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Depletion of *DNTTIP2* Induces Cell Cycle Arrest in Pancreatic Cancer Cells

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Abstract. *Background/Aim: Pancreatic cancer is one of the most lethal malignant cancers worldwide and the seventh most common cause of cancer-related death in both sexes. Herein, we analyzed open access data and discovered that expression of a gene called deoxynucleotidyltransferase terminal-interacting protein 2 (DNTTIP2) is linked to prognosis of pancreatic ductal adenocarcinoma (PDAC). We then elucidated the role of DNTTIP2 in the proliferation of pancreatic cancer cells in vitro. Materials and Methods: A WST-8 assay, cell cycle analysis, Annexin-V staining, quantitative reverse transcription-PCR, and western blot analysis were conducted to assess cell proliferation, cell cycle, apoptosis, and expression of DNTTIP2 mRNA and protein, respectively, in DNTTIP2-depleteted MIA-PaCa-2 and PK-1 cells. Results: Depletion of DNTTIP2 induced G_1 arrest in MIA-PaCa-2 cells by decreasing expression of special AT-rich sequence binding protein 1 (SATB1) and cyclin-dependent kinase 6 (CDK6). In addition, depletion of DNTTIP2 induced G_2 arrest in PK-1 cells by decreasing expression of CDK1. Depletion of DNTTIP2 did not induce apoptosis in MIA-PaCa-2 or PK-1 cells. Conclusion: DNTTIP2 is involved in proliferation of pancreatic cancer cells. Thus, DNTTIP2 is a potential target for inhibiting progression of pancreatic cancers.*

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Key Words: Pancreatic cancer, *CDK1, CDK6, SATB1*, cells, *DNTTIP2*.

Pancreatic cancer, one of the most lethal malignant cancers worldwide, is the seventh most common cause of cancer-related death in both sexes (1). Pancreatic cancer is classified broadly into two types: pancreatic ductal adenocarcinoma (PDAC) and pancreatic endocrine tumors. PDAC accounts for more than 90% of all pancreatic tumors (2). Survival rates for patients with pancreatic cancer are extremely poor because it rarely causes symptoms during the early stages; indeed, it is locally advanced in more than one third of patients, and has already metastasized in another half of patients, by the time it is diagnosed (3). Patients with metastatic PDAC receive first-line treatment with gemcitabine (GEM) (4). Between 40% and 50% of these patients receive second-line treatment after disease progression, but the optimal therapy in this setting has not been established (4).

The Cancer Genome Atlas (TCGA) is a publicly funded project that aims to catalogue and identify major cancer-causing genomic alterations. This database contains a huge amount of publicly available data, giving researchers around the world an immeasurable source of knowledge regarding the genetic and epigenetic profiles of different cancers (5). In the present study, we analyzed the vast amount of cancer data accumulated in TCGA and utilized it to investigate molecules that affect the prognosis of pancreatic cancer.

Analysis of this open access data revealed that expression of a gene called deoxynucleotidyltransferase terminal-interacting protein 2 [*DNTTIP2*; also known as terminal deoxynucleotidyltransferase (TdT) interacting factor 2 (*TdIF2*)] plays a role in the prognosis of PDAC patients. The *DNTTIP2* (*TdIF2*) gene is located on chromosome 1 (6). *TdIF2*, which is localized within the nucleolus where it binds to DNA and core histones, contains an acidic amino acid-rich region within its C-terminus; therefore, it is thought to function as a histone chaperone within the nucleus (7). A previous report examined the association between *DNTTIP2* expression and the malignant characteristics of low-grade glioma (8), but there are no other reports regarding other cancers.

Herein, we conducted this study to elucidate the role of the *DNTTIP2* gene in cell proliferation in pancreatic cancer cells. We also investigated whether the *DNTTIP2* gene could be a

potential target molecule for pancreatic cancer treatment, for which no effective molecular target therapy exists yet.

Materials and Methods

Extraction of target genes from large-scale RNA interference (RNAi) datasets. A large-scale RNAi screening dataset (9) ("D2_combined_gene_dep_scores.csv") was downloaded from the DepMap (10) portal (https://depmap.org/portal/download/all/). The median value of the DEMETER2 score (9, 10) for each gene was calculated using R software (version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria). Genes with a median DEMETER2 score <-1 were extracted as genes involved in cancer cell growth.

Analysis of gene expression and overall survival (OS) using a pancreatic adenocarcinoma patient dataset. A total of 177 PDAC patients from the TCGA database were included in the study. Raw clinical and genomic data for Pancreatic Adenocarcinoma (TCGA, Firehose Legacy, paad_tcga.tar.gz) were downloaded from cBioPortal (11, 12) (https://www.cbioportal.org/). The "paad_tcga.tar.gz" was unzipped and the Files "data_bcr_clinical_data_patient.txt" and "data_RNA_Seq_v2_mRNA_median_Zscores.txt" were analyzed using R software. Expression of each gene extracted from each PDAC patient was examined, and the median expression value was calculated by R software. PDAC patients with a gene expression value higher than the calculated median were classified into the high expression group, and those with a gene expression value lower than the calculated median were classified into the low expression group. Kaplan-Meier plots and survival (OS) curve analyses were performed for patients in each of these groups.

Relationship between DNTTIP2 and SATB1. The relationship between *DNTTIP2* and *SATB1* was investigated using "GSE83744 (13)" data downloaded from the "Gene Expression Omnibus (GEO) (14)" and "GeneMANIA" database (http://genemania.org). When given a single query gene, GeneMANIA identifies genes likely to share function with it based on their interactions (15).

Comparison of SATB1 mRNA expression levels in MIA-PaCa-2 and PK-1 cells. Expression of *SATB1* mRNA in MIA-PaCa-2 and PK-1 cells was investigated by analyzing "23 RNA HPA cell line gene data" downloaded from "The Human Protein Atlas" (16) database version 22.0 (https://v22.proteinatlas.org/about/download).

Evaluation of SATB1 function as a transcription factor. The role of *SATB1* as a transcription factor for *CDK1* and *CDK6* was investigated using the "ChIP-Atlas (17, 18) (Peak Browser)" database (https://chip-atlas.org), which visualizes protein binding at a given genomic loci using Integrative Genomics Viewer (IGV) genome browser (19).

Cell culture. The human pancreatic cancer cell line MIA-PaCa-2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human pancreatic cancer cell line PK-1 was purchased from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University (Sendai, Japan). MIA-PaCa-2 cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and PK-1 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, FUJIFILM Wako Pure Chemical Corporation). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Tokyo, Japan) and 1% penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation). Cells were maintained in a fully humidified incubator at 37˚C/5% CO_2. All cell lines were tested using the Venor GeM Classic Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) and were confirmed negative for mycoplasma contamination.

Knockdown of DNTTIP2 and SATB1 using small interfering RNAs (siRNAs). SiRNAs targeting *DNTTIP2* (s26947, s26949) or *SATB1* (s12480, s12481), as well as a negative control siRNA (product number: 4390846), were purchased from Thermo Fisher Scientific. Cancer cells were seeded on a 6-well plate (Thermo Fisher Scientific) or a 6 cm dish (Thermo Fisher Scientific) and transfected with 5 nM of each siRNA in Opti-MEM (Thermo Fisher Scientific) containing lipofectamine RNAiMAX (Thermo Fisher Scientific). Herein, the *DNTTIP2* siRNA (s26947, s26949), *SATB1* siRNA (s12480, s12481), and negative control siRNA are referred to as siDNTTIP2 #1, siDNTTIP2 #2, siSATB1 #1, siSATB1 #2, and siCtrl, respectively. SiRNAs sequences used in this study are shown in Table I.

Cell proliferation assay. The effect of siDNTTIP2 #1 and #2 on cell proliferation was evaluated in a WST-8 assay using Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan) and GloMax EXPLORER (GloMax, Promega, Madison, WI, USA). We performed the WST-8 assay with n=three independent biological replicates, each containing four technical replicates. Briefly, MIA-PaCa-2 (3.0×10^2 cells in 100 μl of medium) or PK-1 cells (1.0×10^3 cells in 100 μl of medium) were seeded into each well of a flat-bottomed 96-well plate (Thermo Fisher Scientific) and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 72 h. After 69 h, 10 μl of CCK-8 reagent was added to each well. Three hours later, absorbance at 450 nm was measured using GloMax.

Cell cycle analysis. Cell cycle analysis was performed using propidium iodide (PI) staining, followed by analysis using an LSRFortessa X-20 cytometer [Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA]. Data were analyzed by FlowJo software (version 10.4.2; BD). We performed the cell cycle analysis with n=three independent biological replicates, each containing one technical replicate. Briefly, MIA-PaCa-2 cells were seeded at 6.0×10^4 cells in 3 ml of medium into 6 cm dishes and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 48 h. PK-1 cells were seeded at 6.0×10^4 cells in 3 ml of medium into 6 cm dishes and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 72 h. The cells were then analyzed as described previously (20).

Apoptosis measurement. Apoptosis was measured using the eBioscience Annexin V-FITC Apop Kit (Thermo Fisher Scientific) and an LSRFortessa X-20 cytometer. Data were analyzed using FlowJo software. We performed the apoptosis measurement with n=three independent biological replicates, each containing one technical replicate. MIA-PaCa-2 cells were seeded at 3.0×10^4 cells in 3 ml of medium into 6 cm dishes and PK-1 cells were seeded at 6.0×10^4 cells in 3 ml of medium into 6 cm dishes. Seeded MIA-PaCa-2 and PK-1 cells were treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 72 h. Apoptosis analysis was performed in accordance with the instructions in the kit.

Table I. *Small interfering RNAs (siRNAs) sequences used in this study.*

Target	siRNA ID	Sense (5' -> 3')	Antisense (5' -> 3')
DNTTIP2	s26947	CGAAAGUAACUCCAACAAAtt	UUUGUUGGAGUUACUUUCGgc
DNTTIP2	s26949	GAGUAUGCAGAGGAAAUUAtt	UAAUUUCCUCUGCAUACUCct
SATB1	s12480	CGAAUAUACCAGGACGAAAtt	UUUCGUCCUGGUAUAUUCGgt
SATB1	s12481	GCAUUGCUGUCUCUAGGUUtt	AACCUAGAGACAGCAAUGCca

DNTTIP2: Deoxynucleotidyltransferase terminal-interacting protein 2; *SATB1*: special AT-rich sequence binding protein 1.

Table II. *Primer sequences used for quantitative reverse transcription-PCR in this study.*

Target	Forward sequences (5' -> 3')	Reverse sequences (5' -> 3')
CDK1	AACTACAGGTCAAGTGGTAGCC	AAGCACATCCTGAAGACTGACTA
CDK4	AATGTTGTCCGGCTGATGGA	CTGGCGCATCAGATCCTTGA
CDK6	TGATCAACTAGGAAAAATCTTGGA	GGCAACATCTCTAGGCCAGT
DNTTIP2	ATGAGAGCCAGCATGGACC	ACTTCCTTCGGTTGTATCTTCTGA
GAPDH	GAGTCCACTGGCGTCTTCAC	GTTCACACCCATGACGAACA
SATB1	TCGACCAACACAGAGGTGTC	AGCTTCCGGTAACTGCAAGAA

CDK1: Cyclin-dependent kinase 1; *CDK4*: cyclin-dependent kinase 4; *CDK6*: cyclin-dependent kinase 6; *DNTTIP2*: deoxynucleotidyltransferase terminal-interacting protein 2; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *SATB1*: special AT-rich sequence binding protein 1.

RNA extraction and cDNA synthesis. RNA extraction and cDNA synthesis were performed using the NucleoSpin RNA (Takara Bio Inc., Kusatsu, Japan) and a ReverTra Ace qPCR RT Kit (TOYOBO CO., LTD., Osaka, Japan), respectively. Briefly, MIA-PaCa-2 cells were seeded at 4.0×10^4 cells in 2 ml of medium into each well of a 6-well plate and treated with siCtrl, siDNTTIP2 #1, siDNTTIP2 #2, siSATB1 #1, or siSATB1 #2 (5 nM each) for 48 h. PK-1 cells were seeded at 8.0×10^4 cells in 2 ml of medium into each well of the 6-well plate and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 48 h. PK-1 cells were seeded at 4.0×10^4 cells in 2 ml of medium into each well of the 6-well plate and treated with siCtrl, siDNTTIP2 #1, siDNTTIP2 #2, siSATB1 #1, or siSATB1 #2 (5 nM each) for 72 h. RNA extraction and cDNA synthesis were then performed in accordance with the instructions supplied with the kits.

Quantitative reverse transcription-PCR (*qRT-PCR*). Human *CDK1*, *CDK4*, *CDK6*, *DNTTIP2*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *SATB1* mRNA transcript levels were measured by real-time PCR using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and the Thermal Cycler Dice Real Time System II (TaKaRa Bio Inc.). We performed the qRT-PCR with n=three independent biological replicates, each containing three technical replicates. *GAPDH* was used as a loading control. Oligo DNAs for each gene were designed using primerBLAST (21) and purchased from Thermo Fisher Scientific. To evaluate *SATB1* mRNA transcripts in PK-1 cells, 80 ng of cDNA was used in qRT-PCR reaction comprising 50 cycles. Other mRNA transcripts in PK-1 cells and mRNA transcripts in MIA-PaCa-2 cells were evaluated using 5 ng of cDNA and a qRT-PCR reaction comprising 40 cycles. Primer sequences used for qRT-PCR in this study are shown in Table II.

Western blot analysis. MIA-PaCa-2 cells were seeded at 4.0×10^4 cells in 2 ml of medium into each well of a 6-well plate and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM each) for 48 h. PK-1 cells were seeded at 4.0×10^4 cells in 2 ml of medium into each well of a 6-well plate and treated with siCtrl, siDNTTIP2 #1, or siDNTTIP2 #2 (5 nM, respectively) for 72 h. Cells were then treated as described previously (22, 23). We performed the western blot analysis with n=three independent biological replicates, each containing one technical replicate. Briefly, samples (containing 20 μg of protein) were first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with primary antibodies specific for the following proteins: CDK1 (65182-1-Ig, Proteintech, Rosemont, IL, USA), CDK6 (#13331, Cell Signaling Technology, Danvers, MA, USA), DNTTIP2 (HPA044502, Atlas Antibodies, Zurich, Switzerland), Vinculin (sc-73614, Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (#2118, Cell Signaling Technology). GAPDH and Vinculin were used as loading controls.

The study flow chart. We showed the study flow chart including key nodes of the experimental processes undertaken (Figure 1).

Statistical analysis. Data in bar graphs are presented as the mean+standard deviation (SD; n=three independent biological replicates) or as the mean+standard error of the mean (SEM; n=three independent biological replicates). All analyses were conducted using R software. OS was assessed using the Kaplan-Meier method and data were compared using the log-rank test. Differences between three groups were compared using one-way analysis of variance (ANOVA), and multiple comparisons of means were performed using Holm's correction (24). $p<0.05$ was considered significant.

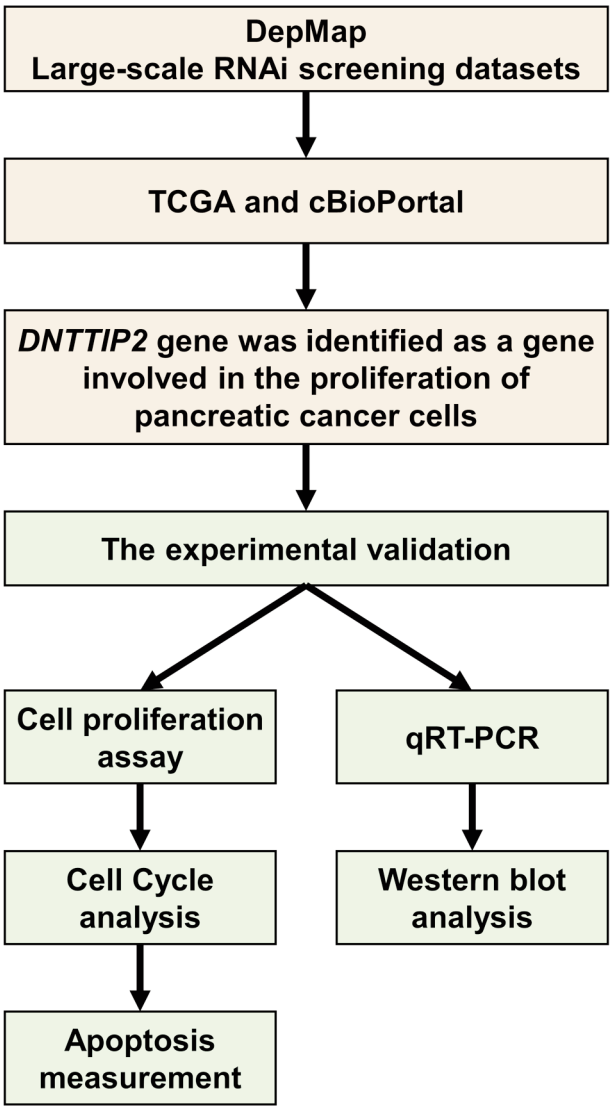

Figure 1. *Study flow chart. We used open access databases to identify DNTTIP2 as a molecule that plays a significant role in the proliferation of pancreatic cancer cells. We performed the experimental validation to reveal the role of DNTTIP2 gene in the proliferation of pancreatic cancer cells.*

Results

Selection of the DNTTIP2 gene. We analyzed large-scale RNAi screening datasets ("D2_combined_gene_dep_scores.csv") and extracted genes with median DEMETER2 scores <-1; these genes were thought to be involved in cancer cell proliferation. We extracted 157 genes from PDAC patients, constructed Kaplan-Meier plots, and performed survival (OS) curve analyses. Patients were divided into groups showing high or low expression of each gene. The results showed that the prognosis of the *DNTTIP2* low expression group (N=88) was significantly better than that of the *DNTTIP2* high expression group (N=89, p=0.0004, Figure 2A). The relationships between expression of *DNTTIP2* mRNA and clinicopathological characteristics are shown in Table III. After analyzing the data, we decided to focus our research on *DNTTIP2*; our hypothesis

Table III. *Relationship between DNTTIP2 mRNA expression and clinicopathological characteristics.*

Characteristics		*DNTTIP2* mRNA expression	
	N	High-expression group (N=89)	Low-expression group (N=88)
Age			
≥65	95	42	53
<65	82	47	35
Sex			
Male	97	43	54
Female	80	46	34
Stage of cancer			
Discrepancy	2	0	2
Not available	1	0	1
Stage I	1	0	1
Stage IA	5	2	3
Stage IB	15	6	9
Stage IIA	28	12	16
Stage IIB	118	63	55
Stage III	3	2	1
Stage IV	4	4	0

DNTTIP2: Deoxynucleotidyltransferase terminal-interacting protein 2.

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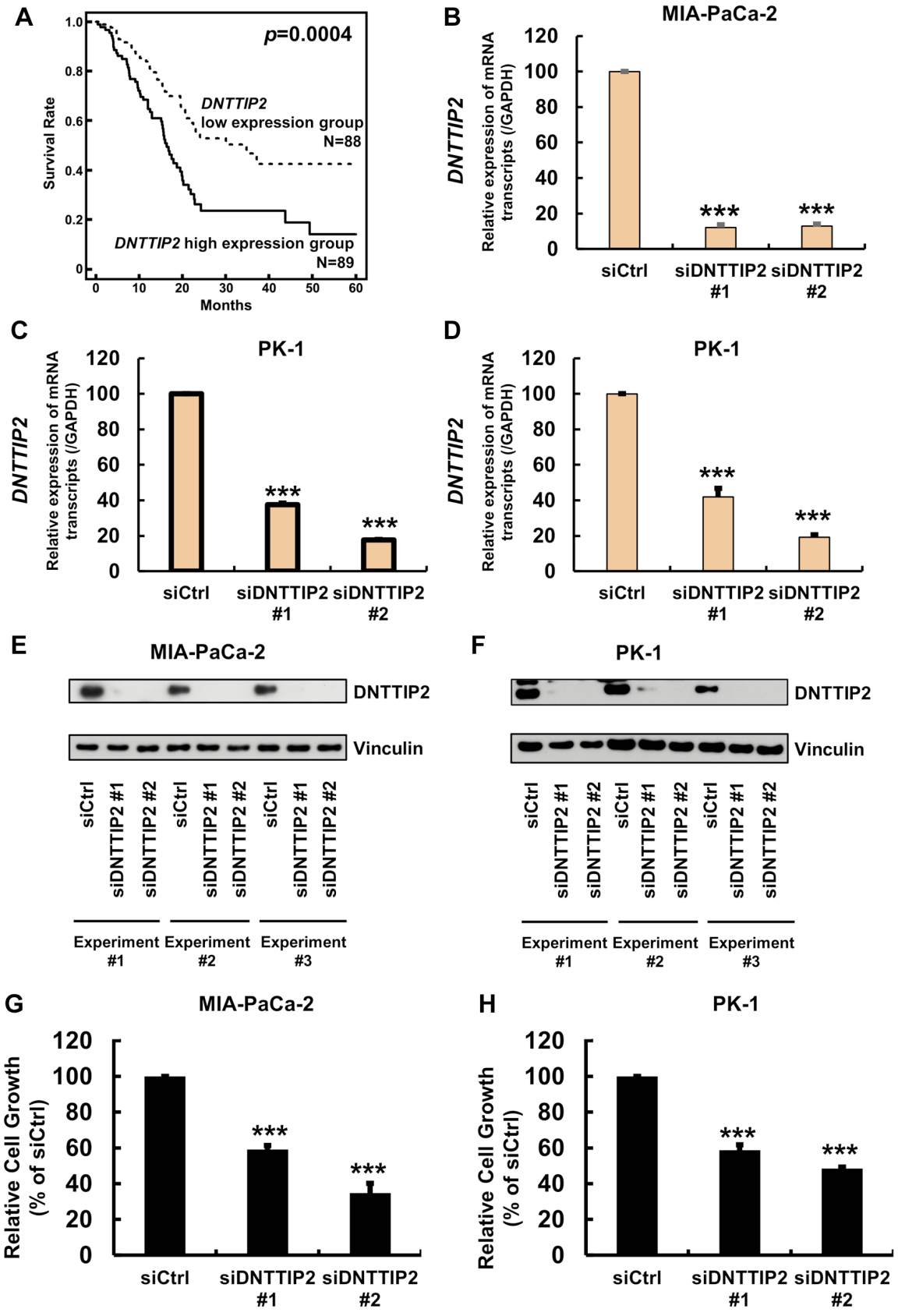
Figure 2. *Effect of DNTTIP2 on survival of patients with PDAC, and the effects of depleting DNTTIP2 on proliferation of pancreatic cancer cells. (A) Survival of the DNTTIP2 high expression (N=89) and low expression (N=88) groups of PDAC patients. OS was assessed using the Kaplan-Meier method, and data were compared using the log-rank test (p=0.0004). (B) Expression of DNTTIP2 mRNA in MIA-PaCa-2 cells treated with DNTTIP2 siRNA s26947 (siDNTTIP2 #1) and DNTTIP2 siRNA s26949 (siDNTTIP2 #2) for 48 h. Data are presented as the mean+standard error of the mean (SEM) of three independent biological replicates, each with three technical replicates. ***p<0.001 [vs. negative control siRNA (siCtrl)]. (C, D) Expression of DNTTIP2 mRNA transcripts in PK-1 cells treated with siDNTTIP2 #1 and #2 for 48 and 72 h. Data are presented as the mean+SEM of three independent biological replicates, each with three technical replicates. ***p<0.001 (vs. siCtrl). (E, F) Expression of DNTTIP2 protein by MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h, and by PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. Data of three independent biological replicates, each with one technical replicate, are shown. (G, H) Proliferation of MIA-PaCa-2 and PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. Data are presented as the mean+SEM of three independent biological replicates, each with four technical replicates. ***p<0.001 (vs. siCtrl).*


A
p=0.0004
DNTTIP2
low expression group
N=88
DNTTIP2 high expression group
N=89
Survival Rate
Months
B
MIA-PaCa-2
DNTTIP2
Relative expression of mRNA transcripts (/GAPDH)
siCtrl
siDNTTIP2 #1
siDNTTIP2 #2
C
PK-1
D
PK-1
E
MIA-PaCa-2
DNTTIP2
Vinculin
Experiment #1
Experiment #2
Experiment #3
F
PK-1
G
MIA-PaCa-2
Relative Cell Growth (% of siCtrl)
H
PK-1

was that reduced expression of *DNTTIP2* would inhibit proliferation of pancreatic cancer cells.

SiDNTTIP2 #1 and #2 inhibit proliferation of MIA-PaCa-2 and PK-1 cells. qRT-PCR and western blotting were conducted to verify whether siRNA (siDNTTIP2 #1 and #2) knocked down *DNTTIP2*. Knockdown of *DNTTIP2* was confirmed by measurement of mRNA in MIA-PaCa-2 cells after 48 h of exposure to siRNAs (Figure 2B), and in PK-1 cells after exposure to siDNTTIP2 #1 and #2 siRNA for 48 and 72 h (Figure 2C and D). Knockdown of *DNTTIP2* was confirmed by measuring expression of protein in MIA-PaCa-2 cells after 48 h (Figure 2E) and in PK-1 cells after 72 h (Figure 2F). Next, we performed a WST-8 assay to investigate whether siDNTTIP2 #1 and #2 affected proliferation of MIA-PaCa-2 and PK-1 cells. Proliferation of MIA-PaCa-2 and PK-1 cells was inhibited significantly after 72 h of treatment with siDNTTIP2 #1 or #2 (Figure 2G and H). Thus, depleting *DNTTIP2* inhibits proliferation of pancreatic cancer cells.

SiDNTTIP2 #1 and #2 induce G_1 arrest in MIA-PaCa-2 cells and G_2 arrest in PK-1 cells. We next analyzed the effects of siDNTTIP2 #1 and #2 on the cell cycle of MIA-PaCa-2 and PK-1 cells. siDNTTIP2 #1 and #2 induced G_1 arrest in MIA-PaCa-2 cells after 48 h (Figure 3A). We exposed PK-1 cells to siDNTTIP2 #1 and #2 for 72 h because no change was observed after exposure for 48 h (data not shown). G_2 arrest was observed after 72 h (Figure 3B). We concluded therefore that depleting *DNTTIP2* induces cell cycle arrest in MIA-PaCa-2 and PK-1 cells.

SiDNTTIP2 #1 and #2 did not induce the apoptosis in MIA-PaCa-2 and in PK-1 cells. Next, we analyzed the effect of siDNTTIP2 #1 and #2 on induction of apoptosis in MIA-PaCa-2 and PK-1 cells. Apoptosis was not induced significantly in MIA-PaCa-2 and PK-1 cells after 72 h of treatment with siDNTTIP2 #1 and #2 (Figure 3C and D). Although there were some significant differences in induction, the increase in the number of cells that underwent apoptosis was small. We concluded therefore that depleting *DNTTIP2* does not induce apoptosis effectively in MIA-PaCa-2 or PK-1 cells.

SiDNTTIP2 #1 and #2 inhibit expression of CDK1 and CDK6 in MIA-PaCa-2 and PK-1 cells. We evaluated the expression of *CDK1* and *CDK6* mRNA and protein in MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h, and in PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. *CDK4/6* regulates G_1/S phase transition, whereas *CDK1* regulates G_2/M phase transition. qRT-PCR revealed a significant reduction in *CDK1* and *CDK6* mRNA transcripts in MIA-PaCa-2 treated with siDNTTIP2 #1 and #2 for 48 h and in PK-1 cells treated with siDNTTIP2 #2 for 72 h (Figure 4A and B). We also found no significant reduction in expression of mRNA transcripts of *CDK4* in *DNTTIP2*-depleted MIA-PaCa-2 and PK-1 cells (Figure 4C). Furthermore, we evaluated expression of CDK1 and CDK6 protein in MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h, and in PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h by Western blotting. Expression of CDK1 and CDK6 protein decreased in *DNTTIP2*-depleted MIA-PaCa-2 and PK-1 cells (Figure 4D and E). Thus, depleting *DNTTIP2* reduces expression of *CDK1* and *CDK6* in MIA-PaCa-2 and PK-1 cells.

SiDNTTIP2 #1 and #2 decrease expression of SATB1 mRNA transcripts in MIA-PaCa-2 and PK-1 cells. We then analyzed data from "GSE83744" to find out which molecules have a relationship with *DNTTIP2*. We found a relationship between *DNTTIP2* and *SATB1* in the SALE immortalized lung cell line (data not shown). Next, we searched the "GeneMANIA" database (which is used to investigate relationships between genes) and found that expression of *DNTTIP2* is associated with that of *SATB1* (data not shown). Therefore, we next evaluated expression of *SATB1* mRNA in MIA-PaCa-2 and PK-1 cells treated with siDNTTIP2 #1 and #2 for 48 h. qRT-PCR revealed that expression of *SATB1* mRNA transcripts decreased in *DNTTIP2*-depleted MIA-PaCa-2 and PK-1 cells (Figure 5A and B).

SiSATB1 #1 and #2 decrease expression of CDK6 mRNA transcripts in MIA-PaCa-2 and PK-1 cells. The "ChIP-Atlas (Peak Browser)" database was used to investigate whether *SATB1* is a transcription factor for *CDK1* or *CDK6*. The results showed that *SATB1* is not a transcription factor for *CDK1*; however, it is a transcription factor for *CDK6* (data not shown). Therefore, we evaluated expression of mRNA transcripts of *SATB1*, *CDK1*, and *CDK6* in MIA-PaCa-2 and PK-1 cells treated with siSATB1 #1 and #2. Expression of *SATB1* and *CDK6* mRNA fell significantly in MIA-PaCa-2 cells treated with siSATB1 #1 and #2 for 48 h (Figure 5C and D). Expression levels of *CDK1* mRNA also decreased

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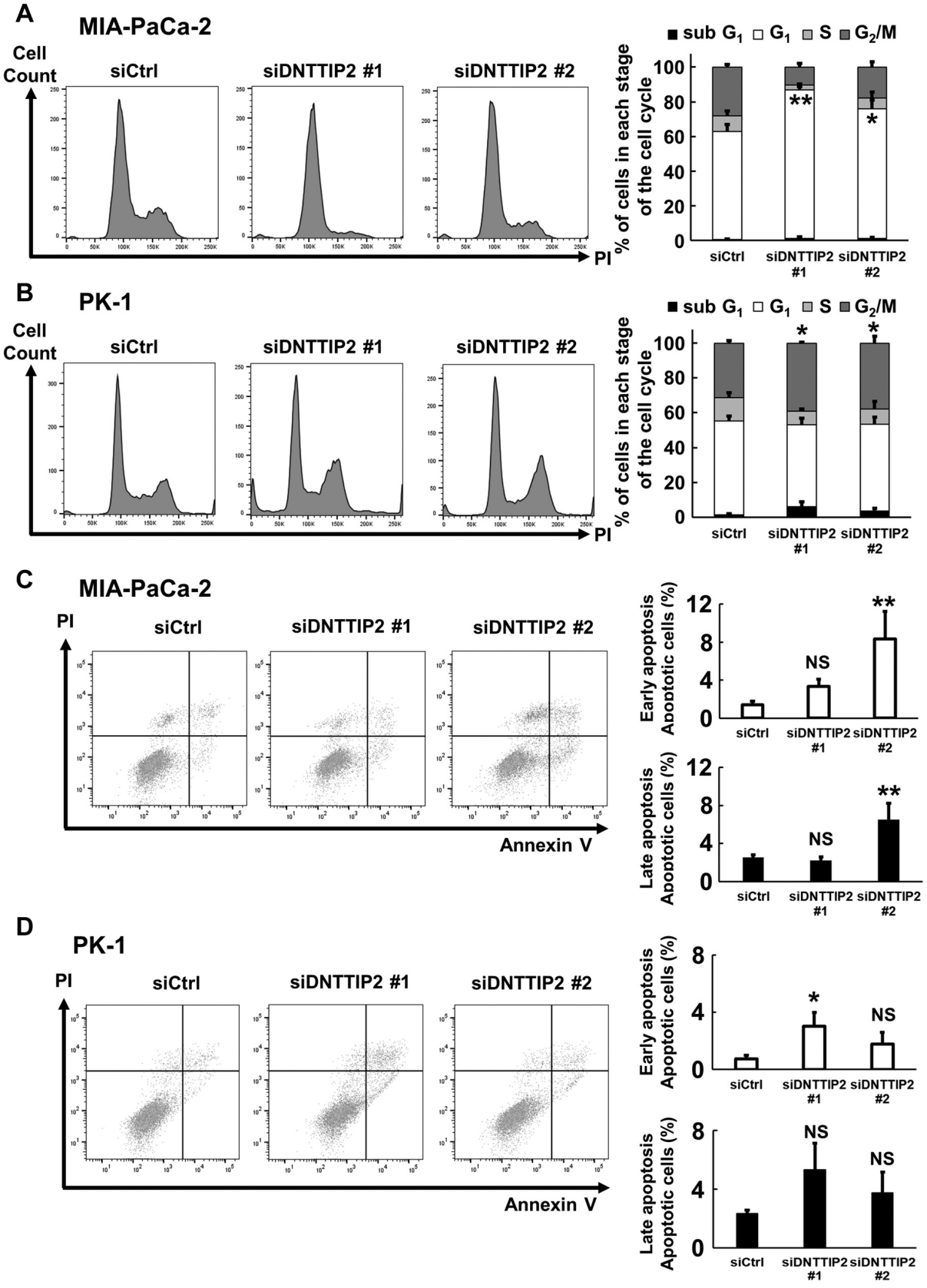
Figure 3. *Cell cycle and apoptosis analysis after depletion of DNTTIP2 from pancreatic cancer cells. (A) Cell cycle analysis of MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h. The data in histograms are representative of three independent experiments. Data in the bar graphs are expressed as the mean+standard deviation (SD) of three independent biological replicates, each with one technical replicate. *p<0.05; **p<0.01 (vs. siCtrl). (B) Cell cycle analysis of PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. The data in the histograms are representative of three independent experiments. Data in the bar graphs are presented as the mean+SD of three independent biological replicates, each with one technical replicate. *p<0.05 (vs. siCtrl). (C, D) Apoptosis of MIA-PaCa-2 and PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. Data in the dot plots are representative of three independent experiments. Data in the bar graphs are presented as the mean+SD of three independent biological replicates, each with one technical replicate. NS: Not significant; *p<0.05; **p<0.01 (vs. siCtrl).*


A
MIA-PaCa-2
Cell Count
siCtrl
siDNTTIP2 #1
siDNTTIP2 #2
PI
sub G1 G1 S G2/M
% of cells in each stage of the cell cycle
siCtrl
siDNTTIP2 #1
siDNTTIP2 #2
B
PK-1
C
MIA-PaCa-2
Annexin V
Early apoptosis Apoptotic cells (%)
Late apoptosis Apoptotic cells (%)
NS
D
PK-1

significantly, but to a lesser extent than that of *CDK6* (Figure 5E). PK-1 cells were treated with siSATB1 #1 and #2 for 72 h because we noted no change in expression of *CDK6* mRNA transcripts after 48 h (data not shown). After 72 h, we noted a significant decrease in the amounts of *SATB1* and *CDK6* mRNA transcripts (Figure 5F and G), but not in the amounts of *CDK1* mRNA transcripts (Figure 5H). In addition, depletion of *SATB1* by siSATB1 decreased the amount of *CDK6* mRNA, but not that of *CDK1* mRNA. These data suggest that *DNTTIP2* regulates *CDK6* through *SATB1* but regulates *CDK1* directly.

Discussion

Studies are being conducted that focus on the expression of specific genes and proteins to predict cancer prognosis, metastasis, and recurrence in pancreatic cancer studies (25, 26). We concentrated on PDAC patients with high or low expression of specific genes and conducted the study to investigate whether the expression of specific genes was involved in the prognosis of PDAC patients.

Herein, we used open access databases to identify *DNTTIP2* as a molecule that plays a major role in the prognosis of PDAC. Depletion of *DNTTIP2* led to reduced expression of *CDK1* and *CDK6*, and induced G_1 arrest in MIA-PaCa-2 cells and G_2 arrest in PK-1 cells.

Since depletion of *DNTTIP2* induced G_1 arrest and did not induce G_2 arrest in MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h, we evaluated the expression of *CDK1* and *CDK6* [*CDK1* regulates G_2/M phase transition (27) and *CDK6* regulates G_1/S phase transition (28)] mRNA and protein in MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h. We did not find evidence of cycle arrest in PK-1 cells treated with siDNTTIP2 #1 and #2 for 48 h; rather, these cells arrested in G_2 after treatment with siDNTTIP2 #1 and #2 for 72 h. Therefore, we also evaluated expression of *CDK1* and *CDK6* mRNA and protein in PK-1 cells treated with siDNTTIP2 #1 or #2 for 72 h.

We also found that depletion of *DNTTIP2* decreased expression of *SATB1* mRNA. *SATB1* is a nuclear protein, chromatin organizer, and transcription factor (29). Studies of human cancer suggest that *SATB1* is an important factor that promotes growth and invasion of breast cancers (30), colorectal cancers (31), and pancreatic cancers (32). Depletion of *SATB1* from Primary Human Epidermal Keratinocytes neonatal (HEKn) reduces expression of CDK6 protein (33). Furthermore, we showed that depletion of *SATB1* decreases expression of *CDK6* mRNA, but not that of *CDK1*. These results suggest that *DNTTIP2* suppresses proliferation of pancreatic cancer cells by regulating *CDK6* through *SATB1*, and by regulating *CDK1* directly (Figure 6). Depletion of *DNTTIP2* induced G_1 arrest in MIA-PaCa-2 cells and G_2 arrest in PK-1 cells. To explain this discrepancy, we estimated the expression levels of *SATB1* mRNA in MIA-PaCa-2 and PK-1 cells using "The Human Protein Atlas" database. The results showed that expression of *SATB1* mRNA is much higher in MIA-PaCa-2 cells (11.9 normalized transcripts per million (TPM)) than in PK-1 (1.0 normalized TPM) cells. Therefore, we speculate that differences in expression of *SATB1* mRNA in these two cell lines are the reason for cell cycle arrest at different phases.

Although depletion of *DNTTIP2* inhibited proliferation of the two pancreatic cancer cell lines, we did not observe apoptotic cell death. Depletion of *DNTTIP2* decreased expression of *SATB1* mRNA. The previous report proposes that *SATB1* plays a role in the GEM resistance of PDAC cells (34); thus, GEM resistance might be overcome by depletion of *DNTTIP2*. Future studies should evaluate the effects of GEM on *DNTTIP2*-depleted pancreatic cancer cells. *SATB1* is also involved in invasion and migration of various cancers (31, 32, 35). For example, *SATB1* regulates expression of the *MYC* oncogene in pancreatic cancer cells (32) and plays a crucial role in regulating genes that control epithelial-mesenchymal transition (EMT) in bladder cancer (35). Based on these previous observations, we speculate that metastasis of pancreatic cancer cells might be suppressed by depletion of *DNTTIP2*. Future studies should evaluate the anti-metastatic effects of *DNTTIP2*-depletion both *in vitro* and *in vivo*.

Conclusion

In conclusion, depleting *DNTTIP2* induces cell cycle arrest in MIA-PaCa-2 cells by inhibiting expression of *CDK6*, and in PK-1 cells by inhibiting expression of *CDK1*. Taken together, these findings strongly suggest that *DNTTIP2* plays a major role in proliferation of pancreatic cancer. *DNTTIP2* is a potential target for inhibiting progression of pancreatic cancer.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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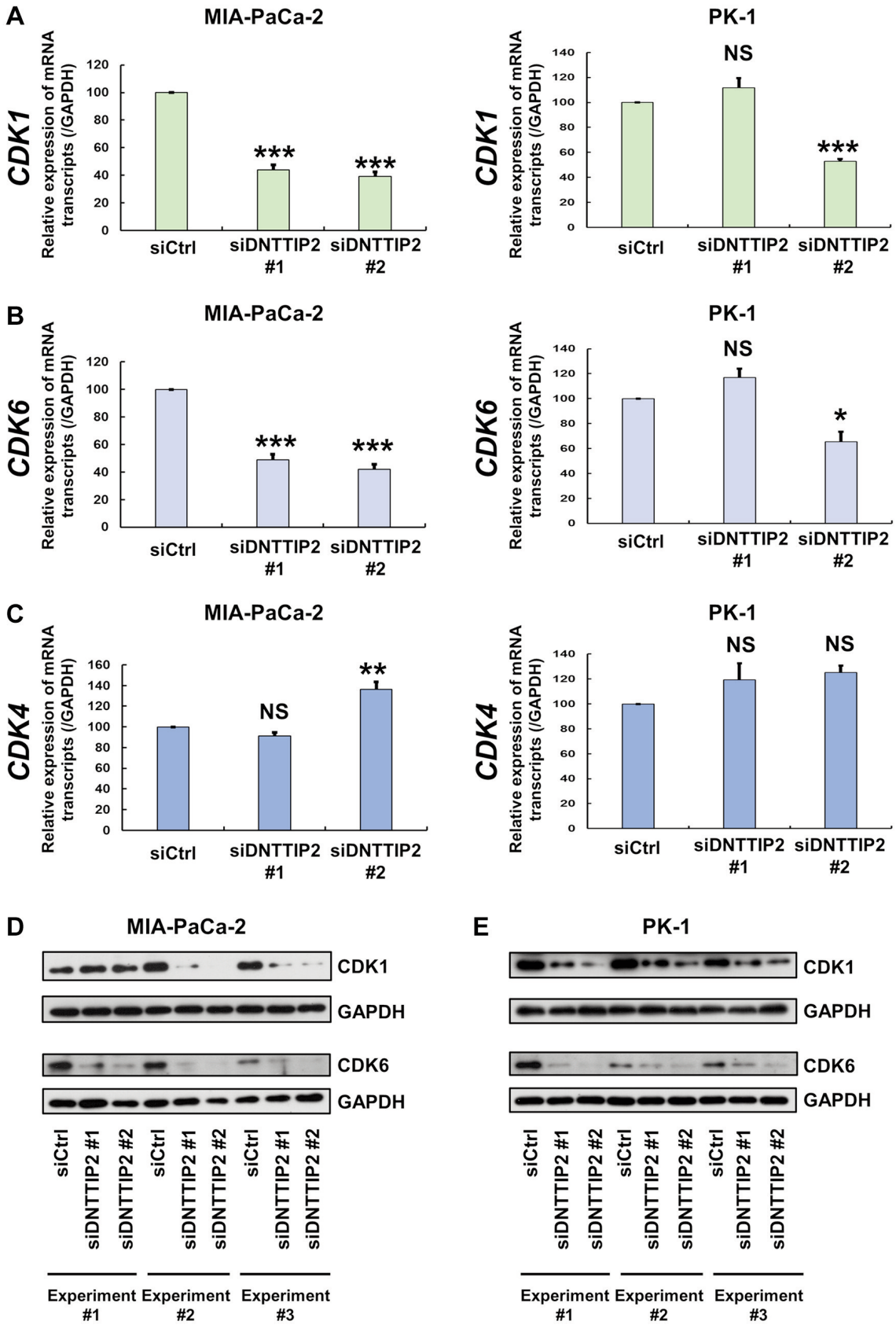
Figure 4. *mRNA and protein levels of cell cycle-regulating molecules in pancreatic cancer cells depleted of DNTTIP2. Evaluation of (A) CDK1, (B) CDK6, and (C) CDK4 mRNA by qRT-PCR. MIA-PaCa-2 cells were treated with siDNTTIP2 #1 and #2 for 48 h, and PK-1 cells were treated with siDNTTIP2 #1 and #2 for 72 h. Data are presented as the mean+SEM of three independent biological replicates, each with three technical replicates. NS: Not significant; *p<0.05; **p<0.01; ***p<0.001 (vs. siCtrl). (D) CDK1 and CDK6 protein expression in MIA-PaCa-2 cells treated with siDNTTIP2 #1 and #2 for 48 h. Data of three independent biological replicates, each with one technical replicate, are shown. (E) CDK1 and CDK6 protein expression in PK-1 cells treated with siDNTTIP2 #1 and #2 for 72 h. Data of three independent biological replicates, each with one technical replicate, are shown.*


A
MIA-PaCa-2
CDK1
Relative expression of mRNA transcripts (/GAPDH)
120
100
80
60
40
20
0
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siDNTTIP2 #1
siDNTTIP2 #2
PK-1
CDK1
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siDNTTIP2 #2
B
MIA-PaCa-2
CDK6
Relative expression of mRNA transcripts (/GAPDH)
120
100
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siCtrl
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PK-1
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siDNTTIP2 #2
C
MIA-PaCa-2
CDK4
Relative expression of mRNA transcripts (/GAPDH)
160
140
120
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siCtrl
siDNTTIP2 #1
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PK-1
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Experiment #1
Experiment #2
Experiment #3

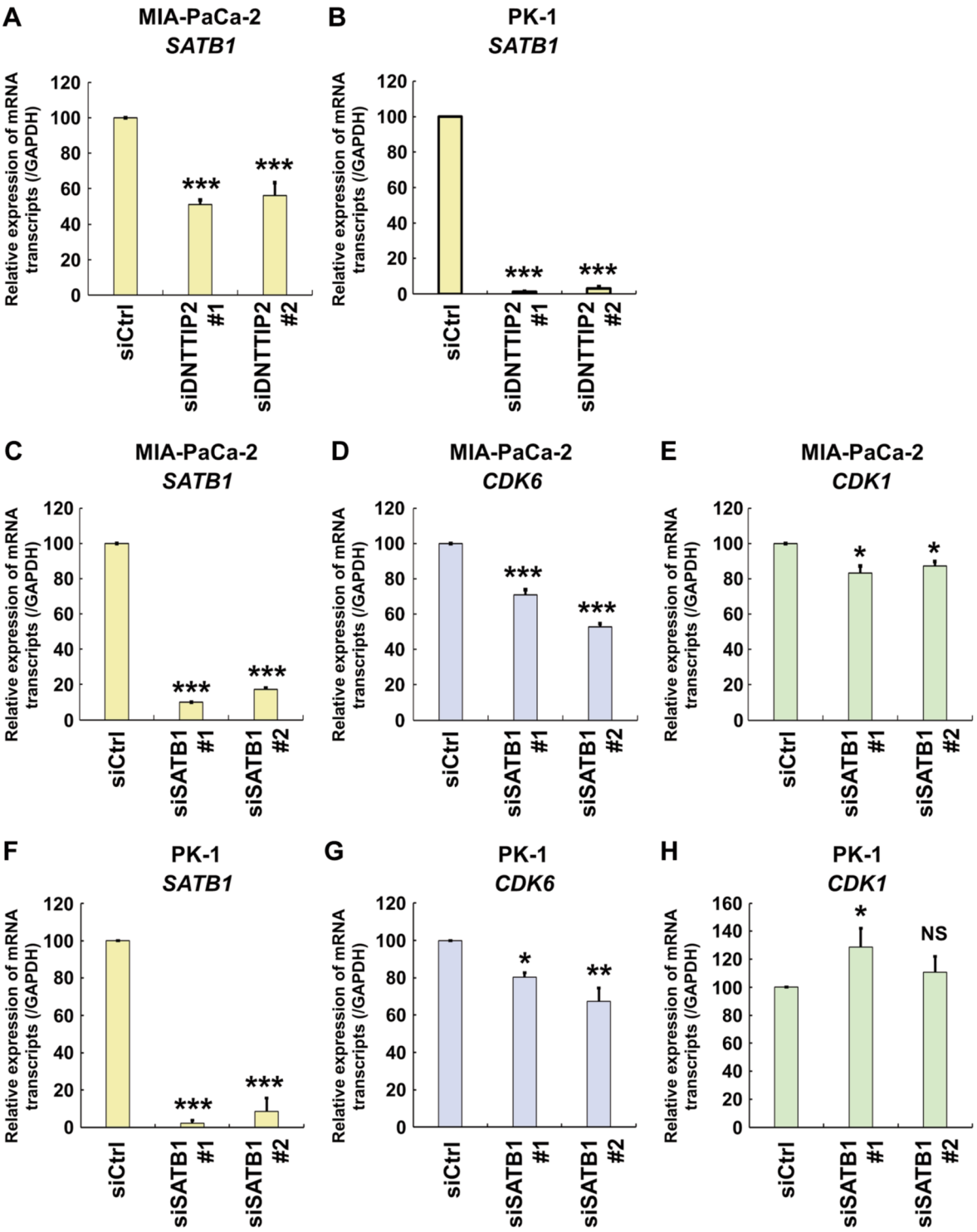

Figure 5. *Relationship between DNTTIP2 and SATB1. (A, B) Expression of SATB1 mRNA, as assessed by qRT-PCR. MIA-PaCa-2 and PK-1 cells were treated with siDNTTIP2 #1 and #2 for 48 h. Data are presented as the mean+SEM of three independent biological replicates, each with three technical replicates. ***p<0.001 (vs. siCtrl). MIA-PaCa-2 cells were treated with SATB1 siRNA s12480 (siSATB1 #1) and SATB1 siRNA s12481 (siSATB1 #2) for 48 h, and expression of SATB1 (C), CDK6 (D), and CDK1 (E) mRNA was assessed by qRT-PCR. Data are presented as the mean+SEM of three independent biological replicates, each with three technical replicates. *p<0.05; ***p<0.001 (vs. siCtrl). PK-1 cells were treated with siSATB1 #1 and #2 for 72 h, and expression of SATB1 (F), CDK6 (G), and CDK1 (H) mRNA was evaluated by qRT-PCR. Data are presented as the mean+SEM of three independent biological replicates, each with three technical replicates. NS: Not significant; *p<0.05; **p<0.01; ***p<0.001 (vs. siCtrl).*

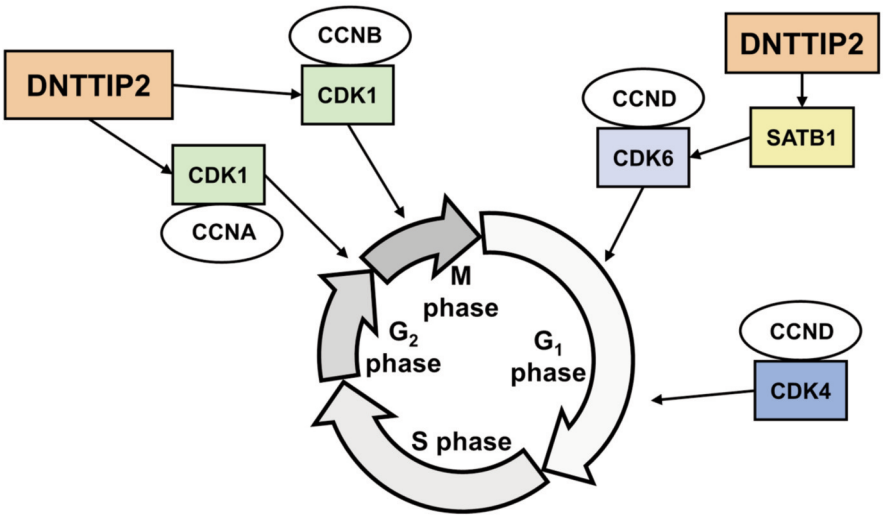

Figure 6. *Proposed mechanism by which DNTTIP2 regulates CDK1 and CDK6. DNTTIP2 regulates CDK6 through SATB1 and regulates CDK1 directly. DNTTIP2 also regulates SATB1. CCNA: Cyclin A; CCNB: cyclin B; CCND: cyclin D; CDK1: cyclin-dependent kinase 1; CDK4: cyclin-dependent kinase 4; CDK6: cyclin-dependent kinase 6; DNTTIP2: deoxynucleotidyltransferase terminal-interacting protein 2; OS: overall survival; PDAC: pancreatic ductal adenocarcinoma; qRT-PCR: quantitative reverse transcription-PCR; RNAi: RNA interference; SATB1: special AT-rich sequence binding protein 1; TCGA: the cancer genome atlas.*

Authors' Contributions

M.Y. performed the experiments and analyzed data; A.S. instructed M.Y. on how to culture pancreatic cancer cell lines; A.S. and E.A. provided intellectual guidance; M.Y. and E.A. wrote the manuscript.

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