RFTS-deleted DNMT1 enhances tumorigenicity with focal hypermethylation and global hypomethylation

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Abbreviations: TSGs, tumor suppressor genes; DNMT1, DNA methyltransferase 1; RFTS, replication foci targeting sequence; HELP, HpaII tiny fragment enrichment by ligation-mediated PCR; KEGG, Kyoto Encyclopedia of Genes and Genomes; COSMIC, catalog of somatic mutation in cancer

Site-specific hypermethylation of tumor suppressor genes accompanied by genome-wide hypomethylation are epigenetic hallmarks of malignancy. However, the molecular mechanisms that drive these linked changes in DNA methylation remain obscure. DNA methyltransferase 1 (DNMT1), the principle enzyme responsible for maintaining methylation patterns is commonly dysregulated in tumors. Replication foci targeting sequence (RFTS) is an N-terminal domain of DNMT1 that inhibits DNA-binding and catalytic activity, suggesting that RFTS deletion would result in a gain of DNMT1 function. However, a substantial body of data suggested that RFTS is required for DNMT1 activity. Here, we demonstrate that deletion of RFTS alters DNMT1-dependent DNA methylation during malignant transformation. Compared to full-length DNMT1, ectopic expression of hyperactive DNMT1-D^RFTS caused greater malignant transformation and enhanced promoter methylation with condensed chromatin structure that silenced DAPK and DUOX1 expression. Simultaneously, deletion of RFTS impaired DNMT1 chromatin association with pericentromeric Satellite 2 (SAT2) repeat sequences and produced DNA demethylation at SAT2 repeats and globally. To our knowledge, RFTS-deleted DNMT1 is the first single factor that can reprogram focal hypermethylation and global hypomethylation in parallel during malignant transformation. Our evidence suggests that the RFTS domain of DNMT1 is a target responsible for epigenetic changes in cancer.

Introduction

Methylation of cytosine bases in CpG dinucleotides is an epigenetic modification in mammals involved in development, imprinting and X chromosome inactivation, which becomes dysregulated in carcinogenesis. Increased DNA methylation in promoter regions is highly associated with transcriptional silencing and hypermethylation-mediated silencing of tumor suppressor genes (TSGs) is a critical epigenetic driver of cancer. However, in addition to site-specific hypermethylation, a reduction of total cytosine methylation of the genome, targeted in part to repetitive elements, is also common in malignancies. Global hypomethylation may be a driver of cancer by promoting genomic instability and oncogene activation. Although both hypermethylation and hypomethylation are found in cancer, the molecular mechanisms that link these alterations are not clear.

DNA methyltransferase 1 (DNMT1) is the enzyme most responsible for the maintenance of DNA methylation patterns during DNA replication. In addition to maintenance methylation activity, DNMT1 can perform de novo DNA methylation as do DNMT3A and DNMT3B. Dysregulated DNMT1 is capable of promoting hypermethylation or hypomethylation and, indeed, DNMT1 has been reported to be up-regulated or down-regulated in different types of cancer. The methyltransferase activity of DNMT1 has been considered the critical effector in reprogramming cancer methylation patterns. However, enforced overexpression of DNMT1 produces hypermethylation of TSGs and increases methylation of the whole genome. Conversely, deficient DNMT1 causes global hypomethylation without site-specific hypermethylation. Thus, changing the expression of DNMT1 alone is insufficient to recapitulate the linked and vectorially opposite epigenetic alterations observed in cancer.

DNMT1 consists of a series of globular domains N-terminal to the catalytic domain, which are implicated in functions essential for catalytic activity, protein association and target specificity. By interacting with different proteins, DNMT1 may be enriched in or dissociated from specific genomic loci with the distribution of DNMT1 determining the methylation status of specific target sites. For example, the oncogenic PML-RAR fusion
protein recruits DNMT1 to the RARβ2 promoter to stimulate methylation, whereas disruption of the DNMT1-proliferating cell nuclear antigen (PCNA) interaction results in hypomethylation of cellular DNA. Seemingly, both enzyme activity and chromatin occupancy of DNMT1 are important for DNMT1-dependent changes in DNA methylation observed during tumorigenesis.

The replication foci targeting sequence (RFTS) was defined as an N-terminal domain required for associating DNMT1 with replication foci. Multiple lines of evidence suggested that RFTS is a positively acting domain required for DNMT1 function. Surprisingly, recombinant forms of DNMT1 including the RFTS domain bind DNA poorly, while specific deletion of RFTS activates DNA binding. Moreover, using a fluorogenic methylation assay, we showed that deletion of RFTS leads to a 640-fold increase in methylation activity due to relief of DNA-competitive inhibition by RFTS. Further biochemical and structural experiments support RFTS as a DNA-competitive endogenous inhibitor of DNMT1 that must be removed from the DNA active site for DNMT1 activity. In contrast to our biochemical insights into negative regulation by RFTS, CXXC was proposed as a nonmethylated DNA-binding inhibitor of DNMT1 activity. Human genetics has the potential to help resolve DNMT1 domain function because an autosomal dominant hereditary sensory and autonomic neuropathy (HSAN1E) maps to the RFTS domain. This mutation is associated with genomic hypomethylation and promoter hypermethylation.

In this study, we investigate the functional impact of 2 DNMT1 regulatory domains, RFTS and CXXC. Here we show that expression of full-length and various deletion mutants resulted in degrees of promoter hypermethylation, chromatin condensation, and transcriptional repression. Ectopic expression of DNMT1-DΔRFTS—at a level equivalent to endogenous DNMT1—led to the most malignant phenotype, which required the presence of the CXXC domain for oncogenic activity. Surprisingly, DNMT1-DΔRFTS cells further showed a global hypomethylation phenotype reminiscent of the malignant state. These data suggest that tumor-specific modulation of RFTS function may mediate the commonly observed linkage of focal hypermethylation and global hypomethylation.

**Results**

Deletion of RFTS enhances the oncogenic activity of DNMT1

To dissect the functional role of 2 regulatory domains in human DNMT1, we established stable cell lines in non-malignant human bronchial epithelial cells (HBEC3) that expressed full-length DNMT1 (DNMT1 cells), RFTS-deleted DNMT1 (DNMT1-DΔRFTS cells), CXXC-deleted DNMT1 (DNMT1-DΔCXXC cells), RFTS/CXXC double-deleted DNMT1 (DNMT1-DΔR/C cells), or vector alone (vector cells). mRNA and protein validation showed that each DNMT1 construct was expressed at levels equivalent to that of endogenous DNMT1 in HBEC3 (Fig. 1A). Because DNMT1 has protooncogenic properties, we evaluated transformation markers of the resulting cell lines. There was no significant proliferation difference between vector cells and DNMT1-expressing cells, with or without supplementation of epidermal growth factor (EGF) (Fig. S1A). We then examined whether DNMT1 overexpression could alter invasive activity, a hallmark of cancer metastasis (Fig. S1B). Although invasion was slightly enhanced in all DNMT1-expressing cells, only DNMT1-DΔRFTS and DNMT1-DΔR/C cells showed a significant difference compared to vector cells, suggesting that deletion of the RFTS domain provides an invasive advantage.

To further analyze the oncogenic properties of these cells, we examined anchorage-dependent growth (Fig. 1B). Overexpression of all forms of DNMT1 increased the number of colonies.
observed. However, whereas DNMT1 cells produced 2-fold more colonies, DNMT1–ΔRFTS cells produced 3-fold more colonies. We further tested the anchorage-independent colony-forming activity of these cells by soft-agar colony formation (Fig. 1C). Because HBEC3 is a non-malignant cell line, vector cells produced fewer than 5 colonies per dish. However, DNMT1 cells produced 30 colonies and DNMT1–ΔRFTS cells produced 130 colonies in soft agar. Interestingly, though DNMT1–ΔRFTS cells elevated soft-agar colony-forming activity, removing the CXXC domain in DNMT1–ΔRFTS cells completely abolished the effect. These data indicate that the CXXC domain is essential for the oncogenic activity of DNMT1–ΔRFTS. Thus, DNMT1 acts as weak oncoprotein, whose transforming activity is enhanced by deletion of the inhibitory RFTS domain.

Promoter hypermethylation and transcriptional silencing of DAPK and DUOX1 is driven by DNMT1

Several TSGs are subject to methylation-associated silencing in lung cancer (Table S1). To test whether introduction of ectopic DNMT1 alleles is sufficient to alter DNA methylation of these genes, we examined the methylation status of promoter-associated CpG islands and transcript levels for each of 24 TSGs. Of these genes, DAPK41,42 and DUOX143 were consistently hypermethylated and silenced by all DNMT1 alleles. By performing methylated DNA immunoprecipitation (MeDIP), we found a 2-fold increase in methylation of the DAPK promoter in all DNMT1-expressing cells (Fig. 2A). Bisulfite sequencing indicated that there were few methylated CpGs in vector cells (1.6%) (Fig. 2B). DNMT1 and DNMT1–ΔRFTS overexpression upregulated methylation more than 10-fold to 17.6% and 24.6%, respectively. Although there was no apparent difference between DNMT1 and DNMT1–ΔRFTS from MeDIP analysis, bisulfite sequencing showed that DNMT1–ΔRFTS expression drove greater methylation of the DAPK promoter than did wild-type DNMT1. Furthermore, we analyzed the association between DNMT1 and the DAPK promoter region by DNMT1 chromatin immunoprecipitation (ChIP) (Fig. 2C). We found increased chromatin occupancy of DNMT1 at the DAPK promoter in all DNMT1-expressing cells.

The transcript levels of DAPK were consistent with its increased promoter methylation. Compared to the vector control, expression of DAPK was decreased in all DNMT1-expressing cells, among which DNMT1–ΔRFTS cells showed the greatest repressive effect (Fig. 2D). A similar correlation in DNA methylation, DNMT1 chromatin occupancy and mRNA expression were also found in DUOX1 in all DNMT1-expressing cells (Fig. 2). In agreement with the transformation result, DNMT1–ΔRFTS overexpression resulted in the greatest effect on hypermethylation-mediated silencing of DAPK and DUOX1, indicating that RFTS-deleted DNMT1 is a gain of function mutant. Deletion of the 2 different regulatory domains of DNMT1 did not change target preference and chromatin occupancy of DNMT1. In DNMT1-expressing cells, the same target genes were affected with different degree of methylation, which could be attributed to differences in DNA methyltransferase activity.31

**Figure 2.** DNMT1–ΔRFTS promotes increased methylation and silencing of the DAPK and DUOX1 genes. (A) The methylation levels of DAPK (left) and DUOX1 (right) promoter-associated CpG islands were analyzed by qPCR. Methylated DNA was analyzed using the MethylMiner kit and amplified with specific primers. (B) Bisulfite sequencing results for DAPK (left) and DUOX1 (right) promoters. White squares represent unmethylated cytosines and black squares represent methylated cytosines in CpG sites. The percentage of methylated CpG dinucleotides from 8 independent clones is indicated. (C) DNMT1 chromatin occupancy was analyzed using DNMT1 ChiP and qPCR. (D) mRNA levels of DAPK (left) and DUOX1 (right) were analyzed by RT-qPCR and normalized to vector cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001 in comparison to vector cells.

Strong alleles of DNMT1 condense chromatin structure at the DAPK and DUOX1 promoters

To test whether RFTS and CXXC domains alter the ability of DNMT1 to condense chromatin structure, we analyzed the sensitivity of the DAPK and DUOX1 promoter regions to DNase
DNA demethylating agent 5-aza-deoxycytidine (5-aza-dC) reverses gene silencing and diminishes the transformation ability of strong DNMT1 alleles

We have demonstrated that the expression of DAPK and DUOX1 is inhibited concurrently with an increase in promoter methylation. We tested whether this methylation-mediated repression is reversible by treating cells with the DNA demethylating agent 5-aza-dC and analyzing expression of DAPK and DUOX1. As expected, 5-aza-dC treatment significantly increased DAPK and DUOX1 levels in DNMT1-expressing cells (Fig. 4A). Moreover, 5-aza-dC-treated DNMT1 and DNMT1-ΔRFTS cells completely lost activity in anchorage-independent growth (Fig. 4B), indicating that increased methylation-dependent gene regulation is responsible for the oncogenic properties of DNMT1 and DNMT1-ΔRFTS and is reversible.

Genome-wide promoter methylation analysis reveals that DNMT1-ΔRFTS cells produce a methylation pattern similar to DNMT1 cells, though more intense

Targeted screening of TSGs indicated that DAPK and DUOX1 promoters are hypermethylated in all DNMT1-expressing cells. DNA from vector, DNMT1 and DNMT1-ΔRFTS cells were used in the high-resolution HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) assay. This genomic methylation array of 720,001 probes was designed to focus on CpG islands near transcription start sites. The Pearson Correlation test showed that the majority of methylation intensity signals was similar in all cell lines (Fig. S2A). We then analyzed DNA methylation profiles by pairwise comparisons of DNMT1, DNMT1-ΔRFTS and vector control cells. As seen in the volcano plots (Fig. 5A and S2B), there were hyper- and hypomethylated sequences in both DNMT1-expressing cells, with DNMT1-ΔRFTS cells exhibiting greater changes. To systematically analyze methylation targets, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to examine the top 1,000 hypermethylated or hypomethylated genes in DNMT1-ΔRFTS cells compared to vector cells (Table S2). Pathway analysis indicated that DNMT1-ΔRFTS drove increased methylation of specific genes involved in cell adhesion, migration and signaling pathways, which are highly related to carcinogenesis. In addition, hypomethylated gene targets in DNMT1-ΔRFTS cells were enriched in lysosomal functions and enzymes in phenylalanine and tyrosine metabolism.

We generated heat maps to compare methylation differences in all 3 sample sets by selecting fragments with more than 4-fold signal changes between DNMT1-ΔRFTS and vector cells (Fig. 5B). This big-data visualization analysis showed that DNMT1 and DNMT1-ΔRFTS cells share similar methylation targets and that DNMT1-ΔRFTS cells produces a higher degree of methylation. The DNMT1 and DNMT1-ΔRFTS cells clustered together, suggesting that the RFTS domain does not control locus specificity but limits the degree of DNA modification, just as it limits in vitro activity.

DNMT1-ΔRFTS cells exhibit genomic hypomethylation

The epigenetic signature of cancer is regional hypermethylation with global hypomethylation. We thus analyzed fractional methyl cytosine content by HPLC (Fig. 6A). Previous studies have shown that highly overexpressed DNMT1 causes genomic hypermethylation. However, in our DNMT1 cells in which ectopic expression is at endogenous levels, there was not a...
significant increase in genomic DNA methylation. In contrast, DNMT1-DRTFS cells, which displayed the highest levels of focal hypermethylation, exhibited reduced overall methyl cytosine content in comparison to vector or other DNMT1-expressing cells. We considered that the main site of demethylation might occur in Satellite 2 repeat sequences (SAT2) for 3 reasons. First, the RFTS domain mediates association of DNMT1 to pericentromeric heterochromatin to maintain dense methylation\(^47,48\) and SAT2 is the most abundant repeat in the region.\(^49\) Second, SAT2-specific hypomethylation has been found in DNMT1-null cells\(^50,51\) and in patients with RFTS-mutated DNMT1.\(^38\) Third, DNA hypomethylation and RNA up-regulation of SAT2 are highly associated with various cancers and contributes to genomic instability.\(^49\) To test whether SAT2 is hypomethylated in our cell lines, we performed bisulfite sequencing (Fig. 6B and C). SAT2 methylation was significant reduced from 66% (vector) and 63% (DNMT1) to 48% in DNMT1-DRTFS cells. Further, we analyzed chromatin occupancy of DNMT1 on SAT2 loci by DNMT1 ChIP (Fig. 6D). The data indicate that DNMT1-DRTFS expression reduced association between DNMT1 and SAT2 loci. Unlike what was observed for SAT2, there was no significant methylation change in LINE1 DNA repeats (Fig. S3). Therefore, specific demethylation of SAT2 might be due to the impaired association between DNMT1-DRTFS and the pericentromeric region. Our results suggest that demethylation of SAT2 and promoter hypomethylation detected in the HELP assay might both contribute to reduced genomic methylation observed in DNMT1-DRTFS cells. Moreover, we investigated whether SAT2 DNA hypomethylation is associated with transcription. Indeed, expression of SAT2 non-coding RNA was increased in DNMT1-DRTFS cells, but was not altered in cell lines with alleles of DNMT1 that are weaker (Fig. 6E).

DNMT1-DRTFS expression has similar effects in H358 lung cancer cells

To rule out a cell-specific effect, we established stable cell lines in H358 cells to determine the effect of DNMT1-DRTFS expression in malignant cells (Fig. 5A). DNMT1-DRTFS expression enhanced the proliferation, invasion and soft-agar colony growth of the cells, while full-length DNMT1 expression behaved similarly to vector control cells (Fig. S4B-D). Moreover, ectopic expression of DNMT1-DRTFS slightly inhibited expression of...
DNMT1 in immortalized HBEC3 cells to examine their oncogenic potential and alteration in DNA methylation. Full-length DNMT1 expression triggered cellular transformation while DNMT1-ΔRFTS functioned as a stronger oncoprotein. The oncogenic effects of DNMT1-ΔRFTS depended on the presence of the CXXC domain, which is apparently a positive factor. Deletion of either regulatory domain resulted in the same apparent target preference; expression of all DNMT1 alleles increased methylation of DAPK and DUOX1 promoters. DNMT1 and DNMT1-ΔRFTS cells share similar hypermethylated targets in genome-wide analysis as well, though deletion of RFTS increased the degree of DNA methylation.

Given previous findings that overexpressed or activated DNMT1 caused non-specific genomic hypermethylation, 15-17 we surprisingly discovered that DNMT1-ΔRFTS expression at endogenous levels led to demethylation of SAT2 and in the genome. This finding is consistent with a previous study on RFTS-mutated DNMT1, 38 in which point mutations in the RFTS domain caused SAT2 and genomic hypomethylation. The study also showed that mutations in the RFTS domain of DNMT1 impaired binding with heterochromatin. 38 We confirmed the impaired association between DNMT1 and SAT2 loci in DNMT1-ΔRFTS cells by DNMT1 ChIP. Because DNMT1 may function as an oligomeric complex, 52 RFTS-deleted DNMT1 and endogenous DNMT1 may form a heterooligomer that is impaired in association with pericentromeric heterochromatin. Thus, we suggest a model in which deletion of the RFTS domain activates DNMT1 for euchromatic DNA-binding, but decreases chromatin occupancy of DNMT1 to heterochromatic SAT2 loci by virtue of a missing protein interaction, leading to passive DNA demethylation (Fig. 7). Searching the catalog of somatic mutation in cancer (COSMIC) database, 26 mutation sites within the RFTS domain of DNMT1 were found (Table S3). These DNMT1 mutants might promote malignancy by increasing DNA methyltransferase binding and activity on euchromatic DNA while being disadvantaged in pericentromeric SAT2 association and methylation.

RFTS-targeted DNMT1 associated proteins (RAPs) are likely to participate in these mechanisms. To our knowledge, there are at least 3 known RAPs including ubiquitin-like containing PHD and RING finger domain protein 1 (UHRF1), 29,30,35 ubiquitin-specific-processing protease 7 (USP7) 53 and N-α-acetyltransferase 10 NatA catalytic subunit (NAA10). 54 These proteins have been shown to recruit DNMT1 to specific loci and stimulate its methylation activity, causing site-specific hypermethylation. Moreover, these proteins were found up-regulated in lung cancers, 54-56 suggesting that release of RFTS domain inhibition might drive cancer formation via hypermethylayion. If this is the case, targeting of these binding partners could be a promising therapeutic strategy to limit DNMT1-dependent hypermethylation in cancer.

Because of the link between hyperactive DNMT1 and transcriptional repression of TSGs, DNMT1-mediated DNA hypermethylation is emerging as a crucial therapeutic target. 57,58 The current approach is to inhibit expression or hyperactivity of DNMT1. 59,61 However, demethylating agents lead to unavoidable non-specific genomic demethylation.

Discussion

We proposed that the RFTS domain is inhibitory by virtue of DNA-competitive association with the DNMT1 active site, 61 while other data suggested that the CXXC domain is DNA-competitive and inhibitory. 37 Here, we hypothesized that expression of hyperactive DNMT1 lacking an autoinhibitory domain could enhance transformation by altering DNA methylation. Thus, we modestly expressed full-length and deletion forms of DNMT1 in immortalized HBEC3 cells to examine their

![Figure 6. Genomic hypomethylation is found in DNMT1-ΔRFTS cells. (A) 5-methylcytosine (mC) content of the total cytosine pool was determined by HPLC. (B) Bisulfite sequencing of SAT2. White squares represent unmethylated CpGs, black squares represent methylated CpGs, and gray squares represent undetermined sites. Each row is an independent sequencing result. (C) Quantitation of SAT2 bisulfite sequencing. (D) DNMT1 chromatin occupancy was analyzed using DNMT1 ChIP and qPCR. (E) Expression of SAT2 non-coding RNA was analyzed by RT-qPCR and normalized to vector cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001 in comparison to vector cells.](www.landesbioscience.com)
causing genomic instability, DNA damage, oncogene reactivation, and promote opportunities for cancer progression.62-65 Because the RFTS domain functions as a key regulator of DNMT1 function, targeting RFTS interactions may revert euchromatin-associated DNMT1 activation while also normalizing pericentromeric DNA methylation.

In conclusion, our study reveals the functional roles of the RFTS domain of DNMT1 in maintenance of a nontransformed epigenome. We have demonstrated that deletion of RFTS enhanced the oncogenic potential of DNMT1 by increasing promoter methylation of TSGs such as \textit{DAPK} and \textit{DUOX1}. However, DNMT1-\DeltaRFTS also decreased association of DNMT1 with the pericentromeric region, causing SAT2 demethylation. Because DNMT1-\DeltaRFTS was able to reprogram the overall methylation pattern of epithelial cells in a manner that is common in cancer, the data suggest that RFTS may be a target of tumor-specific dysregulation.

\section*{Materials and Methods}

\subsection*{Cell Culture}

HBEC3 and stable cell lines were grown in KSFM media supplemented with bovine pituitary extract and recombinant human EGF. H358 cells (CRL-5807, ATCC) and stable cell lines were grown in RPMI1640 media with 10\% serum.

\subsection*{Establishment of Stable Cell Lines}

To establish full length or deletion mutant human DNMT1 stable lines, a full-length DNMT1 cDNA clone (SC325419, Origene) was used as template to amplify full-length or deletion mutant DNMT1 fragments. PCR fragments were first T/A cloned into pGEM-T easy vector (A1360, Promega) and then subcloned into pLenti6/V5 vector (K4950–00, Invitrogen). Viral production and transduction was performed using the ViralPower Bsd Lentiviral Support Kit (K4950–00, Invitrogen).

\subsection*{Proliferation and Invasion Assay}

To analyze proliferation, 1,000 (HBEC3) or 5,000 (H358) cells were seeded in replicates of 6 in KSFM media with or without EGF supplementation in 96-well plates. Relative cell numbers were analyzed using Resazurin (R7017, Sigma) 72 hrs after seeding. Data were collected from 4 independent experiments. Cell invasion was analyzed using the CultreCoat \textregistered 24 Well Low BME Invasion Assay kit (3481–024–01, Trevigen).

\subsection*{RT-qPCR}

Total RNA was extracted using the RNeasy Mini Kit (74106, Qiagen) with DNase treatment (79254, Qiagen) to eliminate DNA contamination. Equal amounts of RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher) and qPCR was performed using the 7500 Real Time PCR System (ThermoFisher). Primer pairs used for plasmid constructions are provided in Table S4.
transcribed to generate cDNA using the QuantiTect Reverse Transcription Kit (205314, Qiagen). Specific primer pairs were then used to amplify targets (Table S4). qPCR reactions were conducted with IQ SYBR Green Supermix (170–8884, Bio-Rad). All data were collected from 3 or 4 independent experiments.

Immunoblotting
Protein extracts from each stable cell line were prepared in RIPA buffer (89900, Thermo Scientific) according to the manufacturer’s instructions. Equal amounts of protein were separated in 10-cm culture dishes in KSFM media without EGF, and allowed to grow for 12 days, followed by 4% methylene blue (M9140, Sigma) staining. Colonies were counted. For soft-agar colony formation, 10,000 (HBEC3) or 5,000 (H358) cells were resuspended in media with 0.4% agarose and plated over a layer of 0.6% agarose. Cells were incubated at 37°C for 6 (HBEC3) or 4 (H358) weeks and then colonies were stained with MTT (M5655, Sigma). Colony images were acquired with ChemiDoc XRS (Bio-Rad) and quantified using Quantity One software (Bio-Rad). All data were collected from 2 or 3 independent experiments, each performed in triplicate.

Methylation assay
Genomic DNA was extracted using DNeasy Blood & Tissue Kit (69506, Qiagen). Promoter methylation analysis was performed using MethylMiner Methylated DNA Enrichment Kit (ME10025, Invitrogen) or bisulfite sequencing. For MethylMiner experiments, genomic DNA was first fragmented by sonication to an average size of 400 bp. Methylated DNA was captured following the manufacturer protocol. The methylation level was analyzed using specific primer sets with qPCR (Table S4). Ten percent of input DNA was used as a control. All data were presented as mean ± SD. One-way ANOVA was used to calculate P-values and determine significance. P-values lower than 0.05 were considered statistically significant.

Statistical analysis
All data were presented as mean ± SD. One-way ANOVA was used to calculate P-values and determine significance. P-values lower than 0.05 were considered statistically significant.

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

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Supplemental Materials
Supplemental data for this article can be accessed on the publisher’s website.

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