THYROID
Volume 33, Number 8, 2023

DOI: 10.1089/thy.2022.0687

The *RCAN1.4* Metastasis Suppressor Is Hypermethylated at Intron 1 in Thyroid Cancer

Tilak Khanal,* Neel Rajan,* Wei Li, Sandya Liyanarachchi, and Matthew D. Ringel

Background: Regulator of calcineurin 1.4 (RCAN1.4) is a functionally downregulated metastasis progression suppressor (MPS) in thyroid cancer; however, the mechanisms for RCAN1.4 loss in thyroid cancer have not yet been reported. The *RCAN1.4* promoter and gene contain several cytosine-guanine (CG)-rich regions, some of which are reported to be hypermethylated in nonthyroid tissues. We, therefore, hypothesized that RCAN1.4 downregulation in thyroid cancer was in part due to hypermethylation.
Methods: Studies were performed in 5 thyroid cancer cell lines (TPC1, FTC133, BCPAP, C643, and 8505C) with different genetic drivers, and in 18 paired normal and thyroid cancer human thyroid cancer tissues. Basal RCAN1.4 messenger RNA (mRNA) and protein levels were assessed in all of the cell lines. Cell lines with lowest RCAN1.4 expression levels were treated with the DNA methyl transferase inhibitor, decitabine. Normal/tumor tissue pairs were analyzed for methylation of three CG-rich regions both by capture of methylated DNA by MBD2 protein and by methylation-specific polymerase chain reaction (MSPCR).
Results: In all assessed cell lines, RCAN1.4 mRNA and protein levels increased after decitabine treatment. *In silico* analysis of the RCAN1.4 gene identified 3 CG-rich regions as possible methylation targets: 1 in the proximal promoter and 2 in intron 1. Hypermethylation of the intron 1 CG-rich regions was identified by both the capture method and MSPCR. In contrast, hypermethylation of the CG-rich region of the proximal promoter was not identified. Gene expression confirmed that hypermethylation in thyroid cancer samples in intron 1 of *RCAN1.4* was associated with lower levels of RCAN1.4 mRNA. Finally, the cancer samples demonstrated increased NFE2L3 expression, a downstream marker of functional RCAN1.4 loss.
Conclusions: The MPS gene, RCAN1.4, is downregulated in thyroid cancer cells and human thyroid cancer in part by hypermethylation of CG-rich regions in intron 1.

Keywords: NFE2L3, metastatic dormancy, gene regulation

Introduction

Thyroid cancer is the most common malignancy of a classical endocrine organ, accounting for an estimated 43,800 new cases and 2230 deaths in 2022 in the United States.[1] The progressive increase in frequency of thyroid cancer diagnosis has stabilized in recent years with adherence to new guidelines.[2,3] However, the number of individuals who die from thyroid cancer has not reduced, mostly due to progressive disease and metastasis.[2] It is well recognized that many patients with thyroid cancer distant metastasis have nonprogressive disease for decades, and that clinically silent distant metastases are common on autopsy.[4–6]

Thus, thyroid cancer is an important model to study ''gatekeepers'' of progression, including gain of secondary drivers and loss of metastasis progression suppressor (MPS) genes, which might serve as predictive biomarkers of disease-specific mortality and/or as new therapeutic targets.[4,7]

The multiple steps involved in cancer metastasis have been extensively studied and modeled.[8,9] For most tumor types, a subset of cancer cells undergo an epithelial-to-mesenchymal transition, enabling local invasion and

Division of Endocrinology, Diabetes, and Metabolism, Departments of Internal Medicine and Molecular Medicine and Therapeutics, The Ohio State University College of Medicine and Comprehensive Cancer Center, Columbus, Ohio, USA.
Presented in part at Endocrine Society 2022 Annual Meeting, Atlanta, GA (June 11–14, 2022).
*Both these authors contributed equally to this study.

motility.[10–12] Individual or clusters of cancer cells invade, intravasate into the vasculature, circulate, and a subpopulation extravasates and survives in the metastatic environment (disseminated tumor cells [DTCs]).[13,14] The process also involves release of factors from the primary tumor that support development of a premetastatic niche.[15–17]

Surviving metastatic DTCs can immediately proliferate leading to cancer progression or enter dormancy in which they survive in a quiescent state.[18] These ''dormant'' cells are relatively therapeutically resistant, thus, when they escape from dormancy, they are difficult to treat.[19] Understanding the factors that lead cancers to emerge from dormancy represents a key translational need to properly target therapies at the time of cancer progression. In metastatic thyroid cancer, despite the tendency for prolonged dormancy, new driver gene mutations unique to newly invasive primary cancers or growing metastases are relatively uncommon.[20,21]

This feature has enabled the use of primary tumor genomics to predict response to targeted therapies,[20,22] but also defines the need for a deeper understanding of factors leading to cancer progression to improve outcomes since current therapies are not curative. Due to this relative paucity of new driver mutations in progressive thyroid cancer, we posited that loss of MPSs may be important in thyroid cancer progression.[4,19] MPS genes encode proteins that when lost facilitate primary cancer growth, invasion, metastasis, and progression, but not transformation separating them from tumor suppressors.[7,23]

We initially identified regulator of calcineurin 1.4 (RCAN1.4) as a potential MPS in an objective screen for downstream pathways of the KiSS1/GPR54 MPS pathway.[24,25] Subsequent studies demonstrated its MPS function *in vitro* and *in vivo* and confirmed reduced levels in advanced thyroid cancer samples and metastatic lesions.[24,26] RCAN1.4 is one of two expressed isoforms of the RCAN1 gene (*RCAN1.1* and *RCAN1.4*) expressed with unique promoters from the same gene that originally was identified as the Down's syndrome critical region 1 (DSCR1) gene on chromosome 21.[27–30]

RCAN1.4 was identified as the primary inducible RCAN1 transcript. RCAN1.4 functions as a negative regulator of calcineurin/NFAT signaling, thereby reducing cell proliferation, migration, and apoptosis.[29,31–34] Interestingly, individuals with Down's syndrome who express 3 copies of chromosome 21 have very low incidence of solid tumors.[35] This reduced cancer incidence can be partially attributed to the three copies of *RCAN1* in mouse model systems.[33,36–38]

Using transcriptomics, we identified that RCAN1.4 loss also results in an increase in NFE2L3 levels, a member of the Cap N' Collar family of basic leucine zipper transcription factors, and that this gene is functionally required for RCAN1.4 loss-mediated cancer progression.[26] An anti-progression role for RCAN1.4 subsequently has been confirmed for renal cell carcinoma,[39] hepatocellular cancer,[32] osteosarcoma,[31] pancreatic cancer,[40] and breast cancer.[36] RCAN1.4 also was reported to be a functional target of the prometastatic mIR-619-5p that suppresses RCAN1.4 levels, leading to angiogenesis and metastasis nonsmall cell lung cancer.[41]

Hypermethylation of the *RCAN1.4* proximal promoter or intron 1 has been reported in liver fibrosis and hepatocellular carcinoma cells, respectively,[32,42] but has not been studied in the thyroid cancer. This study aims to test the hypothesis that RCAN1.4 levels are reduced in thyroid cancer in part due to hypermethylation. Our results demonstrate that RCAN1.4 levels are reduced in thyroid cancer by methylation of cytosine-guanine (CG)-rich regions of intron 1 in association with increased expression of NFE2L3.

Materials and Methods

Cell lines, cell culture, in vitro *drug reagent*

Five thyroid cancer cell lines were used with mutational information detailed by Landa et al.,[43] 8505C (anaplastic thyroid cancer [ATC]; $BRAF^{V600E}$), TPC1 (papillary thyroid cancer [PTC]; RET/PTC1), BCPAP (PTC; $BRAF^{V600E}$), FTC-133 (follicular thyroid cancer, PTEN null), and C643 (ATC; HRAS). All cell lines were independently DNA fingerprinted for identification. 8505C, BCPAP, and C643 cells were cultured in RPMI with TPC1 and FTC-133 cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. RPMI, DMEM, and FBS were obtained from ThermoFisher Scientific (118775119, Gibco; 11960069, Gibco; 26140079, Gibco, respectively).

Cells were split approximately every 3–4 days using 0.05% trypsin–EDTA (25300120; Gibco). For experiments, cells were split, washed, and placed in culture conditions as already mentioned except with 1% FBS for 24 hours. The DNA methyltransferase inhibitor decitabine (S1200; Selleckchem) diluted in 0.2% dimethyl sulfoxide (DMSO), or diluent alone, was added at the shown concentrations.

Western blot

Cells were plated on 10 cm dishes and allowed to incubate for 24 hours. Medium was changed to 1% FBS and allowed to incubate for 24 hours. Plates were treated with decitabine at 0, 5, and 10 μM concentrations for 72 hours; cells were isolated in phosphate-buffered saline (PBS; 10010-023; Gibco); and lysed with MPER buffer (78501; ThermoFisher). Protein concentrations were measured and quantified by Pierce BCA Protein Assay Kit (23225; ThermoFisher).

Protein was combined with NuPAGE LDS sample buffer ($4\times$NP0007; ThermoFisher), DTT, and ddH_2O, boiled for 5 minutes, and 20 μg protein per well for Western blot. Samples were run, transferred, and blocked with 5% bovine serum albumin (BSA) for 1 hour. Membranes were incubated in primary antibody overnight at 4°C, washed 3 times with PBS, and incubated with secondary antibody at room temperature for 1 hour. After washing, blots were imaged using the Odyssey CLx LI-COR Imager and ImageStudio software (LI-COR). Primary antibodies were the following: anti-DSCR1 (RCAN; 1:1000, D6694-200uL; Millipore) and β-Actin (1:5000, sc-8432; Santa Cruz).

Human samples, DNA extraction, and sequencing

Eighteen papillary thyroid tumor and opposite lobe histologically normal tissues from consecutive unselected patients were collected after obtaining informed consent as part of an Ohio State University IRB-approved protocol (2006C0047) in accordance with the Declaration of Helsinki (revised 2013). Deidentified clinical/pathological information is listed

in Supplementary Table S2. DNA was isolated from the paired thyroid tissue samples and five cell lines using phenol–chloroform extraction. RNase A (12091021; Invitrogen) was applied during the extraction procedure. Methods for BRAF and TERT promoter sequencing are included in Supplementary Methods.

DNA methylation analysis

Capture of methylated DNA by MBD protein. DNA fragmentation was obtained by restriction digestion using *Mse*I; efficiency was confirmed by agarose gel electrophoresis. Three DNA fragment positions are: chr21: 34527031–34527182, chr21:34525269–34525646, and chr21: 34524894–34525268 (hg38). Methylated DNA was isolated using CpG MethylQuest DNA Isolation Kit (Cat. No. 17-10035; Millipore) according to the manufacturer's protocol. In brief, 400 ng *Mse*I-digested DNA fragments were incubated with CpG MethylQuest beads. The beads were washed three times and eluted to isolate the methylated DNA.

Primers were designed to amplify the CG-rich regions in RCAN1.4 proximal promoter and the two CG-rich regions of intron 1 individually. Quantitative polymerase chain reaction (qPCR) was performed with methylated DNA using Fast SYBR Green Master mix (ThermoFisher), and the percentage of methylation then was calculated as the amount over input DNA. Primers are given in Supplementary Table S1.

Qualitative methylation-specific polymerase chain reaction. DNA was converted by using EpiTect Bisulfite Kit (Cat. No. 59104; Qiagen) as per the manufacturer's protocol. Methylated and unmethylated DNA-specific primers for intron 1 are from Jin et al.[32] and are listed in Supplementary Table S1. Forty cycles of polymerase chain reaction (PCR) were performed and results were analyzed qualitatively. Methylation-specific PCR (MSPCR) was not possible in the proximal promoter region, and there were not suitable primers that could amplify the two intron 1 regions separately. Thus, this method was used for intron 1 methylation encompassing both regions.

RNA preparation, messenger RNA analysis, quantitative real-time PCR

Thyroid tissue total RNA was extracted by using TRIzol (15596018; Invitrogen). One microgram RNA was treated with DNA-*free* DNA Removal Kit (AM1906; ThermoFisher) and reverse transcribed to complementary DNA (cDNA; High-Capacity cDNA Reverse Transcription Kit; 4368814, Applied Biosystems). qPCR was performed with Fast SYBR Green Master mix for RCAN1.4 and TaqMan Fast Universal PCR Master Mix (4352042; ThermoFisher) for NFE2L3. GAPDH was used as an internal control to calculate the relative expression levels for gene RCAN1.4 and NFE2L3. PCR primers are shown in the Supplementary Table S1.

Data analyses

All cell line experiments were repeated at least three times. For real-time PCR (RT-PCR) and methylation experiments, technical replicates were performed for each independent experiment. One-way analysis of variance was used to determine the significant differences ($p < 0.05$) between groups in protein and messenger RNA (mRNA) data. After stabilizing methylation proportions by the arcsine square root transformation, nonpaired or paired Student *t*-tests were applied appropriately to compare groups.

Quantitative real-time PCR (qRT-PCR) relative expression data were $\log_2$ transformed and tumor versus normal comparison analyses were performed by applying paired Student *t*-tests. Western blots were quantified using ImageJ (1.53a; National Institute of Health). Statistical analysis and graph generation were done using Prism GraphPad (9.2.0) and R software.

Results

Decitabine upregulates RCAN1.4 mRNA and protein levels in thyroid cancer cells

After initial time course studies, the 5 thyroid cancer cell lines were treated with decitabine for 48 hours and RNA was extracted. Then, 1, 5, and 10 μM doses were used along with a nontreatment control. qRT-PCR was performed. Results from 3 independent experiments show RCAN1.4 RNA levels increase with increasing drug concentration, with significant increases after treatment with 5 and 10 μM decitabine for all cell lines (Fig. 1B).

To assess effect of demethylation on RCAN1.4 protein levels, after initial time course studies, thyroid cancer cells were treated with decitabine (0, 1, 5, and 10 μM) for 72 hours. RCAN1.4 protein levels significantly increased in all cell lines after decitabine treatment in three independent experiments (Fig. 1C, D). RCAN1.1 protein levels did not show an increase, suggesting that the regulatory regions for *RCAN1.1* gene expression that are unique from *RCAN1.4*[30] are not hypermethylated. β-actin was used as loading control for all experiments.

Hypermethylation was detected at RCAN1.4 intron 1 with thyroid cancer cell lines and PTC tissues

Previous studies reported hypermethylation in the 5′ regulatory region and intron 1 of *RCAN1.4*.[32] To determine whether RCAN1.4 downregulation in thyroid cancer tissues is associated with DNA methylation at these sites, methylation analysis at the three potential methylation sites in *RCAN1.4* promoter and intron 1 was performed (Fig. 1A).

Using the quantitative capture assay, we did not detect methylation in the CG-rich region proximal to the *RCAN1.4* promoter region in the five thyroid cancer lines (Fig. 2A, B). MSPCR was not possible in the promoter region as there were not suitable sequences for primers. To assess methylation in intron 1, qualitative methylation-specific PCR identified methylation in the five cell lines. Subsequent studies demonstrated that the intron 1 methylation was partially reversed with decitabine (Fig. 3A).

This method was not able to be performed separately for the two adjacent GC-rich regions in intron 1. To confirm and extend the data quantitatively for each region, subsequent studies using the quantitative capture assay confirmed hypermethylation in both regions of intron 1. They also demonstrated quantitative reversal with decitabine treatment more consistently in region 2 versus region 3 (Fig. 3B).

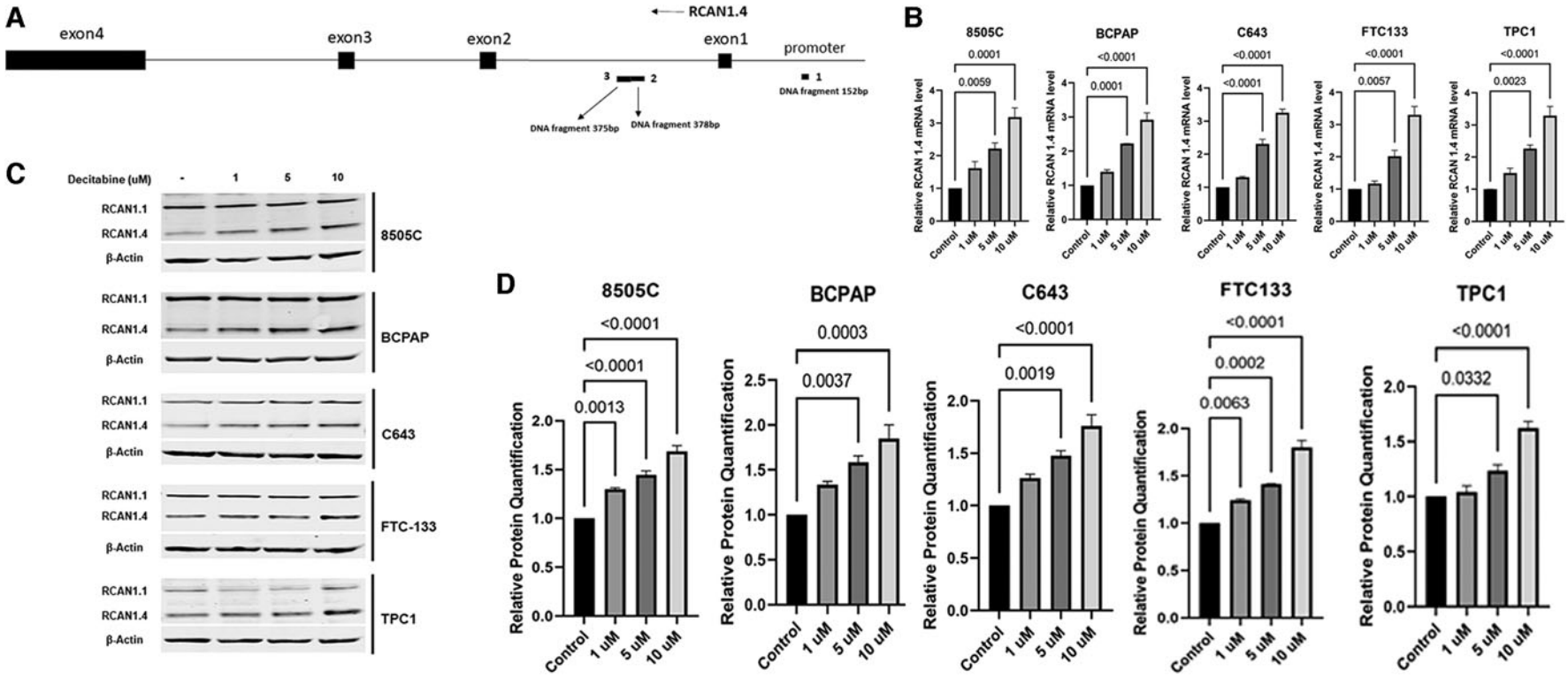

FIG. 1. Decitabine increases protein and mRNA levels. (**A**) Diagram of *RCAN1.4* showing the three CG-rich regions in the promoter and intron 1 with the sizes of the DNA fragments. (**B**) Cells were treated with increasing doses of decitabine (0, 1, 5, and 10 μM) for 48 hours, mRNA levels were measured; RCAN1.4 levels increased after treatment with 5 or 10 μM decitabine. (**C**, **D**) Cells were treated with increasing doses of decitabine (0, 1, 5, and 10 μM) for 72 hours. RCAN1.4 protein levels increased in all cell lines with 5 or 10 μM dosing. β-actin was used as loading control, $n=3$ for all experiments. Statistical comparisons were performed using one-way ANOVA; $p<0.05$ is significant. ANOVA, analysis of variance; mRNA, messenger RNA; RCAN1.4, regulator of calcineurin 1.4.

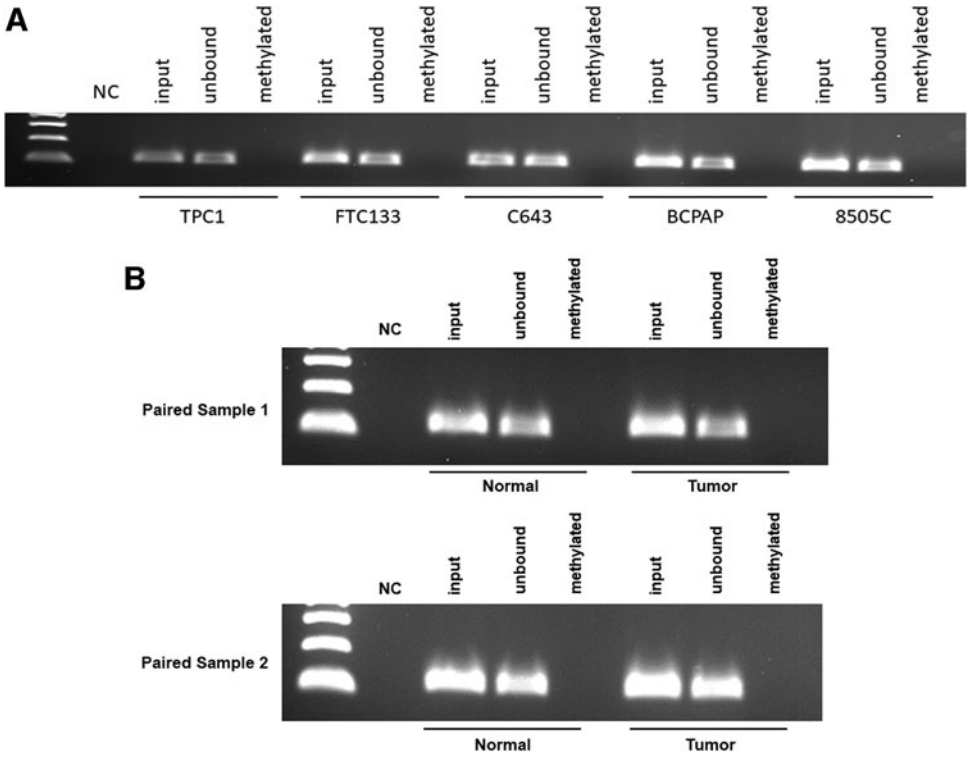

FIG. 2. (**A**) Promoter region shows no hypermethylation in all five cell lines. (**A**) DNA was isolated from five cell lines and quantitative capture of methylated DNA by MBD2 protein bead precipitation and PCR was performed, $n=3$. (**B**) DNA from 18 paired normal and tumor thyroid samples were analyzed by quantitative capture of methylated DNA by MBD2 protein bead precipitation. Two samples shown in Figure 2, all samples are shown in Supplementary Figure S1. NC, negative control; PCR, polymerase chain reaction.

To validate the findings in human thyroid cancer tissues, we performed DNA methylation analysis with 18 paired PTC normal and tumor tissue samples. As for the cell lines, there was no evidence of methylation of the proximal promoter region (Supplementary Figs. S1 and S2). Qualitative analysis of intron 1 by MSPCR in 14 of the sample pairs suggested a relative increase in methylation in most of the samples (Fig. 4A). Four of the samples did not have adequate DNA from complete pairs for both methods and we prioritized the quantitative region-specific capture assay.

Using this assay, higher methylation in both intron 1 regions in tumor versus normal tissue was identified (region 2: $p=1.13e-06$, region 3: 1.03e-04) (Fig. 4B). Region 2 effects

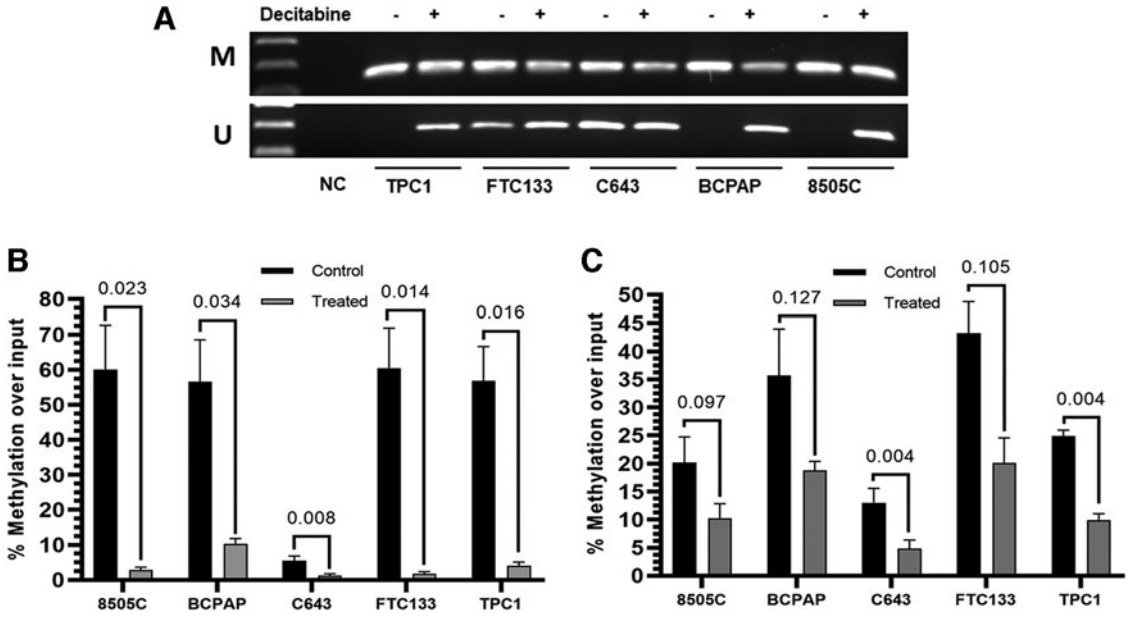

FIG. 3. - Decitabine decreases *RCAN1.4* intron 1 methylation in thyroid cancer cell lines (**A**) Cells were treated with decitabine (10 μM) for 72 hours and DNA isolated. Qualitative methylation-specific PCR for both regions of intron 1 was performed and demonstrated relatively less methylation after decitabine treatment for most samples. (**B**, **C**) Quantitative capture of methylated DNA by MBD2 protein bead precipitation and PCR was performed for region 2 (**B**) and region 3 (**C**). Significant reductions of methylation were identified for region 2 in all cell lines and in 2 of 5 cell lines for region 3 ($n=3$). Student *t*-tests were used to compare results in (**B**, **C**), $p<0.05$ is considered significant. M, methylated; U, unmethylated.

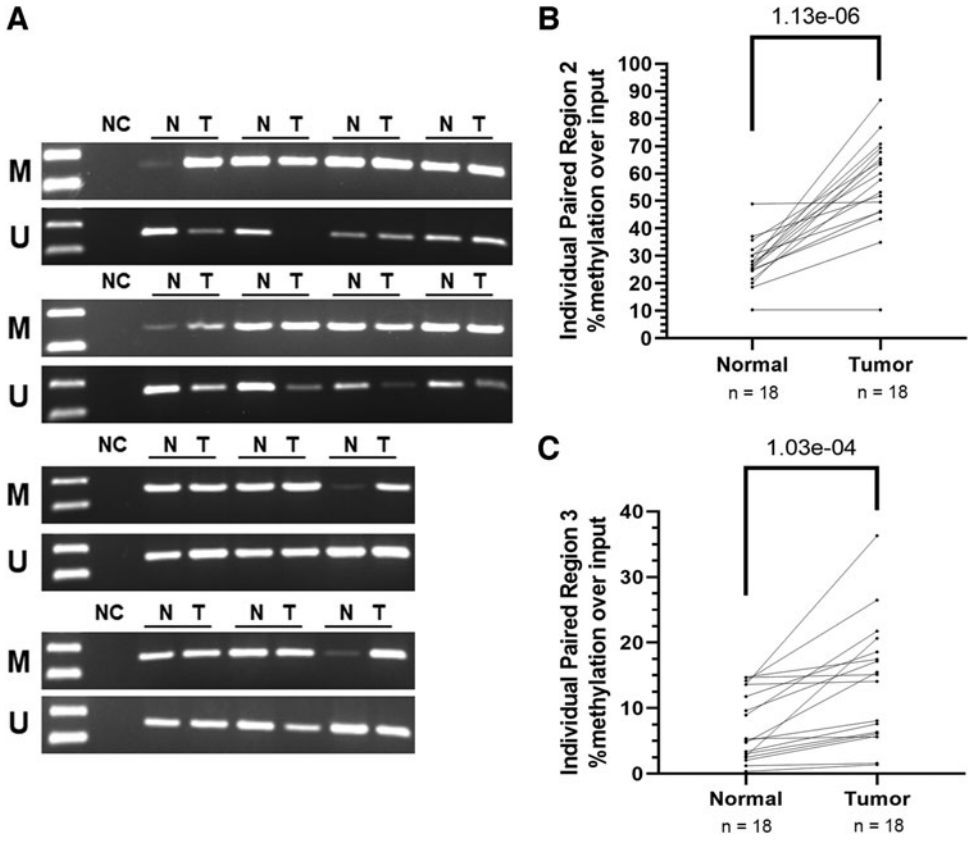

FIG. 4. *RCAN1.4* intron 1 is hypermethylated in tumor PTC vs. paired normal tissue samples. Paired thyroid cancer normal and tumor samples were analyzed for *RCAN1.4* methylation of intron 1. (**A**) Qualitative methylation-specific PCR was performed to assess methylation of intron 1 (both regions) in 14 PTC normal and tumor paired samples with adequate DNA with results suggesting relatively higher methylated: unmethylated DNA in tumor samples. (**B**) Quantitative capture of methylated DNA by MBD2 at *RCAN1.4* intron 1 for all 18 pairs confirmed higher levels of methylation in both regions 2 and 3 in thyroid cancer samples. Paired Student *t*-test was used for statistical analysis; $p < 0.05$ is significant. N, normal; PTC, papillary thyroid cancer; T, tumor.

were more consistent than region 3 effects (higher in 16/18 tumors vs. normal), a result that is similar to the thyroid cancer cell lines.

RCAN1.4 *expression is lower and* NFE2L3 *expression is higher in PTC samples*

To determine whether the human tumor samples had lower levels of RCAN1.4 versus normal tissue we performed qRT-PCR and identified lower levels in the tumor samples versus paired normal samples (Fig. 5, $p = 2.18e\text{-}05$). As a measure of biological effects of RCAN1.4 loss in the paired thyroid cancer samples, we analyzed gene expression for *NFE2L3* to assess for the predicted inverse results based on our prior functional studies.[26] Results in the paired samples show the predicted higher levels of NFE2L3 in tumor versus the normal samples (Fig. 5, $p = 1.41e\text{-}06$).

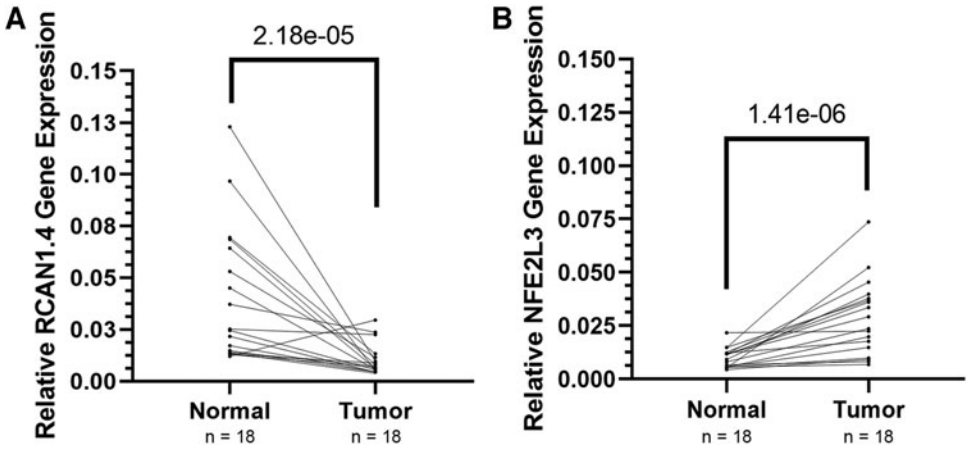

FIG. 5. RCAN1.4 gene expression is downregulated while NFE2L3 gene expression is upregulated in PTC tissues. RCAN1.4 and NFE2L3 gene expression levels in 18 paired papillary thyroid cancer normal and tumor samples were assessed by qRT-PCR. (**A**) Reduced levels of RCAN1.4 mRNA were found in 16 of 18 samples ($p = 2.18e\text{-}05$). (**B**) Increased levels of NFE2L3 were identified in 17 of 18 samples ($p = 1.41e\text{-}06$). Graphs represent relative gene expression compared with normal tumor samples, with each normal sample paired with the corresponding tumor sample. Data were $\log_2$ transformed and analyses were performed using paired Student *t*-tests; $p < 0.05$ is significant. qRT-PCR, quantitative real-time PCR.

Discussion

Differentiated thyroid cancer typically is characterized by relatively slow growth rates and prolonged stability in both primary and distant metastatic sites. However, local invasion and progression of distant metastases are the most common causes of thyroid cancer morbidity and mortality. Thus, understanding the molecular causes of progression of thyroid cancer represents an important opportunity to identify new biomarkers and therapeutic targets (reviewed in Summers et al.,[19] Ringel,[44] and Ganesh and Massagué[45]). We previously identified and characterized RCAN1.4 as a bona fide functional MPS in thyroid cancer that is downregulated in some primary and most metastatic lesions, a finding that has been confirmed in a number of other malignancies.[24–26,31,32,36,39,40]

Although RCAN1.4 is known to regulate calcineurin signaling, and does so in thyroid cancer cells,[24,30] we identified a new functional downstream target, NFE2L3 (Nrf3), for RCAN1.4 loss in cancer cells.[26] NFE2L3 is a member of the Cap N' Collar family of transcription factors known to be important in promoting cancer development and progression.[46–50] In addition, association studies confirmed a progressive increase in NFE2L3 levels in more aggressive thyroid cancers and metastatic lesions.[26] Although these data defined a new RCAN1.4/NFE2L3 regulatory pathway for cancer progression, the causes of the reduction of RCAN1.4 levels in thyroid cancers had not been addressed.

In this study, we hypothesized that the reduced levels of RCAN1.4 in thyroid cancer would be, at least in part, due to hypermethylation of regulatory sequences. This hypothesis is built on an initial analysis of the *RCAN1.4* promoter and introns for CG-rich regions and published data demonstrating regulation of *RCAN1.4* in the proximal promoter or in intron 1 in hepatic stellate cells or hepatocellular carcinoma cells *in vitro*.[32,42] Our data demonstrate that the proximal promoter is not hypermethylated either in tested thyroid cancer cell lines or in human thyroid cancers. In contrast, hypermethylation was identified in in intron 1 in the two predicted regions, most convincingly in region 2 (Fig. 1A).

The intron 1 hypermethylation was reversed with decitabine in concert with the predicted increase in RCAN1.4 levels. Importantly, human thyroid cancer samples (mostly PTC) showed similar *RCAN1.4* methylation and gene expression patterns. Functional activation of the pathway is suggested by the increased expression of NFE2L3 in association with the RCAN1.4 loss.[26]

Epigenetic methylation is important in regulating expression of genes important for tumor progression.[51,52] For instance, clustered and single circulating tumor cells express differential methylation patterns in accordance with upregulated proliferation and stem cell genes.[53] Experiments are ongoing to identify the upstream regulators of the hypermethylated intron 1 regions. Intriguing recent data identified a superenhancer in intron 1 of *RCAN1.4* in breast cancer.[36] It is possible that hypermethylation reduces access to the superenhanced region, thereby downregulating RCAN1.4 expression after cellular stress.

This hypothesis requires further evaluation of superenhancer activity in the intron 1 sites in the thyroid cell context. Finally, RCAN1.4 has been reported to be phosphorylated by several kinases in some cellular contexts.[54–57] It is possible that these modifications, and others, regulate activity and/or stability of the protein in thyroid cells. Thus, hypermethylation may not account for all downregulation of RCAN1.4 activity.

We identified the hypermethylated regions in thyroid cancer cell lines and validated the findings in human thyroid cancers. These results provide a basis for future studies assessing a potential role for RCAN1.4 methylation as a predictive or prognostic biomarker in thyroid cancer. Although MPS gene reductions can enable primary cancer progression as well as metastases, it is possible that the quantitative level of RCAN1.4 intron 1 hypermethylation may increase in metastatic lesions versus paired primary tumor tissue.

This may be suggested by the progressive increase in NFE2L3 in metastatic lesions we identified in our prior studies.[26] Further studies using paired samples from patients with metastatic lesions are planned to assess this possibility. It is of interest that all of the human tumor samples in this study harbored $BRAF^{V600E}$ mutations. While the cell lines with hypermethylation were not specific for this mutation, analysis of larger and different histologies will be needed to determine whether this association is related to $BRAF^{V600E}$.

In conclusion, we demonstrated for the first time that intron 1 of *RCAN1.4* is hypermethylated at CG-rich regions in thyroid cancer cells. We also report that a methyltransferase inhibitor reverses the methylation and increases RCAN1.4 mRNA and protein. Our results also demonstrate hypermethylation of this same region in human PTC in association with reduced expression of RCAN1.4. The reduced RCAN1.4 is inversely correlated with NFE2L3 expression, a pattern consistent with functional loss of RCAN1.4 that also is consistent with prior association data.[26] These results demonstrate that reduction of the RCAN1.4 MPS gene in thyroid cancer is in part due to hypermethylation of intron 1, thereby providing a regulatory mechanism for loss of this key regulator of thyroid cancer progression.

Authors' Contributions

T.K. contributed to methodology, investigation, analysis, and writing; N.R. was involved in methodology, investigation, analysis, and writing; W.L. carried out methodology, investigation, analysis, and writing; S.L. took charge of analysis and writing; M.D.R. was in charge of conceptualization, methodology, investigation, analysis, and resources.

Author Disclosure Statement

The authors all declare that there are no conflicts of interest.

Funding Information

This study was supported by grants R01CA240302 and R01CA227847 to M.D.R. and in part by the OSUCCC Core Grant P30CA0166058. T.K., N.R., W.L., and S.L. were supported by the mentioned grants to M.D.R.

Supplementary Material

Supplementary Methods
Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2

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Address correspondence to:
Matthew D. Ringel, MD
Division of Endocrinology, Diabetes, and Metabolism
Departments of Internal Medicine
and Molecular Medicine and Therapeutics
The Ohio State University College of Medicine
and Comprehensive Cancer Center
McCampbell Hall, Room 565; 581 Dodd Drive
Columbus, OH 43210
USA

E-mail: matthew.ringel@osumc.edu