Molecular Cancer Research

# ETV4 Facilitates Cell-Cycle Progression in Pancreatic Cells through Transcriptional Regulation of Cyclin D1

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## Abstract

The ETS family transcription factor ETV4 is aberrantly expressed in a variety of human tumors and plays an important role in carcinogenesis through upregulation of relevant target gene expression. Here, it is demonstrated that ETV4 is overexpressed in pancreatic cancer tissues as compared with the normal pancreas, and is associated with enhanced growth and rapid cell-cycle progression of pancreatic cancer cells. ETV4 expression was silenced through stable expression of a specific short hairpin RNA (shRNA) in two pancreatic cancer cell lines (ASPC1 and Colo357), while it was ectopically expressed in BXPC3 cells. Silencing of ETV4 in ASPC1 and Colo357 cells reduced the growth by 55.3% and 38.9%, respectively, while forced expression of ETV4 in BXPC3 cells increased the growth by 46.8% in comparison with respective control cells. Furthermore, ETV4-induced cell growth was facilitated by rapid transition of cells from $G_1$- to S-phase of the cell cycle. Mechanistic studies revealed that ETV4 directly regulates the expression of *Cyclin D1 CCND1*, a protein crucial for cell-cycle progression from $G_1$- to S-phase. These effects on the growth and cell cycle were reversed by the forced expression of *Cyclin D1* in ETV4-silenced pancreatic cancer cells. Altogether, these data provide the first experimental evidence for a functional role of ETV4 in pancreatic cancer growth and cell-cycle progression.

**Implications:** The functional and mechanistic data presented here regarding ETV4 in pancreatic cancer growth and cell-cycle progression suggest that ETV4 could serve as a potential biomarker and novel target for pancreatic cancer therapy. *Mol Cancer Res; 16(2); 187–96. ©2017 AACR.*

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## Introduction

Pancreatic cancer is the third leading cause of cancer-related death in the United States with approximately 8% five-year survival postdiagnosis (1). According to an estimate by American Cancer Society, approximately 53,670 patients are expected to be diagnosed with pancreatic cancer this year and about 43,090 people will succumb to this disease (1). Furthermore, it is expected to become the second leading cause of cancer-related death by the year 2030 or even earlier, if no breakthrough is made in approaches to its clinical management (2). Clearly, there is an urgent need to make efforts at several fronts to tackle this extremely lethal malignancy effectively. In that regard, identification of aberrantly expressed and functionally involved genes in pancreatic cancer pathogenesis is highly significant from the perspective of the development of novel approaches for early detection and therapy.

ETV4, also known as PEA3 and E1AF, is an ETS-oncogene family transcription factor (3). It is broadly expressed in tissues during embryogenesis; however, its expression is low or highly restricted to specific organs in most adult tissues (4). During early development, ETV4 promotes morphogenesis of epithelial organs like kidney, lungs, and mammary gland (5–7). Moreover, ETV4 transcription factor also plays a role in cellular growth, proliferation, migration, and apoptosis (3). An aberrant expression of ETV4 is also reported in various cancer tissues including, breast, gastric, prostate, colon, and ovarian cancers at both mRNA and protein levels (8–12). The overexpression of ETV4 is associated with advanced and more aggressive forms of the tumor with worse prognosis (9, 12). Moreover, a role of ETV4 has also been established in anchorage-independent growth (13), enhanced motility and invasiveness (14), epithelial to mesenchymal transition (10), and tumorigenesis (10, 15).

In this study, we examined the expression and functional significance of ETV4 in pancreatic cancer. A high expression of ETV4 was reported in pancreatic tumor tissues as compared with the normal pancreas. Furthermore, high ETV4 expression was associated with increased growth and rapid cell-cycle progression in a *Cyclin D1*-dependent manner. ETV4 regulated *Cyclin D1* expression by directly binding to its gene promoter, and the

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**Note:** Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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effects of ETV4 silencing in pancreatic cancer cells were partly abrogated by the rescue of *Cyclin D1* expression. Taken together, these findings suggest an important role of ETV4 in pancreatic cancer pathogenesis.

## Materials and Methods

### Cell lines and pancreatic cancer tissue specimens

The pancreatic cancer cell lines, Panc10.05, Panc02.03, Panc03.27, Capan1, SW1990, HPAF, MiaPaCa, CFPAC, ASPC1, Colo357, Panc1, and BXPC3, were procured from ATCC and maintained under normal culturing conditions as described previously (16). All the cells were tested and determined to be free from mycoplasma contamination every alternate month. Frozen pancreatic tissue samples (normal and malignant) were obtained through cooperative human tissue network (CHTN) at the University of Alabama at Birmingham (UAB) under an Institutional Review Board (IRB)-approved protocol.

### Microarray data analysis

Oncomine cancer microarray database (ref. 17; http://www.oncomine.org) was used to study the gene expression of ETV4 in human pancreatic cancer and normal pancreas tissue samples. A gene was considered as overexpressed when its mean value in tumor samples was significantly higher than mean value in the normal tissue counterpart using a Student *t* test ($P < 0.05$).

### Antibodies and plasmids

Antibody against *Cyclin D1* (rabbit polyclonal) was purchased from Cell Signaling Technology. Anti-ETV4 (rabbit polyclonal) antibody and respective anti-rabbit horseradish peroxidase–conjugated secondary antibodies were procured from Santa Cruz Biotechnology. The β-actin (mouse monoclonal) antibody was purchased from Sigma-Aldrich. ETV4 knockdown short hairpin RNA (shRNA) expression constructs (pGFP-V-RS-shETV4) along with scrambled control (pGFP-V-RS-NTScr) and ETV4 overexpression construct (pCMV6-ETV4) and scramble control (pCMV6-Neo) were purchased from OriGene. *Cyclin D1* overexpression construct (pCMV6-CCND1) and control vector (pCMV6) were also purchased from OriGene.

### Constructs, transfections, and treatments

For ETV4 silencing, Colo357 and ASPC1 cell lines were transfected with pGFP-V-RS-shETV4, and for ectopic overexpression of ETV4, BXPC3 cell lines were transfected with pCMV6-ETV4, along with their respective control plasmids, using X-tremeGENE HP DNA Transfection reagent (Roche) as per the manufacturer's instructions. Stable pooled population of transfected cells were selected in media containing puromycin (2 μg/mL; for shRNA) or G148 (200 μg/mL; for overexpression), expanded, and examined for stable ETV4 silencing or overexpression. The construct -962 human *Cyclin D1* promoter pGL3Basic (Addgene plasmid #32727) was from Frank McCormick's laboratory and was procured through Addgene.

### Luciferase reporter assays

Cells were cultured at a density of $1 \times 10^6$ cells/well in 6-well plate and transfected with -962 human *Cyclin D1* promoter pGL3Basic and mutant variation of -962 human *Cyclin D1* promoter pGL3Basic reporter constructs. After 24 hours of transfection, the cells were harvested in passive lysis buffer and luciferase activity was measured using the Dual Luciferase Assay System (Promega).

### Site-directed mutagenesis

Site-directed mutagenesis was performed using QuickChange Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The *Cyclin D1* luciferase reporter vector was used as template and the predicted target site A sequence 5′- GGATGGCT-3′ was mutated to 5′-CGTTGCCA-3′ by PCR using the following primers: FP: GCAGGCGGGCGCCTCAGGGATGCCATTTGGGCTCTGC; RP: GCAGAGCCCAAATGGCATCCCTGAGGCGCCCGCCTGC. The mutagenized plasmids were isolated using the Qiagen Miniprep Kit (Qiagen) and DNA sequencing (Eurofins) confirmed mutations of the region containing the mutation.

### Gene expression analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described in detail previously (18). The list of primer pairs is presented in Supplementary Table S1.

### Western blot analysis

Proteins isolated from pancreatic cancer cells and frozen tissues were estimated using DC Protein Assay Kit (Bio-Rad), resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against various proteins (19). Primary antibodies were detected with respective anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology). The signal of secondary antibodies was detected using ECL plus Western Blotting detection kit (Thermo Scientific) and LAS-3000 image analyzer (Fuji Photo Film Co.).

### Growth kinetics assay

Cells ($1 \times 10^4$/well) were seeded in 6-well plates (in triplicate) and allowed to grow for 8 days. The growth rate was determined every day by counting the number of cells on a hemocytometer. Cell population doubling time (*dt*) was calculated during exponential growth phase (120–168 hours) using the following formula: $dt = 0.693\ t/ln\ (Nt/N0)$, where *t* is time (in h), *N*t is the cell number at time *t*, and *N*0 is the cell number at initial time (20). The percent growth inhibition on the 8th day was calculated using the formula: $100-[(N_{shETV4}$ or $N_{neo}/N_{NTScr}) \times 100]$, where $N_{shETV4}$, $N_{neo}$, and $N_{NTScr}$ are the number of cells on the 8th day (20).

### Colony formation assay

For anchorage-dependent colony formation, cells ($1 \times 10^3$/well) were seeded in 6-well plates. After two weeks, colonies were fixed with methanol, stained with crystal violet, photographed, and counted using Image analysis software (Gene Tools) as described in detail previously (21).

### Cell-cycle analysis

Cell-cycle analysis was executed on synchronized cells by culturing them in serum-free media for 72 hours, and then incubated in regular media for 24 hours. Cells were washed, trypsinized, and fixed with 70% ethanol overnight at 4°C. The next day, cells were washed with cold PBS, stained with propidium iodide using PI/RNase staining

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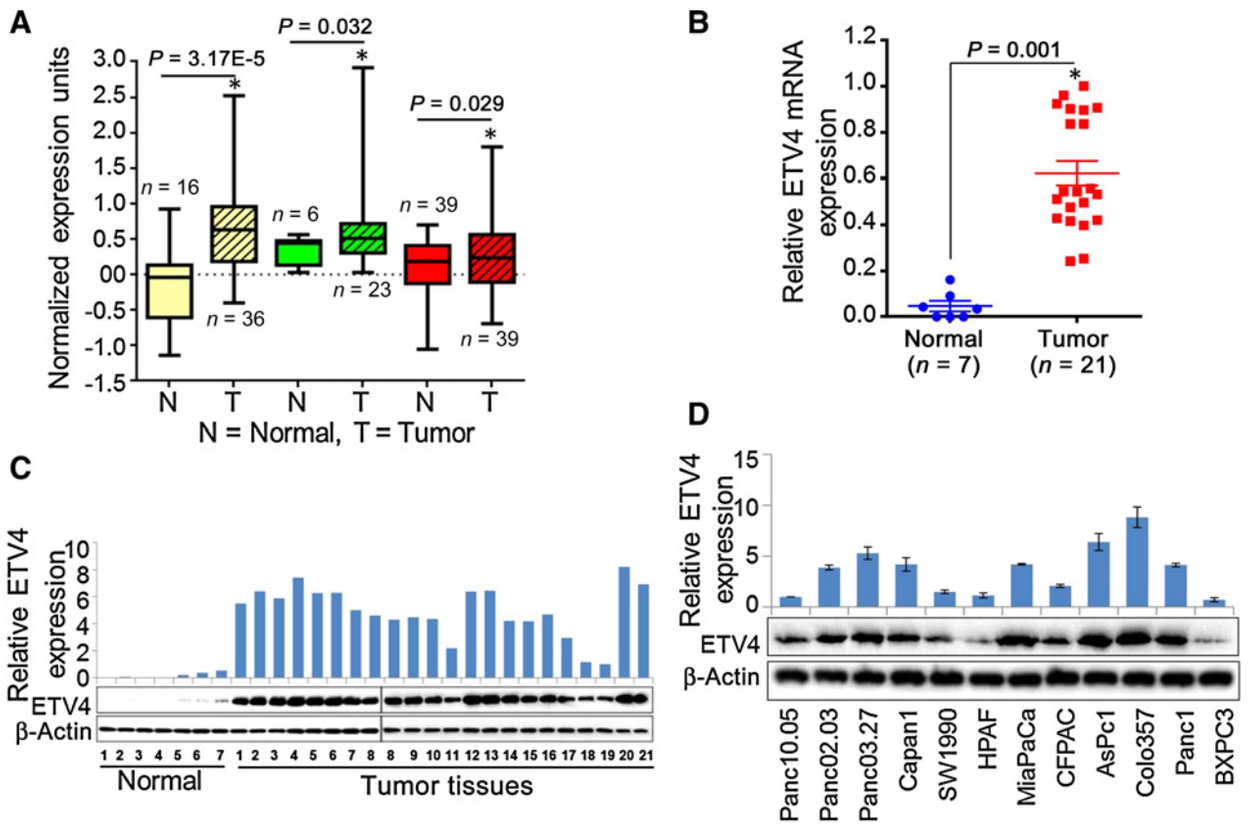


**Figure 1.**

Expression of ETV4 in pancreatic cancer. **A,** Oncomine database analysis for the expression of ETV4. Analysis of ETV4 expression in pancreatic cancer ($n = 21$) and normal pancreas ($n = 7$) tissue samples at mRNA level by qRT-PCR ($P < 0.5$; **B**) and at the protein level by immunoblot analysis ($n = 1$; **C**). GAPDH and β-actin were used as internal controls. **D,** ETV4 expression in pancreatic cancer cell lines. Bars, mean ± SD ($n = 3$; *, $P < 0.05$).

buffer for 1 hour at 37°C, and analyzed by flow cytometry (Becton Dickinson).

### Chromatin immunoprecipitation assay

ChIP assay was performed using a ChIP-IT enzymatic kit as described previously (22). Briefly, cells were fixed with paraformaldehyde (37%) for DNA-protein cross-linking, lysed, and sonicated to obtain small chromatin fragments and subjected to immunoprecipitation using anti-ETV4 or normal rabbit IgG (as control) antibodies. Subsequently, immunoprecipitates were subjected to reverse cross-linking and DNA was isolated. ChIPed DNA was analyzed by PCR using specific primers: *Cyclin D1* promoter site (-343), CCND1-Chip-F-5′-ACCGGACTACAGGGGCAAC-3′, CCND1-Chip-R-5′-AAACGCCGGGAGCAGCGA-3′; *Cyclin D1* promoter site (-794), CCND1-Chip-F-5-CTTGGGCATTTGCAACGACG-3′, CCND1-Chip-R-5′-CACACAACCCCTGTGCAAGT-3′; *Cyclin D1* promoter site (-941), CCND1-Chip-F-5-GAAGAGTCTCCAGGCTAGAAG-3′, CCND1-Chip-R-5′-GCAGCAGCCCAAGATGGTG-3′; *Cyclin D1* promoter site (-1038), CCND1-Chip-F-5-CAGAGGTGTGTTTCTCCCG-3′ and CCND1-Chip-R-5′-CTTCCTACCTTGACCAGTCG-3′. Input DNA (without immunoprecipitation) and normal IgG-precipitated DNA were used as positive and negative controls, respectively.

### Statistical analysis

All the experiments were performed at least three times, independently, and all data are expressed as "mean ± SD." Wherever appropriate, the data were also subjected to unpaired two-tailed Student *t* test. $P < 0.05$ was considered statistically significant.

## Results

### ETV4 is aberrantly expressed in pancreatic cancer clinical specimens and cell line models

We searched the ONCOMINE databases for the expression of ETV4 in pancreatic cancer tissues. In three microarray expression studies (23–25), ETV4 mRNA expression level was higher in pancreatic cancer tissues than in normal pancreas tissues (Fig. 1A). To investigate the role of ETV4 in pancreatic cancer pathogenesis, we first tested the expression of ETV4 in human pancreatic cancer tissue specimens ($n = 20$) along with normal ($n = 7$) pancreatic tissue samples using quantitative real-time RT-PCR and Western blot analysis. The expression of ETV4 at mRNA level in pancreatic cancer tissue specimens was significantly higher ($P < 0.01$) than in normal samples (Fig. 1B). In addition, we observed an aberrantly high expression of ETV4 in all pancreatic cancer tissues, whereas no or minimal expression was observed in normal pancreatic

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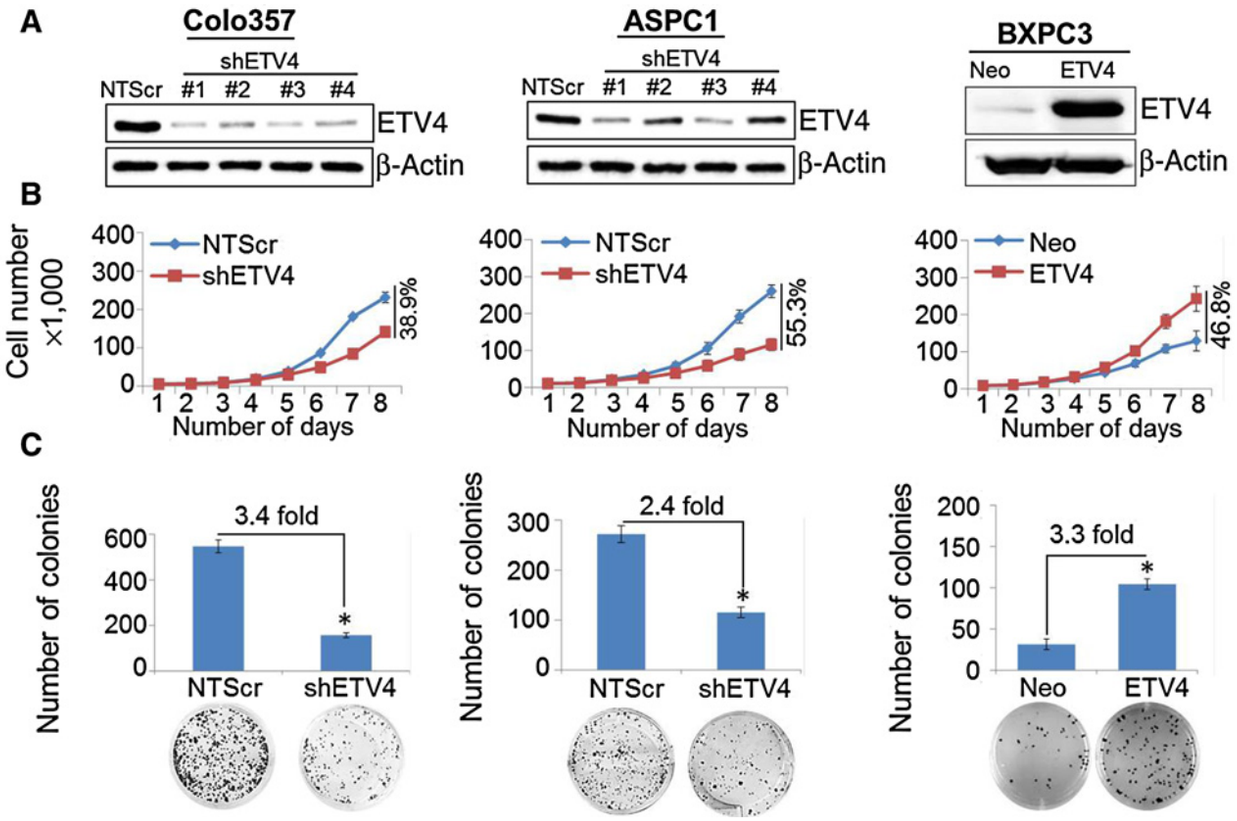


**Figure 2.**

ETV4 enhances pancreatic cancer cell growth and clonogenic ability. **A,** Stable ETV4-silenced (Colo357-shETV4 and ASPC1-shETV4) and overexpressed (BXPC3-ETV4) cell lines with their scrambled control (Colo357-NTScr and ASPC1-NTScr and BXPC3-Neo) cells were generated, and ETV4 expression was examined by immunoblot analyses. **B,** For growth kinetics, cells ($1 \times 10^4$) were seeded in 6-well plates and growth was monitored by counting the cell number up to 8 days. **C,** For anchorage-dependent colony formation assay, cells were seeded at low density ($1 \times 10^3$ cells/well) in regular media. After 2 weeks, colonies were stained with crystal violet and visualized and photographed using imaging system. Bars, mean ± SD ($n = 3$; *, $P < 0.05$).

tissue specimens (Fig. 1C). To observe the staining pattern of ETV4, we performed IHC on a small set of human pancreatic tumor tissues and normal pancreas ($n = 5$, each). We detected an intense nuclear staining of ETV4 with some diffuse staining in the cytoplasm in all pancreatic cancer tissues, while no or minimal staining was observed in normal tissue samples (Supplementary Fig. S1). We also examined the expression of ETV4 in a panel of pancreatic cancer cell lines of varying tumorigenic and metastatic potential (26) and detected high expression of ETV4 in majority of the cell lines. BXPC3, a poorly tumorigenic cell line (27), showed minimal expression of ETV4 (Fig. 1D). These findings imply that ETV4 is overexpressed in pancreatic cancer and might play a role in pancreatic cancer pathogenesis.

### ETV4 expression is associated with the growth of pancreatic cancer cells

To explore the functional role of ETV4 in pancreatic cancer, we silenced ETV4 in two high ETV4-expressing tumorigenic cell lines, Colo357 and ASPC1, while overexpressed it in a poorly tumorigenic and low ETV4-expressing cell line, BXPC3. We used four shRNA constructs to knockdown ETV4 expression, of which two (shETV4 #1 and shETV4 #3) were able to cause significant ETV4 silencing in both cell lines (Fig. 2A). Therefore, we performed subsequent functional studies with their resulting transfectants and/or their pooled population (Colo 357-shETV4 and ASPC1-shETV4). Moreover, in a converse approach, we forcefully overexpressed ETV4 in poorly tumorigenic BxPC3 cell line by stable transfection (Fig. 2A). Subsequently, we studied the growth kinetics of high (Colo357-Scr, ASPC1-Scr, and BxPC3-ETV4) and low (Colo357-shETV4, ASPC1-shETV4, and BxPC3-Neo) ETV4-expressing pancreatic cancer cells. Our findings reveal that silencing of ETV4 led to a decrease in the growth of Colo357 and ASPC1 cells, while its ectopic expression promoted the growth of BXPC3 cells. After 8 days, the cell growth reduced by 38.9% in Colo357-shETV4 and 55.3% in ASPC1-shETV4 cells as compared with their respective control cells, while we recorded 46.8% enhancement in the growth of ETV4-overexpressing cells (BXPC3-ETV4) as compared with BXPC3-Neo cells (Fig. 2B). These changes in growth were due to altered population doubling times (dt), which increased from 21.4 to 32.1 hours and 29.7 to 40.1 hours in ETV4-silenced Colo357-shETV4 and ASPC1-shETV4 cells, respectively, as compared with their control sublines. In contrast, population doubling time of BXPC3 cells was decreased

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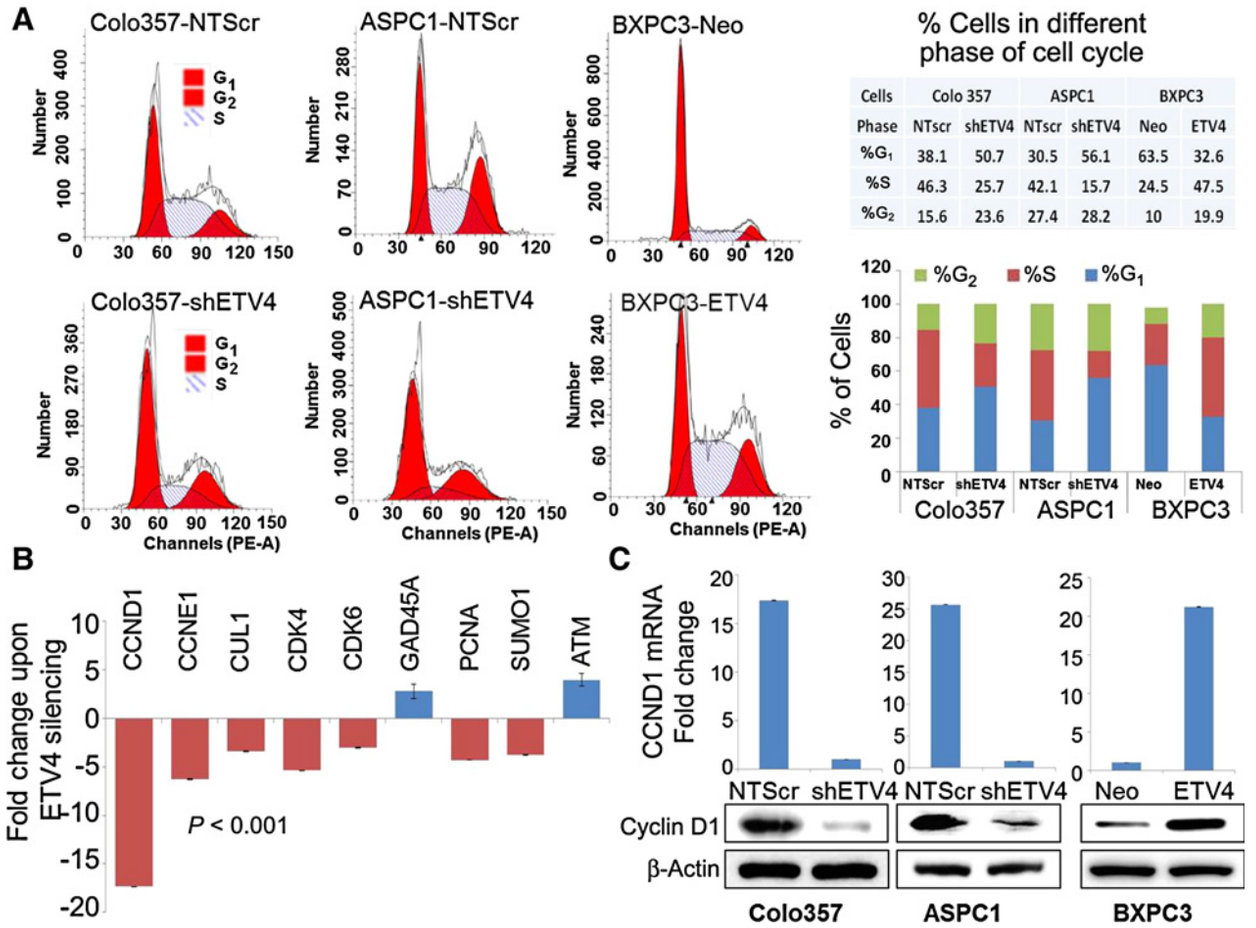


**Figure 3.**

ETV4 silencing arrests cell cycle in $G_1$–S phase by regulating the expression of genes involved in regulation and progression of cell cycle. **A,** ETV4-silenced and -overexpressing cells were synchronized by culturing them in serum-free media for 48 hours and then incubated in regular medium for 24 hours. Subsequently, cells were fixed in methanol and stained with propidium iodide. The distribution of cells in different phases of cell cycle was analyzed by flow cytometry. **B,** Expression profile of selected cell-cycle–associated genes by quantitative RT-PCR upon ETV4 silencing in Colo357 cells and (**C**) expression of Cyclin D1 at mRNA and protein level in low and high ETV4-expressing Colo357, ASPC1, and BXPC3 cells. GAPDH and β-actin were used as loading controls.

from 36.1 to 27.2 hours upon ectopic expression of ETV4 (Fig. 2B). To monitor the effect of ETV4 on growth in the long term, we performed the colony-forming assay. Overexpression of ETV4 enhanced the clonogenic ability by 3.3-fold in BXPC3-ETV4 cells while ETV4-silenced (Colo357-shETV4 and ASPC1-shETV4) cells showed diminished clonogenic ability (3.4- and 2.4-fold, respectively) compared with their respective control cells (Fig. 2C). These findings thus suggest that ETV4 plays an important role in the growth of pancreatic cancer cells.

### ETV4 promotes $G_1$–S phase transition by modulating cell-cycle–associated genes

Having observed a role of ETV4 in the growth of pancreatic cancer cells, we next examined the effect of ETV4 expression on cell-cycle progression. For this, we first synchronized cells by culturing them in serum-free media for 72 hours and then cultured in regular serum-containing medium for 24 hours. Cells were processed for staining with propidium iodide using the PI/RNase Kit and analyzed by flow cytometry. The data from flow cytometry analysis indicate that ETV4-silencing causes a $G_1$ phase cell-cycle arrest in pancreatic cancer cells, while a greater fraction of ETV4-overexpressing cells is detected in S-phase (Fig. 3A). In low ETV4-expressing (Colo357-shETV4, ASPC1-shETV4 and BXPC3-Neo) cell lines, enhanced fraction of cells in $G_1$ phase (50.7%, 56.1%, and 63.5% respectively), were observed as compared with high ETV4-expressing [Colo357-NTScr (38.1%), ASPC1- NTScr (30.5%), and BXPC3-ETV4 (63.5%)] cells. Furthermore, drastic reduction in the fraction of cells in S-phase in cells exhibiting low expression of ETV4 (Colo357-shETV4 (25.7%), ASPC1-shETV4 (15.7%), and BXPC3-Neo (24.5%) was seen as compared with Colo357-NTScr (46.3%), ASPC1- NTScr (42.1%), and BXPC3-ETV4 (47.5%) cells (Fig. 3A). These data indicate a role of ETV4 in cell-cycle progression by facilitating $G_1$ to S-phase transition. To decipher the unbiased mechanistic basis of ETV4-induced cell-cycle progression, we next examined the expression profile of 48 genes implicated in cell-cycle regulation in high and low ETV4-expressing pancreatic cancer cells by quantitative RT-PCR. We observed that 6 genes (*CCND1*, *CCNE1*,

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**Table 1.** List of selective cell-cycle–regulatory genes with symbol, functions, and folds change in high and low ETV4-expressing pancreatic cancer cells

| Gene | Function | Fold change | $P^a$ |
|---|---|---|---|
| *CCND1* | CCND1 interacts with CDK4 and CDK6 and control cell cycle at the $G_1$–S transition | −17.3643 | 0.000172144 |
| *CCNE1* | CCNE1 interacts with CDK2 and control cell cycle at the $G_1$–S transition | −6.28749 | 0.000477381 |
| *CUL1* | CUL1 regulate cell cycle at the $G_1$–S transition by ubiquitinylation of Cyclins D1 and E | −3.42371 | 0.001956218 |
| *CDK4* | Interacts with Cyclin D1 | −5.36261 | 1.44007E-05 |
| *CDK6* | Interacts with Cyclin D1 | −3.02619 | 0.001454547 |
| *GADD45A* | GADD45A inhibit entry of cells into S phase | 2.81315 | 0.051256666 |
| *PCNA* | PCNA controls DNA replication | −4.28021 | 0.000373706 |
| *SUMO1* | SUMO1 stablize CDK6 by SUMOylation in $G_1$ phase and control cell cycle through $G_1$–S transition | −3.77065 | 0.001100455 |
| *ATM* | ATM activates checkpoint and phosphorylate p53 | 3.980067 | 0.015487116 |

[a]$P < 0.001$ was considered significant.

*CUL1*, *CDK4*, *CDK6*, and *SUMO1*) primarily involved in $G_1$ phase and $G_1$–S-phase transition (Table 1) of cancer cells were significantly altered (fold $\geq 2$; $P < 0.001$) and *Cyclin D1* was the most downregulated gene exhibiting approximately 18-fold decreased expression in ETV4-silenced Colo357 pancreatic cancer cells (Fig. 3B). The expression of *Cyclin D1* was further confirmed at mRNA and protein level in all the three pancreatic cancer cell lines engineered for low and high expression of ETV4 (Colo357-NTScr/-shETV4, ASPC1-NTScr/-shETV4 and BXPC3-Neo/-ETV4). As expected, we detected reduced expression of *Cyclin D1* in low ETV4-expressing pancreatic cancer cells, while its high expression was observed in ETV4-overexpressing BXPC3 cells (Fig. 3C). Taken together, these findings suggest that the ETV4 facilitates cell-cycle progression of pancreatic cancer cells by modulating the expression of genes associated with the $G_1$–S transition phase of cell cycle.

### *Cyclin D1* is a direct transcriptional target of ETV4

Having observed the transcriptional regulation of *Cyclin D1* by ETV4, and *Cyclin D1* being an essential cell-cycle–regulatory protein required for rapid progression of cell cycle from $G_1$–S-phase of the cycle (28, 29), we next examined whether *Cyclin D1* is under direct transcriptional control of ETV4. For this, we first performed an *in silico* analysis of approximately 1-kb DNA region 5′ upstream of coding DNA sequence (CDS) of *CCND1* (GenBank accession number Z29078) using available online analytic applications (TF-Bind, JASPAR and ALGGEN-PROMO). Our analysis revealed four putative ETV4-binding sites within the promoter region of *CCND1* (Fig. 4A). To confirm the direct binding of ETV4 to the *CCND1* promoter, we performed ChIP analysis using ETV4-specific antibody. Normal IgG was used as negative control. The ChIPed DNA was subjected to the PCR amplification using primer sets covering putative ETV4-binding sites (Fig. 4A). The data demonstrated that ETV4 most strongly bound to the predicted promoter region −343 to −336 that lies in the closest proximity to the transcriptional initiation site of *Cyclin D1*, while its binding to other sites was less significant (Fig. 4B). To determine whether the predicted target site (−343 to −336) for ETV4 binding is a functional target site we mutated the ETV4-binding sequence 5′-GGATGGCT-3′ to 5′-CGTTGCCA -3′ (Fig. 4C) using site-directed mutagenesis kit and mutation was confirmed by DNA sequencing of the region containing the mutation. The pancreatic cancer cells, Colo357 and ASPC1, were transfected with mutant or wild-type *Cyclin D1*, and a control promoter–reporter constructs. Estimation of relative luciferase activity demonstrated 70.1% and 72.4% reduction in mutant construct-transfected Colo357 and ASPC1 cells, respectively (Fig. 4C and D) suggesting that the mutated sequence in the *CCND1* promoter represents the functional target site of ETV4. In accordance to these findings, we also detected reduced PCR amplification signal for *Cyclin D1* in ETV4-silenced cells. The relative ETV4 binding to *CCND1* promoter at −343 to −336 site is reduced by 12.1-fold in Colo357-shETV4 and 12.7-fold in ASPC1-shETV4 cells, supporting the specificity of ETV4-dependent chromatin pull-down (Fig. 4E). Together, these data suggest that ETV4 positively regulates *Cyclin D1* at the transcriptional level in pancreatic cancer cells.

### ETV4 regulates cell-cycle progression and overall growth, at least in part, through upregulation of Cyclin D1

To determine the involvement of *Cyclin D1* in ETV4-induced effect on the PC cells, low ETV4-expressing (Colo357-shETV4 and ASPC1-shETV4) cells were transiently transfected with *CCND1* overexpression construct and the effect on cell cycle and growth was examined. First, we confirmed the overexpression of *Cyclin D1* by immunoblot analysis in ETV4-silenced (Colo357 and APSC1) cells transiently transfected with *CCND1* construct (pCMV6-CCND1; Fig. 5A). The findings were further confirmed by reading the transcriptional activity of *Cyclin D1* promoter by luciferase reporter assay (Fig. 5B). Next, we tested whether overexpression of Cyclin D1 in ETV4-silenced pancreatic cancer cells could reverse the growth-inhibitory effect of ETV4-silencing in pancreatic cancer cells. Our data revealed that overexpression of *Cyclin D1* significantly abrogated the growth-inhibitory effect of ETV4 silencing in both Colo357 and APSC1 cells (Fig. 5C). Furthermore, overexpression of *Cyclin D1* in ETV4-silenced cells released the cells from $G_1$–S-phase arrest (Fig. 5D) and partially rescued the long-term inhibitory effect on growth (Supplementary Fig. S2) of pancreatic cancer cells. These data suggest that ETV4 promotes pancreatic cancer cell growth and cell-cycle progression, at least in part, through upregulation of *Cyclin D1*.

## Discussion

This study revealed, for the first time, the oncogenic involvement of ETV4 in pancreatic cancer. Specifically, using malignant and normal pancreatic tissues and established cancer cell lines, we were able to show the aberrant expression of ETV4 in pancreatic cancer. These data are also supported by analysis of ONCOMINE datasets from three independent studies (23–25). Oncomine datasets analyses, however, also showed one contradictory report citing a downregulation of ETV4 at the mRNA level in pancreatic cancer and pancreatitis tissues as compared with the normal pancreas (30). This could either be sample-specific or tumor subset–specific as the study used a very small sample size ($n = 5$). In a small pilot study, we also performed

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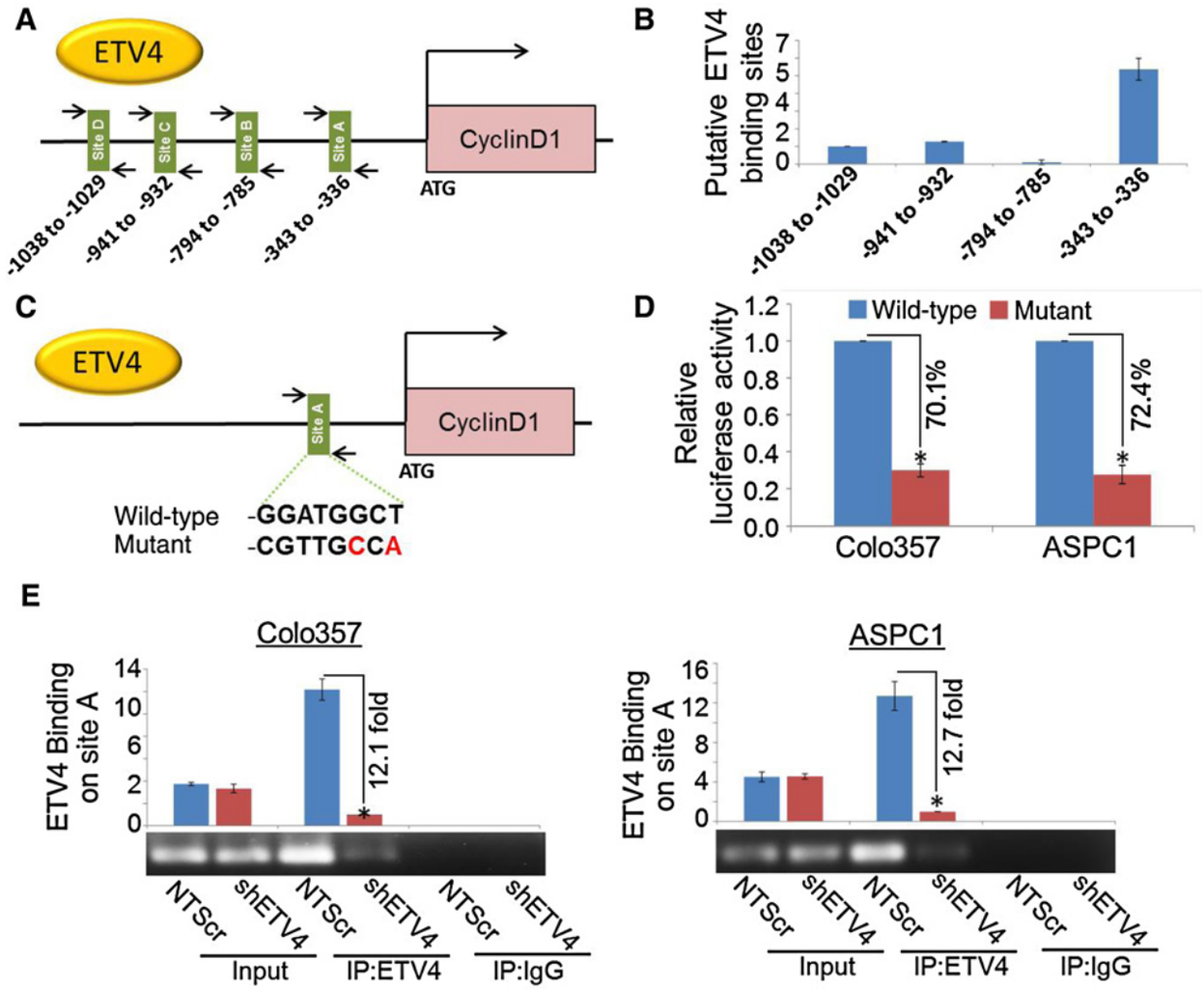


**Figure 4.**

ETV4 transcriptionally upregulates cyclin D1 via direct binding to its promoter region. **A,** Schematic diagram of human cyclin D1 promoter showing putative ETV4-binding sites. Arrows indicate the position and orientation of forward and reverse primers. The number below the bars represents the position of putative binding sites. **B,** The direct binding of ETV4 to Cyclin D1 promoter was shown using ChIP assay. PCR was performed using specific primers as indicated. **C,** Site A (−343 to −336) sequence 5′- GGATGGCT-3′ was mutated to 5′-CGTTGCCA-3′ using site-directed mutagenesis kit. **D,** The wild-type and mutated cyclin D1 promoter construct was transfected into pancreatic cancer cells and luciferase assay was performed 24 hours after transfection using the dual Luciferase Reporter Assay kit to determine the luciferase activity. **E,** PCR amplification signal in low and high ETV4-expressing Colo357 and ASPC1 cells, suggesting the specificity of ETV4-dependent chromatin pull-down. Input DNA (without immunoprecipitation) and normal IgG-precipitated DNA were used as positive and negative controls, respectively. Bars, mean ± SD ($n = 3$; *, $P < 0.05$).

immunostaining for ETV4 to examine its *in situ* expression and subcellular localization in normal and malignant pancreatic tissues ($n = 5$ each). None of the normal tissues exhibited ETV4 staining (except some faint background staining), while varying levels of ETV4 were detected in malignant tissues of mixed grades suggesting its tumor-specific expression. However, clear determination of its specificity as well as overall incidence and extent of dysregulated expression will require a study that examines its expression in larger set of normal, adjacent normal, and cancerous tissues of pancreas. It would also be interesting to investigate whether ETV4 expression exhibits any correlation with increasing tumor grades. Regardless, there are numerous studies in the literature that report an overexpression of ETV4 in a variety of other tumor types as well, including breast, lung, colon, and prostate, etc., which clearly suggests its important roles in cancer pathogenesis (8–12, 31).

Pancreatic cancer is characterized by several genetic aberrations (32). Of which, genetic mutations in K-Ras (33, 34), inactivating mutation or deletion of TP53 (35, 36), homozygous deletion of *SMAD4* (37, 38), *INK4* (39), *PTEN* (40) are frequently detected, and their roles in etiology and progression of pancreatic cancer have also been reported (32–40). However, precise mechanisms through which gene aberrations promote pancreatic cancer initiation and progression remain to be clearly understood. It is believed that the accumulation of these genetic changes leads to altered expression of several tumor-specific genes through

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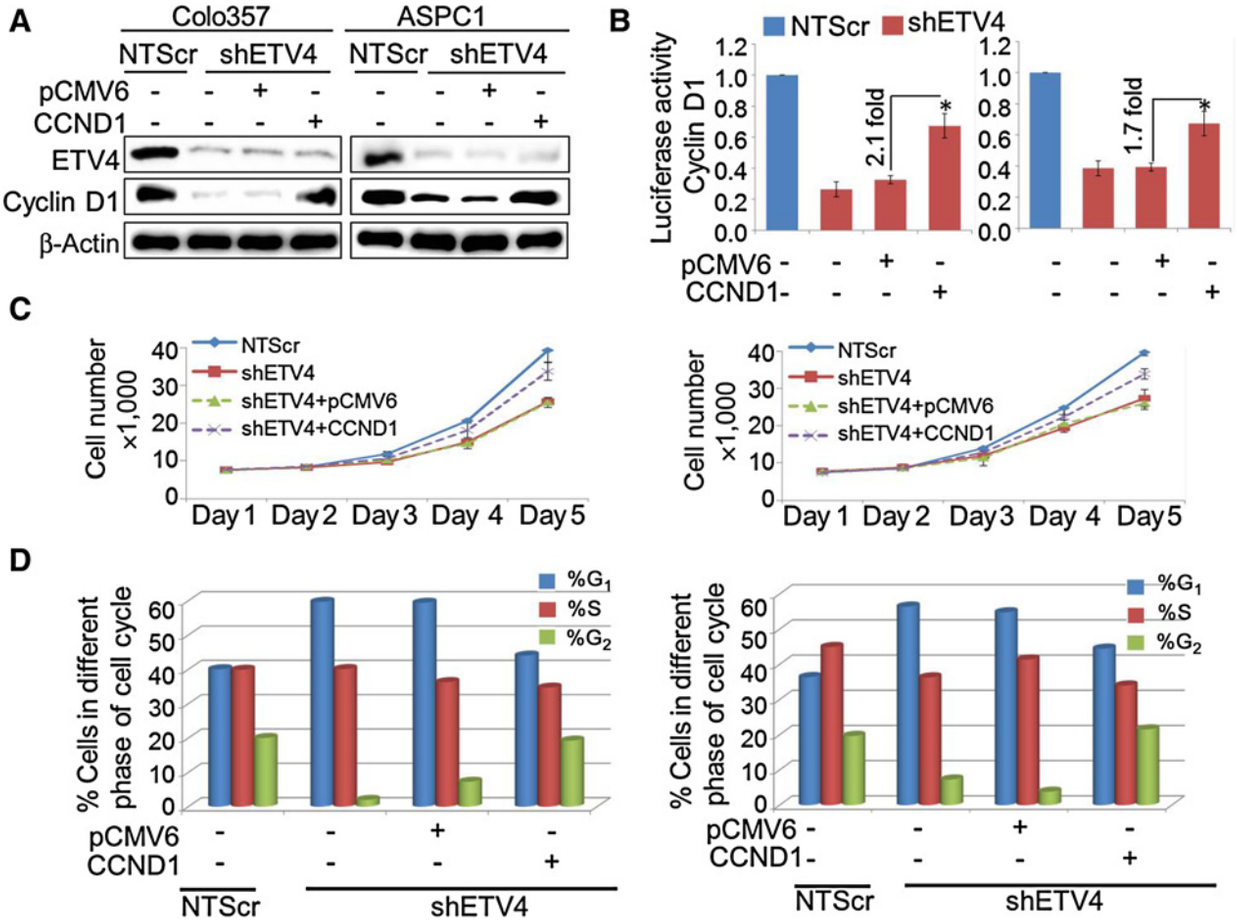


**Figure 5.**

ETV4 regulates pancreatic cancer cell growth through cyclin D1. **A,** Cyclin D1 cDNA construct (pCMV6-CCND1) was transfected in ETV4-silenced Colo357 and ASPC1 cells for its overexpression and confirmed by immunoblot analyses. **B,** After 48 hours of transfection, cells further transfected with Cyclin D1 promoter reporter construct and transcriptional activity of Cyclin D1 was examined. Data are presented as normalized relative luciferase activity (mean ± SD; $n = 3$, $^*$, $P < 0.05$). **C** and **D,** Overexpression of Cyclin D1 in ETV4-silenced cells rescued the inhibitory effect of ETV4-silencing on growth (**C**) and cell-cycle progression (**D**) of pancreatic cancer cells.

activation of oncogenic signaling pathways. Importantly, a regulatory connection of K-Ras signaling with ETV4 expression has also been reported (41). Furthermore, ETV4-mediated regulation of PI3-kinase and Ras signaling has also been reported in a mouse model of advanced prostate cancer (42). A role of ETV4 as a downstream effector of MET signaling pathway has also been reported, whereby it is involved in the regulation of migration and invasiveness (14). In another report, a positive correlation of ETV4 with HER2/Neu overexpression, tumor grade, and higher recurrence in human breast cancer patients is also demonstrated (43) suggesting a pathologic involvement of ETV4 in multiple cancer types.

Our data suggested an oncogenic function of ETV4 in pancreatic cancer by demonstrating its role in growth and cell-cycle progression of pancreatic cancer cells. Similarly, earlier studies have also documented a pathologic role of ETV4 overexpression in variety of other cancers. Pellecchia and colleagues (2012) reported that inhibition of ETV4 retarded the proliferation of prostate cancer cells (10). Moreover, ETV4 is shown to promote growth, proliferation, and cell-cycle progression in breast cancer cells as well (15). Uncontrolled cell proliferation is one of the hallmarks of cancer. This is achieved by the tumor cells typically through abnormal expression and functions of genes involved in cell-cycle regulation. In this regard, we also observed the cell-cycle–regulatory role of ETV4 in pancreatic cancer, whereby it promoted cell-cycle progression by facilitating transition of cells from $G_1$–S phase. Furthermore, we also observed changes in expression of genes associated with $G_1$ or $G_1$–S-phase transition.

*Cyclin D1* is a critical cell-cycle regulator, which is rapidly synthesized in $G_1$ phase and degraded as the cells enter into the S-phase suggesting its important role in $G_1$–S progression (28, 29). Under normal scenario, expression of *Cyclin D1* is tightly regulated; however, high expression of *Cyclin D1* is frequently reported in cancer cells leading to dysregulation of cell cycle and overall growth of the cells (44). Several lines of evidence support that aberrant overexpression of *Cyclin D1*, either through genetic (gene amplification) or nongenetic (epigenetic, transcriptional) mechanisms, is a common event in a variety of cancers including pancreatic cancer (45–47). Cyclin D1 is overexpressed in approximately 42%–82% human pancreatic cancers. However, its gene

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amplification is reported only in 25% cases (48) suggesting that its overexpression is likely regulated through other mechanisms in pancreatic cancers. One such mechanism may be the enhanced transcription of *Cyclin D1* in pancreatic cancer cells. Our findings are quite intriguing, as we have identified a novel mechanism of *Cyclin D1* regulation in pancreatic cancer by ETV4. We observed that ETV4 binds to the promoter region of *Cyclin D1* and upregulates the transcription of *Cyclin D1*. Our findings are in agreement with the previous study that demonstrated that ETV4 transcriptionally activates *Cyclin D3*, a member of the *Cyclin D* family, in breast cancer cells (15). We also observed that *Cyclin D1* was required, at least in part, for ETV4-induced pancreatic cancer cell growth and cell-cycle progression.

Having observed the pathologic significance of ETV4 in PC in terms of cell-cycle regulation, it would be of interest to conduct additional studies in animal model systems to investigate its role in pancreatic tumorigenicity and metastasis. In a previous related study, ETV4 has been shown to be pathologically involved in tumorigenesis and metastasis of prostate cancer (42). In other reports, overexpression of ETV4 was also shown to drive metastasis in lung, mammary and colorectal carcinomas (49–51). Prognostic significance of ETV4 has also been reported in some solid malignancies (9, 43, 52). Considering that ETV4 is able to influence pancreatic cancer progression, it can also potentially exhibit an association with patient's prognosis, and be used as a biomarker for disease progression and therapeutic planning. Moreover, from the mechanistic standpoint, more comprehensive studies, such as ChIP-seq and RNA-seq, need to be performed in pancreatic cancer model systems to define its global as well as promoter-specific impact on gene regulation. Resulting data may not only identify novel ETV4-regulated gene targets, but also yield important mechanistic insights into pancreatic tumor biology to support future ETV4-focussed basic and translational studies.

In summary, our study established the role of ETV4, for the first time, in pancreatic cancer growth and cell-cycle progression through transcriptional activation of *Cyclin D1*. These results give new insights into the role of ETV4 in pancreatic cancer and suggest that it could serve as a novel biomarker for pancreatic cancer stratification at the molecular level and exploited as a target for pancreatic cancer therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** N. Tyagi, S.K. Deshmukh, S.K. Srivastava, S. Azim, A.P. Singh, J.E. Carter, B. Wang, S. Singh

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**Study supervision:** A.P. Singh, S. Singh

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