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Keywords: chromatin, DNA methylation, DNMT1, RFTS domain, tumorigenicity

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-ΔRFTS 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.[1] 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.[2,3] 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.[4-6] 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.[7]

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.[8-10] 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.[11–14] 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.[15-17] Conversely, deficient DNMT1 causes global hypomethylation without site-specific hypermethylation.[18,19] 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[20-26] with the distribution of DNMT1 determining the methylation status of specific target sites. For example, the oncogenic PML-RAR fusion

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protein recruits DNMT1 to the *RARβ2* promoter to stimulate methylation,[20] whereas disruption of the DNMT1-proliferating cell nuclear antigen (PCNA) interaction results in hypomethylation of cellular DNA.[24,25] 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.[27] Multiple lines of evidence suggested that RFTS is a positively acting domain required for DNMT1 function.[27-30] Surprisingly, recombinant forms of DNMT1 including the RFTS domain bind DNA poorly, while specific deletion of RFTS activates DNA binding.[31] Moreover, using a fluorigenic 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.[32–36] In contrast to our biochemical insights into negative regulation by RFTS, CXXC was proposed as a nonmethylated DNA-binding inhibitor of DNMT1 activity.[37] 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.[38] This mutation is associated with genomic hypomethylation and promoter hypermethylation.[38]

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-ΔRFTS—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-ΔRFTS 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.

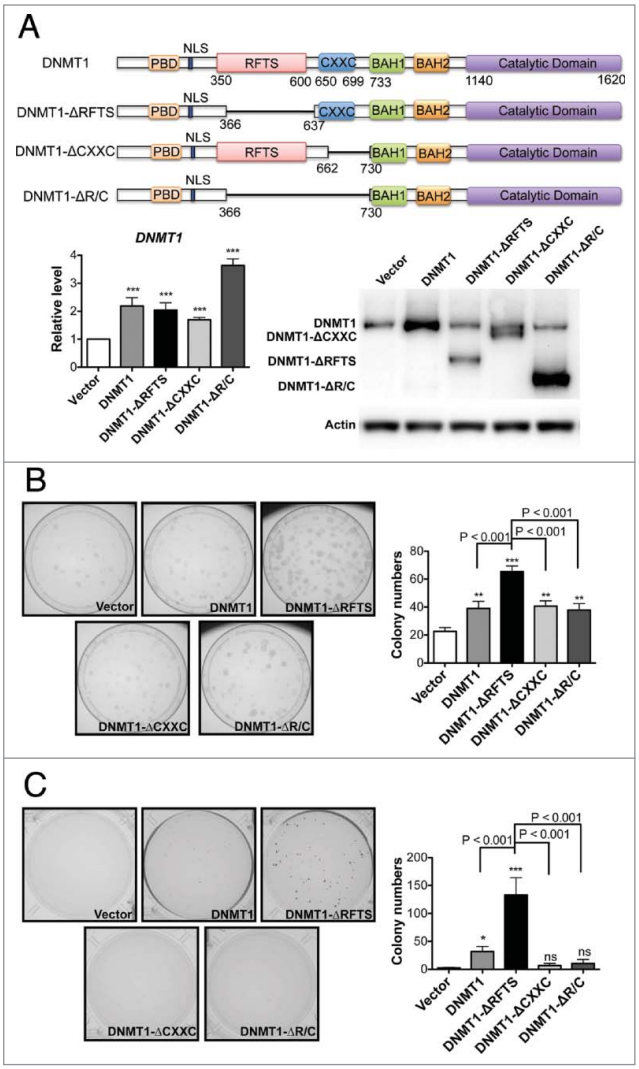

Figure 1. Deletion of RFTS enhances the oncogenic activity of DNMT1. (**A**) HBEC3 stable cell lines were established to express full-length and DNMT1 deletion forms near endogenous DNMT1 levels. The levels of DNMT1 were determined by RT-qPCR (left) and western blotting (right). Data were normalized to vector cells. (**B**) Adherent colony formation. (**C**) Soft-agar colony formation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, indicates no significant difference in comparison to vector cells.

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 nonmalignant human bronchial epithelial cells (HBEC3)[39] that expressed full-length DNMT1 (DNMT1 cells), RFTS-deleted DNMT1 (DNMT1-ΔRFTS cells), CXXC-deleted DNMT1 (DNMT1-ΔCXXC cells), RFTS/CXXC double-deleted DNMT1 (DNMT1-ΔR/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,[15,16,40] 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-ΔRFTS and DNMT1-ΔR/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-ΔR/C 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, *DAPK*[41,42] and *DUOX1*[43] 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]

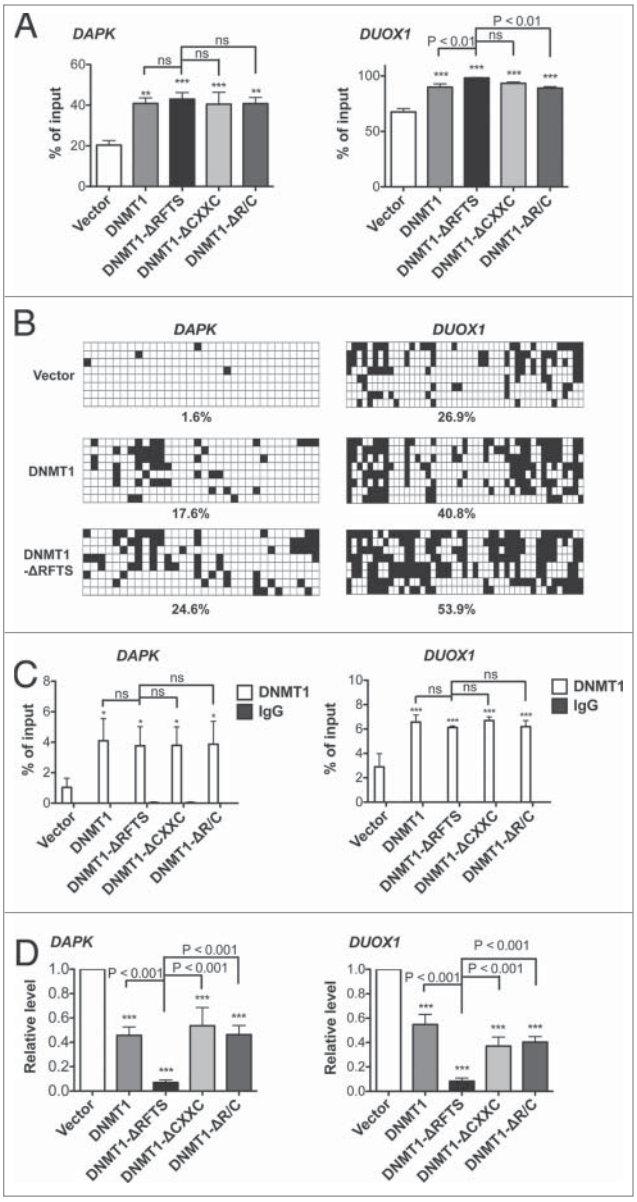

Figure 2. DNMT1-ΔRFTS promotes increased methylation and silencing of the *DAPK* and *DUOX1* genes. (**A**) The methylation levels of *DAPK* (left) and *DUOX1* (right) promoter-associated CpG islands were analyzed by qPCR. Methylated DNA was analyzed using the MethylMiner kit and amplified with specific 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

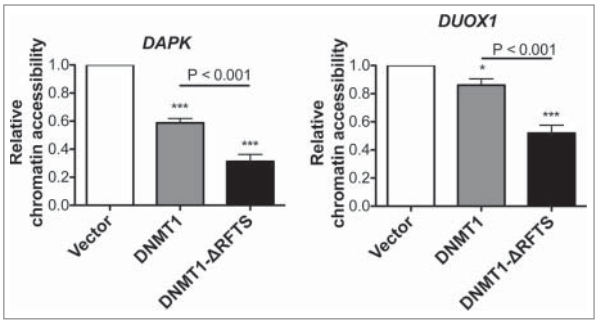

Figure 3. DNMT1-ΔRFTS decreases chromatin accessibility at *DAPK* and *DUOX1* promoters. Cells were treated with or without DNA nuclease for 1 hr, prior to detection of promoter DNA by qPCR. The index of chromatin accessibility = $2^{((Ct\ DNase\ treated)-(Ct\ Untreated))}$. *, $p < 0.05$; ***, $p < 0.001$ in comparison to vector cells.

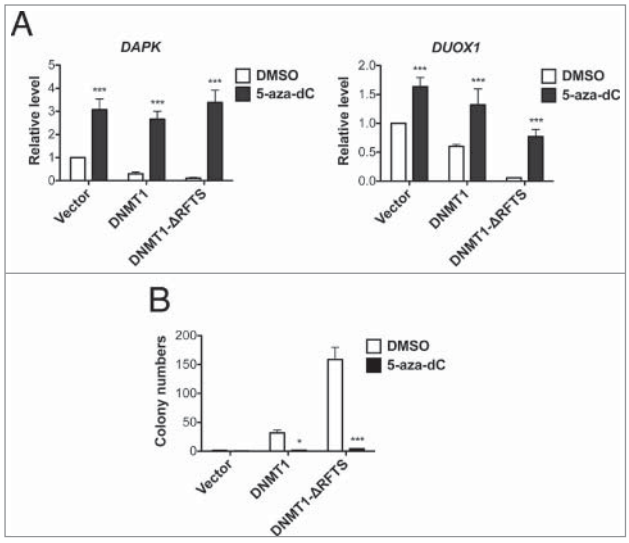

Figure 4. 5-aza-dC treatment reactivates TSG expression and suppresses DNMT1-dependent transformation. (**A**) mRNA levels of *DAPK* (left) and *DUOX1* (right) were analyzed by RT-qPCR after 100nM 5-aza-dC treatment for 5 d and normalized to vector cells treated with DMSO. (**B**) Soft-agar colony formation after 5-aza-dC treatment. ***, $p < 0.001$ in comparison to the DMSO treated control.

treatment (**Fig. 3**). Our data indicate that expression of DNMT1 or DNMT1-ΔRFTS reduced chromatin accessibility at both promoters compared to vector, with DNMT1-ΔRFTS producing the greatest reduction of DNase sensitivity. Thus, silencing of *DAPK* and *DUOX1* is due to DNMT1-dependent alteration of chromatin, and can be increased by deletion of the inhibitory RFTS domain.

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 concurrent 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.[44] This genomic methylation array of 720,001 probes was designed to focus on CpG islands near transcription start sites. The Pearson Correlation test[45] 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.[31]

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**).[46] Previous studies have shown that highly overexpressed DNMT1 causes genomic hypermethylation.[15-17] 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-ΔRFTS 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-ΔRFTS cells. Further, we analyzed chromatin occupancy of DNMT1 on *SAT2* loci by DNMT1 ChIP (**Fig. 6D**). The data indicate that DNMT1-ΔRFTS 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-ΔRFTS 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-ΔRFTS cells. Moreover, we investigated whether *SAT2* DNA hypomethylation is associated with transcription. Indeed, expression of *SAT2* non-coding RNA was increased in DNMT1-ΔRFTS cells, but was not altered in cell lines with alleles of *DNMT1* that are weaker (**Fig. 6E**).

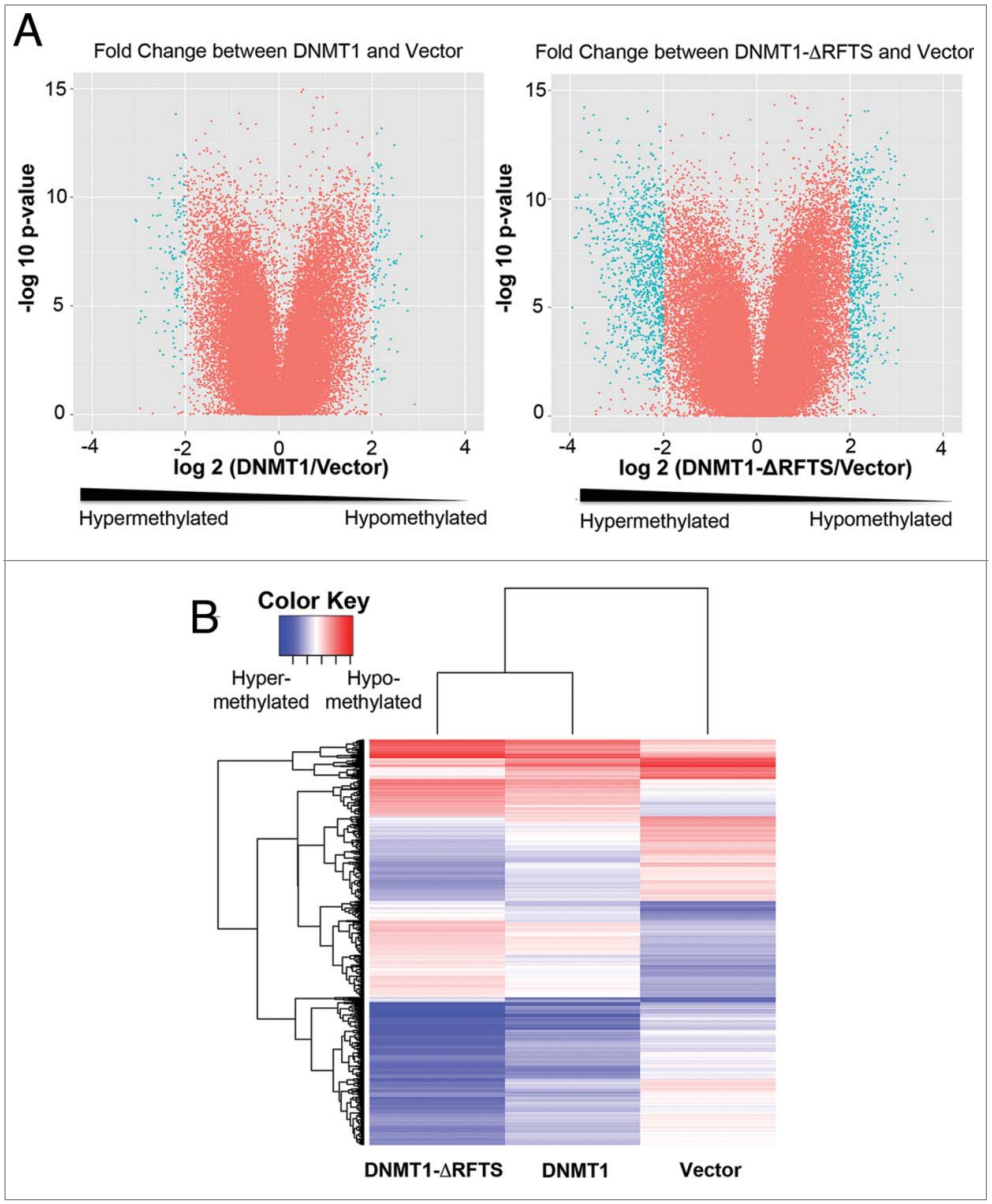

Figure 5. DNMT1-ΔRFTS expression enhances global DNMT1 methylation changes. (**A**) Genome-wide promoter DNA methylation profiles were obtained using the HELP assay. In volcano plots, the x-axis scores probe-specific methylation ratios and the y-axis scores p-values for the confidence of measurements. The plots allow visualization of methylation differences between vector and DNMT1 cells as well as the differences between vector and DNMT1-ΔRFTS cells. Probes sets that showed significant hyper- or hypomethylation ($p < 0.05$ for methylation changes (log2(HpaII/MspI)) > 2) are shown in cyan. All other probes are shown in red. (**B**) Heat map illustration of HpaII-enrichment fragments with methylation changes (log2(HapII/MspI)) > 2 between vector and DNMT1-ΔRFTS cells.

DNMT1-ΔRFTS 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-ΔRFTS expression in malignant cells (**Fig. S4A**). DNMT1-ΔRFTS 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-ΔRFTS slightly inhibited expression of

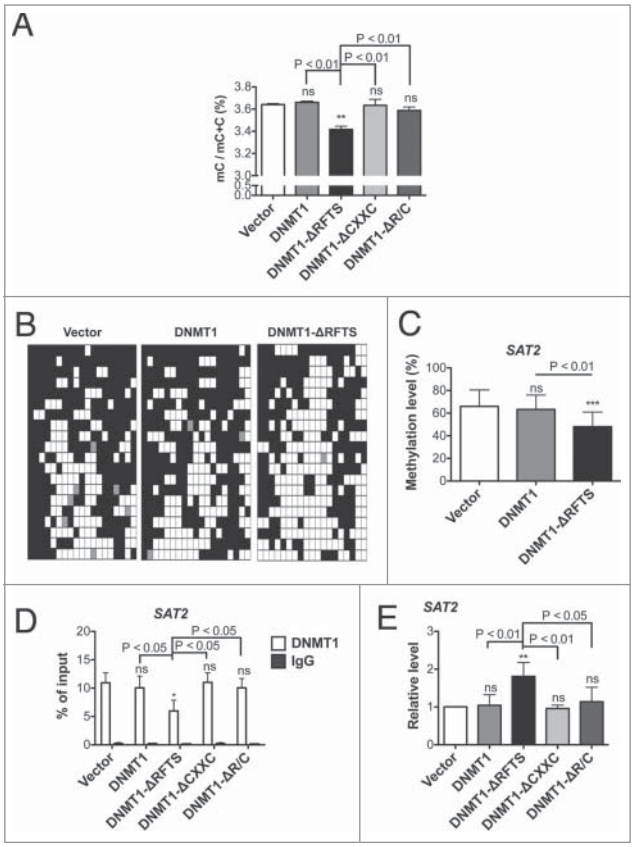

Figure 6. Genomic hypomethylation is found in DNMT1-ΔRFTS cells. (**A**) 5-methylcytosine (mC) content of the total cytosine pool was determined by HPLC. (**B**) Bisulfite sequencing of *SAT2*. White squares represent unmethylated CpGs, black squares represent methylated CpGs, and gray squares represent 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.

DAPK and *DUOX1* (Fig. S5A). Although DNMT1-ΔRFTS cells did not express significantly more *SAT2* RNA transcripts (**Fig. S5A**), there was a notable methylation reduction of *SAT2* in DNMT1-ΔRFTS cells (**Fig. S5B and C**). These data suggest that the biological function of DNMT1-ΔRFTS is not cell-specific.

Discussion

We proposed that the RFTS domain is inhibitory by virtue of DNA-competitive association with the DNMT1 active site,[31] 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 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

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 *DAPK* and *DUOX1*. However, DNMT1-ΔRFTS also decreased association of DNMT1 with the pericentromeric region, causing *SAT2* demethylation. Because DNMT1-ΔRFTS 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.

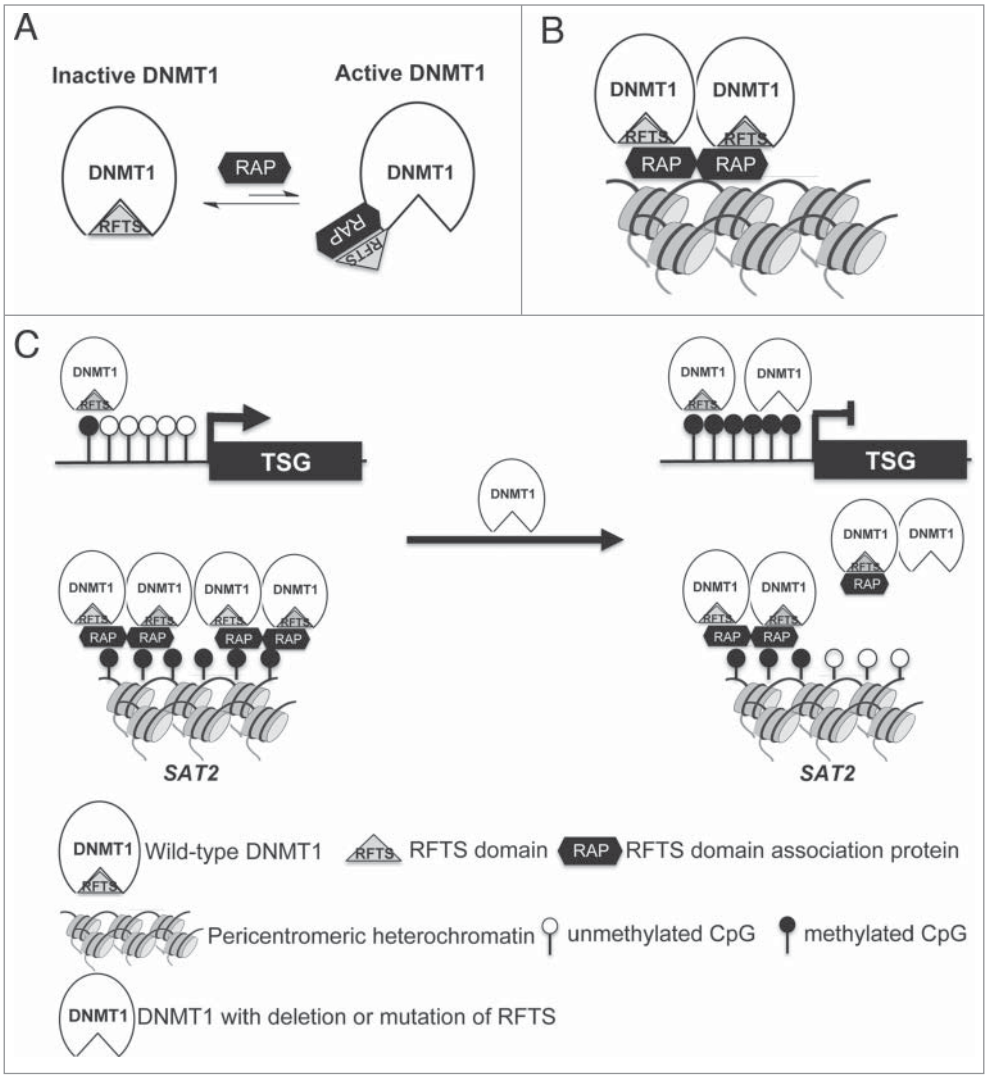

Figure 7. Dual roles for RFTS domain in DNMT1-dependent DNA methylation. (**A**) RFTS-targeted DNMT1 associated proteins (RAP) are proposed to relieve inhibition of DNMT1 for access to euchromatin. (**B**) The RFTS domain mediates association between DNMT1 and pericentromeric heterochromatin. (**C**) In cancer, overexpression of RAPs or mutation of RFTS is proposed to relieve DNMT1 inhibition, thereby increasing methylation and silencing of TSGs. However, because the RFTS domain is required for association with heterochromatic *SAT2* sequences, DNMT1 with mutant RFTS may be less associated with such sequences, accounting for global hypomethylation.

Materials and Methods

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.

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). Primer pairs used for plasmid constructions are provided in **Table S4**.

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® 24 Well Low BME Invasion Assay kit (3481–024–01, Trevigen).

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 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 using NuPAGE® Novex® 3–8% Tris-Acetate Gels and transferred to 0.2 μm nitrocellulose membranes at 4°C overnight. DNMT1 (WH0001786M1, Sigma) and actin (ab3280, Abcam) were detected using specific antibodies and visualized by Super-Signal West Femto Substrate (34096, Thermo Scientific).

Adherent and soft-agar colony formation

For adherent colony formation, 200 cells were seeded on 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 >3 mm 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. Data were collected from 3 independent experiments. For bisulfite sequencing, genomic DNA was treated with bisulfite using EpiTect Bisulfite kit (59124, Qiagen). Bisulfite treated DNA was then used as a template and PCR was performed using specific primer pairs (**Table S4**). Final PCR products were gel purified and cloned into the pGEM-T easy vector (A1360, Promega). Independent clones were subjected to sequencing. Global methylation was analyzed by HPLC. Genomic DNA was digested to nucleosides using DNA Degradase Plus Kit (E2021, Zymo Research). One μg digested DNA was separated on a 25 cm × 4.6 μm C18 Supelco column using 7.5 mM ammonium phosphate and methanol. Retention time of each nucleoside was determined by nucleotide standards. Data were collected from 3 independent experiments, each performed in duplicate.

ChIP

ChIP was performed with Magna ChIP HiSens chromatin immunoprecipitation kit (17–10461, Millipore), DNMT1 antibody (IMG-261A, Imgenex), and analyzed using qPCR. Ten percent of input DNA was used as a control. All data were collected from 3 independent experiments.

Nuclease-protection assay

Chromatin accessibility was assessed using EpiQ™ Chromatin Preparation Kit (172–5402, Bio-Rad). Primers used are listed in **Table S4**. All data were collected from 3 independent experiments, each performed in triplicate.

HELP assay and data analysis

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (69506, Qiagen). The HELP assay was conducted in the Epigenomics Core Facility of Weill Cornell Medical College. Samples were hybridized to a custom oligonucleotide array (Roche NimbleGen, Madision, WI; design name: 100128_HG19_MKF_HELP_ChIP_HX3). HELP data were processed using the HELP package in R from Bioconductor. Primary data are available from the NCBI GEO public database (accession number: GSE57829).

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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