RNA-binding protein DND1 participates in migration, invasion, and EMT of prostate cancer cells by degrading CLIC4

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RNA-binding protein DND1 participates in migration, invasion, and EMT of prostate cancer cells by degrading CLIC4

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Running title: Role of DND1 and CLIC4 in prostate cancer.

Abstract
Dead-End 1 (DND1) is an RNA-binding protein (RBP) with regulatory functions in multiple cancers, including gastric and colorectal. Nevertheless, the role that DND1 plays in prostatic cancer (PCa) as well as the hidden molecular mechanism is still obscure. The gene expression of DND1 and survival analyses in PCa were analyzed by the UALCAN database. Expression of DND1 and chloride intracellular channel 4 (CLIC4) were detected by qRT-PCR and western blot analysis. The Cell Counting Kit-8 assay and EDU staining were employed for the estimation of cell viability. The capabilities of cells to migrate and invade were appraised by the wound healing assay
as well as the Transwell assay, while epithelial-mesenchymal transition (EMT) was measured by immunofluorescence and western blot assay. The interaction of DND1 and CLIC4 was predicted by PCTA, linkedomics, and RPISeq databases. It was discovered that DND1 expression was elevated in PCa cells. DND1 silencing had suppressive impacts on cells’ proliferative, migrative, and invasive capabilities as well as EMT in DU145 and 22Rv1 cells. Mechanistically, bioinformatic analysis demonstrated that DND1 was negatively correlated with CLIC4 and that DND1 protein could bind to CLIC4 mRNA. Additionally, the CLIC4 level was reduced in PCa cells. CLIC4 depletion counteracted the suppressive impacts of DND1 deficiency on the capabilities of DU145 and 22Rv1 cells to proliferate, migrate, and invade as well as the process of EMT. These results suggested that DND1 silencing repressed the proliferation, migration, invasion, and EMT in PCa by regulating the mRNA level of CLIC4.

Keywords: Prostate cancer, Dead-End 1, RNA-binding protein, Chloride intracellular channel 4, Bioinformatics analysis

Introduction

Being a prevalent malignancy of the reproductive system in elderly men worldwide, prostate cancer (PCa) has featured an increasing trend in incidence and mortality over the past few decades (Komura et al., 2018; Schatten, 2018). PCa threatens long-term health and is the major cause of cancer death in men (Carlsson and Vickers, 2020). There is no definite cause of PCa and huge studies have investigated the association of PCa with behavioral, physiological, environmental, and genetic factors (Nguyen-Nielsen and Borre, 2016). At present, the current standard of care consists of prostatectomy and radiation therapy, which may often be supplemented with hormonal therapies (Ritch and Cookson, 2018). Also, several approved new drugs have significantly improved the overall survival of patients with metastatic castration-resistant PCa, including docetaxel, cabazitaxel, abiraterone, and Enzalutamide (Achard et al., 2022). However, the recurrence of PCa is common, and traditional treatments for
many metastatic PCas have moderate efficacy (Uhr et al., 2020). With a deeper understanding of the molecular mechanisms of PCa, unprecedented progress has been achieved in developing new therapeutic targets (Kretschmer and Tilki, 2017; Sha et al., 2020; Talkar and Patravale, 2021). Several targeted therapeutic agents have been developed and clinically used for the treatment of solid tumors, such as breast cancer, non-small cell lung cancer, along with renal cancer (Rahman and Sakr, 2012; Hirsch et al., 2017; Chowdhury and Drake, 2020). Thus, it is of critical importance for us to explore the molecular mechanisms involved in PCa and develop more effective targeted therapeutic agents for PCa treatment.

Dead-End 1 (DND1) is an evolutionary conserved RNA-binding protein (RBP) that contains two RNA recognition motifs (RRMs) in tandem, spanning approximately aa residues 58–136 and 138–218 (Zhang et al., 2021). DND1 regulates protein expression by stabilizing or degrading mRNA, thus exerting its translation regulatory effects (Xu et al., 2021). DND1 has been regarded as an important regulator in mediating germ-cell activity, germ-cell tumor formation, and male germ-cell development (Ketting, 2007). Previous studies have discovered that DND1 is an anti-proliferative and pro-apoptotic tumor suppressor in a variety of cancers (Kedde et al., 2007; Suzuki et al., 2016). These findings indicate that DND1 not only mediates germ-cell development but also acts as a critical player in tumor development. However, the role that DND1 plays in PCAs is obscure. Thus, we aimed to explore DND1 expression in PCAs as well as resolve its hidden mechanism.

Materials and methods

Bioinformatic analysis

The gene expression of DND1 and PCa survival analyses were analyzed by the UALCAn (http://ualcan.path.uab.edu) database. The PCTA database (http://www.thepcta.org) was used to analyze the relationship between DND1 and the Gleason (GS) score. In addition, the LinkedOmics database (http://www.linkedomics.org) was utilized to characterize the correlation between DND1 and chloride intracellular channel 4 (CLIC4). RPISeq
(http://pridb.gdcb.iastate.edu/RPISeq) was used to predict the RNA-protein binding sites of DND1 and CLIC4.

**Cell culture and treatment**

Human normal prostate epithelial cells RWPE-1 and PCa cell lines (22Rv1, DU145 LNCaP), provided by BeNa Culture Collection (Henan, China), were cultured in DMEM (Gibco, Shanghai, China) enhanced with 10% FBS (Biological Industries, Israel) and 1% antibiotics (Gibco) and placed in a humid incubator in the presence of 5% CO₂ at 37°C.

**Cell transfection**

Small interfering RNAs (siRNAs) targeting DND1 (siRNA-DND1-1/2), CLIC4-specific siRNAs (siRNA-CLIC4), and the corresponding negative control (siRNA-NC) were constructed by Gene Pharma (Shanghai, China). By Lipofectamine 2000 reagent (Shanghai Aiyang Biotechnology Co., Ltd.), the transfection of these recombinants into DU145 and 22Rv1 cells was implemented following standard instructions. At 48h post-transfection, DU145 and 22Rv1 cells were harvested for ensuing experiments.

**The Cell Counting Kit-8 (CCK-8) assay**

Initially, DU145 and 22Rv1 cells were seeded in 96-well plates and cultured. Afterwards, 10 µl CCK-8 solution was put into each well to culture cells for another 2h. Using a microplate reader, the OD value was read at λ=450 nm.

**5-Ethynyl-2’-deoxyuridine (EDU) assay**

First, DU145 and 22Rv1 cells were seeded in 96-well plates and cultured overnight at 37°C. Subsequently, DU145 cells were exposed to 4% polyformaldehyde for 1h and cultured with 0.5% Triton X-100 for 15 min. Then, cells were reacted with the Cell-Light™ EdU Cell Proliferation Detection Assay (Life, USA) and subjected to 10 min of DAPI counterstaining. The photographs of stained cells were captured by means of a fluorescent microscope.
**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The quantification of RNA, which was isolated from sample cells utilizing Trizol reagent (Renold Biotechnology Co., Ltd; Suzhou, China) following the standard protocol, was performed with a NanoDrop 2000 (Quawell, San Jose, CA, USA) at 260 and 280 nm. The cDNA was obtained with a cDNA Synthesis Kit (TaKaRa, Japan) to reverse transcribe 2 µg RNA. Afterward, the cDNA was amplified via qRT-PCR utilizing the SYBR Premix Ex Taq™ II kit (Takara, Shiga, Japan). The comparative Ct method was adopted for the determination of relative gene expression.

**Actinomycin D Treatment**

For Actinomycin D treatment, DU145 and 22Rv1 cells transfected with siRNA-DND1 were administered Actinomycin D (GlpBio, Shanghai, China) at a concentration of 2 mg/mL for 0, 6, 12, and 18h. qRT-PCR was used to determine CLIC4 mRNA expression.

**Wound healing assay**

The migrative capabilities of DU145 and 22Rv1 cells were appraised utilizing the wound healing assay. First, transfected DU145 and 22Rv1 cells were inoculated into a six-well plate and then cultured until 90% cell fusion was achieved. The wounds in cell monolayers were made by means of white pipette tips. After 24h of incubation, the migration rate was calculated based on the formula: (wound width at 0h - wound width at 24h)/wound width at 0h×100%. Five randomly selected fields were analyzed.

**Transwell assay**

Transfected cells were suspended in serum-free DMEM and loaded into the upper compartment pre-treated with Matrigel (BD Biosciences, CA, USA). Meanwhile, medium enhanced with 10% FBS was put in the lower compartment. After 24h of incubation, the lower chamber insert was exposed to 100% methanol for fixation and
0.1% crystal violet for staining. The invaded cells were captured by a microscope. The number of invaded cells was counted in randomly selected five fields for each group.

**Immunofluorescence staining**

The contents of E-cadherin and Vimentin were appraised utilizing immunofluorescence staining. Initially, DU145 and 22Rv1 cells were exposed to 4% paraformaldehyde for fixation. Overnight culture with antibodies against E-cadherin and Vimentin (Abcam) was implemented at 4°C, after which they were exposed to the appropriate secondary antibody (Abcam). DAPI solution (Beyotime, China) was applied for staining the nuclei and the visualization of cells was carried out using a fluorescence microscope.

**Western blot analysis**

The quantification of total proteins, isolated from sample cells utilizing RIPA buffer (Auragene, Changsha, China), was performed with the BCA Protein Assay Kit (Dingguo, Beijing, China) following the standard protocol. After separation with 10% SDS-PAGE, the proteins were transferred to PVDF membranes. Overnight incubation of the membranes, blocked with 5% non-fat milk, with primary antibodies against DND1, ZO-1, Slug, CLIC4, and GAPDH was conducted at 4°C, after which they were incubated with anti-mouse or anti-rabbit secondary antibodies (Abcam). Finally, the visualization of protein bands was via an ECL detection system following the standard protocol. ImageJ software (Version 1.49; NIH, Bethesda, MD, USA) was applied for the analysis of band density. The ratio of the target to GAPDH was regarded as the relative concentration of protein expression.

**Statistical analysis**

All data are displayed as mean ±SD and were analyzed utilizing SPSS 22.0 (Chicago, IL) and GraphPad Prism 6 software (San Diego, CA). The demonstration of comparisons among multiple groups was analyzed with one-way ANOVA followed by a Bonferroni post hoc test. P less than 0.05 meant that experimental data were of statistical significance.
Results

DND1 expression is upregulated in PCa tissues and cells
To elucidate the role that DND1 plays in PCa, DND1 expression in PCa tissues and cells was initially appraised. According to the UALCAN database, DND1 was greatly elevated in PCa tissues relative to the healthy control (Fig.1A). The GEPIA database demonstrated that DND1 elevation was closely related to the poor prognosis of patients with PCa (Fig.1B). In addition, data obtained from the PCTA database disclosed that DND1 expression became more elevated with increasing GS scores (Fig.1C). Moreover, qRT-PCR and western blot assay revealed a significant increase in mRNA and protein expression of DND1 in PCa cell lines compared with normal prostate epithelial cells (Fig.1D-E). The highest DND1 expression was displayed in DU145 and 22Rv1 cells, therefore, DU145 and 22Rv1 cells were adopted for ensuing experiments.

DND1 knockdown inhibits the proliferation, invasion, migration, and EMT of PCa cells
To figure out the biological role that DND1 plays in PCa cells, the transfection of siRNA-DND1 into DU145 and 22Rv1 cells was implemented. qRT-PCR as well as western blot was applied for the examination of transfection efficiency (Fig. 2A-B, Fig. S3A-B). CCK-8 was applied for the assessment of cell proliferative ability. Results obtained revealed that DND1 depletion conspicuously suppressed the capability of DU145 and 22Rv1 cells to proliferate relative to the negative control group (Fig. 2C, Fig. 3C). EDU assay showed that the stained cell number was specifically decreased after transfection with siRNA-DND1 (Fig. 2D, Fig. 3D). In addition, the capacities of DU145 and 22Rv1 cells to invade and migrate were suppressed because of DND1 deficiency (Fig. 2E-F, Fig. 3E-F). EMT remains a common event during the process of PCa that deeply alters tumor cell features, leading to loss of epithelial markers (i.e., E-cadherin, ZO-1) and an increase in mesenchymal markers (i.e., N-cadherin, Slug, and vimentin). We also found that the level of E-cadherin was increased and Vimentin was decreased after cells were transfected with siRNA-DND1 (Fig. 4A-B, Fig. 5A-B).
Furthermore, the downregulation of DND1 enhanced the level of ZO-1 but reduced the level of Slug in DU145 and 22Rv1 cells (Fig. 4C, Fig. 5C).

**DND1 silencing promotes the expression of CLIC4 in PCa cells**

The mechanism underlying the regulatory role of DND1 in PCa cells was further discussed in the section. Based on the linkedomics database, DND1 had a negative correlation with CLIC4 in PCa (Fig. 6A-B). The RPISeq website also predicted that the probability that DND1 could bind to the mRNA of CLIC4 (positive) reached 0.98. Moreover, the mRNA and protein levels of CLIC4 were both downregulated in PCa cells compared with the normal prostate epithelial cells (Fig. 6C-D). DND1 silencing increased the mRNA and protein levels of CLIC4 in DU145 and 22Rv1 cells (Fig. 6E-F). Also, knockdown of DND1 reduced CLIC4 mRNA stability in DU145 and 22Rv1 cells (Fig. 6G).

**DND1 deletion regulates the proliferation, migration, invasion, and EMT of PCa cells through binding to CLIC4**

To study the role of CLIC4 in DND1-mediated PCa progression, CLIC4 expression in DU145 and 22Rv1 cells was silenced and qRT-PCR as well as western blot was employed for the examination of transfection efficiency (Fig. 7A-B, Fig. 8A-B). Results from the CCK-8 assay revealed that CLIC4 silencing evidently increased the OD values of DU145 and 22Rv1 cells transfected with siRNA-DND1 (Fig. 7C, Fig. 8C). Depletion of CLIC4 enhanced the decreased number of EDU-positive cells compared with that of DND1-silenced DU145 and 22Rv1 cells (Fig. 7D, Fig. 8D). Additionally, siRNA-CLIC4 reversed the inhibitory effects of DND1 silencing on the invasion and migration of DU145 and 22Rv1 cells (Fig. 7E-F, Fig. 8E-F). Moreover, immunofluorescence assay displayed a remarkable decrease in E-cadherin levels and an increase in Vimentin levels in cells co-transfected with siRNA-DND1 and siRNA-CLIC4 compared with those in cells transfected with siRNA-DND1 and siRNA-NC (Fig. 9A-B, Fig. 10A-B). Additionally, western blot assay manifested that CLIC4
silencing reversed the effects of DND1 silencing on the protein levels of ZO-1 and Slug in transfected DU145 and 22Rv1 cells (Fig. 9C, Fig. 10C).

Discussion

Being a prevalent cancer in men, PCa is a major contributor to death worldwide (Rawla, 2019). The incidence and mortality of PCa are closely related to age and older men are more susceptible (Rebbeck, 2017). The most common symptoms of PCa are difficulty urinating, increased urinary frequency, and nocturia, all of which may result from an enlarged prostate (Mayor de Castro et al., 2018). Since the axis skeleton is the most common site of metastatic bone disease, urinary retention and back pain may occur in the advanced stages of the disease (Zhang, 2019). Thanks to the advancement of novel genetic technologies, the genetic and epigenetic changes in PCa have been comprehensively analyzed (Haffner et al., 2021). Studies of targeted therapies have helped to identify key signaling pathways incidentally associated with PCa initiation and progression (Tsujino et al., 2021; Vietri et al., 2021). In this study, we demonstrated that RNA binding protein DND1 was highly expressed in PCa tissues as well as cell lines. DND1 deficiency restrained the capabilities of PCa cells to proliferate, migrate, and invade, along with EMT. In addition, DND1 bound to CLIC4 and regulated CLIC4 expression in DU145 and 22Rv1 cells. Downregulation of CLIC4 reversed the effects of DND1 knockdown on DU145 and 22Rv1 cell proliferation, migration, invasion, and EMT. Taken together, our study demonstrated that DND1 might be a therapeutic target of PCa and that DND1 regulated PCa development by binding to CLIC4.

RBPs comprise over 2000 proteins that interact with transcripts in a variety of RNA-driven processes (Smith et al., 2021). It is known that the mechanisms by which RBP binds and regulates RNA are very diverse (Zhang and Li, 2021). In zebrafish and mammals, DND1 is critical for the normal development of primordial germ cells (PGCs), suggesting that 3’ UTR interactions of RBP with mRNA play a critical role in developmental decisions (Gonzalez-Rodriguez et al., 2016). DND1, which is a component of the RBP family, acts as a positive or negative regulator of mRNAs (Ruthig et al., 2019). Thus, DND1 plays both positive and negative regulatory roles in
different types of cancers (Zhang et al., 2022). A recent study reported that the RBP DND1 restrains miRNA-dependent inhibition of Bim expression, thus exerting inhibitory effects on breast cancer cells (Cheng et al., 2017). It was also discovered that silencing of DND1 can reduce the capabilities of DU145 cells to proliferate, migrate, and invade but enhances chemosensitivity in gastric cancer (Jiang et al., 2020). In this study, we revealed that DND1 expression was increased in PCa tumor tissues as well as cell lines. In addition, DND1 knockdown imparted these suppressive impacts on DU145 and 22Rv1 cell proliferative, migrative, and invasive capabilities, which agrees with previous bioinformatic analysis results.

EMT, which refers to the conversion of epithelial cells to mesenchymal cells by losing cell–cell junction and apical-basal polarity while gaining high motility and an invasive phenotype, remains a crucial event related to PCa metastasis (Montanari et al., 2017; Odero-Marah et al., 2018). During the EMT process, the expression of epithelial proteins, such as E-Cadherin, are repressed to reinforce the destruction of adherent junctions, and the expression of mesenchymal markers, such as N-Cadherin and Vimentin, are elevated to promote migration (Paolillo and Schinelli, 2019). Tight junctions in epithelial cells function in an adhesive manner and preventing the disassembly of epithelial cell-cell contacts is the first barrier that cancer cells must overcome for metastasis (Nehme et al., 2023). ZO-1 is a critical epithelial tight junction marker that functions as a critical participant in calcitonin-stimulated PCa metastasis (Aljameeli et al., 2016; Aljameeli et al., 2017). Slug can downregulate E-cadherin and thus cause EMT activation through binding to the E-boxes located in the E-cadherin promoter (Sterneck et al., 2020). Slug has been reported to be overexpressed in PCa and correlates with aggressive tumor features and poor outcomes in PCa patients (Børretzen et al., 2021). Intriguingly, DND1 has been reported to suppress EMT in hepatocellular carcinoma (Xu et al., 2017). However, our present work demonstrated that deficiency of DND1 elevated the expression of E-cadherin and ZO-1 yet reduced the expression of Vimentin and Slug, implying a suppressive role for DND1 ablation in the EMT process of PCa cells.
CLIC4 is a p53- and tumor necrosis factor-alpha (TNF-α)-regulated chloride channel protein localized in the mitochondria and cytoplasm of mouse and human keratinocytes (Abdul-Salam et al., 2019). A previous study has shown that CLIC4 expression is reduced in PCa tissues, and its downregulation is associated with the poor prognosis of PCa (Zou et al., 2022). Meanwhile, CLIC4 expression and subcellular localization are significantly altered during early tumorigenesis. CLIC4 inhibits the growth of squamous carcinoma, and decreased CLIC4 expression is associated with reduced nuclear retention and alters the redox status of tumor cells, contributing to transforming growth factor-β (TGF-β) resistance and tumor progression (Suh et al., 2012). Wang et al. reported that CLIC4 levels are negatively correlated with the clinical stage of gastric cancer. Overexpression of CLIC4 reduces the expression of cancer stem cell markers CD44 and OCT4 and abates cell migration, invasion, and EMT (Wang et al., 2020). To predict the interaction between DND1 and CLIC4, we first used the linkedomics database to analyze the correlation and found that DND1 was negatively correlated with CLIC4. Also, the RPISeq website predicted that the protein sequence of DND1 bound to the FASTA sequence of CLIC4, with an interaction probability of 0.98. Additionally, the mRNA and protein levels of CLIC4 were upregulated in DU145 and 22Rv1 cells after DND1 was knocked down. Actinomycin D assay verified that silencing of DND1 decreased the mRNA stability of CLIC4. Furthermore, we also found that CLIC4 silencing reversed the suppressive effects of siRNA-DND1 in DU145 and 22Rv1 cells via enhancing DND1 silencing-inhibited cell proliferation, migration, invasion, and EMT, suggesting that CLIC4 might function as a tumor suppressor in PCa and participate in the regulatory functions of DND1 in PCa.

In summary, our findings demonstrated that DND1 was increased in PCa and was closely related to poor prognosis. DND1 modulated PCa cells’ proliferative, migrative, and invasive capabilities as well as EMT, and could bind to CLIC4 mRNA. Rescue experiments revealed that CLIC4 silencing countervailed the impacts of DND1 knockdown on PCa cells, implying the potential of DND1 as a novel therapeutic target for PCa.
Declarations

Ethical Statement
Not applicable.

Consent for publication
All the authors agreed to be published.

Availability of data and materials
The analyzed data sets generated during the present study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare no potential conflict of interest.

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Authors' contributions
Hua Zhu and Bing Zheng conceptualized the experiment. Wei Zhang, Qian Xu, Chunmei Shi, Xinfeng Chen, Cheng Shen and Yong Zhang performed the experiment. Wei Zhang, Hua Zhu, and Bing Zheng wrote the article. Hua Zhu and Bing Zheng processed the experimental data and ensured the authenticity and accuracy of the experimental data. All authors agreed to the publication of the article.
Figure legends

Figure 1
DND1 expression is upregulated in PCa tissues and cells. A, Analysis of DND1 expression in PCa by the UALCAN database. B, Association between DND1 expression and overall survival by the UALCAN database. C, Association between DND1 expression and GS score by the PCTA database. mRNA expression (D) and protein level (E) of DND1 in PCa cell lines were detected by qRT-PCR and western blot assay. Data are expressed as mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 2
DND1 knockdown inhibits the proliferation, invasion, and migration of DU145 cells. mRNA expression (A) and protein level (B) of DND1 in DU145 cells after transfection with siRNA-DND1 were detected by qRT-PCR and western blot assay. CCK-8 assay (C) and EDU staining (D) were used to assess cell proliferation. E, Wound healing assay was performed to evaluate cell migration. F, Transwell assay was used to examine cell invasion. Data are expressed as mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 3
DND1 knockdown inhibits the proliferation, invasion, and migration of 22Rv1 cells. mRNA expression (A) and protein level (B) of DND1 in 22Rv1 cells after transfection with siRNA-DND1 were detected by qRT-PCR and western blot assay. CCK-8 assay (C) and EDU staining (D) were used to assess cell proliferation. E, Wound healing assay was performed to evaluate cell migration. F, Transwell assay was used to examine cell invasion. Data are expressed as mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 4
DND1 silencing suppresses EMT in DU145 cells. The levels of E-cadherin (A) and Vimentin (B) were detected by immunofluorescence staining. C, Western blot assay was performed to detect the protein levels of ZO-1 and Slug in transfected DU145 cells. Data are expressed as mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$. 
Figure 5
DND1 silencing suppresses EMT in 22Rv1 cells. The levels of E-cadherin (A) and Vimentin (B) were detected by immunofluorescence staining. C, Western blot assay was performed to detect the protein levels of ZO-1 and Slug in transfected 22Rv1 cells. Data are expressed as mean ± SD. ***P<0.001.

Figure 6
DND1 regulates the expression of CLIC4 in DU145 and 22Rv1 cells. A-B, Association between DND1 expression and CLIC4 in PCa by the linkedomics database. mRNA expression (C) and protein level (D) of CLIC4 in several PCa cell lines were detected by qRT-PCR and western blot assay. mRNA expression (E) and protein level (F) of CLIC4 in DU145 and 22Rv1 cells after transfection with siRNA-DND1 were detected by qRT-PCR and western blot assay. G, Actinomycin D treatment was used to identify the mRNA stability of CLIC4 in DU145 and 22Rv1 cells transfected with siRNA-DND1. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 7
DND1 deletion regulates the proliferation, migration, and invasion of DU145 cells through binding to CLIC4. mRNA expression (A) and protein level (B) of CLIC4 in DU145 cells after transfection with siRNA-CLIC4 were detected by qRT-PCR and western blot assay. CCK-8 assay (C) and EDU staining (D) were used to assess cell proliferation. E, Wound healing assay was performed to evaluate cell migration. F, Transwell assay was used to examine cell invasion. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 8
DND1 deletion regulates the proliferation, migration, and invasion of 22Rv1 cells through binding to CLIC4. mRNA expression (A) and protein level (B) of CLIC4 in 22Rv1 cells after transfection with siRNA-CLIC4 were detected by qRT-PCR and
western blot assay. CCK-8 assay (C) and EDU staining (D) were used to assess cell proliferation. E, Wound healing assay was performed to evaluate cell migration. F, Transwell assay was used to examine cell invasion. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 9
Deletion of DND1 restrained EMT in DU145 cells through binding to CLIC4. The levels of E-cadherin (A) and Vimentin (B) were measured by immunofluorescence staining. C, Western blot assay was performed to detect the protein levels of ZO-1 and Slug in transfected DU145 cells. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 10
Deletion of DND1 restrained EMT in 22Rv1 cells through binding to CLIC4. The levels of E-cadherin (A) and Vimentin (B) were measured by immunofluorescence staining. C, Western blot assay was performed to detect the protein levels of ZO-1 and Slug in transfected 22Rv1 cells. Data are expressed as mean ± SD. **P<0.01, ***P<0.001.

References
Am. 104, 1051-1062.


Carcinogenesis 41, 841-849.
Expression of DND1 in PRAD based on sample types

Effect of DND1 expression level on PRAD patient survival

Mean of expression

Relative DND1 mRNA expression

Relative DND1 protein expression
A. Relative DND1 mRNA expression

B. Western blot analysis of DND1 and GAPDH expression

C. OD value at 450nm

D. EDU and DAPI staining

E. Cell migration assay

F. Cell invasion assay
A

Control  |  siRNA-NC  |  siRNA-DND1
---|---|---
**Merge**

**E-cad**

**DAPI**

B

Control  |  DU145  |  siRNA-DND1
---|---|---
**Merge**

**Vimentin**

**DAPI**

C

**ZO-1**

**Slug**

**GAPDH**

DU145

Relative ZO-1 protein expression

Relative Slug protein expression

DU145

***

**

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HISTOLOGY AND HISTOPATHOLOGY

**A**

![Image of tissue sections]  

**B**

Pearson Correlation: 0.3917  
P-value: 1.143e-19  
Sample Size: (N = 497)

**C**

Relative CLIC4 mRNA expression

- RWPE-1  
- 22RV1  
- DU145  
- LNCaP

**D**

Clinc4  
GAPDH  

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**E**

Relative CLIC4 protein expression

- DU145  
- 22RV1

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**F**

Clinc4  
GAPDH  

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**G**

Relative CLIC4 mRNA remaining

- DU145  
- 22RV1

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**HISTOLOGY AND HISTOPATHOLOGY**

**A**

![Graph showing relative CLIC4 mRNA expression](image)

**B**

![Western blot showing CLIC4 and GAPDH expression](image)

**C**

![Graph showing cell viability at 450nm](image)

**D**

![Images of cell cultures](image)

**E**

![Images of cell migration assay](image)

**F**

![Images of cell invasion assay](image)
A

**siRNA-NC**

**Merge**

**E-cad**

**DAPI**

**siRNA-DND1**

**Merge**

**Vimentin**

**DAPI**

**siRNA-DND1 + siRNA-NC**

**siRNA-DND1 + siRNA-CLIC4**

B

**DU145**

**siRNA-NC**

**Merge**

**Vimentin**

**DAPI**

**siRNA-DND1**

**siRNA-DND1 + siRNA-NC**

**siRNA-DND1 + siRNA-CLIC4**

C

**ZO-1**

**Slug**

**GAPDH**

**DU145**

**Relative ZO-1 protein expression**

**Relative Slug protein expression**