in Supplemental table. For miR-29b expression, qPCR was conducted by TaqMan microRNA assays (4427975) and normalized by U44/48.31,41

In vivo tumor growth and metastasis assays
C57BL/6 and athymic nude mice (male, 4–6 weeks old) were purchased from the Harlan Laboratories. All animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee. In co-depletion or co-expression experiments, the transfected LL/2 (0.5×10⁶) or H1975 (1×10⁶) cells were injected into C57BL/6 or nude mice, respectively. In therapeutic experiments, LL/2 (0.5×10⁶) were injected into C57BL/6 mice. Drugs were prepared just prior to administration by dissolving it in ethanol to give a clear solution and diluting with PEG400 and saline (ratio 15:38:47). When the tumor size approached 50 mm³, the tumor-bearing mice were given by intraperitoneal injection three times per week of 25 mg/kg decitabine or 5 mg/kg PKC412 (suboptimal) as single or in combination form for 2 weeks. Mice were sacrificed at indicated time points and excised lungs were fixed with 10% formaldehyde. The tumors were fixed in 10% neutral buffered formalin for 24 hours at 4°C and transferred to phosphate buffered saline for paraffin embedded blocks. The histopathological examination was performed as previously described.29,41
Statistical analysis

The quantification for target changes was performed using the Student’s t test. The percentage of comparison between low and high gene expression in TMAs was calculated using the χ² test. Correlation data were determined by Pearson correlation coefficients. Differences were considered statistically significant at P < 0.05. All P values were determined by unpaired, two-tailed Student’s t test (details in Supplementary Methods).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by Hormel Foundation (S. Liu) and National Cancer Institute (Bethesda, MD) grants R01CA149623 (S. Liu) and R21CA155915 (S. Liu).

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Figure 1.
DNMTs and KIT are frequently overexpressed and positive correlated in lung cancer. (a) IHC staining for human NSCLC TMA. Representative micrographs with the H-score for each group are shown (magnification ×200). (b) Graph indicates gene expression levels at the different stages of lung cancer. Data were analyzed using one-way ANOVA followed by Dunnett’s test; Horizontal bars indicate the mean±SE. (c) Scatter plot shows the correlation of KIT with DNMT1. (d) Scatter plot shows the correlation of DNMT1 with 5mC. (e and f) qPCR for the expression of KIT and DNMT1 in fresh-frozen human lung cancer tissues (n=30, e) or in human lung cancer cell lines (f). (g) Scatter plot showing the correlation of KIT with DNMT1 derived from (e). (h) Scatter plot showing the correlation of KIT with DNMT1 derived from (f). Note: In b, c and d, Y- or X-axis represents the H-Score derived from IHC staining in (a); In c, d, g and h, R: Pearson correlation coefficients; R²: means
“the goodness of fit”. Statistical significance was calculated by Pearson correlation coefficients.
Figure 2.
DNMT1 positively regulates KIT expression by Sp1/miR-29b network. (a and b) H1975 and H1299 cells were transfected with DNMT1 siRNA (a, left), shRNA vectors (a, right) or expression plasmids (b) for 48 hours and the total cell lysates were subjected to Western blot. (c) NSCLC TMA was stained with anti-Sp1. Representative micrographs with the H-score for each group are shown (×200). (d) Western blot in H1975 and H1299 cells transfected with DNMT1 siRNA (left) or expression vector (right) for 48 hours. (e) H1975 and H1299 cells were transfected with Sp1 siRNA and subjected to Western blot. (f) qPCR assessing miR-29b expression in H1975 and H1299 cells transfected with DNMT1 siRNA or treated with decitabine (left) or in H1975 cells transfected with DNMT1 wild-type or mutant expression vectors (right) for 48 hours. *P<0.05, **P<0.01. (g) H1975 cells were transfected with DNMT1 siRNA for 48 hours and subjected to immunoprecipitation followed by Western blot. Input, the whole cell lysates. (h) Western blot in H1975 cells transfected with miR-29b for 48 hours. (i) qPCR to assess Sp1 expression in H1975 and H1299 cells transfected with DNMT1 expression vector for 24 hours and miR-29b for another 24 hours. Note: si, siRNA; sh, shRNA.
Figure 3.
KIT kinase activity contributes to DNA methylation. (a and b) H1975 and H1299 cells were transfected with KIT siRNA (a) or expression vector (b) for 48 hours and subjected to Western blot. (c) H1975 and H1299 cells were transfected with KIT siRNA (left) or expression vectors (right) for 48 hours and the genomic DNA was subjected to Dotblot. **P<0.01. (d) qPCR for p15INK4B expression in H1975 cells transfected with KIT siRNA (left) or expression vectors (right) for 48 hours. (e) Bisulfite sequencing of p15INK4B promoter in H1975 cells transfected with KIT siRNA (upper) or expression vectors (lower) for 48 hours. CpG locations are indicated as vertical bars in the promoter and first exon of p15INK4B.
*p15<sup>INK4B</sup>*. Arrows indicate the bisulfite sequencing region (transcription start site +147 to +221). Open circles indicate unmethylated CpG sites, solid circles indicate methylated CpG sites. Results of 10 clones are presented. (f) Western blot in H1975 cells transfected with STAT3 siRNA (left) or expression vectors (right) for 48 hours. (g) H1975 cells were treated with 30 μM NSC74859 for 24 hours and subjected to Western blot. (h) ChIP assay in H1975 cells transfected with STAT3 siRNA (upper) or expression vector (lower) for 48 hours. The change of STAT3 binding in *DNMT1* promoter was assessed by qPCR. (i) left: H1975 cells were transfected with pGL3-*DNMT1* alone or plus STAT3 vectors for 48 hours; right: H1975 cells were transfected with pGL3-*DNMT1* vector 24 hours and NSC74859 was added for further 24 hours, which was followed by the measurement of luciferase activity. (j) Dotblot to measure global DNA methylation in H1975 cells transfected with STAT3 siRNA (upper) for 48 hours or treated with NSC74859 for 24 hours (lower). Note: pSTAT3, phosphorylated STAT3; si, siRNA; Con, loading control.
Figure 4.

DNMT1 and KIT cooperatively regulate mobility and aggressiveness of lung cancer cells in vitro. a-d, H1975 cells were transfected with scramble, DNMT1, KIT or both siRNA, (a) Western blot using total protein lysates; (b) Representative pictures of Dotblot (lower) and the relative densitometric intensities (upper) using genomic DNA; (c) Representative pictures of colony-forming assay (lower) and the quantification of colonies (upper); (d) Representative pictures of wound-healing assay (lower) and the quantification of distance migrated (upper). e-h, H1975 cells were transfected with empty, KIT, DNMT1 or both expression vectors, (e) Western blot; (f) Representative pictures of Dotblot (lower) and the relative densitometric intensities (upper); (g) Representative pictures of colony-forming assay (lower) and the quantification of colonies (upper); (h) Representative pictures of wound-healing assay (lower) and the quantification of distance migrated (upper). Error bars represent SD. *P<0.05; **P<0.01. In b to d, f to h, the abbreviations of SC, EM, D, K and D+K are scramble, empty, DNMT1, KIT and DNMT1+KIT; Con, loading control; d and h, scale bars, 1 mm.
Figure 5.
Co-overexpression of DNMT1 and KIT confers enhanced tumorigenicity. (a) H1975 cells were transfected with empty, DNMT1, KIT, or both expression vectors. At 6 hours after transfection, the transfected cells (1×10^6) were subcutaneously injected into nude mice (n=6 tumors/group). Pictured are the representative external views of tumor-bearing nude mice at day 28 after implantation. (b) Pictured are the tumors dissected at day 28 after implantation. Total 6 tumors from each group are shown. Scale bar, 5mm. (c) The graph represents the mean tumor volume at the indicated days during the experiment (left). The tumor weight graphs derived from nude mice injected with transfected H1975 cells (right), n=6 tumors/group. (d) Representative images of lungs and H&E staining for boxed lesion. Arrows indicate focal tumor nodules on lung surfaces (n=3 mice/group). (e) The quantification of lesion area (left) and tumor nodules (right) from lungs (n=3 mice/group). (f) Representative H&E or IHC staining of tumor sections from tumor-bearing nude mice. Note: For c and e, values represent the mean+SD. *P<0.05.

Oncogene. Author manuscript; available in PMC 2018 March 04.
Figure 6.
Co-depletion of KIT and DNMT1 synergistically contributes to in vivo reduced tumorigenic potential and metastatic ability. (a) LL/2 cells were transfected with scramble, DNMT1, KIT and both siRNA. At 6 hours after transfection, the transfected cells (0.5×10^6) were subcutaneously injected into the C57BL/6 mice. The photographs of tumors derived from transfected LL/2 cells and dissected at day 20 after implantation (n=6 tumors/group). Scale bar, 5mm. (b) Growth curves (left) and weight graph (right) of tumors derived from the indicated gene types, n=6 tumors/group. Error bars represent SD. *P<0.05. (c) left: representative images of lung from mice injected with transfected LL/2 cells. Arrows indicate focal tumor nodules on the lung surfaces; right: H&E staining images of lung sections. The boxed regions are magnified (×200) and shown beside, n=6 mice/group. (d) Graphs show the quantification of lesion area (upper) and metastatic foci (lower) in the
different group of treatments reported in (e). Horizontal bars indicate the mean±SD. (e) representative H&E or IHC staining of tumor sections.