LncRNA CANT1 suppresses retinoblastoma progression by repelling histone methyltransferase in PI3Kγ promoter

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Abstract
Retinoblastoma (RB) is the most common malignant intraocular tumor of childhood. Recent studies have shown that long noncoding RNAs (lncRNAs), which are longer than 200 bp and without protein-coding ability, are key regulators of tumorigenesis. However, the role of lncRNAs in retinoblastoma remains to be elucidated. In this study, we found that the expression of lncRNA CASC15-New-Transcript 1 (CANT1) was significantly downregulated in RB. Notably, overexpression of CANT1 significantly inhibited RB growth both in vitro and in vivo. Furthermore, lncRNA CANT1, which was mainly located in the nucleus, occupied the promoter of phosphoinositide 3-kinase gamma (PI3Kγ) and blocked histone methyltransferase hSET1 from binding to the PI3Kγ promoter, thus abolishing hSET1-mediated histone H3K4 trimethylation of the PI3Kγ promoter and inhibiting PI3Kγ expression. Furthermore, we found that silencing PI3Kγ either by lncRNA CANT1 overexpression or by PI3Kγ siRNA, reduced the activity of PI3K/Akt signaling and suppressed RB tumorigenesis. In summary, lncRNA CANT1 acts as a suppressor of RB progression by blocking gene-specific histone methyltransferase recruitment. These findings outline a new CANT1 modulation mechanism and provide an alternative option for the RB treatment.

Introduction
Long noncoding RNAs (lncRNAs) are transcripts longer than 200 bp with no apparent protein-coding ability, that are involved in numerous important biological phenomena such as X chromosome inactivation, chromosome conformation shaping, and DNA damage repair1–4. The functional roles and mechanism of action of some classically defined lncRNAs are well understood. For instance, lncRNA XIST coats the X chromosome, is expressed only from the inactive X chromosome (Xi), and is essential for the silencing process5,6. LncRNA-ROR is an important factor for the reprogramming process because its depletion or overexpression results in reduced or increased efficiency of reprogramming fibroblasts to iPSCs7. LncRNA non-coding RNA activated by DNA damage (NORAD) interacts with proteins involved in DNA replication and repair in steady-state cells and localizes to the nucleus upon replication stress or DNA damage stimulation8,9. However, the functions of the majority of lncRNAs are unknown, and it is necessary to explore the functions of lncRNAs.

As lncRNAs play a key role in the maintenance of homeostasis, aberrant lncRNA expression may be an important trigger for a variety of diseases. LncRNA BACE1-AS levels have been found to be increased along with amyloid β levels across different regions in postmortem brains from Alzheimer’s disease patients, and BACE1-AS protects BACE1 mRNA from degradation10. LncRNA myocardial infarction associated transcript
LncRNA GALNT8 activates the expression of its target gene of their promoters. Thus, correcting lncRNA-guided activators is a novel tumor suppressor in uveal melanoma and a pathway by directly binding to the promoters of lncRNAs. In addition, epigenetic alterations strongly with tumor cell survival. These findings reveal the mechanisms underlying rapid retinoblastoma progression following RB1 inactivation and provide a basis for further investigation of new regulatory mechanisms and promising therapeutic approaches for RB tumor progression.

Here, we successfully identified that lncRNA CANT1 functions as a noncoding RB suppressor. Using epigenetic approaches, we found that lncRNA CANT1 acts as a necessary suppressor playing a vital regulatory role in RB tumorigenesis and we identified a novel type of histone modification that inhibits PI3Kγ transcription. The retinoblastoma cell lines Y79 (obtained from ATCC), Weri-Rb1 (obtained from ATCC), and RB44 (kindly provided by Heping Xu, Central South University, Changsha, China) were cultured in RPMI-1640 medium (GIBCO, USA). All media were supplemented with 10% fetal bovine serum (GIBCO, USA), 1% penicillin and streptomycin, and cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

Bioinformatics analysis
LncRNA profiling data are available via the Gene Expression Omnibus (GEO): GSE111168. Total RNA from lncRNA CANT1-knockdown RB and control RB cells was isolated and quantified. The concentration of each sample was measured by a NanoDrop 2000 (Thermo Scientific, USA). The quality was assessed by an Agilent2200 (Agilent, USA). The sequencing library of each RNA sample was prepared by using an Ion Proton Total RNA-Seq Kit v2 according to the protocol provided by the manufacturer (Life Technologies, USA).

Real-time quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted using TRIzol Reagent (ThermoFisher Scientific, USA) according to the manufacturer’s instructions. For the analysis of mRNA expression, cDNA synthesis was performed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biomedical Technology, Beijing, China). PCR analysis was performed using KlenTaq I mix, and amplified PCR products were quantified and normalized using GAPDH as a control. The PCR cycle parameters for lncRNA CASC15 and lncRNA CANT1 expression were as follows: 40 cycles of denaturation at 95 °C for 30 s, 65 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. RT qPCR was performed using the SYBR Premix ExTaq (Takara Biomedical Technology, Beijing, China) under standard conditions according to the manufacturer’s instructions and also normalized using GAPDH as a control. The primers are listed in Supplementary Table S1.

Plasmid construction and lentivirus packaging
The lncRNA CANT1 plasmid was constructed as previously described. The 293T cells were transfected using Lipofectamine 2000 reagent (Invitrogen, USA) with 3 μg CMV-CANT1, 3 μg pMD2.D, and 6.0 μg PsPax. The medium was replaced with 10 mL of fresh DMEM after 6 h. The virus-containing supernatants were collected at 48 and 72 h after transfection and then mixed and filtered through a 0.45 μm cellulose acetate filter (Sartorius, German). The viral supernatants were concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore, USA) and spun at 5000 rpm for 30 min. Colonies were
selected for subsequent culturing after incubation with 4 μg/mL puromycin for 4 weeks.

Western blot analysis
Total protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentration was determined using a BCA Kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked with 5% nonfat milk for 1 h and incubated with primary antibodies overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. Protein bands were detected using a BIO-RAD imaging system (BIO-RAD, Hercules, CA, USA). The following antibodies were used: anti-PI3Kγ (1:1000 dilution; CST 54055), anti-AKT (1:1000 dilution; CST 46915), anti-phospho-AKT (Ser473) (1:2000; CST 4060S), and anti-GAPDH (1:10000 dilution; Sigma G9295).

siRNA transfection
PI3Kγ and negative control siRNAs were designed and synthesized by Biomics (Shanghai, China). Y79 and Weri-Rb1 cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s protocol. Briefly, the cells were seeded at 2 × 10^5 cells per well in 6-well plates and transfected with 125 pmol of siRNA (target gene or negative control) using Lipofectamine 2000 in Opti-MEM I Reduced Serum Medium (GIBCO, USA). After 48 h of transfection, the cells were harvested and centrifuged for Western blotting. Twenty-four hours after transfection, the cells were harvested by centrifugation and used for tumor assays.

CCK8 cell viability assay
Cells were seeded at a density of 2000 cells per well in flat-bottomed 96-well plates. At the end of the incubation time, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo) solution was added to each well. After 4 h, the optical density at 450 nm was determined using a microplate reader (Varioskan Flash; Thermo, USA), and the absorbance values were normalized to the values of the cells at 0 h.

Soft agar assay
A volume of 1 mL of 0.6% agar in the complete medium was spread in each well of a 6-well plate; 5000 cells were suspended in 1.0 mL of 0.3% agar complete medium and seeded into the upper layer. The cells were cultured with 300 μL of complete medium for 4 weeks. The colonies in soft agar were stained with 0.005% crystal violet and then photographed.

Mouse xenograft experiments
All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were cared for according to the guiding principles in the care and use of animals. The animal experiments were approved by the Animal Care and Use Committee at Shanghai JiaoTong University School of Medicine. All experiments conform to the guide for care and use of laboratory animals published by the National Institutes of Health (NIH Publication 85-23, revised in 1996).

For xenograft experiments, 4-week-old male BALB/c nude mice were used. Mice were randomly divided into two groups: the Weri-Rb1 group (N = 7 eyes) and the Weri-Rb1-CANT1 group (N = 7 eyes). The method for the inoculation of tumor cells into the posterior segments of the eye was as follows: nude mice were anaesthetized by intraperitoneal injection of a ketamine (final concentration: 10 mg/mL) and xylazine (final concentration: 1 mg/mL) mixture (0.01 mL/g mouse weight) and with alcaline ocular surface anesthesia. Under a surgical microscope, a sharp 30-gauge needle was used to make two holes through the sclera, one into the intravitreal space to reduce intraocular pressure and one tangentially through the sclera into the subretinal space for injection. RB cells (1 × 10^6) were injected into the second hole into the choroid and subretinal space using a 1.5 cm, 33-gauge blunt end microinjection needle (7803-05, Hamilton, Reno, NV, USA). After the injection, eyes were covered with ophthalmic bacitracin ointment and buprenorphine was administered for relieving pain relief.

Immunohistochemistry (IHC)
Tissues were embedded in paraffin, deparaffinized with xylene and rehydrated. Antigen retrieval was performed by heating in sodium citrate buffer (pH 6.0). The sections were blocked with 3% hydrogen peroxide for 20 min and then in 10% goat serum for 5 min. For tissue microarray immunohistochemical staining, tissues sections were incubated at 4 °C overnight with a rat anti-human PI3Kγ antibody (CST, USA) at a dilution of 1:100. Tissues were then rinsed in PBST (PBS containing 0.05% Triton X-100), and biotinylated anti-rat secondary antibody was added at a 1:500 dilution and incubated at room temperature for 1 h. After washing twice with PBST, the slides were incubated with streptavidin–horseradish peroxidase (BD Biosciences, USA) and diaminobenzidine substrate for colorimetric development.

Cytoplasmic and nuclear RNA isolation
Cytoplasmic and nuclear RNA was extracted using Thermo Fisher BioReagents (Thermo Fisher, USA) according to the manufacturer’s instructions. RT-qPCR was performed to amplify the localized IncRNA CANT1.
as follows: 1 μL of 3× Klen-Taq I Mix, 1 μL of cDNA, and 0.5 μL of each 10 μM primer were combined under liquid wax. After incubation at 95 °C for 2 min, the cDNA was amplified with 40 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s (extension), and with a final extension at 72 °C for 5 min.

**RNA Fish**

RNA FISH was performed using a fluorescent in situ hybridization kit (RiboBio, China) following the manufacturer’s instructions. The lncRNA CANT1 probes were designed and synthesized by the RiboBio Company. Briefly, cells were collected after transfection with the corresponding vector for 48 h and subsequently seeded on glass coverslips. Fluorescence detection was performed with a microscope (BX41; Olympus, Japan).

**Chromatin oligonucleotide precipitation (ChOP)**

The ChOP assay was performed as previously described. Cells were fixed using 1% formaldehyde (Sigma-Aldrich, USA) for 10 min at room temperature and centrifuged at 3000 rpm for 15 min. The pellet was suspended in 300 μL of buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% v/v NP-40) and incubated for 15 min on ice. The nuclei were harvested in 150 μL of buffer B (50 mM Tris-HCl, pH 7.9, 0.5 mM PMSF, 0.1% SDS, 10 mM EDTA,100 U/mL RNase, and protease inhibitors) and incubated on ice for 10 min. An equal volume of buffer C (15 mM Tris-HCl, pH 7.9, 1 mM EDTA,1% Triton X-100, 150 mM NaCl, 0.5 mM PMSF, 100 U/mL RNase, and protease inhibitors) was added, and the samples were sonicated (10 s on, 15 s off, output 30%, 4 min). Sonicated DNA was found to be enriched in the range of 200–500 bp. After centrifugation, 150 μL aliquots of sonicated chromatin were combined with 100 pmol of either biotinylated antisense oligo against the target RNA or biotinylated scrambled oligos, incubated at an appropriate annealing temperature for 5 min and then slowly cooled to room temperature. A 50 μL volume of beads was used to capture the biotinylated DNA/RNA complexes for 25 min at room temperature with gentle rotation. After 3 washes, 150 μL of diethyl pyrocarbonate (DEPC) water was used for elution at 70 °C for 5 min. After crosslink reversal and purification, the samples were ready for PCR. A TaqMan assay using the ABI 7500 Real-Time PCR System was performed to detect the quality of the lncRNA CANT1 pulled down by Dynabeads MyOne Streptavidin C1 beads. Primers and probes labeled at their 5’ and 3’ ends with FAM and minor groove binder or black hole quencher-1 were designed to target lncRNA CANT1. The amplification reactions were optimized individually for all of the probes and associated primers. Each reaction was conducted in a total volume of 10 μL consisting of 0.25 μL of 10 mM dinucleotide triphosphates (dNTPs), 0.1 μL of the TaqMan probe, 0.6 μL of 25 mM MgCl₂, 2 μL of 5× Q buffer, 0.1 μL of 5 U/μL Hotstart, 0.1 μL of the reference ROX dye, 0.25 μL of each 10 μM primer, and 4 μL of the template.

**Chromatin immunoprecipitation (ChiP)**

ChiP was performed using an EZ-Magna ChiP A/G kit (Millipore) according to the manufacturer’s instructions. Anti-hSET1 was purchased from Abcam and anti-H3K4me3 was purchased from Millipore. Anti-RNA polymerase-II (pol-II; Millipore) was used as a positive control antibody and normal mouse IgG was used as a negative control. See Supplementary Table S1 for a list of primers for ChiP-qPCR.

**Statistical analysis**

Experiments were performed in triplicate when indicated, and the data were presented as the means ± SEM. The comparative threshold cycle method was applied in the RT-qPCR assay according to the ΔΔ threshold cycle method. The differences between two groups were analyzed using the unpaired two-sided Student’s t test. A P value <0.05 was considered statistically significant.

**Results**

**CANT1 lncRNA expression is downregulated in retinoblastoma**

A genome-wide RNA-sequencing analysis of three retinoblastoma tissues and paired normal tissues that we previously reported can be accessed via GEO: GSE11116814. The bioinformatics analysis showed that the level of CASC15 lncRNA was lower in tumor tissues than that in normal tissues (Fig. 1a). We next identified which transcript existed on the chromosome 6p22.3 locus in different tissues, for example, CASC15 is 1902 bp in length with 12 exons according to databases in the University of California, Santa Cruz (UCSC) and National Center for Biotechnology Information (NCBI) databases, and lncRNA CANT1 (GenBank: KP981381.1) is 1114 bp with 7 exons, which was confirmed by our previous research. Therefore, we designed two isoform-specific primers to differentiate the transcripts (Supplementary Fig. S1A). The data showed that lncRNA CANT1 expression was significantly decreased in RB cells compared with normal ARPE19 cells, however, CASC15 was weakly expressed in both ARPE19 and RB cell lines (Fig. 1b). We also further confirmed CANT1 expression by RT-PCR (Fig. 1c). To determine the clinical relevance of CANT1 in RB, we collected human RB tissue samples (Table S2) to examine CANT1 expression. The expression of CANT1 was markedly reduced in RB tissues compared with normal tissues (Fig. 1d). These data show that lncRNA CANT1 is alternatively spliced from chromosome.
Fig. 1 (See legend on next page.)
6p22.3 and is likely to play an unknown role in RB tumorigenesis.

To explore the potential role of CANT1 in RB, we overexpressed IncRNA CANT1 in RB cell lines. Therefore, we constructed a plasmid containing the full-length CANT1 sequence and packaged it in a lentivirus for transfection into Y79 and Weri-Rb1. As expected, IncRNA CANT1 was successfully overexpressed in Y79 and Weri-Rb1 cells (Fig. 1e).

CANT1 modulates RB tumorigenesis in vitro and in vivo

We next investigated whether the RB tumor characteristics were altered after CANT1 overexpression. In a colony formation assay, the number of colonies of CANT1-overexpressing Y79 cell (Fig. 1f upper and Fig. 1g) and Weri-Rb1 cell (Fig. 1f bottom and Fig. 1h) colonies was significantly reduced. In addition, we used a CCK8 assay to evaluate tumor cell growth. As expected, the RB cell growth rate was significantly lower than the wide-type RB cell growth rate (Fig. 2a, b). Next, we used a classical soft agar assay to examine tumor formation ability in vitro. We observed that tumor colonies were markedly smaller than those formed by wild-type tumor cells and mock cells (Fig. 2c). In addition, the statistical analysis confirmed that the number of colonies formed by the two tumor cells types was greatly reduced by CANT1 overexpression in vitro (Fig. 2d).

To examine the ability of CANT1 to suppress tumor formation in vivo, we inoculated CANT1-overexpressing Weri-Rb1 cells and mock cells into the posterior segments of the eyes of nude mice. Extraocular tissue was removed, and the tumor-bearing eye mass was measured. As expected, the eye mass weight in the CANT1-overexpressing group was reduced by 48% (n = 7, *P < 0.05; Fig. 2e, Supplementary Fig. S2A), and the eye mass exhibited a marked reduction in size (Fig. 2f). These results demonstrate that the IncRNA CANT1 serves as a tumor suppressor that modulates tumor formation in RB.

CANT1 modulates PI3K/Akt signaling in RB

To elucidate the mechanism underlying the suppressive role of CANT1 in RB expansion, we employed RNA transcriptome-sequencing (GEO Accession number: GSE141327) to analyze CANT1-overexpressing cells and control Y79 and Weri-Rb1 cells. We found differentially expressed genes (fold change ≥ 1.5, false discovery rate < 0.05) in both cell lines, with 12 genes upregulated and 454 genes downregulated (Fig. 3a). The Gene Ontology (GO) analysis showed that the most significantly overrepresented biological processes included pathways involved in oxygen transport, regulation of cell population proliferation, as well as signal transduction (Supplementary Fig. S3A). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that several pathways, including the NF-κB signaling pathway and PI3K/Akt signaling pathways, were altered (Fig. 3b). Then, we focused on the differentially expressed genes in PI3K/Akt signaling pathway (Fig. 3c) and examined the expression of PI3Kγ (also called PIK3CG, gene ID: 5294; one of the most important genes in PI3K/Akt signaling) in tumor cells and in normal ARPE19 cells. The RNA-sequence analysis showed that there was almost no transcription at the PI3Kγ locus in CANT1-overexpressing RB cells (Supplementary Fig. S3B). As expected, at the mRNA level, PI3Kγ expression was upregulated more than 10-fold in Y79 cells and approximately 20-fold in Weri-Rb1 cells, compared with that in normal ARPE19 cells (Fig. 3d). The Western blot data confirmed that the protein expression of PI3Kγ in RB tumor cells was markedly increased compared to that of normal ARPE19 cells, which displayed extremely weak PI3Kγ expression (Fig. 3e, lane1, lane 4, and lane 7). However, in CANT1-overexpressing RB cells, both mRNA (Fig. 3d) and protein (Fig. 3e, lane 2 and lane 5) levels of PI3Kγ were significantly reduced. We also examined the activation of Akt, downstream of PI3Kγ. The Akt phosphorylation level decreased in accordance with the PI3Kγ expression level. An IHC staining was then performed to detect PI3Kγ protein expression in RB tissues compared with normal retina. The results clearly showed that PI3Kγ protein was remarkably increased in the human RB tissues (Fig. 3f). The staining of PI3Kγ in the mouse Weri-Rb1-mock eyes was also darker than that in the Weri-Rb1-CANT1 eyes obtained from the mouse experiments (Supplementary Fig. S3C). Taken together, these data demonstrate that PI3Kγ may act as a potential target of the IncRNA CANT1.
Fig. 2 Functional roles of CANT1 IncRNA in RB. a, b A CCK8 assay was performed to measure the cell growth rate of CANT1-overexpressing Y79 and Weri-Rb1 cells. The experiments were performed in triplicate, and the absorbance at day 1 was set as 100%. *P < 0.05 compared with the control and mock. c, d A soft agar assay was used to assess the tumor formation ability in vitro. c Small colonies were observed and counted under the microscope. d Colony count statistics showed a significant reduction in colonies formed by CANT1-overexpressing cells. Colony numbers were counted in three independent soft agar plates. All of the data are presented as the mean ± SEM. *P < 0.05 compared with the control and mock. Scale bar: 600 μm. e, f Eye weight of the orthotropic xenograft formed by Weri-Rb1 cells injected into the vitreous cavity with or without CANT1 overexpression at 40 days after implantation; n = 7, *P < 0.05 compared with the mock. f Representative images of H&E staining for the evaluation of tumor formation in vivo. Scale bar: 200 μm.
Fig. 3 Regulatory targets of CANT1 IncRNA in RB. a The overlapping genes presenting differential expression between the two RB cell lines are shown. Twelve genes were co-upregulated, and 454 genes were co-downregulated (fold change ≥ 1.5, FDR < 0.05). b KEGG analysis for all genes with differential expression. c Heatmap of genes in the PI3K/Akt signaling pathway. d Real-time PCR was performed to measure the PI3Kγ mRNA level in tumor and normal cells. All of the data are presented as the mean ± SEM. *P < 0.05: compared with the control and mock. e Western blot showing the protein levels of PI3Kγ, p-AKT, and pan-AKT in tumor and normal cells. GAPDH was used as internal control. f Immunohistochemical staining of PI3Kγ in RB and normal tissues. PI3Kγ expression in tumor sections was higher than normal tissues. Scale bar: 200 μm. g Two siRNAs were used to silence PI3Kγ, as examined by Western blot.
**PI3Kγ is required for RB tumorigenesis**

To further investigate the role of PI3Kγ in RB, we used the classic RNAi method to silence PI3Kγ expression in RB cells. Three siRNAs (siPI3Kγ-1, siPI3Kγ-2, and siPI3Kγ-3) were designed to test the efficiency of silencing. The results showed that both siPI3Kγ-1 and siPI3Kγ-2 worked and an siPI3Kγ-mix that combined siPI3Kγ-1 and siPI3Kγ-2 was more efficient than the two siRNAs alone in knocking down PI3Kγ expression at the mRNA transcript level (Supplementary Fig. S4A, B). siPI3Kγ-1, siPI3Kγ-2, and siPI3Kγ-mix were able to knock down the PI3Kγ protein and silence PI3K/Akt signaling in the two RB cell lines (Fig. 3g). Intriguingly, IncRNA CANT1 expression was not notably changed when PI3Kγ was depleted (Supplementary Fig. S4C, D). These data support our hypothesis that diminished PI3Kγ expression is triggered by CANT1 upregulation and PI3Kγ acts as a regulatory target of IncRNA CANT1.

To further define the role of PI3Kγ in tumor formation, we chose siPI3Kγ-mix for subsequent experiments. In the CCK8 assay, the proliferation of PI3Kγ-knockdown RB cells was found to be significantly decreased (Fig. 4a, b). A colony quantification assay showed that the number of colonies was markedly reduced by siRNA treatment in Y79 and Weri-Rb1 cells (Fig. 4c, d). Furthermore, through a soft agar assay, we found that the in vitro colony formation ability of RB cells was significantly reduced by PI3Kγ inactivation (Fig. 4e, f). These data show that PI3Kγ may act as an oncogene in RB progression and its attenuation may mediate the antitumor effect of CANT1 in RB tumorigenesis.

**CANT1 directly binds to the PI3Kγ promoter**

Using U2 snRNA as a positive control for nuclear fraction and GAPDH as a control for the cytosolic fraction, we found that CANT1 was located mainly in the nucleus in CANT1-overexpressing Y79 and Weri-Rb1 cells (Fig. 5a). RNA fluorescence in situ hybridization (RNA FISH) further confirmed that CANT1 was mainly distributed in the nucleus (Fig. 5b). In addition, RNA FISH demonstrated that CANT1 RNA was abundant in the cell nucleus of human retina tissues, whereas very weak signals were observed in human retinoblastoma tissues (Fig. 5c). These data suggest that CANT1 is a nuclear IncRNA in RB cells and might guide PI3Kγ regulation via a chromosome-related mechanism. To explore the mechanism by which CANT1 regulates the expression of PI3Kγ, we used a classical ChOP assay. We designed two biotin-labeled, short oligonucleotides aligned with CANT1 (Fig. 5d, upper). Site a (300 bp upstream of the PI3Kγ TSS) was used to detect the promoter region of PI3Kγ and site b was used as nonspecific promoter region (Fig. 5d, bottom). After pull-down, we found that CANT1 bound strongly to the PI3Kγ promoter in two CANT1-overexpressing cell lines (Fig. 5e, f, lane 3 and lane 6), whereas this DNA–RNA interaction was not observed in the control or mock-transfected cells (Fig. 5e, f, lane 2, lane 4, lane 5, and lane 7). We also used a random oligonucleotide as a negative control because it did not bind to the PI3Kγ promoter in either cell line (Fig. 5e, f, lanes 8–10). In addition, to further validate the binding of CANT1 to the PI3Kγ promoter, we performed an RT-qPCR analysis to quantitate the enrichment of CANT1 at the PI3Kγ promoter. The analysis showed that CANT1 binding to the promoter of PI3Kγ was increased compared with that in the controls and mocks (Fig. 5g, h). Taken together, these results demonstrate that CANT1 might regulate PI3Kγ expression by directly binding to key DNA regulatory regions of its promoter.

**CANT1 modulates PI3Kγ expression by abolishing histone H3K4 methylation**

Next, we explored whether epigenetic modifications were altered and whether the histone methylation status was changed at the promoter of the PI3Kγ gene after CANT1 overexpression. Site X was 6 kb upstream of the PI3Kγ transcription start site (TSS), site Y was 300 bp upstream of the PI3Kγ TSS, and site Z was the promoter of GAPDH (Fig. 6a). Through a ChIP assay, we found that the H3K4 trimethylation status at the PI3Kγ promoter was significantly decreased after CANT1 overexpression (Fig. 6c). The H3K4 trimethylation status at 6 kb upstream of the PI3Kγ TSS (site X) was minimal, whether CANT1 was overexpressed or not (Fig. 6b). H3K4 modification at the GAPDH promoter (site Z) was used as a positive control because GAPDH expression was stable in all cell lines (Fig. 6d). The above results were further confirmed by a quantitative ChIP-PCR assay (Fig. 6e, f). Furthermore, we found that there was no significant change in H3K4me3 expression after CANT1 overexpression (Supplementary Fig. S4E), indicating that CANT1 itself does not regulate the activity of H3K4 methyltransferase in cell lines and likely regulates H3K4me3 of the PI3Kγ promoter by modulating the binding of H3K4 methyltransferase to the target regions of the genome.

**CANT1 competes with hSET1 methyltransferase in vitro**

Because hSET and MLL1–4 are H3K4 methyltransferases, we sought to identify which participates in the above process. We used a ChIP assay and found that hSET1 bound to the nearby PI3Kγ promoter in control and mock cells (Fig. 7b, lane 2, lane 4, lane 5, and lane 7), but the recruitment of hSET1 to the PI3Kγ promoter was markedly inhibited in the two CANT1-overexpressing cell lines (Fig. 7b, lane 3 and lane 6). We used negative ChIP Site X (6 kb upstream of the PI3Kγ TSS) to exclude nonspecific interactions. As expected, hSET1 binding to the PI3Kγ promoter could not be measured regardless of the CANT1 expression status at site X (Fig. 7a). Similarly, the
ChIP-qPCR data were consistent with these data (Fig. 7c, d). We also performed an RNA IP assay and found that \textit{CANT1} does not bind to the hSET1 protein. Taken together, these results demonstrate that \textit{CANT1} blocks the hSET1 methyltransferase from binding to the \textit{PI3K\gamma} promoter.

**Discussion**

Considering that more than 80\% of the genome is transcribed and that the majority of the transcription across the genome is non-coding, the chances of identifying a functional potential noncoding RNA are high\textsuperscript{27}. A set of characterization studies has identified the critical roles played by long noncoding RNAs in gene expression regulation, cytoplasmic or nuclear complexes scaffolding, and pairing with other RNAs\textsuperscript{3,28,29}. However, the functions of the vast majority of these transcripts remain unknown and deserve further investigation. Herein, we revealed that a novel inactivated IncRNA, \textit{CANT1}, at chromosome 6p22.3 modulates RB tumorigenesis through the epigenetic activation of \textit{PI3K\gamma} expression, thus enhancing PI3K/Akt signaling and accelerating tumor progression (Fig. 7e).
Fig. 5 (See legend on next page.)
**Fig. 5 CANT1 binds to the promoter of its targets.**

- **a** The location of mature CANT1. CANT1 is mainly located in the nucleus. U2 RNA was used as a positive control for nuclear RNA, and GAPDH served as a positive control for the cytoplasmic RNA.
- **b** Representative RNA FISH images showed that the CANT1 signal overlapped with DAPI staining in ARPE19 cells. The scale bars represent 10 μm.
- **c** RNA-FISH was performed with CANT1 oligos on clinical retinoblastoma samples and normal control samples. Scale bar: 20 μm.
- **d** Schematic diagram of lncRNA CANT1 and the PI3Kγ promoter region. CANT1 oligo-1 and oligo-2 indicate the biotinylated antisense oligonucleotides targeting IncRNA CANT1. Random oligo indicates the scrambled oligonucleotide used as a negative control in the ChOP assay.
- **e, f** PCR examination of the binding of CANT1 to the PI3Kγ promoter in the ChOP assay. Site a: CANT1 interacts with the PI3Kγ promoter. Site b: a negative control locus. Input: Total RNA was reverse transcribed before incubation with labeled CANT1 fragments and amplified with primers for site a and site b.
- **g, h** Quantification of the binding of CANT1 to the PI3Kγ promoter in the ChOP assay by real-time qPCR. The data are presented as mean ± SEM. *P < 0.05.

**Fig. 6 CANT1 modulates PI3Kγ transcription by abolishing H3K4 methylation.**

- **a** Schematic diagram of the PI3Kγ and the GAPDH promoter regions. Arrow: transcriptional direction; sites X–Z: different sites used in this assay.
- **b–d** PCR examination of histone H3K4 trimethylation changes in the PI3Kγ promoter (c) and GAPDH promoter (d) upon CANT1 overexpression in Y79 and Weri-Rb1 cells. IgG was used as a negative control. Input: Total RNA was reverse transcribed before incubation and amplified with primers for site a and site b.
- **e, f** Quantiﬁcation of the binding of CANT1 to the PI3Kγ promoter in the ChOP assay by real-time qPCR. The data are presented as mean ± SEM. *P < 0.05.

Comparing the control and mock.
Fig. 7 CANT1 competes with hSET1 at the PI3Kγ promoter in vitro. a, b ChIP assays demonstrated that CANT1 blocks the recruitment of hSET1 to the PI3Kγ promoter. IgG was used as a negative control. Sites X, Y: ChIP detection sites. Input: total RNA was reverse transcribed before incubation and amplified with primers. M: marker. c, d Real-time qPCR examination of hSET1 changes in the PI3Kγ promoter. All data are presented as the means ± SEM. *P < 0.05: compared with the control and mock. e Model of CANT1 regulation in tumorigenesis. In parent cancer cells, CANT1 IncRNA is inactivated and the hSET1 methyltransferase freely modifies the PI3Kγ promoter, providing histone H3K4 methylation to induce PI3Kγ expression; however, in CANT1-overexpressing cells, CANT1 occupies the PI3Kγ promoter and blocks the hSET1 interaction with the PI3Kγ promoter. Then, free hSET1 fails to methylate the PI3Kγ promoter and silences PI3Kγ expression, thus decreasing PI3K/Akt signaling and inhibiting tumor growth.
It has been shown that chromosome 6p22.3 is a tumor susceptibility locus that impacts tumor initiation and progression. Ensemble annotation predicted six CASC15 lncRNA isoforms, two of which (CASC15-003 and CASC15-004) are widely expressed in neuroblastoma, predicting an improved clinical outcome with increased expression. Otherwise, the CASC15 isoform exerts oncogenic functions in the cutaneous melanoma progression. We previously reported that lncRNA CANT1 triggers a CANT1-3P/FTX-XIST long noncoding pathway to suppress uveal melanoma progression. Due to the etiology of eye neoplasms differing markedly from cutaneous melanomas and neuroblastomas, it is not surprising that the CANT1 isoform exists in RB and acts as a tumor suppressor that affects the characteristics of RB.

Studies have shown that class 1 phosphoinositide 3-kinases (PI3Ks), consisting of PI3Ka, PI3Kβ, PI3Kδ, and PI3Kγ, are a family of dual-specificity lipid and protein kinases that phosphorylate the inositol ring of phosphoinositides and then activate Akt, a serine/threonine kinase that directly phosphorylates a wide variety of targets participating in cell growth and survival. PI3Kγ is a gene that plays different roles in various human malignancies. PI3Kγ is frequently deleted in myeloid malignancies and is evaluated as a candidate myeloid tumor suppressor gene. However, in T-cell acute lymphoblastic leukemia, PI3Kγ can alone support leukemogenesis in the absence of PTEN phosphatase tumor suppressor function and serves as a nonclassical oncogene. Others have reported that PI3Kγ may represent a suitable molecular target for therapeutic intervention in Kaposi’s sarcoma and claudin-low breast cancer. Study has shown that down-regulation of PI3Kγ expression and hypermethylation at CpG sites of the promoter regions were also detected in primary colon cancers. It has been shown that PI3Kγ and PI3Kδ are inhibitors of PI3Kβ and PI3Kγ, respectively. PI3Kγ is overexpressed in RB cells, PI3Kγ can alone suppress leukemogenesis in the absence of PTEN phosphatase tumor suppressor function and serves as a nonclassical oncogene. Others have reported that PI3Kγ may represent a suitable molecular target for therapeutic intervention in Kaposi’s sarcoma and claudin-low breast cancer. However, PI3Kγ may alone support leukemogenesis in the absence of PTEN phosphatase tumor suppressor function and serves as a nonclassical oncogene. Others have reported that PI3Kγ may represent a suitable molecular target for therapeutic intervention in Kaposi’s sarcoma and claudin-low breast cancer. Further genetic or epigenetic alterations, such as MYCN amplification, inactivating mutations of BCL-6 co-repressor (BCOR), the aberrant expression of SYK, DNA copy number gains of KIF14 and noncoding RNAs, likely contribute to the malignant transformation. In addition, we previously reported for the first time that lncRNA GAU1 is a novel oncorRNA that promotes RB tumorigenesis. In this study, through the genomic analysis of our previous profiling data, we identified that lncRNA CANT1 expression is markedly downregulated in RB tissues compared to that in normal tissues. Low expression levels of CANT1 correlated with some clinicopathological factors, such as tumor growth rate and increased tumor size, suggesting that CANT1 might be a potential therapeutic target and prognostic indicator of RB.

In aggregate, our data suggest a novel mechanism in which lncRNA CANT1 serves as a tumor suppressor and outline a new pattern of histone modification in RB tumorigenesis. It is possible that CANT1 may block target gene expression by binding to DNA elements. Understanding the various roles of lncRNAs in tumor progression enables us to better explain the disease phenotype, improve the treatment regimens and provide reliable lncRNA-based prognostic markers.
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Conflict of interest

The authors declare that they have no conflict of interest.

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