ORIGINAL ARTICLE

UNC5D, suppressed by promoter hypermethylation, inhibits cell metastasis by activating death-associated protein kinase 1 in prostate cancer

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Prostate cancer (PCa) death primarily occurs due to metastasis of the cells, but little is known about the underlying molecular mechanisms. This study aimed to evaluate the expression of UNC5D, a newly identified tumor suppressor gene, analyze its epigenetic alterations, and elucidate its functional relevance to PCa metastasis. Meta-analysis of publicly available microarray datasets revealed that UNC5D expression was frequently downregulated in PCa tissues and inversely associated with PCa metastasis. These results were verified in clinical specimens by real-time PCR and immunohistochemistry assays. Through methylation analysis, the downregulated expression of UNC5D in PCa tissues and cell lines was found to be attributable to the hypermethylation of the promoter. A negative correlation was observed between methylation and UNC5D mRNA expression in PCa samples. The ectopic expression of UNC5D in PCa cells effectively reduced their ability to migrate and invade both in vitro and in vivo, and siRNA-mediated knockdown of UNC5D yielded consistent results. UNC5D can recruit and activate death-associated protein kinase 1, which remained to be essential for its metastatic suppressor function. In conclusion, these results suggested that UNC5D as a novel putative metastatic suppressor gene that is commonly down-regulated by hypermethylation in PCa.

KEYWORDS
expression, metastasis, methylation, prostate cancer, UNC5D
Prostate cancer is one of the most commonly diagnosed malignancies among men and is the fourth most common cancer among men worldwide.\(^1\) Mortality occurs due to metastasis of PCa that is present either at the time of diagnosis or after failure of primary treatment. Metastatic disease indicates poor prognosis in men with PCa, and correlates with significant mortality.\(^2\) Most of the metastatic PCa cases eventually develop resistance to primary androgen deprivation therapy, a condition known as metastatic castration-resistant prostate cancer.\(^2\) However, little is known about the molecular mechanisms involved in the metastasis of PCa cells, which ultimately limits the development of effective therapies that aimed to prevent the spread of PCa cells. Therefore, identification of novel genes that are functionally involved in PCa development and progression or new markers that have the capability to predict or classify metastatic disease in the early stage could help to investigate potential diagnostic and therapeutic targets.

In recent years, rapid development has been observed in next-generation sequencing technology, which indicates that genetic and epigenetic changes are the primary drivers of PCa.\(^3\) For human PCa, epigenetic alterations are not only involved in the initial tumorigenesis process, but also in the continuous regulation of subsequent progression of the malignancy.\(^4,5\) An in-depth understanding regarding the epigenetic changes in PCa provides new opportunities for the discovery of biomarkers for screening, diagnosis, and risk stratification of PCa.\(^6\) Methylation of the promoter DNA leads to the silencing of the genes involved, and plays crucial roles in tumorigenesis. In addition, abnormal DNA methylation occurs even earlier than genetic events, such as loss of heterozygosity and microsatellite instability,\(^7,8\) and much earlier than the changes of protein biomarkers. This in turn produced DNA methylation abnormality as a biomarker that is more sensitive and valuable than genetic aberrations and protein biomarkers.

Members of the UNC5 family, including four homologues (UNC5A-D), were originally identified as netrin receptors and are considered to participate in the regulation of cell migration and morphogenesis during development.\(^9\) The expression of UNC5 homologues was downregulated in many human malignancies due to genetic and epigenetic alterations.\(^10\) UNC5 receptors have been reported to function as “dependence receptors” owing to their dependence on the availability of netrin-1 for cell survival.\(^10,11\) Although UNC5H receptors share high homology with each other, their functions are not exactly the same.\(^10,12,13\) UNC5D/H4 is the most recently identified member of UNC5 family.\(^14\) Like other members of this family, UNC5D has also been characterized as a tumor suppressor gene in several cancers, such as neuroblastoma,\(^15,16\) renal cell carcinoma,\(^17\) and bladder cancer.\(^18\) UNC5D has been observed with high expression in the human prostate tissue, but underexpressed in the castration-resistant stage of prostate cancer.\(^17,19\) However, little is known about the role and underlying mechanism of UNC5D in prostate cancer pathogenesis and progression.

In the present study, we evaluated the expression status of UNC5D in normal prostate and primary and metastatic PCa, and clarified whether the downregulated expression of UNC5D was mainly attributable to the methylation alterations on the CpG island in the promoter. Both in vitro and in vivo functional assays were applied to characterize the inhibitory effects of UNC5D on the metastasis of PCa cells. Molecular mechanisms for the suppressive function of UNC5D were also explored in this study.

### 2 | MATERIALS AND METHODS

#### 2.1 | Prostate cancer clinical specimens

Prostate cancer and corresponding noncancerous tissues were obtained from 82 PCa patients, including 60 patients with primary tumor and 22 patients with metastatic tumor, who underwent surgery at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). All PCa patients gave written informed consent on the use of clinical specimens for medical research. All procedures undertaken in studies involving human participants were in accordance with the 1964 Helsinki Declaration ethical standards and approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

#### 2.2 | Cell lines, Abs, and drug treatments

All PCa cell lines used in this study were obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China), and maintained in RPMI-1640 supplemented with 10% FBS (Gibco-BRL, Gaithersburg, MD, USA) and 1% penicillin/streptomycin. Antibodies specific to UNC5D, \(\beta\)-actin, and DAPK1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho-DAPK1 Ab (Ser-308) was purchased from Cell Signaling Technology (Beverly, MA, USA).

For demethylation assays, cell lines were treated with 10 mmol/L 5-aza-2-deoxycytidine (Sigma-Aldrich) for 3 days with exchange of reagents and medium every 24 hours. For experiments using the DAPK1 inhibitor ((4Z)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one; Sigma-Aldrich), cells were plated in 24-well plates, pretreated for 60 minutes with 25 \(\mu\)mol/L DAPK inhibitor, and then maintained with DAPK1 inhibitor at the concentration of 1 \(\mu\)mol/L (in media with 0.1% DMSO). Media alone with 0.1% DMSO was used as vehicle control.

#### 2.3 | Methylation analysis of UNC5D

Genomic DNA (500 ng) was bisulfite converted following EZ DNA-Methylation Gold kit instructions (Zymo Research, Irvine, CA, USA). Methylation-specific PCR and bisulfate genomic sequencing analysis were carried out as described previously.\(^20\) Methylation negative or positive was determined by the presence or absence of the electrophoresis band. Polymerase chain reaction for MethyLight assays and the calculation for the percentage of methylated reference was
FIGURE 1  Expression of UNC5D is downregulated in primary and metastatic prostate cancer (PCa). A, meta-analysis of UNC5D expression in prostate nontumor tissues and primary and metastatic PCa tissues through the publicly available microarray datasets, Gene Expression Omnibus (GEO) and Oncomine. a, GEO datasets; log_{10} scale at Y-axis; b and c, Oncomine datasets; log_{2} scale at Y-axis. Mean and SD values are shown. B, Expression of UNC5D mRNA in 5 PCa cell lines, nontumor prostate tissue as the positive control, and water as the negative control. C, Scatterplots of the relative expression of UNC5D detected by quantitative RT-PCR in prostate nontumor tissues and primary and metastatic PCa tissues. Linear scale at Y-axis, mean and SD values are shown. D, Representative immunohistochemistry staining of UNC5D expression in adjacent nontumor, primary tumor, and metastatic tumor, respectively (magnification, 200×). E, Comparison of the relative protein levels of UNC5D in prostate nontumor tissues, primary and metastatic PCa tissues as measured by immunohistochemistry; Fisher’s exact test was used. *P < .05; **P < .01; ***P < .001.
undertaken as described previously.\textsuperscript{21} Sequence information for the primers and probes used is listed in Table S1.

### 2.4 | Immunohistochemistry and scoring

Tissue sections were incubated with anti-UNC5D Ab (Sigma-Aldrich) at 1:200 dilution overnight at 4°C. The sections were then washed and subsequently incubated with a universal secondary Ab for 1 hour at room temperature. The scoring method that combined intensity and percentage of positivity was previously described,\textsuperscript{22} and extent and intensity measures for each core were combined as weak (score 1), moderate (score 2), and strong (score 3).

### 2.5 | Transfection, infection, and western blot analysis

Adenoviruses expressing \textit{UNC5D}, \textit{UNC5D-ΔDD} (UNC5D without the death domain\textsuperscript{17}), or MOCK were packaged by the Vector Gene Technology Company (Beijing, China). Prostate cancer cell lines were infected with 20 MOI of the adenoviral vector. JetPRIME transfection reagent (Polyplus-transfection, Illkirch, France) was used for all transfections in this study. All western blots were detected by electrochemiluminescence (GE Healthcare Life Sciences, Uppsala, Sweden). Beta-actin (Sigma-Aldrich) was used as the internal control.

### 2.6 | Knockdown of UNC5D

Two UNC5D-specific Stealth siRNA, targeting the noncoding region of UNC5D and a negative control siRNA, were purchased from Invitrogen (Carlsbad, CA, USA). Transfection was carried out using the jetPRIME transfection reagent (Polyplus-transfection) according to the manufacturer’s instructions. After transfection for 72 hours, the cells were harvested for further analysis. Sequence information for the siRNA used is listed in Table S1.

### 2.7 | Immunoprecipitation

The IP was carried out as described previously.\textsuperscript{23} Cells were lysed in lysis buffer for cell IP (Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes on ice. After sufficient centrifugation, the supernatant was incubated with anti-DAPK1, anti-UNC5D, or anti-IgG Affinity Gel (Sigma-Aldrich) at 4°C overnight. The beads were washed 3 times with 25 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, and 1% (w/v) Triton X-100. Approximately 5% of the whole lysate (Input) was used as a positive control. The precipitates were analyzed by SDS-PAGE and immunoblotting.

### 2.8 | Wound healing assay

Cell motility was determined by measuring the movement of cells to close an artificial wound. Cells were seeded in 24-well plates at 80% confluence. Cells were wounded with a 200-μL pipette tip, washed with PBS, and incubated with medium containing 1% FBS. The distance traveled by cells was monitored by phase-contrast microscopy (Olympus, Tokyo, Japan) at indicated time points.

### 2.9 | Cell migration and invasion assay

For cell migration assay, PCa cells were seeded into Transwell inserts (8 μm pore; BD Biosciences, San Jose, CA, USA). The bottom chamber contained medium with 10% FBS. After the cells were incubated for 20 hours, the noninvading cells remaining on the upper surface of the membrane were removed by scraping and cells attaching to the bottom of the membranes were fixed in 10% formalin for 30 minutes and stained with 0.05% crystal violet. For the cell invasion assay, cells were seeded in the Matrigel-coated Transwell inserts (BD Biosciences) and the invasion time was extended to 36 hours.

### 2.10 | In vivo metastasis assay

For lung metastasis assays, the androgen-independent DU145 cells were infected with adenoviruses expressing UNC5D or MOCK. Twenty-four hours after infection, the cells were trypsinized and washed with PBS. A total of one million cells in 100 μL were injected into the tail vein of nude mice. At 6 weeks after injection, the animals were killed and lungs were harvested and fixed in 10% formaldehyde/PBS before being assessed for evidence of metastases by H&E staining. In vivo experiments, the female Nu/Nu nude mice at age of 4 weeks were maintained under specific pathogen-free conditions. All animal protocols were in accordance with guidelines for animal care and were approved by the Animal Ethics Committee of Tianjin Medical University.

### 2.11 | Statistical analysis

Quantitative data were expressed as data plots or mean ± SD. Mann-Whitney U test was applied to compare the variables of the 2 specimen groups. For multiple comparison, one-way ANOVA analysis followed by Tukey’s multiple comparison test. Differences between the mean values of 2 experiment groups were analyzed by Student’s t test. To compare the relative protein levels of UNC5D prostate non-tumor tissues and primary and metastatic PCa tissues, and to analyze the association between promoter methylation and the expression of UNC5D, Fisher’s exact test was used. The Pearson correlation coefficients method was used to evaluate the association between the percentage of methylated reference and the expression of UNC5D. All statistical tests were undertaken using SPSS (version 13.0; SPSS, Chicago, IL, USA). Value of \( P < .05 \) was taken as statistical significance.

### 3 | RESULTS

#### 3.1 | Downregulated expression of UNC5D in primary and metastatic PCa

First, a meta-analysis of UNC5D expression was carried out through the publicly available microarray datasets GEO (https://www.ncbi.nlm.nih.gov/geo).
(A) GC percentage of CpG island (B) Methylation status of PC3, LNCap, and DU145 cell lines (C) 5-Aza-dC treatment effects on UNC5D and GAPDH expression in DU145 and LNCap cells (D) Patient 101 and Patient 105 methylation analysis (E) Methylation status in primary tumors (n=60)
FIGURE 2 Promoter methylation contributes to the suppressed expression of UNC5D in cell lines and primary prostate cancer (PCa). A, Schematic diagram of the CpG island of UNC5D and detection primers. B, Methylation status of UNC5D promoter in PCa cell lines. Left, results of methylation-specific PCR (MSP) analysis; right, results of bisulfate genomic sequencing (BGS) analysis. Circles, CpG sites analyzed; row of circles, an individual promoter allele that was cloned, randomly selected, and sequenced; filled circle, methylated CpG sites; open circle, unmethylated CpG site. C, Results of UNC5D expression (right) and BGS analysis (left) in DU145 and LNCap cells after demethylation (5-aza-2-deoxycytidine [5-Aza-dC]) treatment. D, UNC5D promoter methylation was evaluated by MSP and BGS analysis in primary PCa and adjacent nontumor tissues. Results from 2 representative pairs are shown. E, Association between the expression of UNC5D and the aberrant methylation of the UNC5D promoter (**P < .01, Fisher’s exact test).

FIGURE 3 Methylation level of UNC5D is positively correlated with the metastasis of prostate cancer (PCa). A, Scatterplots of the methylation levels of UNC5D percentage of methylated reference (PMR) detected by MethyLight assay in prostate nontumor tissue and primary and metastatic PCa tissues. Log_{10} scale at Y-axis, mean values are shown. B, Correlation between the expression of UNC5D and the methylation levels of UNC5D promoter (r = −0.448, P < .0001, Pearson’s correlation coefficients method). The nontumor tissues and primary and metastatic PCa tissues are displayed separately as indicated. **P < .01; ***P < .001.

Promoter hypermethylation of UNC5D in cell lines and primary PCa

The mechanism for the downregulated expression of UNC5D in PCa was explored. Methylation detection primers against the UNC5D promoter were designed to analyze the methylation status of UNC5D in PCa cell lines and tissues (Figure 2A). Methylation-specific PCR and bisulfate genomic sequencing analysis revealed that the UNC5D promoter was highly methylated in DU145 and LNCap, not expressing UNC5D, but much less methylated in PC-3, expressing UNC5D (Figure 2B). To further determine the silence effects of methylation, DU145 and LNCap were treated with DNA methyltransferase inhibitor 5-aza-2-deoxycytidine. Restoration of UNC5D expression was observed in those cells, accompanied by significantly declined methylation levels (Figure 2C). Methylation analysis was further extended to primary tumors. The results showed that the primary PCa tissues have higher levels of methylation than the paired nontumorous tissues (Figure 2D). The mean mRNA expression level of UNC5D was selected to classify patients with primary PCa into the UNC5D high-expression group (n = 31) and UNC5D

nm.nih.gov/geo/) and Oncomine (https://www.oncomine.org/resource/main.html). Datasets meeting the following criteria were included: (a) the datasets were about human prostate cancer; (b) nontumor gland, primary tumor, and metastatic tumor were included; (c) the mRNA expression of UNC5D was measured in these databases; and (d) the number of cases exceeds 100. Finally, a total of 3 independent human prostate microarray databases (one GEO database, GSE6919; two Oncomine databases, Grasso and Taylor Prostate) were enrolled in this analysis, including 115 nontumor glands adjacent to tumor or not, 254 primary PCa, and 79 metastatic PCa samples. Compared with nontumor tissue, the relative level of UNC5D was significantly lower in the primary tissue in all the three datasets (Figure 1A). The results showed a more notable decline in the expression of UNC5D in metastatic PCa compared to the primary tumor (Figure 1A).

Then we examined the expression of UNC5D in 5 prostate cancer cell lines. Complete lack of UNC5D expression was observed in NCI-H660, VCaP, LNCap, and DU145 cells. Only PC-3 was detected with relatively weak UNC5D expression compared with the normal prostate tissue (Figure 1B). Real-time PCR (Figure 1C) and IHC staining (Figure 1D) assays yielded consistent results with the meta-analysis. The IHC staining was further scored by taking into account the staining intensity and percentage of cells showing positive staining. The specimens were divided into 3 groups (nontumor, primary tumor, and metastatic tumor), and the percentages with strong, medium, and weak expression levels were calculated separately. As shown in Figure 1E, the UNC5D protein was significantly downregulated in primary PCa tissues compared to nontumorous tissues, and least expression was observed in the metastatic tumors.

3.2 Promoter hypermethylation of UNC5D in cell lines and primary PCa

The mechanism for the downregulated expression of UNC5D in PCa was explored. Methylation detection primers against the UNC5D promoter were designed to analyze the methylation status of UNC5D in PCa cell lines and tissues (Figure 2A). Methylation-specific PCR and bisulfate genomic sequencing analysis revealed that the UNC5D promoter was highly methylated in DU145 and LNCap, not expressing UNC5D, but much less methylated in PC-3, expressing UNC5D (Figure 2B). To further determine the silence effects of methylation, DU145 and LNCap were treated with DNA methyltransferase inhibitor 5-aza-2-deoxycytidine. Restoration of UNC5D expression was observed in those cells, accompanied by significantly declined methylation levels (Figure 2C). Methylation analysis was further extended to primary tumors. The results showed that the primary PCa tissues have higher levels of methylation than the paired nontumorous tissues (Figure 2D). The mean mRNA expression level of UNC5D was selected to classify patients with primary PCa into the UNC5D high-expression group (n = 31) and UNC5D
low-expression group (n = 29). Ten of 31 in the UNC5D high-expression group, and 19 of 29 in the UNC5D low-expression group were positively methylated. UNC5D expression was significantly associated with promoter hypermethylation (Figure 2E; \( P = .0009 \), Fisher’s exact test).

### 3.3 Positive correlation between methylation level of UNC5D and the metastasis of PCa

MethyLight assay was used to analyze the methylation in an accurate and quantitative manner. This detection system containing 9
CpG sites (3 in the forward primer, 2 in the reverse primer, and 4 in the TaqMan probe; Table S1), and the oligonucleotides with all these sites methylated will be annealed and amplified. All the metastatic tumors, primary tumors, and paired noncancerous tissues were detected. As shown in Figure 3A, the methylation levels in the promoter of UNC5D were the highest in metastatic tumors, followed by primary tumors, and the lowest in adjacent nontumor tissues. In addition, a significant inverse correlation between UNC5D expression and promoter methylation was observed for PCa samples (Figure 3B, \( r = -0.448, P < .0001 \)). Taken together, these data indicated that the promoter methylation was the major cause of UNC5D downregulation in PCa.

### 3.4 Restoration of UNC5D expression suppresses the migration and invasion of PCa cell lines

Frequent downregulation of UNC5D in PCa tissues, especially metastatic tumor tissues, suggests a potential role for UNC5D in the tumorigenesis and metastasis of PCa. To test this possibility, expression of UNC5D was restored in DU145 and LNCap cells, and the capabilities of tumor cells in migration and invasion were monitored. In vitro wound healing assay revealed that the motility of DU145 (Figure 4A) and LNCap (Figure 4B) cells has been significantly weakened. In vitro Transwell migration (C,D) and Matrigel invasion (E,F) assays were undertaken to compare cell motilities between the two groups. The number of migrated or invaded cells was calculated and the results are expressed as mean ± SD of 3 independent experiments. G, In vivo lung metastasis assay was carried out to evaluate the effect of UNC5D on tumor metastasis. Representative images of lungs from nude mice injected with DU145-MOCK (a) or DU145-UNC5D (b) are shown. Tumors on the surface of the lung are indicated by arrows. Representative images of H&E staining of lung sections with tumors on the surface (c) and internal (d) of the lung are shown (magnification, 200×). The numbers of all metastatic nodules are summarized (right). Mean and SD values are shown. *\( P < .05 \); **\( P < .01 \); ***\( P < .001 \)
Input IgG  

**IP**

**IB**: DAPK1

Input IgG  

**IP**

**IB**: UNC5D

(A)

(B)

Mock  

UN5CD  

UN5CD - ΔDD

Anti-UNC5D  

Anti-pDAPK1  

Anti-DAPK1  

Anti-actin

(C)

(D)

Distance (nm)

Time (h)

0 h 20 h

0 4 8 12 16 20

**MOCK**  

**UN5CD**  

**UN5CD - ΔDD**

0 h 24 h

0 4 8 12 16 20

(E)

(F)

MOCK + vehicle  

UN5CD + vehicle

MOCK + inhibitor  

UN5CD + inhibitor

MOCK + vehicle  

UN5CD + vehicle

MOCK + inhibitor  

UN5CD + inhibitor

MOCK + vehicle  

UN5CD + vehicle

MOCK + inhibitor  

UN5CD + inhibitor

**MOCK**  

**UN5CD**  

**UN5CD - ΔDD**

Number of migrating cells/field

0 100 200 300 400 500

Mock + vehicle  

UN5CD + vehicle  

Mock + inhibitor  

UN5CD + inhibitor
found that enforced expression of UNC5D significantly inhibited the migratory and invasive abilities of DU145 (Figure 4C,E) and LNCap (Figure 4D,F) cells compared with control cells. To test the suppressive effects of UNC5D on metastasis in vivo, DU145-MOCK and DU145-UNC5D cells were injected into the tail vein of nude mice (10 per group). Lung metastatic nodules were counted at 6 weeks after injection. The number of metastatic nodules formed on the surface and inside the lung were significantly less in mice injected with DU145-UNC5D cells compared to those injected with DU145-MOCK cells (Figure 4G).

3.5 | Silencing of UNC5D enhances the metastatic ability of PCa cells

To further confirm the inhibitory effects of UNC5D on PCa cell metastasis, UNC5D expression was knocked down by siRNA in PC-3 cells, showing endogenous expression of UNC5D to a certain extent. Knockdown effects were confirmed by western blot analysis after 48 hours of transfection of siRNA (Figure 5A). Wound healing assay showed that knockdown of UNC5D significantly increased the cell motility in PC-3 cells compared with the control cells transfected with scrambled-siRNA (Figure 5B). The effect of UNC5D knockdown in PC-3 cells was also confirmed by in vitro Transwell migration and Matrigel invasion assays (Figure 5C,D). This experiment further confirmed the metastatic-suppressive effect of UNC5D in PCa.

3.6 | DAPK1 is essential for the metastasis suppressor function of UNC5D

UNC5D shares high homology with other members that were discovered previously, in both sequence and structure.10 UNC5A, UNC5C, and especially UNC5B are capable of binding with the serine/threonine kinase, DAPK1,24 which is a key intracellular kinase with both apoptosis-inducing25,26 and motility-inhibitory functions.27,28 The apoptosis-inducing biological effect of UNC5B is achieved by recruiting and activating DAPK1.24,25 This subsequently led us to speculate whether the antimetastatic effect of UNC5D in PCa is mediated by DAPK1.

The expression of DAPK1 in PCa tissues and cell lines was analyzed. Three publicly available microarray datasets (2 from GEO, both from GSE6919; 1 from Oncomine, Taylor Prostate) were selected to undertake the meta-analysis of DAPK1 expression. Although usually reported as a tumor suppressor gene, expression of DAPK1 was actually upregulated in the PCa tissues compared with the nontumor prostate tissue (Figure S1A–C). Moreover, the expression level of DAPK1 was even higher in metastatic PCa than in primary tumors, according to the two datasets (Figure S1B,C). These results suggested that DAPK1 could play a regulatory role in the tumorigenesis and metastasis of PCa. The expression of DAPK1 in PCa cell lines was also evaluated by western blot analysis. DU145 and VCap cells showed relatively strong expression of UNC5D, whereas LNCap, PC-3, and HCl-H660 cells showed lower UNC5D expression (Figure S1D).

We then investigated whether UNC5D could recruit and activate DAPK1. The co-IP assays were carried out in DU145 cells infected with adenoviruses expressing UNC5D. As shown in Figure 6A, UNC5D and DAPK1 were IP with each other. Decreased levels of phosphorylation at Ser-308 showed the active state of DAPK1.29,30 Western blot analysis indicated that DAPK1 was obviously activated in DU145-UNC5D cells more than DU145-MOCK cells (Figure 6B). As reported, the death domains of UNC5 family members remained crucial for their biological function in tumor cells.10 Therefore, DU145 cells were also infected with adenoviruses expressing UNC5D-ΔDD. As shown in Figure 6B, UNC5D-ΔDD was observed with significantly diminished capability of activating DAPK1. To determine the indispensable role of the death domain for UNC5D, in vitro wound healing and cell Transwell migration assays were carried out. The antimetastatic effect of UNC5D-ΔDD was significantly diminished in both wound healing or Transwell migration assays (Figure 6C,D). These data support that the death domain of UNC5D was essential for the metastatic-suppressive function of UNC5D, possibly due to its indispensable role in activating DAPK1. Finally, DAPK1 inhibitor was used to further confirm the pivotal role of the activation of DAPK1 in the functioning of UNC5D. In vitro wound healing and cell Transwell migration assays (Figure 6E,F) showed that the antimetastatic effects of UNC5D were significantly reduced by DAPK1 inhibitor. Taken together, these data illustrated that DAPK1 could interact with UNC5D and might mediate its inhibitory function.

4 | DISCUSSION

Epigenetic changes are common features of PCa, and play key roles in the initiation and progression of cancer.5 As for UNC5D, hypermethylation alterations at the promoter region are one of the main causes for its repression in a variety of tumors,15-18 and are the same...
in PCa cells according to our study. However, there are seldom reports on the methylation status of UNCSD in metastatic tumors. Our data indicated that the methylation changes exist not only in primary PCa, but also in metastatic tumors, and in a more reinforced manner, accounting for further downregulation of UNCSD in the metastatic tumors. Large-scale cancer genome sequencing studies in the genome of invasive PCa showed catastrophic hypermethylation alterations, involving hundreds to thousands of CpG islands, and the majority of these were highly methylated across the metastasis process within the individuals. Thus, cancer metastasis could be driven by cumulative expression disorders, where hypermethylation occurs in genes that are key components in the metastatic pathways. In this study, we tried to determine whether UNCSD might act as one such key component.

Recently, it has been reported that UNC5 family receptors act as dependent receptors for netrin-1, and these receptors induce apoptosis when not engaged with their ligand. However, it is not always the case. Except from apoptosis induction, the UNC5 homolog family also plays other important roles that are independent of netrin-1. UNC5B could interact with FLRT3 and Rnd1 to modulate cell adhesion in Xenopus embryos, and the UNCSD/FLRT2 complex regulates the radial migration of cortical cells. These findings indicated that whether the dependent receptors induce apoptosis depends on various factors both intracellularly and extracellularly. For tumor cells, the factors that influenced apoptosis are considered to be more complicated. It is well known that many malignant cells gain resistance to apoptosis to evade chemotherapy. Typical examples include renal cell carcinoma cells and advanced PCa cells. Both UNCSC and UNCSD have no apoptosis-inducing effects on renal carcinoma cells, whereas UNCSD exerted tumor suppressive effects mainly by cell cycle arrest in renal carcinoma cells. In this study, we showed that UNCSD has the capability to suppress the metastatic ability of PCa cells. Moreover, the suppressive function of UNCSD was dependent on DAPK1 in the downstream. DAPK1 is a key intracellular kinase that has both apoptosis-inducing and motility-inhibitory functions. In apoptosis-sensitive cells, DAPK1 participates in a wide range of apoptotic signals. In tumor cells that are resistant to DAPK-induced apoptosis, DAPK has a motility-inhibitory effect and functions as a determining factor in tumor cell invasion.

DAPK1 has been reported as a tumor suppressor gene, with promoter hypermethylation and downregulated expression in various tumors. However, interestingly, our work revealed that DAPK1 was significantly upregulated in PCa. This explains, to some extent, that the PCa cells were classified as cells resistant to DAPK-induced apoptosis, making DAPK1 a cell motion regulator. As a death-associated protein kinase, DAPK1 could be activated by the death domain of UNC5 homologues. UNCSD-ADD, UNCSD without death domain, did not activate DAPK1 and the metastatic inhibitory effects of UNCSD in PCa cells were diminished significantly. Therefore, activation of DAPK1 remains to be essential for the function of UNCSD in PCa. Although the underlying mechanisms for the upregulated expression of DAPK1 are still unknown, considering its indispensable role for the metastatic inhibitory effects of UNCSD, high expression of DAPK1 induces UNCSD as a potential target for PCa therapy.

Collectively, we have identified UNCSD as a novel candidate metastasis suppressor that is silenced by promoter hypermethylation in PCa. We present clinical evidence that UNCSD expression is negatively associated with its methylation level, as well as PCa metastasis. In addition, we validated the metastasis suppressor function of UNCSD in PCa cells, which is dependent on the downstream activation of DAPK1. Our findings proposed that UNCSD could be a potential diagnostic biomarker and therapeutic target for metastatic PCa.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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