MYC Targeted Long Noncoding RNA DANCR Promotes Cancer in Part by Reducing p21 Levels

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Abstract

The MYC oncogene broadly promotes transcription mediated by all nuclear RNA polymerases, thereby acting as a positive modifier of global gene expression. Here, we report that MYC stimulates the transcription of DANCR, a long noncoding RNA (lncRNA) that is widely overexpressed in human cancer. We identified DANCR through its overexpression in a transgenic model of MYC-induced lymphoma, but found that it was broadly upregulated in many human cancer cell lines and cancers, including most notably in prostate and ovarian cancers. Mechanistic investigations indicated that DANCR limited the expression of cell-cycle inhibitor p21 (CDKN1A) and that the inhibitory effects of DANCR loss on cell proliferation could be partially rescued by p21 silencing. In a xenograft model of human ovarian cancer, a nanoparticle-mediated siRNA strategy to target DANCR in vivo was sufficient to strongly inhibit tumor growth. Our observations expand knowledge of how MYC drives cancer cell proliferation by identifying DANCR as a critical lncRNA widely overexpressed in human cancers.

Significance: These findings expand knowledge of how MYC drives cancer cell proliferation by identifying an oncogenic long noncoding RNA that is widely overexpressed in human cancers.

Cancer Res; 78(1); 64–74. © 2017 AACR.

Introduction

MYC is a frequently amplified human oncogene that encodes the transcription factor MYC, which dimerizes with MAX to regulate both the activation and repression of gene transcription (1–9). Although MYC could have nontranscriptional roles, many of its activities could be experimentally linked to regulation of transcription (2). To understand and appreciate MYC’s biological roles, early studies identified key MYC transcriptional targets that are involved in metabolism, cell growth, cell-cycle regulation, and apoptosis (1, 2). In subsequent studies, we documented that MYC could also regulate microRNAs to modulate many different cellular processes and surmised that MYC would also have a key role in regulating biologically important long noncoding RNAs (lncRNA; refs. 1, 10–13). LncRNAs have now been documented to play important roles in cancers (14, 15). Although we were performing the studies reported herein, a number of MYC-regulated lncRNAs or lncRNAs that regulate MYC function were recently reported to have biological activities (16–22).

Here, we report our identification of MYC-regulated lncRNAs and a highly responsive MYC-inducible lncRNA, termed DANCR, in a model of MYC-inducible human B lymphoma. DANCR was first described as a lncRNA that blocks differentiation of the epidermal progenitor cells (23). Intriguingly, we also found that a conserved murine DANCR homolog (miDancr) is highly expressed in an MYC-induced transgenic model of lymphoma, suggesting that DANCR is involved in MYC-induced lymphomagenesis. We document the expression of MYC-regulated lncRNAs across the spectrum of human cancers, the direct regulation of DANCR by MYC and its role in prostate and ovarian tumorigenesis. We found that loss of DANCR is associated with increased p21 (CDKN1A) expression and decreased S-phase entry and cell proliferation in prostate and ovarian cancer cell lines. Importantly, we found that nanoparticle delivered siRNAs against DANCR in vivo significantly diminished tumor growth in an orthotopic ovarian xenograft model (24–27). These studies document a significant role for MYC in the regulation of cell growth through the expression of lncRNAs, particularly DANCR, adding to its broad biological influence over the entire transcriptome and cell proliferation.

Materials and Methods

Patients and specimens

Human tumor-derived samples were studied according to the U.S. Common Rule, collected with informed consent, and
approved for use by the Johns Hopkins Institutional Review Board (approval No. NA00048544). RNA samples were isolated using TriZol from frozen sections of matched prostate tumor and benign samples (tumor/normal pairs) obtained at radical prostatectomy at The Johns Hopkins Hospital. Tumor samples were highly enriched (generally greater than 80% tumor purity). Gleason scores ranged from 6 to 9 and pathological stage ranged from T2N0 to T3B1N1. Patient ages ranged from 46 to 69.

Cell lines and cell culture
P493-6 cell was a generous gift of Dr. Eick at the Institute of Molecular Biology and Tumors Genetics, GSF-Research Centre, Munich, Germany. This Mycoplasma-free cell line is unavailable from the ATCC and has been continuously used in the Dang laboratory and authenticated by the presence of a highly inducible human MYC transgene. The PC3 and DU145 cell lines were obtained from the ATCC and are Mycoplasma free. Ovarian cancer OVCA432 cells was a kind gift from Dr. Kwong K. Wong, MD Anderson Cancer Center (Houston, TX). Cells are generally unavailable from the ATCC and has been continuously used in the Dang laboratory and authenticated by the presence of a highly inducible human MYC transgene. The PC3 and DU145 cell lines were obtained from the ATCC and are Mycoplasma free. Ovarian cancer OVCA432 cells was a kind gift from Dr. Kwong K. Wong, MD Anderson Cancer Center (Houston, TX). Cells are generally used for 5 to 10 passages from the initial expansion and freeze-down. Cells were maintained in a log phase of cell growth by culturing in RPMI-1640 supplemented with 10% FBS and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA) in a humidified incubator at 37°C in the presence of 5% CO2 and 95% air. Cells were routinely screened for Mycoplasma and authentication of cells was done by the characterized Cell Line Core Facility at the UTMDACC. Cells were cultured in DMEM or RPMI-1640 with 10% FCS and 1% penicillin/streptomycin. To repress MYC, the DANC-DA in P493-6 cells, cultures were supplemented with 0.1 μg/mL tetracycline (Sigma) for 48 hours and harvested. Human ES cells (H1 and H9 clones) were grown on mouse embryo fibroblasts (MEF) in culture medium that consisted of 80% DMEM/F12 supplemented with 20% knock-out serum replacement (Invitrogen), 1 mmol/L L-glutamine, 0.1 mmol/L 2-mercaptoethanol, and 1% nonessential amino acids (Sigma-Aldrich).

Vivo-Morpholino studies
The DANC mRNA was analyzed, two exon-intron junctions were inspected. The boundaries were submitted to the Gene Tools design service for morpholino design (http://www.gene-tools.com/). The DANC exon1-intron1 Vivo-Morpholino sequence is 5′-AAAGACTGGAGATGCACCTCAACGCCCG-3′. The DANC exon2-intron2 Vivo-Morpholino sequence is 5′-CGCTCAAGGCTGAGAAGATCG-3′. The standard control nontargeting Vivo-Morpholino is the sequence designed and published by Gene Tools (5′-CTGGTTACCCACTGTAC-3′). Vivo-Morpholino treatment of P493-6 cells. Knockdown of DANC was carried out with two DANC-targeting antisense Vivo-Morpholino obtained from Gene Tools. Equal amount of PBS and standard control (nontargeting) Vivo-Morpholino (Gene Tools) were used as negative controls. Different amount of Vivo-Morpholinos (to achieve 0.5, 1.0, and 1.5 μmol/L concentrations) or vehicle (PBS) were added to cell culture and cells were harvested at subsequent time points (24, 48, and 72 hours).

RNA-seq analysis
Total RNA from P493-6 cells was extracted using TRizol (Invitrogen) and an RNaseasy Plus Mini Kit (Qiagen). Extracted RNA samples underwent quality control assessment using an Agilent Bioanalyzer (Agilent). RNA samples underwent quality control assessment using the Agilent Bioanalyzer (Agilent) and all RNA samples submitted for sequencing had a RNA Integrity Number (RIN) >9, with a minimum of 1 μg input RNA. To normalize the expression values, we imported the aligned reads of each BAM file to Cufflinks RNA-Seq workflow to obtain the expression levels for genes by summarizing the fragments per kb per million mapped fragments (FPKM) values. Poly-A enriched library preparation and sequencing were performed at the Penn Next-Generation Sequencing Core following standard protocols. Briefly, mRNA was purified using poly(A) selection or RNA depletion before the RNA was chemically fragmented and converted into single-stranded cDNA using random hexamer priming. The second strand was generated to create double-stranded cDNA. Library construction began with the generation of blunt-end DNA fragments from ds-cDNA. Then, A-bases were added to blunt-ends to prepare them for the ligation of sequencing adapters using the TruSeq RNA Sample Preparation Kit (Illumina). Fragments of approximately 300 to approximately 400 bp were selected by gel electrophoresis, followed by 15 cycles of PCR amplification. The prepared libraries were then sequenced using an Illumina HiSeq 2000, which generated approximately 50 to approximately 100 million unfiltered reads per sample.

For RNA-seq analysis of MYC-ON versus MYC-OFF states, the P493-6 cells, which were engineered to have tetracycline-repressible ectopic MYC, were supplemented with 0.1 μg/mL tetracycline (Sigma) for 48 hours before harvesting for the MYC-OFF state. The RNA from these cells were compared with MYC-ON cells that had continuously expressed MYC for 48 hours grown contemporaneously with the MYC-OFF cells.

Annotation of lncRNA, protein-coding genes, and pseudogenes
The GENCODE lncRNA annotation version 22 and the GENCODE whole genome comprehensive annotation version 22 were downloaded from the official GENCODE ftp repository: ftp://ftp.sanger.ac.uk/pub/GENCODE/. The GENCODE lncRNA annotation (V22), a manually curated and evidence-based lncRNA annotation containing 15,900 genes, was used to define lncRNA genes. The GENCODE whole genome annotation (V22) was used to define protein-coding genes (PCG) and pseudogenes, resulting in a PCG set containing 19,814 genes; a pseudogene set containing 14,477 genes; and an ‘other genes’ set containing 10,292 genes.

The Cancer Genome Atlas and Cancer Cell Line Encyclopedia RNA-seq data
The poly(A)+ RNA-seq (Illumina) data in FASTQ format of 496 human prostate tumor specimens and 52 matched normal adjacent tissue specimens were generated by University of North Carolina (UNC) as part of the The Cancer Genome Atlas (TCGA) project. The poly(A)+ RNA-seq (Illumina) data in BAM format of 933 human cancer cell lines across 21 cancer types were generated by the Cancer Cell Line Encyclopedia (CCLE) project, a collaboration between the Broad Institute, the Novartis Institutes for Biomedical Research, and the Genomics Institute of the Novartis Research Foundation. RNA-seq files were downloaded from the Cancer Genomics Hub (http://cghub.ucsc.edu). The poly(A)+ RNA-seq (Illumina) data in FASTQ format of Ei-Myc mouse were generated by Dr. Bruno Amati laboratory. RNA-seq files were downloaded from Gene Expression Omnibus (GSE51011). All FASTQ files were aligned to the reference human genome (hg38, GRCh38) or reference
mouse genome (mm10, GRCm38) via RNA-STAR-based ENCODE long RNA-Seq–processing pipeline. The BAM files from CCLE project were converted to FASTQ format using Picard Tools (Broad Institute) before alignment. We imported the aligned reads of each BAM file to Cufflinks RNA-Seq workflow to obtain the expression levels for genes by summarizing the fragments per kb per million mapped fragments (FPKM) values. GENCODE annotations were used to define lncRNAs, PCGs, and pseudogenes. The log-transformed FPKM values of genes were further analyzed using BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) according to the instructions provided.

qRT-PCR
Total RNA was extracted using TRIzol Reagent (Invitrogen) and reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer’s instructions. cDNA was quantified by real-time RT-PCR using an ABI Viia 7 System (Applied Biosystems). PCR was performed using SYBR Green reagents (Applied Biosystems) according to the manufacturer’s instructions. GAPDH was used as an internal control.

Chromatin immunoprecipitation assay
A total of 1 x 10^6 cells were cross-linked in 1% formaldehyde for 10 minutes, and the cross-linking was terminated with 0.125 mol/L glycine. The nuclear protein and fragmented chromatin was extracted by sequential incubation in cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 2 mmol/L EDTA, 0.2% Sarkosyl, 0.5% IGEPAI, 1 mmol/L PMSF) and nuclear lysis buffer (50 mmol/L Tris-HCl pH 8.0, 10 mmol/L EDTA, 1% SDS and protease inhibitor cocktail), followed by optimized sonication. The nuclear extract was subjected to immunoprecipitation with anti-MYC antibody (sc-764, Santa Cruz Biotechnology). The immunoprecipitate was washed sequentially in Dialysis buffer (10 mmol/L Tris-HCl pH 7.5, 10 mmol/L NaCl, 3 mmol/L MgCl2, 0.5% IGEPAI, 1 mmol/L PMSF) and Dialysis buffer (10 mmol/L Tris-HCl pH 9.0, 3 mmol/L MgCl2, 0.5% IGEPAI, 1 mmol/L PMSF). The cross-linking of chromatin was reversed and DNA purified by sequential incubation in cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 0.125 mol/L glycine. The nuclear protein and fragmented chromatin was extracted by sequential incubation in cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 2 mmol/L EDTA, 0.2% Sarkosyl) and dialysis buffer (100 mmol/mL Tris-HCl pH 9.0, 500 mmol/L LiCl, 1% NP-40, 1% Deoxycholic acid). The cross-linking of chromatin was reversed and DNA purified by sequential incubation in cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 2 mmol/L EDTA, 0.2% Sarkosyl) and dialysis buffer (100 mmol/mL Tris-HCl pH 9.0, 500 mmol/L LiCl, 1% NP-40, 1% Deoxycholic acid). The cross-linking of chromatin was reversed and DNA purified by sequential incubation in cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 2 mmol/L EDTA, 0.2% Sarkosyl) and dialysis buffer (100 mmol/mL Tris-HCl pH 9.0, 500 mmol/L LiCl, 1% NP-40, 1% Deoxycholic acid).

Affymetrix microarray
Total RNA (300 ng) was amplified using a WT Expression Kit (Ambion) according to the manufacturer’s instructions. cRNA (10 μg) was used for reverse transcription and 5.5 μg cDNA was hybridized to a Human Exon 1.0 ST (Affymetrix) according to the manufacturer’s protocol. After overnight hybridization, the chips were terminally labeled and scanned using an Affymetrix GeneChip 3000 7G scanner.

Gene ontology and gene set enrichment analyses
Gene ontology (GO) analysis was performed by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/). Gene set enrichment analysis (GSEA) algorithm (http://www.broadinstitute.org/gsea/index.jsp) was used to identify the pathways that were significantly enriched between DANCR low and high tumor samples.

SNP array data processing and copy-number analysis
The TCGA SNP array (Affymetrix Genome-Wide Human SNP Array 6.0) data in CEL format of 496 patients’ paired tumor and germline-derived DNA specimens of prostate cancer were downloaded from the TCGA Data Portal (https://gdc.cancer.gov/). The CEL files were imported to the Partek Genomic Suite to perform segmentation and calculate the predicted copy number for each given gene. A predicted copy number larger than 2.3 or smaller than 1.7 was considered as a copy-number gain or loss for each gene, respectively.

siRNA transfection
siRNA oligonucleotides targeting DANCR were ordered from Sigma. Cells were seeded in 6-well plates in antibiotic-free media overnight, and then transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). Cells were incubated in the media containing the transfection mixture. All transfection experiments were performed with 50 mmol/L of each siRNA. In control experiments, cells were transfected with an equivalent amount of nontargeting (NT) siRNA.

Cell-cycle assay
The cell cycle was analyzed using an in situ cell proliferation kit FLIOS (Roche) according to the manufacturer’s instruction. Briefly, cells were labeled with bromodeoxyuridine (BrdUrd) for 40 minutes before trypsinization. Cells were rinsed with PBS, and then fixed by adding 70% ice-cold ethanol. Fixed cells were incubated with 4 mol/L HCl at room temperature for 30 minutes then washed with PBS. The cells were then incubated with anti-BrdUrd-FLIOS antibody at room temperature for 45 minutes, washed with PBS, and resuspended in 0.1 mL PBS. Cell suspensions were incubated with 7-AAD for 5 minutes and immediately analyzed by flow-cytometry. The data were analyzed by FlowJo software (Tree Star).

Liposomal nanoparticle preparation
siRNA oligonucleotides targeting DANCR for in vivo delivery were encapsulated into 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC; Avanti Polar Lipids, Inc.) DOPC-nano delivery system. Briefly, DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA:DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20: siRNA–DOPC. The mixture was vortexed, frozen in an acetone/dry ice bath, and lyophilized. Before in vivo administration, this preparation was hydrated with PBS at room temperature at a concentration of 250 μg/mirRNA per kilogram per mouse injection (each mouse received 200 μL of DOPC–siRNA–PBS solution by the intraperitoneal route).

In vivo ovarian cancer model and tissue processing
Female athymic nude mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD) and maintained according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the MD Anderson Cancer Center Institutional Animal Care and Use Committee. All animals used were between 8 and 12 weeks of age at the time of injection. To determine the therapeutic efficacy of DANCR LncRNA silencing, we used a...
well-characterized orthotopic model of ovarian carcinoma. To establish the tumors, OVCA432 ovarian cancer cells (2.0 × 10^6) were trypsinized, suspended in 200 μL of Hanks Balanced Salt Solution (Gibco) and injected into the intraperitoneal cavity of mice on day 1. Seven days after cell injection, mice were randomly divided into 3 groups (n = 10 mice/group): (i) Control siRNA/DOPC or (ii) DANCR1 siRNA/DOPC or (iii) DANCR2 siRNA/DOPC. To assess tumor growth, treatment began by injecting siRNA/DOPC nanoparticles twice weekly (150 μg/kg body weight) through intraperitoneal injection. Mice were monitored daily for adverse effects of therapy and tumor volume of each group was quantified by the IVIS-200 bioluminescence and fluorescence imaging system (Caliper Life Sciences Inc.) every week. At the time of sacrifice, mouse weight and tumor weight were recorded. Tumor tissue was harvested and either snap-frozen in liquid nitrogen for lysate preparation or fixed in formalin for paraffin embedding, or frozen in optimum cutting temperature medium (Miles, Inc.) to prepare frozen slides. The individuals who performed the necropsies, optimum cutting temperature medium (Miles, Inc.) to prepare

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**Results**

**Intergenic lncRNA, DANCR, responsive to MYC is broadly expressed in multiple cancers types**

In our early studies of MYC-regulated transcriptomes using exon microarrays (28), we found a lncRNA (then termed KIAA0114) that was induced by MYC that was later annotated as DANCR (Supplementary Fig. S1). As such, we sought to determine the global effect of MYC on lncRNA expression in the P493-6 cell line that was engineered from a peripheral human B lymphocyte, immortalized by an Epstein-Barr viral genome and harbors a tetracycline-regulated MYC transgene. The addition of tetracycline to these cells shuts off MYC expression and renders P493-6 cells nonproliferative (29). We cultured P493-6 cells with (MYC-OFF) and without tetracycline (MYC-ON). The RNAs from both conditions were harvested and subjected to RNA sequencing (RNA-seq) analysis. To estimate the RNA expression level of a given lncRNA, we calculated the fragments per kilobase of transcript per million mapped reads (FPKM) value for each lncRNA, given lncRNA, we calculated the fragments per kilobase of transcript per million mapped reads (FPKM) value for each lncRNA.

Among the 238 MYC-regulated intergenic lncRNAs, only five were broadly expressed in nearly all CCLE cell lines with high FPKM value (the average FPKM > 5): ENSG00000226950 (DANCR), ENSG00000260260, ENSG00000198106 (SNX29P2) was only highly expressed in large B lymphoma cell lines but not other cancer types (Fig. 1C and D). Among the 238 MYC-regulated intergenic lncRNAs, only five were broadly expressed in nearly all CCLE cell lines with high FPKM value (the average FPKM > 5): ENSG00000226950 (DANCR), ENSG00000260260, ENSG00000198106 (SNX29P2), and ENSG00000255198 (Fig. 1C). Then, we asked whether the expression levels of these five MYC-regulated intergenic lncRNAs identified from P493-6 cells were correlated with the MYC expression in CCLE cell line panel. In this regard, the expression levels of three MYC genes (MYC, MYCL, and MYCN) were retrieved from CCLE RNA-seq and a multivariate linear regression analysis indicated that the expression of all three MYC genes were significantly and positively correlated with the expression levels of 4/5 intergenic lncRNAs except ENSG00000255198 (P < 0.001, Fig. 1E). Notably, we found that MYC was the strongest factor affecting the expression levels of these four intergenic lncRNA than MYCL and MYCN (Fig. 1E). These observations suggest that these four intergenic lncRNAs are regulated by MYC broadly among many cancer cell types. Because DANCR is the intergenic lncRNA with highest expression level across CCLE cell lines, is strongly correlated with MYC expression, and has been previously reported to be involved in cell differentiation (23), we chose this intergenic lncRNA for further study.

**MYC directly regulates DANCR**

Given the significant induction of DANCR by MYC in a model cell line and the correlation of their expression across a broad panel of human cancers, we sought to determine whether DANCR is a direct MYC target. The hallmark of direct MYC target genes include documentation of MYC binding by chromatin immunoprecipitation (ChIP) and an alteration of RNA levels. We corroborated the genome-wide RNA-seq study with RT-PCR that DANCR is one of the most highly induced MYC-responsive genes with a >15-fold induction [as compared with reported MYC targets: ODC1 (4.6-fold), LDHA (7.5-fold), NCL (5.8-fold), and NPM1 (6.7-fold)] in the P493-6 human B-cell model (Fig. 2A). Because the prostate cancer cell line PC3 is an MYC-dependent cell line, and the known link between MYC and human prostate cancer (31), we sought to determine whether expression of DANCR in PC3 cells depends on MYC. Indeed, knocking down MYC expression with siRNA resulted in diminished DANCR expression (Fig. 2A). Using available genome-wide MYC ChIP data (6), we found that DANCR is robustly bound by MYC upon MYC induction in the P493-6 cells in a time-dependent manner (Fig. 2B). The...
peak of MYC binding in intron 2 corresponds to a conserved canonical 5′CACGTG3′ Myc consensus-binding site (UCSC Genome Browser). In addition to validating the ChIP-seq data in P493-6 cells, IncRNAs analyzed as genic and intergenic inset. The percentage of IncRNAs that were coregulated with the nearest PCGs of the MYC-regulated genic IncRNAs (left) and intergenic IncRNAs (right). C, Left, heatmap of the expression of intergenic IncRNAs that are significantly altered due to the change of MYC expression in P493-6 cells. The experiment was repeated. Each row represents a lncRNA; each column represents a condition. The rows above the black line are IncRNAs upregulated by MYC; those below the black line are IncRNAs downregulated by MYC. Yellow, high expression; blue, low expression. Middle, heatmap of the FPKM value of MYC-regulated IncRNAs (from left) in CCLE cell lines. Each row represents an intergenic IncRNA, and each column a cell line. The cell lines are grouped by cancer types, which is color-coded and numbered at the bottom of the panel. Red, high expression; white, low or nondetected expression. Right, heatmap of the FPKM value of MYC-regulated IncRNAs (from left) in CCLE cell line collections. Five IncRNAs were expressed at high levels across multiple cancer types. D, The box plot of FPKM of SNX29P2, DANCR, and MYC in different cancer types. SNX29P2 is a B-cell cancer-specific intergenic IncRNA. MYC expression was also included at the top of the panel. E, The correlation between the expressions of MYC family genes and each of the five ubiquitously expressed intergenic IncRNAs in CCLE cell line panel. Left, the P value of multivariate linear regression analysis for the correlation of MYC/MYCL/MYCN and five intergenic IncRNAs. Right, heatmap of the fraction of variance of MYC-regulated intergenic IncRNA expression explained by three MYC family members. The MYC expression level showed strongest efforts on 4/5 intergenic IncRNAs (red).
IncRNA DANCER and Cell Proliferation

Figure 2.
DANCER is a direct MYC target gene. A. RT-PCR analysis reveals induction of DANCER in P493-6 cells by MYC activation and reduction of DANCER expression with MYC knockdown in PC3 cells. Cells were transfected with siRNA over 3 hours and cells were collected 24 hours after transfection for analysis. B. MYC ChIP-seq histograms illustrating MYC binding to DANCER as a function of time after induction of MYC in P493-6 cells (data from GEO GSE36554). C. ChIP-qPCR illustrating the binding of MYC to the DANCER locus in P493-6, PC3, and human H1 and H9 embryonic stem cells. Control represents ChIP signals from a locus located approximately 1.9 kb away from the DANCER transcriptional start site. D. RNA-seq analysis reveals induction of mDancr in Eu-myc transgenic mouse model. E. MYC ChIP-seq histograms illustrating MYC binding to mDancr as a function of time after induction of MYC in Eu-myc transgenic mouse model (data from GEO GSE10101). Error bars, SD.

the mDancr as determined by chromatin immunoprecipitation increased across the spectrum from normal to frank tumor (Fig. 2E).

Elevation of MYC and DANCER in human prostate cancers
In addition to loss of tumor suppressors such as PTEN, human prostate cancers have been linked to elevated MYC expression. In fact, we documented that MYC protein levels correlate extremely well with advanced stages of prostate cancer but not with metastatic disease (31). MYC has also been used in mouse models, illustrating its power to drive prostate tumorigenesis in vivo (32).

Because of MYC’s significant role in prostate cancer, we examined the expression of MYC and DANCER among human prostate cancer and found that both are elevated in tumor versus normal pairs from TCGA (Fig. 3A). We corroborated these data with RNAs isolated from paired primary human normal and prostate tumors using RT-PCR, illustrating elevated MYC and DANCER mRNA expression in prostate cancer (Fig. 3B). Because MYC is frequently amplified in prostate cancer, we also determined whether DANCER could be independently amplified and found that although MYC amplification is frequent, the DANCER locus is infrequently amplified (Fig. 3C). These observations suggest that elevated MYC expression results from MYC-induction in prostate cancer and not a result of DANCER gene amplification. In this regard, we examined the correlation between MYC and DANCER expression in prostate cancers from TCGA (Fig. 3D) and found a significant correlation.

Functional role of DANCER
To determine the functional role of DANCER, we sought to delineate how its loss of function affects the transcriptome with the hypothesis that DANCER participates in gene silencing similar to other known IncRNAs. Specifically, we ruled out its potential role in cis-regulating nearby genes, by measuring the expression of ERVMER34-1 and USP46 upon knockdown of DANCER by siRNAs (Supplementary Fig. S3). Indeed, we found no significant changes in the expression of these nearby genes. Hence, we performed global gene-expression array analysis and as expected, found that knockdown of DANCER led to up-regulation of genes, but there were also a significant number of downregulated genes (Fig. 4A). Because IncRNAs have been associated with chromatin repressors, we focused on genes that were upregulated by DANCER knockdown. Gene ontology analysis of the upregulated genes demonstrates a significant representation of those involved in cell-cycle regulation and DNA dynamics (Fig. 4B; Supplementary Table S4). Further network analysis reveals key cell cycle and DNA replication hubs (Fig. 4C) are highly represented in the upregulated genes. These findings suggest that DANCER plays a key role in regulating the cell cycle.

We then determined whether loss of DANCER affects the cell cycle and used pulsed BrdUrd-labeling for cell-cycle analysis. As shown in Fig. 4D, knockdown of DANCER resulted in a significant decrease in S-phase entry by the prostate PC3 cancer cells associated with a significant increase in p21 protein induction (Fig. 4E) that correlates with the increase in p21 (CDKN1A) mRNA levels found in gene-expression arrays (Supplementary Table S5). The inhibition of S-phase entry and induction of p21 with loss of DANCER are corroborated by the finding that DANCER expression inversely

Published OnlineFirst November 27, 2017; DOI: 10.1158/0008-5472.CAN-17-0815

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correlates with p21 mRNA expression in human prostate cancers in the TCGA dataset (Fig. 4F). Consistent with these findings, we also knocked down DANCR in P493 cells using Vivo-Morpholinos and found that they could also curb the growth of P493 cells (Supplementary Fig. S4A and S4B). Furthermore, the diminished growth of P493 cells was associated with an increased in p21 (Supplementary Fig. S4C). These observations collectively suggest that MYC induction of DANCR results in the suppression of p21, which is required for cell-cycle progression.

Role of p21 (CDKN1A) in DANCR-mediated cell growth

To further determine the role of p21 in DANCR-mediated proliferation, we examined the effects of DANCR knock down on the prostate cell lines PC3 and DU145 and the ovarian cancer cell line OVCAR432. Using two different siRNAs (siDANCR1 and siDANCR2), we found that the siRNA-treated cells had significantly slowed growth (Fig. 5A). To specifically address whether p21 induction is involved in the slowed growth induced by DANCR knockdown, we determined whether the simultaneous knockdown of p21 could rescue the slowed growth of PC3 cells caused by loss of DANCR. As shown in Fig. 5B, simultaneous knockdown of p21 diminished its expression in PC3 cells treated with siDANCR. Importantly, simultaneously loss of p21 partially rescued slowed growth due to siDANCR, but not to the diminished proliferation caused by knockdown of MYC (siMYC; Fig. 5C). The lack of rescue by loss of p21 in siMYC-treated cells is consistent with the broad effect of MYC on the cancer transcriptome and on cancer cell growth, whereas loss of p21 could partially rescue a more restricted effect of siDANCR. These observations indicate that the induction of p21 following loss of DANCR is functionally significant in slowing DANCR-dependent cell proliferation.

LncRNA DANCR silencing reduces tumor burden in ovarian tumors

On the basis of our in vitro findings, we next asked whether DANCR silencing in vivo would affect the tumor growth. To determine the therapeutic efficacy, we used orthotopic ovarian OVCAR432 tumor model. Markedly lower luciferase activity was detected in DANCR silencing (siRNA packaged in DOPC nanoliposomes) groups compared with the control group (Fig. 6A), illustrating diminished tumorigenesis in DANCR silenced mice. As shown in Fig. 6B, both DANCR1 siRNA and DANCR2 siRNA treated animals showed significant reduction in tumor growth compared with control siRNA treated ones (79% and 81%, P value 0.0005 and 0.0001, respectively; Fig. 6B and C). Similarly, compared with control siRNA-treated animals, both DANCR siRNA-treated groups showed significant reduction in number of tumor nodules (78% and 81%; P value 0.0031 and 0.0028, respectively; Fig. 6C) as well as ascites (P value 0.028; Fig. 6D), suggesting that silencing of DANCR IncRNA played an effective role in reducing the tumor burden. There was no obvious toxicity noted in the animals during therapy experiments, as assessed by changes in behavior, feeding habits, and mobility. The mean body weight was also similar between the treatment groups (Fig. 6E). Consistent with the in vitro effects of siDANCR on the cell cycle, Fig. 6F and G document the reduction of Ki67 (cell cycle) expression in the siDANCR-

Figure 3.
Correlation of MYC and DANCR expression in human prostate cancer. **A**, MYC and DANCR expression levels are elevated in paired tumor versus normal prostate samples from TCGA. **B**, RT-PCR analysis of primary human paired tumor versus normal prostate samples illustrates increased MYC and DANCR expression in tumors. **C**, MYC is frequently amplified in prostate cancers and DANCR is not. Degree of red corresponds to degree of amplification. **D**, MYC mRNA expression correlates with DANCR expression amongst human prostate cancers in TCGA.
treated tumors, which demonstrated increased apoptosis (Fig. 6H and I). These findings illustrate that silencing DANCR in vivo significantly reduces ovarian tumor burden in an orthotopic xenograft model.

**Discussion**

MYC is a human oncogene that is both downstream and upstream of IncRNAs with its expression being regulated by IncRNAs at the transcriptional and translational levels, and its

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**Figure 4.**
Transcriptomic and cell-cycle effects of loss of DANCR expression. **A,** Heatmap of expression of mRNAs in response to knockdown of DANCR by siRNA (siDANCR) as compared with control (Ctrl). Blue, downregulated. **B,** Gene ontology analysis of genes induced upon DANCR knockdown. **C,** Functional hubs of genes induced upon DANCR knockdown as analyzed by DAVID analysis on the basis of gene ontology term. **D,** Pulsed BrdUrd analysis of the cell cycle of control (left) and DANCR (right) knockdown PC3 cells. The proportions of cells in other phases were control (G1, 41%; G2M, 25%) and siDANCR (G1, 56%; G2M, 30%). **E,** Induction of p21 protein with loss of DANCR expression illustrated by immunoblot for p21 at the indicated time points after siDANCR transfection. Tubulin served as loading control. **F,** Inverse correlation of p21 (CDKN1A) mRNA and DANCR mRNA expression in human prostate cancers from TCGA.

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**Figure 5.**
Loss of DANCR expression diminishes growth of prostate and ovarian cancer cell lines. **A,** Effects of two different siRNAs targeting DANCR (siDANCR1; siDANCR2) on growth (cell number) of PC3 and DU145 prostate or OVCAR432 ovarian cancer cells as compared with control siRNA (Ctrl). **B,** Induction of p21 by loss of DANCR expression and the suppression of p21 expression 48 and 72 hours after cotransfecting with siRNA targeting DANCR and p21 in PC3 cells. The immunoblot for p21 is shown with tubulin serving as loading control. **C,** Diminished growth caused by siDANCR, but not by knockdown of MYC (siMYC), could be partially rescued by simultaneous knockdown of p21 expression in PC3 cells.
product MYC in turn regulates lncRNAs in a complex network of target genes. Expression of the MYC proto-oncogene is exquisitely regulated at the transcriptional, translational, and post-translational levels, because deregulated MYC oncogene is a powerful transforming gene. As such, MYC expression is under tight control of many regulatory mechanisms, including lncRNAs and miRNAs. Here, we focus on the ability of oncogenic MYC to drive the expression of lncRNAs, particularly a target termed DANCR, which we document to have a significant role in MYC-mediated cell proliferation.

In our earlier studies using exon arrays, we found KIAA0114 (aka, DANCR) as a very responsive MYC target in the P493-6 cells, leading us to examine the global effect of MYC on lncRNA expression. During the course of our studies, DANCR was identified as an antagonist of keratinocyte differentiation and its anti differentiation role was further reported in osteoblasts, in which its function could be mediated through EZH2—perhaps through direct RNA–protein interaction (23, 33). DANCR was subsequently found to be induced by MYC in the P493-6 cell system among many lncRNAs (34, 35). Furthermore, MYC is reported to induce other lncRNAs that have a variety of biological activities including metastasis and cell cycle regulation (18, 36–38). We focused our work on determining whether DANCR is a bona fide MYC target and further explored its function in MYC-induced cell proliferation. How DANCR functions through affecting the transcriptome, however, was unknown.

Here, we found that DANCR expression is elevated broadly among many human cancer cell lines and document its elevated expression in association with MYC in human prostate cancers. Importantly, we rigorously document DANCR as a direct MYC target gene in several cell types, spanning from mouse lymphoid, and human prostate cancer to human embryonic stem cells. Through gene expression analysis of the prostate cancer cell line PC3, we found that siRNA-mediated loss of DANCR expression resulted in elevation of mRNAs that are involved in the cell cycle, particularly p21. Because both PC3 and DU145 prostate cancer...
cell lines have mutant p53, wild-type p53 does not seem necessary for loss of DANCR to induce p21 expression (39). Functionally, siRNA-mediated diminished DANCR expression resulted decreased cell proliferation of prostate and ovarian cancer cell lines. The diminished proliferation of PC3 cells with DANCR knockdown could be partially rescued with simultaneous reduction of p21 with siRNA. The partial rescue is consistent with the possible multifaceted role of DANCR in cell proliferation, a topic that would require extensive mechanistic future studies. Collectively, we establish DANCR as a direct MYC target that suppresses p21 expression, among other transcriptomic effects, to promote cell proliferation. It is notable the MYC has been shown to inhibit p21 expression through other mechanisms, such as its interference with MIZ1-mediated transactivation of p21, interference with SP1 activation of p21, activation of AP4 that in turn transrepresses p21, or activation of miR-17-92 that in turn represses p21 (40). However, in contrast with DANCR, knockdown of MYC resulted in growth arrest that could not be rescued by knockdown of p21. This observation is consistent with the broad effect of MYC on cell growth and proliferation, and suggests that DANCR may have a more limited role with p21 being an important node for DANCR’s function in influencing cell proliferation. The exact mechanism by which DANCR mediates p21 suppression is beyond the scope of our current study and is deserving of future studies.

The occurrence of DANCR in the cytoplasm of many mammalian cell lines suggests that it may have a cytosolic function independent of transcription (41, 42). As such, the exact mechanism function of DANCR in the cytosol as well as its role in the nucleus will require significant depth of investigation in future studies. Notwithstanding the lack of full mechanistic understanding of DANCR function, the elevation of DANCR expression across a broad spectrum of human cancers, the direct regulation of DANCR by MYC, the effect of DANCR on p21 expression and cell proliferation, and the ability to curb in vivo tumorigenesis with nano-particle–mediated siRNA-targeting DANCR are documented herein and provide a path for further studies of this intriguing IncRNA. Consistent with our results, DANCR was recently reported to increase stemness in liver cancer (43) and correlate with tumor progression in colon cancer (44). Our study further adds to the foundation for the exploration of other MYC-induced IncRNAs that may have roles in tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

We thank the TCGA and CCLE project teams as well as members of the Dang laboratory for input. This work was supported, in whole or in part, by the DoD (W81XWH-15-1-0630 to C.V. Dang), NIH (R01CA051497 to C.V. Dang, R01CA057341 to C.V. Dang, R01CA190415 to L. Zhang; UH5TR000943 to A.K. Sood), the Basser Center for BRCA (to L. Zhang), and the RCK Foundation (to A.K. Sood). Z.E. Stine is supported by a fellowship from F32CA174148. We thank the members of the Johns Hopkins Urological Specimen Repository and Database, supported in part by NIH Prostate SPORE P50CA58236 (A.M. DeMarzo), and the Prostate Cancer Biorepository Network (PCBN), supported by the Department of Defense Prostate Cancer Research Program (W81XWH-14-2-0182, W81XWH-14-2-0183, W81XWH-14-2-0185, W81XWH-14-2-0186, and W81XWH-15-2-0062 to A.M. DeMarzo).

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Received April 17, 2017; revised September 20, 2017; accepted November 3, 2017, published OnlineFirst November 27, 2017.

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MYC Targeted Long Noncoding RNA DANCR Promotes Cancer in Part by Reducing p21 Levels


Cancer Res 2018;78:64-74. Published OnlineFirst November 27, 2017.

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