Identification of senescence-associated circular RNAs (SAC-RNAs) reveals senescence suppressor CircPVT1

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ABSTRACT

Using RNA sequencing (RNA-Seq), we compared the expression patterns of circular RNAs in proliferating (early-passage) and senescent (late-passage) human diploid WI-38 fibroblasts. Among the differentially expressed senescence-associated circRNAs (which we termed ‘SAC-RNAs’), we identified CircPVT1, generated by circularization of an exon of the PVT1 gene, as a circular RNA showing markedly reduced levels in senescent fibroblasts. Reducing CircPVT1 levels in proliferating fibroblasts triggered senescence, as determined by a rise in senescence-associated β-galactosidase activity, higher abundance of CDKN1A/P21 and TP53, and reduced cell proliferation. Although several microRNAs were predicted to bind CircPVT1, only let-7 was found enriched after pulldown of endogenous CircPVT1, suggesting that CircPVT1 might selectively modulate let-7 activity and hence expression of let-7-regulated mRNAs. Reporter analysis revealed that CircPVT1 decreased the cellular pool of available let-7, and antagonizing endogenous let-7 triggered cell proliferation. Importantly, silencing CircPVT1 promoted cell senescence and reversed the proliferative phenotype observed after let-7 function was impaired. Consequently, the levels of several proliferative proteins that prevent senescence, such as IGF2BP1, KRAS and HMGA2, encoded by let-7 target mRNAs, were reduced by silencing CircPVT1. Our findings indicate that the SAC-RNA CircPVT1, elevated in dividing cells and reduced in senescent cells, sequesters let-7 to enable a proliferative phenotype.

INTRODUCTION

Cellular senescence is a state of indefinite growth arrest triggered by exposure of cell to stress-causing stimuli (1). It was first described by Hayflick in 1965 and has been studied extensively in cultured cells (2). When the stress signal arises from successive rounds of replication causing gradual shortening of telomeres, which exposes telomeric DNA and triggers a DNA damage response, the ensuing program is named replicative senescence (3–6). When the stress signal comes from other sources of damage, such as oxidants, radiation, heat, activated oncogenes, or toxins, the ensuing program is named stress-induced senescence (6–9). Senescence is characterized by increased activity of the tumor suppressor TP53, higher levels of its transcriptional target p21 and CDK inhibitor p16/INK4A, and activation of the p16 target retinoblastoma (pRB) (7,8). Senescent cells have a complex impact on human physiology and pathology. Some effects of senescent cells are beneficial, such as tissue remodeling, wound repair, and growth suppression of potentially oncogenic cells (10). However, many effects of senescent cells are believed to be detrimental. Besides causing tissue dysfunction, senescent cells exhibit a senescence-associated secretory phenotype (SASP), whereby they produce and secrete inflammatory cytokines and chemokines, matrix metalloproteases, and growth and angiogenic factors (11). The accumulation of senescent cells has been associated with disease processes such as sarcopenia, arthritis, cancer, diabetes, and neurodegeneration (12–14).

MicroRNAs (miRNAs) are ~22-nucleotide long non-coding (nc)RNAs that form part of the RNA-induced silencing complex (RISC), within which the RNA-binding protein (RBP) AGO2 binds microRNAs directly. MicroRNA–RISC complexes influence protein expression patterns through the interaction of the microRNA with subsets of mRNAs via partial complementarity, generally leading to reduced stability and/or reduced translation of the mRNA (15). By influencing protein expression patterns,
microRNAs have been implicated in key cellular processes, including proliferation, survival, differentiation, immune activation, stress response, and cell senescence. As reviewed recently, microRNAs impact upon numerous pathways that control senescence (16). Indeed, many microRNAs show altered expression levels during senescence (17,18).

A notable class of microRNAs implicated in growth arrest and senescence is the human let-7 family, which consists of 13 different microRNAs with homology to the Caenorhabditis elegans let-7, a regulator of development and aging (19–24). Given that let-7 members are expressed from genomic regions that are deleted in tumors and that they suppress expression of oncogenes and proteins that enhance cell proliferation, the let-7 family has been implicated in tumor suppression (20,25,26). Accordingly, let-7 members have been proposed to promote senescence, as their levels rise during cell senescence and let-7 suppresses the production of proteins that promote proliferation and inhibit senescence (17,32,33). One let-7 target, HMGA2 (high-mobility group AT-hook 2), promotes mesenchymal tumorigenesis, and suppresses cellular senescence (34–39). Other let-7 targets include oncoproteins in the Ras family, specifically NRAS, KRAS and HRAS, which are enriched in endogenous CircPVT1, a eukaryotic splicing regulator of development (early-passage) and in senescent (late-passage) human diploid WI-38 fibroblasts. Among the circRNAs selectively reduced in senescent cells, we focused on CircPVT1, a moderately expressed circRNA generated by circularization of an exon of the PVT1 pre-lncRNA, as silencing CircPVT1 in proliferating cells triggered senescence. Several microRNAs were predicted to bind CircPVT1, but only let-7 was found enriched in endogenous CircPVT1 pulled down from cells, suggesting that CircPVT1 might regulate let-7 activity and influence expression of downstream targets. After obtaining evidence that CircPVT1 decreased the cellular pool of available let-7, we found that silencing CircPVT1 rescued the proliferative phenotype caused by antagonizing let-7. Moreover, several let-7 target mRNAs encoding proliferative proteins that prevent senescence were suppressed by silencing CircPVT1 levels, particularly IGF2BP1, KRAS and HMGA2. In sum, we have found that the senescence-associated circRNA CircPVT1 binds to let-7 and inhibits its actions and thereby influences senescence.

**MATERIALS AND METHODS**

**Cell culture, ionizing radiation (IR), siRNA transfection and SA-β-galactosidase activity**

Human WI-38 fibroblasts, MCF7 breast carcinoma cells, and IMR-90 lung fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics, and non-essential amino acids (Invitrogen). Human breast epithelial MCF10a cells, lung epithelial BEAS-2B cells, and lung adenocarcinoma A549 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. Human non-small cell lung carcinoma H1299 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and antibiotics. Proliferating WI-38 cells were used at population doublings (PDL) between 15 and 25, and senescent fibroblasts were used after additional culture, at PDLs 50–55. Proliferating WI-38 cells were exposed to ionizing radiation (10 Gy) and cultured for 10 days to induce senescence. Ctrl siRNA (AACUAUACAACCUACUACCUCA, Ambion), anti-let-7 antagonist (AACUAUACAACCUACUACCUCA, Ambion), let-7 mimic (UGAGGUAGUAGGUAGUAGUAGU, Ambion), and CircPVT1 siRNA (CUGUCAGCUGCAUGGAGCUUCGU, IDT) were transfected at 100 nM final concentration using Lipofectamine-2000 (Invitrogen). Senescence-associated (SA)β-galactosidase activity in WI-38 cells was assessed using a kit from Cell Signaling Technology.
Western blot analysis

Whole-cell lysates were prepared in RIPA buffer containing protease inhibitors and were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes (Invitrogen iBlot Stack). Incubations with primary antibodies recognizing KRAS (Abcam), IGFB2BP1 (Proteintech), HMGA2 (Abcam), P21 (Millipore), TP53, GAPDH or HSP90 (Santa Cruz Biotech), were followed by incubations with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP; GE Healthcare). Signals were developed using Enhanced Chemiluminescence (ECL).

Antisense oligomer pulldown

For antisense oligomer pulldown of CircPVT1, WI-38 cells were lysed in polysome extraction buffer (PEB; 20 mM Tris–HCl at pH 7.5, 100 mM KCl, 5 mM MgCl2 and 0.5% NP-40) with protease and RNase inhibitors for 10 min on ice and the supernatant was collected by centrifugation (15 000 × g, 10 min, 4°C). The lysates were incubated with 100 pmol of biotin-labeled control oligomers (GCTGTAGAGGGAGCATG-bio) or an oligomer complementary to the junction sequence of CircPVT1 (bio-GCCAAAAGATCAGGCCTCAAGCCCAGCTGA, also known as Biotin-ASO) in 1× TENT buffer (10 mM Tris–HCl at pH 8.0, 1 mM EDTA at pH 8.0, 250 mM NaCl, 0.5% [v/v] Triton X-100) containing protease and RNase inhibitors for 1 h at 25°C with rotation. Streptavidin-coupled Dynabeads (50 µl, Invitrogen), were washed with 1× TENT buffer and incubated with lysate (30 min, 25°C, rotation). After isolating and washing the beads three times with ice-cold 1× TENT buffer, RNA was isolated using TRIzol, and CircPVT1 and associated microRNAs in the pulldown were detected by RT-qPCR analysis.

RNA isolation and RT-qPCR analysis

RNA was isolated from WI-38 cells or from pulldown samples using TRIzol (Thermo Fisher Scientific) following the manufacturer’s procedure. Total RNA was used for gene expression analysis by reverse transcription (RT) followed by quantitative (q)PCR analysis. RT was performed by using random hexamers and reverse transcriptase (Maxima Reverse Transcriptase, Fermentas) and qPCR was carried out using gene-specific primers and SYBR green master mix (Kapa Biosystems) in Applied Biosystems 7300 and 7900 instrument. Data are available at GSE85771.

For circRNA-Seq analysis, adapter contamination was removed from the raw FASTQ files and sequences were aligned to the human genome (hg19) with TopHat2 (v2.1.0), first to identify linear RNA and later to identify fusion transcripts using reads which did not align to the linear RNA. The program CIRCexplorer was run with the fusion transcripts obtained from TopHat2 using Ensembl GRCh37 Release 82 annotation to identify the circular RNAs (Supplementary Table S2). Additionally, the cleaned FASTQ files (after removing adapters) were used for finding circularizing junctions by employing the find_circ software. In short, the reads were aligned to linear RNA using Bowtie2 program and then the unmapped reads were split into two anchors and aligned using Bowtie2 followed by identification of circularizing junctions (Supplementary Table S3). Using CIRCexplorer and find_circ analysis, the combined circRNA junction read number from two proliferating and two senescent samples were normalized to the respective number of mapped reads and represented as ‘reads per million’.

Plasmid constructs and reporter analysis

To prepare plasmid psiCHECK2-let-7, the let-7 antisense (AACTATACACCTACTACCTCA) sequence was cloned downstream of renilla ORF in psiCHECK2 vector. For reporter analyses, HeLa cells were transfected with Ctrl siRNA, CircPVT1 siRNA, anti-let-7 or let-7 mimic using Lipofectamine 2000, and 48 h later with 200 ng of psiCHECK2 and psiCHECK2-let-7 reporter plasmids. Sixteen hours after that, RL and FL activities were measured using Dual-Glo Luciferase Assay System (Promega).

Ribonucleoprotein immunoprecipitation (RIP) analysis

The association of CircPVT1 with endogenous RNA-binding proteins (RBPs) present in WI-38 fibroblasts was analyzed by ribonucleoprotein (RNP) immunoprecipitation (IP) as described (54). Briefly, proliferating WI-38 cells were lysed in polysome extraction buffer (PEB; 20 mM Tris–HCl at pH 7.5, 100 mM KCl, 5 mM MgCl2 and 0.5% NP-40) supplemented with RNase and protease inhibitors. The cytoplasmic lysates were incubated with protein A sepharose beads coated with antibodies recognizing DGCR8, HNRNPA1, KHSRP, LIN28 (Abcam) or control IgG (Santa Cruz Biotech) for 2 h at 4°C with rotation. Following washes with ice-cold NT2 buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl2, 0.05% NP-40), the RNA bound to the RNP complexes was isolated using TRIzol and used for RT-qPCR analysis.
Test of resistance to RNase R digestion and circRNA sequencing

Total RNA (2 μg) isolated from proliferating WI-38 cells was either left untreated (ctrl) or treated with 20 units of RNase R (RNR07250, Epicentre) in the presence of 1× RNase R buffer, 20 units of RiboLock RNase Inhibitor (Thermo Scientific), and incubated for 30 min at 37°C. The digested RNA was isolated using TRIzol, cDNA from the untreated and RNase R-treated RNA was prepared using maxima RT protocol (above), and 0.2 μl of the cDNA reaction was used as template for RT-qPCR analysis. The RT-qPCR product for hsa_circ_PVT1_01 (CircPVT1) was resolved in ethidium bromide-stained, 2.5% agarose gels and visualized on an ultraviolet transilluminator. Forward and reverse primers (Supplementary Table S1) were used to sequence the amplified PCR products and identify the junction sequence.

RT-qPCR analysis of microRNAs

Total RNA from WI-38 fibroblasts or RNA from pulldown samples was extracted using TRIzol following the manufacturer’s protocol. RNA samples were reverse-transcribed using the Mir-X™ microRNA First Strand Synthesis Kit (#638315, Clontech), microRNA-specific forward primer (Supplementary Table S1) and universal reverse primer were used to measure the levels of individual microRNAs by RT-qPCR analysis using Applied Biosystems 7300 and 7900HT instruments and normalized to U6 RNA levels.

3H-Thymidine incorporation assays

Proliferating WI-38 fibroblasts were transfected with or without combinations of Ctrl siRNA, CircPVT1 siRNA, anti-let-7 and let-7 mimic using Lipofectamine 2000; 3 days later, cells were treated with [methyl-3H]-Thymidine anti-let-7 and let-7 mimic using Lipofectamine 2000; 3 without combinations of Ctrl siRNA, CircPVT1 siRNA, Proliferating WI-38 fibroblasts were transfected with or

RESULTS

Senescence-associated circRNAs (SAC-RNAs)

Compared with proliferating, early-passage [population doublings (PDLs) 15–25] WI-38 human diploid fibrob-
Figure 1. Identification and annotation of senescence-associated circRNAs in WI-38 fibroblasts. (A) Micrographs to visualize SA-β-gal activity in proliferating, early-passage, and senescent late-passage WI-38 cells. (B) Western blot analysis of the levels of P53, P21, and loading control HSP90 in proliferating and senescent cells. (C) RT-qPCR analysis of the levels of P16 mRNA, P21 mRNA, and loading control GAPDH mRNA in proliferating and senescent cells. (D) Table of highly expressed circRNAs in proliferating WI-38 cells. (E) Venn diagram depicting the overlap between the two different circRNA prediction algorithms. (F) Number of circRNAs identified by the CIRCexplorer and find_circ algorithms. Data in (C) represent the means ± S.E.M. from three independent experiments. **P < 0.01 (Student’s t-test).
Figure 2. Characterization of senescence-associated circRNAs in WI-38 fibroblasts. (A) RT-qPCR analysis of changes in circRNA expression in proliferating and senescent WI-38 cells. (B) Representative images of SA-β-gal staining in mock-treated and IR-treated (senescent) WI-38 cells. (C) RT-qPCR analysis of circRNA levels in mock-treated and IR-treated (senescent) WI-38 cells. Data in A and C are the means ± S.E.M. from three independent experiments.
other SAC-RNAs, total RNA from proliferating WI-38 cells was treated with RNase R, which degrades the linear RNA without affecting the covalently circularized CircPVT1, and the levels of linear and circular RNAs in WI-38 cells were measured by RT-qPCR analysis using primers designed in a divergent orientation for the circular RNAs (Figure 3A). The CircPVT1 PCR product was assessed on agarose gels to confirm that a specific circRNA species was amplified (Figure 3B), and the amplified PCR product was verified by DNA sequencing to confirm the amplification of the specific circRNA junction (Figure 3C). Droplet PCR analysis revealed that ~50 copies of CircPVT1 are expressed per ng of WI-38 total RNA, while IncRNA PVT1 is expressed at a higher level of ~200 copies per ng RNA (Figure 3D).

When comparing proliferating and senescent cells, the level of PVT1 IncRNA did not change as significantly as did CircPVT1, suggesting that the levels of linear and circular PVT1 transcript were regulated independently during cellular senescence (Supplementary Figure S1B). To further investigate the role of this circRNA in cell senescence, we silenced CircPVT1 in WI-38 cells using an siRNA that lowered CircPVT1 levels without affecting PVT1 IncRNA levels (Figure 3E) (Materials and Methods). Silencing CircPVT1 led to increased levels of senescence marker TP53 (p53) and triggered a flattened and enlarged cell morphology accompanied by increased SA-βgal activity (Figure 3F and G). Together, our results show that CircPVT1 is expressed in proliferating WI-38 cells and suppressed cellular senescence. Given this evidence, we set out to investigate how CircPVT1 might regulate the senescent phenotype.

**Sponging of let-7 by CircPVT1 prevents senescence**

CircRNAs can enhance or suppress gene expression by modulating the availability of microRNAs for target RNAs in the cell (49, 50). As circRNAs have been shown to be highly stable, we hypothesized that CircPVT1 could potentially act as a ‘sponge’ or a ‘ decoy’, inhibiting the activity of associated microRNAs. We examined the microRNAs predicted to target CircPVT1 (Figure 4A, Supplementary Table S4) (58) and investigated if such predicted microRNAs interact with CircPVT1 by pulling down endogenous CircPVT1 using antisense biotinylated oligomers targeting the CircPVT1 junction (Figure 4B and C). The specificity for the pulldown of CircPVT1 by the biotinylated antisense oligomer was confirmed by RT-qPCR analysis in Figure 4C. As shown in Figure 4D, a few microRNAs were enriched in the CircPVT1 pulldown sample compared with the control pulldown samples, supporting the existence of circRNA-microRNA complexes. One of the microRNAs, let-7, was found to be the most highly enriched microRNA in the CircPVT1 pulldown (Figure 4D); moreover, among the four circRNAs most downregulated in senescent cells (Figure 2A), CircPVT1 had the highest density of let-7 sites (not shown). This result confirmed the specific interaction of CircPVT1 with let-7 in WI-38 cells. Given the implication of let-7 in growth arrest and cell senescence (17, 26, 59), we explored this interaction further.

We asked if CircPVT1 might influence cell proliferation and senescence by modulating let-7 availability in the cells. Four days after blocking let-7 activity by transfecting a let-7 antagonist (anti-let-7), the expression levels of CDKN1A mRNA (encoding the senescence marker P21) decreased, while silencing CircPVT1 elevated CDKN1A mRNA expression (Figure 5A). Western blot analysis revealed that anti-let-7 downregulated both P21 and TP53 (Figure 5B). Importantly, silencing CircPVT1, which increases let-7 availability, significantly reversed this loss of P21 and TP53 expression observed with anti-let-7 alone (Figure 5B). SA-βgal activity was elevated in CircPVT1-silenced cells, while anti-let-7 had the opposite effect, reducing SA-βgal activity and increasing proliferation; importantly, the proliferative phenotype seen after antagonizing let-7 was rescued when CircPVT1 was silenced (Figure 5C). Together with the finding that the steady-state levels of let-7 are not significantly changed in proliferating relative to senescent WI-38 cells (Figure 5D), these results suggest that CircPVT1 functions as a negative regulator of let-7 and thus suppresses cellular senescence.

Considering that miRNA function can be regulated at multiple levels, we examined whether CircPVT1 might have a role in the maturation of let-7. For this, we tested by ribonucleaseprotein immunoprecipitation (RIP) analysis if CircPVT1 was capable of associating with several RNA-binding proteins (RBP) implicated in let-7 processing and maturation, including DGCRI8, KHSRP, HNRNPA1, and LIN28. Most of these RBP (except LIN28) did show modest interaction with CircPVT1 (Supplementary Figure S3A). We then tested if CircPVT1 influenced let-7 biogenesis in WI-38 cells using RT-qPCR strategies to distinguish pri-, pre- and mature let-7 (Supplementary Figure S3B). We found that silencing CircPVT1 did not change significantly pri- or pre-let-7 levels (Supplementary Figure S3C), suggesting that CircPVT1 did not affect let-7 transcription or early processing, but it did lower mature let-7 levels slightly. These findings indicate that CircPVT1 does not suppress let-7 activity by preventing let-7 biosynthesis, and further support the notion that reduced CircPVT1 enhances let-7 function as an inhibitor of cell proliferation.

**By acting as a decoy for let-7, CircPVT1 promotes cell proliferation**

The let-7 family of microRNAs has been shown to hinder cell growth by suppressing the expression of proliferative proteins such as IGF2BP1, KRAS and HMGA2 (35, 40, 43). The interaction of CircPVT1 with let-7 prompted us to investigate whether the levels of CircPVT1 affected WI-38 proliferation by sponging or sequestering let-7. Initial evidence in support of this possibility was obtained from experiments examining the correlation between let-7 activity as a function of WI-38 growth. As shown in Figure 5E, inhibition of let-7 by transfection of anti-let-7 increased cell numbers, while silencing CircPVT1 decreased cell numbers, supporting the hypothesis that CircPVT1 promoted cell proliferation by blocking let-7 activity. Interestingly, silencing of CircPVT1 in anti-let-7-transfected cells partially rescued cell proliferation. Likewise, silencing CircPVT1 significantly reduced the incorporation of 3H-thymidine in dividing cells while anti-let-7 elevated the 3H-thymidine incorporation (Figure 5F), and transfection of both Circ-
Figure 3. CircPVT1 inhibits WI-38 cell senescence. (A) RT-qPCR results showing the abundance of circRNAs and linear RNAs in WI-38 cells treated with RNase R. The levels of CircPVT1 and PVT1 lncRNA were normalized to the values measured after mock treatments. (B) The RT-qPCR product of CircPVT1 (± RT, with or without reverse transcription) was visualized by electrophoresis in ethidium bromide-stained 2.5% agarose gels. (C) qPCR products were purified and sequenced to confirm CircPVT1 junction sequences. (D) Absolute quantification for CircPVT1 and PVT1 lncRNA in proliferating WI-38 cells. (E) RT-qPCR analysis of the levels of CircPVT1 and PVT1 lncRNA in proliferating WI-38 cells 4 days after transfection of Ctrl siRNA or CircPVT1 siRNA. (F, G) Western blot analysis of the senescence marker TP53 (F) and SA-βgal staining (G) in WI-38 cells 4 days after transfection with Ctrl siRNA or CircPVT1 siRNA. Data in A, D–F are the means ± S.E.M. from three independent experiments. *P < 0.05 (Student’s t-test).
Figure 4. let-7 associates with CircPVT1 in WI-38 cells. (A) List of microRNAs predicted to target CircPVT1. (B) Schematic of CircPVT1 pulldown for specific detection of microRNAs associated with CircPVT1 in WI-38 cells. (C) RT-qPCR analysis of the enrichment of CircPVT1 in CircPVT1 pulldown compared with control. (D) Enrichment of microRNAs predicted to target CircPVT1 in CircPVT1 pulldown analyzed by RT-qPCR. Data in (C, D) are the means ± S.E.M. from at least three independent experiments.

cPVT1 siRNA and anti-let-7 rescued the reduction in 3H-thymidine incorporation. Together, these results support the notion that CircPVT1 controls the proliferation of WI-38 cells by modulating let-7 activity. Given that let-7 inhibits cell proliferation partly by inhibiting the production of IGF2BP1, KRAS, and HMGA2 (35,36,40,43), we hypothesized that CircPVT1 might alter let-7 activity to promote WI-38 cell proliferation and inhibit cellular senescence.

To investigate whether CircPVT1 influenced let-7 activity, we prepared luciferase reporter vectors derived from the parent plasmid psiCHECK2. A let-7 complementary sequence was cloned downstream of the renilla luciferase (RL) coding sequence, and firefly luciferase (FL) expressed from the same construct served as internal normalization control (psiCHECK2-let-7, Figure 6A, top). Two days after transfecting HeLa cells with CircPVT1 siRNA, anti-let-7 or pre-let-7, cells were transfected with the vector control (psiCHECK2) or with psiCHECK2-let-7. RL and FL activities were measured 16 h later and the ratio of RL activity to FL activity (RL/FL) was calculated. The RL/FL activity ratio in each population (control siRNA, CircPVT1 siRNA) was assessed using the empty vector (psiCHECK2) as reference (Figure 6A). Although pre-let-7-transfected cells showed greater reduction in psiCHECK2-let-7 activity (RL/FL) (45% of control), silencing CircPVT1 (Figure 6B) also significantly lowered RL/FL activity (70% of control),
Figure 5. CircPVT1 regulates cellular senescence by inhibiting let-7 function. (A) Proliferating WI-38 cells were transfected with CircPVT1 siRNA or Ctrl siRNA, in the presence or absence of anti-let-7. The graph shows RT-qPCR measurements of the senescence marker CDKN1A (P21) mRNA in WI-38 cells 4 days after transfection with the two siRNAs above. N.S., not significant (P = 0.06); *P < 0.05; **P < 0.01 (Student’s t-test). (B) Western blot analysis of senescence marker P21, TP53, and loading control GAPDH in WI-38 cells 4 days after transfection with two siRNAs indicated. (C) SA-βgal staining in WI-38 cells 4 days after transfection with the siRNAs shown. (D) RT-qPCR analysis of let-7 levels in proliferating and senescent WI-38 cells. (E, F) Four days after transfection of WI-38 cells with the siRNAs indicated, cell numbers were counted (E) and measurements were taken for [3H]-thymidine incorporation (F). Data in A, D–F are the means ± S.E.M. from three or four independent experiments. N.S., not significant; *P < 0.05; **P < 0.01 (Student’s t-test).
Figure 6. CircPVT1 promotes translation of let-7 targets encoding proliferative proteins. (A) Top, schematic of the dual luciferase reporter plasmids derived from the parent vector psiCHECK2 (psi), which expresses renilla luciferase (RL) and the internal control firefly luciferase (FL), and psiCHECK2-derived plasmids bearing the target sequence of let-7. Bottom, 48 h after transfection of HeLa cells with CircPVT1 siRNA, anti-let-7 or let-7 mimic, reporter plasmid was transfected, and 16 h later the ratio of RL activity to FL activity was measured. The changes in RL/FL ratios after the various small RNA transfections relative to RL/FL ratios of Ctrl siRNA-transfected cells are indicated. (B) RT-qPCR analysis of the levels of CircPVT1 in proliferating WI-38 cells 4 days after transfection with Ctrl siRNA or CircPVT1 siRNA. (C) Western blot analysis of the let-7 targets HMGA2, IGF2BP1, and KRAS, as well as loading control GAPDH in WI-38 cells 4 days after transfection with Ctrl siRNA or CircPVT1 siRNA. (D) RT-qPCR analysis of the levels of CircPVT1 and lncRNA PVT1 in three sets of cancerous and noncancerous lines from lung [BEAS-2B versus A549 (left) and IMR-90 versus H1299 (center)] and breast [MCF10A vs MCF7 (right)]. ACTB mRNA was used as a normalization control. Data in A–D are the means ± S.E.M. from three independent experiments. *P < 0.05; **P < 0.01 (Student’s t-test).
indicating that CircPVT1 reduced the availability of functional let-7 to transcripts bearing a let-7 site (Figure 6A).

Further validation of this hypothesis was sought by investigating if CircPVT1 regulated the expression levels of let-7 targets IGF2BP1, KRAS and HMG2A. As shown in Figure 6C, expression of these proteins was consistently reduced after silencing CircPVT1, in agreement with the notion that silencing CircPVT1 elevated the concentration of functional let-7 (Figure 6A) and led to the suppression of let-7-target mRNAs, including three that encoded proliferative proteins IGF2BP1, KRAS and HMG2A. Interestingly, silencing CircPVT1 in HeLa cells increased significantly the association of let-7 with AGO2, the microRNA-binding RBP component of RISC, indicating that CircPVT1 represses let-7 activity at least in part by reducing the availability of let-7 (Supplementary Figure S3D). Taken together, these results suggest that the SAC-RNA CircPVT1 binds to and functionally inactivates let-7, in turn promoting the translation of IGF2BP1, KRAS and HMG2A in WI-38 cells. Given that let-7 can also suppress tumor growth (20,25–31), we tested whether CircPVT1 might be differentially expressed in cancer and non-cancer cells and further studied if IncRNA PVT1 expression changed proportionately. Measurement of CircPVT1 levels in three sets of cancerous and non-cancerous cell lines (Figure 6D) revealed that both CircPVT1 and PVT1 were higher in lung (A549, H1299) and breast (MCF7) carcinoma cell lines than in non-cancerous cell lines from lung (epithelial BEAS-2B cells and IMR-90 fibroblasts) and breast (MCF10A epithelial cells).

In summary, we propose that in proliferating cells, the relatively abundant CircPVT1 sequesters let-7 from its target mRNAs in order to derepress production of proliferation proteins and impede senescence (Figure 7). Accordingly, the reduction in CircPVT1 levels in senescence allows greater let-7 functional availability in the cell to repress the expression of the let-7-target mRNAs.

DISCUSSION
We have characterized a new function for a circular RNA derived from the PVT1 gene, CircPVT1, on cellular senescence. The decrease in CircPVT1 levels in senescent WI-38 human fibroblasts appeared to be functionally important because knockdown of CircPVT1 alone promoted senescence (Figure 3). In order to elucidate the mechanism whereby CircPVT1 elicited this effect, we investigated whether it interacted with any specific microRNAs. Among the candidate microRNAs that might potentially interact with CircPVT1, let-7 showed the highest enrichment in CircPVT1 pulldown assays (Figure 4). Since let-7 plays a role in promoting senescence by repressing the production of proliferative genes (35,40,43), we investigated a potential function for CircPVT1 as an inhibitor or sponge of let-7. We found that senescence and growth arrest triggered by silencing CircPVT1 were rescued if let-7 was neutralized using an anti-let-7 antagonir (Figure 5). These results were consistent with CircPVT1 modulating the level of functional let-7 in the cell, as revealed by studying a let-7 luciferase reporter. Three proteins encoded by let-7 targets, HMG2A, IGF2BP1 and KRAS, showed reduced levels when CircPVT1 was silenced and thus more let-7 molecules were available to regulate these target transcripts. In sum, we have identified a novel role for CircPVT1 in preventing senescence by binding to and inhibiting let-7.

CircPVT1 is generated through backsplicing of the last exon of the gene that encodes for PVT1, a lncRNA that is upregulated in cancer cells (60). LncRNA PVT1 knockdown reduced cell proliferation in ovarian and breast cancer cells (61) and in hepatocellular carcinoma (62). The genetic locus is in close proximity to the gene that encodes the oncoprotein MYC (63) and this proximity was identified as one of the mechanisms through which PVT1 functions in controlling the stability of MYC protein (64). PVT1 was also proposed to regulate carcinogenesis via the microRNAs that it encodes (reviewed in (65)). Whether IncRNA PVT1 plays a role in senescence, as we propose for CircPVT1 in the present study, is not known. Conversely, however, it is plausible that CircPVT1 promotes proliferation in other cellular states such as cancer, just as it promotes proliferation in nontransformed cells (Figure 5). Although IncRNA PVT1 has been proposed to sponge miR-200 (66), there is no report to-date that it may sponge let-7. It is possible that let-7 binding to PVT1 may cause degradation of the IncRNA and thus binding is prevented by a secondary structure unique to the linear form of PVT1. It will be important to assess directly whether PVT1 is regulated by let-7 or may instead sponge let-7 as does CircPVT1. Although in WI-38 fibroblasts CircPVT1 was 7 or 8 times less abundant than PVT1 (Figure 3D), the relative abundance of the two transcripts has not been assessed in other cell types or tissues. In addition, investigating whether production of the two molecules is coordinately regulated or whether CircPVT1 biogenesis is independent of PVT1 production is also warranted. PVT1 levels did not change significantly in senescent relative to proliferating cells (Supplementary Figure S1B), indicating that CircPVT1 levels may be regulated at the levels of circularization or turnover. Specific RBPs may play a role in these processes and their own abundance or activity might be modulated during senescence, independently of the levels of the parent transcript IncRNA PVT1. In sum, we propose that the linear and circular form of PVT1 may work in the same direction to control cell proliferation, albeit potentially through different mechanisms.

CircRNAs have been known for more than two decades but did not draw much attention until recently, when their high abundance was revealed by transcriptome-wide RNA-sequencing and several circRNAs have been characterized as inhibitors of microRNAs and thus regulators of gene expression (49,50). The regulatory function stems from their ability to bind microRNAs by sequence complementarity and their high stability due to their covalent circle structure. Our pulldown and functional studies corroborated this function, as CircPVT1 was capable of binding selectively to let-7 and inhibiting let-7 function, despite the computational prediction that other microRNAs might also bind. This selectivity may be explained by the fact that CircPVT1 may form a specific secondary structure that makes other putative microRNA sites inaccessible to microRNAs. It is also possible that certain RBPs may bind CircPVT1 and mask microRNA-recognition sequences. Additionally, some computationally predicted targets may simply not
Figure 7. CircPVT1 action model. Proposed model whereby CircPVT1 acts as a competing endogenous RNA, sponging let-7; CircPVT1 promotes the expression of target genes required for cell cycle progression by preventing let-7 from acting on such target mRNAs. The decreased expression levels of CircPVT1 in senescent cells allows higher levels of functional let-7, which in turn suppresses let-7 target expression leading to growth inhibition and cellular senescence.

be expressed in WI-38 fibroblasts, and there are microRNAs that could potentially interact with CircPVT1 but do not share standard sequence complementarity at the microRNA seed region.

Let-7 levels and activity have been shown to increase in senescent cells, and let-7 family members inhibit cell growth by suppressing the expression of target mRNAs encoding proliferative proteins (20,26,33,35). Like most microRNAs, let-7 can suppress gene expression by reducing the stability and/or translation of target mRNAs with which it shares partial complementarity. Given that CircPVT1 can regulate let-7 activity generically and for a few target proteins (IGF2BP1, KRAS and HMGA2; Figure 6), we anticipate that CircPVT1 might also modulate the levels of other let-7 targets, although such a global effect for CircPVT1 remains to be investigated. It was somewhat surprising to find that an abundant microRNA such as let-7 (collectively comprising over 6,000 copies per cell (67)) could be sponged by a circRNA far less abundant (at ∼5 copies per cell, with possibly three let-7 sites per molecule). However, given the vast number of let-7 target mRNAs, let-7-interacting RBPs [e.g. HuR, AUF1 and LIN28 (68,69)], and let-7 sponges [including lncRNAs and mRNAs (67,70)], the number of let-7 molecules bioavailable to repress target transcripts is likely far smaller. The relative sponging of different members of the let-7 family (let-7a, -b, -c, -d, -e, -f, -g, -i, miR-98) by CircPVT1 was not investigated, although let-7a is often the most abundant isofrom. It was also interesting to find that CircPVT1 did not influence early stages of let-7 biogenesis. The slight reduction in mature let-7 levels observed following CircPVT1 silencing further solidifies the view that let-7 function and availability to target mRNAs increase when CircPVT1 levels decline. Finally, we cannot exclude the possibility that other circular RNAs may regulate let-7 in a similar manner. However, among the circular RNAs that we tested for differential expression during senescence, CircPVT1 had the highest density of let-7 sites (data not shown).

Like CircPVT1, a handful of other circRNAs have been found to sponge microRNAs (49–53,71). With rising evidence that some circRNAs can regulate gene expression programs, there is mounting interest in elucidating the mechanisms that generate circRNAs, the molecules with which they interact (RBPs, microRNAs and likely other
molecules as well), the mechanisms that control their subcellular localization, and more broadly, their impact on protein expression patterns, their cell and tissue function, and their influence on physiology and pathology. The discovery that Circ-PVT1 promotes proliferation and prevents senescence paves the way for the analysis of other circRNAs that influence cell metabolism in different physiologic and disease states.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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