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Original Article

δ-catenin promotes Twist1 stabilization in prostate cancer through ubiquitination modification

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Abstract: Prostate cancer generally has a high long-term survival rate; however, metastatic prostate cancer remains largely incurable despite intensive multimodal therapy. Recent research has identified δ-catenin, a member of the catenin family, as playing a crucial role in the progression of prostate cancer. Nonetheless, the extent to which δ-catenin influences transcription factors associated with epithelial-mesenchymal transition (EMT) has not been thoroughly explored. This study aims to investigate the hypothesis that δ-catenin enhances the stability of Twist1, thereby promoting the migratory and invasive capabilities of prostate cancer cells. Clinical data indicate a strong correlation between δ-catenin and Twist1 expression levels. Western blot analysis confirmed that δ-catenin stabilizes Twist1 and induces ectopic expression. Additionally, δ-catenin was found to reduce Twist1 phosphorylation by inhibiting GSK-3β activity. Immunoprecipitation analysis suggested that δ-catenin exerts its effect by competing with Twist1 for binding to ubiquitin (Ub). These results highlight the role of δ-catenin in the ubiquitination modification of Twist1, suggesting that the combined presence of δ-catenin and Twist1 could serve as a biomarker for tumor progression in prostate cancer.

Keywords: δ-catenin, Twist1, ubiquitination, prostate cancer, GSK-3β

Introduction

Prostate cancer (PCa) is the most prevalent noncutaneous cancer globally, with approximately 1.6 million cases and 366,000 deaths annually [1]. Despite advances in medical treatment, PCa continues to pose significant challenges. One major issue is the overtreatment of indolent PCa, resulting in unnecessary interventions and associated side effects. Moreover, there is an urgent need for effective therapies for metastatic PCa, as current treatments often fail to provide satisfactory outcomes for all patients. Addressing these issues necessitates further research and the development of targeted therapies to improve PCa management and outcomes [2]. Established risk factors for PCa include genetics, family history, chronic inflammation, and advanced age [3]. Additionally, emerging evidence indicates that abnormal expression of transcription factors (TFs) involved in epithelial-mesenchymal transition (EMT) plays a significant role in PCa development. Previous studies have shown that δ-catenin inhibits the degradation of hypoxia-inducible factor 1-alpha (HIF-1α), which in turn increases the transcription of downstream factors such as vascular endothelial growth factor (VEGF) [4]. However, the precise mechanisms by which TFs contribute to PCa progression remain poorly understood, highlighting the need for further investigation.

CTNND1 encodes p120ctn, while CTNND2 encodes δ-catenin/NPRAP, belonging to the delta subfamily of catenins [5]. Initially identified for its interaction with Presenilin-1 (PS-1), δ-catenin was later found to share structural characteristics with p120ctn, including 10 armadillo repeats and 42 amino acid motifs first described in a drosophila segment polarity gene. These features suggest δ-catenin's

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involvement in various protein-protein interactions and its critical roles in cell physiology beyond cytoskeleton remodeling. δ-Catenin interacts with several proteins, including E-cadherin, Synaptic Scaffolding Molecule (S-SCAM), P0071, densin-180, and Postsynaptic Density-95 (PSD-95), highlighting its multifunctional nature and importance in cellular processes. Recent studies have identified δ-catenin as a potential biomarker for several cancers, including prostate [6], breast, lung [7], colorectal [8], and ovarian cancers [9], due to its overexpression. In prostate cancer (PCa), δ-catenin overexpression promotes cell growth and progression by inducing the expression of genes related to cell proliferation and survival [10]. Consequently, developing therapeutic strategies that specifically target δ-catenin with minimal side effects holds significant potential for controlling PCa growth and progression. The epithelial-mesenchymal transition (EMT) plays a crucial role in cancer metastasis by inducing loss of cell polarization and homotypic cell adhesion in epithelial cells, leading to a fibroblast-like morphology [11]. Among the various factors involved in EMT, Twist1, a member of the basic helix-loop-helix family of transcription factors, is associated with fibrotic diseases and tumor progression [12-14]. Our previous study revealed that glaucarubinone exerts anti-cancer effects in the hepatocellular carcinoma cell line Huh7 by regulating the EMT-associated transcription factor Twist1 [15]. Phosphorylation of Twist1 at Ser68 by MAPK increases its stability, leading to elevated Twist1 protein levels [16]. Additionally, the upregulation of TMPRSS4 in PCa enhances stem-like properties by upregulating Sox2, mediated through SLUG and TWIST1, underscoring the significant role of Twist1 in PCa development [17]. Mechanistic studies have shown that Twist1 promotes cancer development by protecting cells from oncogene- and chemotherapy-induced apoptosis and senescence, while augmenting cancer invasion and metastasis through the promotion of EMT [18]. Considering the interaction between Twist1 and β/δ-catenin observed during neural tube development, it is important to investigate the regulatory role of δ-catenin and Twist1 in PCa [19].

Protein stability regulation involves essential post-translational modifications, such as ubiquitination, where the 76-amino acid ubiquitin protein is covalently attached to target protein [20]. The diverse array of ubiquitination modifications and ubiquitin-binding proteins forms the basis of selectivity in the ubiquitin system, enabling the transmission of specific signals with precise spatial and temporal coordination [21, 22]. In mammals, GSK-3, a member of the serine-threonine kinase family, exists in two highly homologous forms: GSK-3α and GSK-3β. Previous research demonstrated that GSK-3α and GSK-3β interact with δ-catenin, phosphorylate it, and subsequently promote its ubiquitination and degradation through proteasome-dependent proteolysis [23]. However, the relationship between Twist1, δ-catenin, GSK-3β, and ubiquitination modifications remains uninvestigated. This study aims to explore the role of δ-catenin in ubiquitination modification to regulate Twist1 protein levels. Our findings reveal that δ-catenin enhances the ectopic expression of Twist1 and influences the interaction between GSK-3β and Twist1, an interaction disrupted by MG132 treatment. Furthermore, we observed competitive binding between δ-catenin and Twist1, resulting in reduced ubiquitination modifications. These results suggest that δ-catenin could serve as a prognostic marker associated with Twist1 in PCa metastasis.

Materials and methods

Antibodies and reagents

Cycloheximide (CHX) was obtained from Selleck (Houston, TX, USA), and MG132 (474790) was obtained from Millipore Sigma-Aldrich. Antibodies against Twist1 (Sc-81417), E-cadherin (Sc-7870), GFP (Sc-9996), Lamin B1 (Sc-374015), and β-actin (Sc-47778) were sourced from Santa Cruz Biotechnology (USA). Antibodies against δ-catenin (611537) were obtained from BD Bioscience. Antibodies against GSK-3α/β (#9671s), and p-GSK-3α/β (#9331s) were sourced from Cell Signaling Technology (USA). δ-catenin and Twist1 siRNA were purchased from Limbio biotechnology (CN).

Cell lines and transfection

The human PCa cell lines CWR22Rv-1, PC3, LNCaP, DU145 and Hek-293T were obtained from the American Type Culture Collection (USA). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine

serum at 37.0°C in a 5% CO_2 incubator. To generate stable cell lines, Rv/δ (expressing δ-catenin-GFP) and Rv/C (expressing GFP) were established in Rv-1 cells by transfection with mouse δ-catenin-GFP. The cells were then selected with G418 (200 μg/ml, Sigma-Aldrich) at 37°C in a 5% CO_2 environment [24]. Plasmid transfections were performed using Plus/Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) for 12 hours, following the manufacturer's instructions. Before commencing experimental procedures, the RPMI 1640 medium supplemented with 10% FBS was replaced with serum-free medium and incubated for 2 hours. Subsequently, the cells were treated as previously described [25].

Plasmids

In previous research, constructs of GFP-δ-catenin in pEGFP-C1, along with deletion constructs 1-690, 690-1040, and δN85-325/δC207, were generated through PCR amplification and subsequently cloned into a pEGFP-C1 vector [23, 24]. PCR amplification was used to produce HA-tagged GSK-3α wild type (referred to as GSK-3α WT) and kinase-dead (referred to as GSK-3α KD) constructs, which were then cloned into a cytomegalovirus promoter-derived mammalian expression vector (pCS4-3Myc, -3HA). Constructs of HA-tagged GSK-3β wild type (referred to as GSK-3β WT) and kinase-dead (referred to as GSK-3β KD) were generously supplied by Kang-Yeol Choi from Yonsei University. Additionally, HA-tagged ubiquitin was kindly provided by Professor Kwang Youl Lee from Chonnam National University.

Western blot analysis

Western blot analysis was performed following established protocols [26]. Protein concentration was measured using the Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific, 23228, Waltham, MA, USA). Each sample was loaded with 30 μg of protein and separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine gels, followed by transfer onto polyvinylidene fluoride (PVDF) membranes. The proteins were detected using various antibodies.

Immunoprecipitation

Cells were lysed using a buffer containing 137 mM NaCl, 20 mM Tris-HCl (T5030, Sigma-Aldrich, St. Louis, MO, USA), 1% NP-40, 2 mM EDTA, 50 mM NaF, and 0.1 μM aprotinin (SRE0050, Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation with primary antibodies at 4°C, the cells were incubated with agarose beads (GE Healthcare, Uppsala, Sweden) at 4°C for 6 h. The immunoprecipitated proteins were then washed three times with the lysate buffer. For elution, the immunoprecipitated proteins were heated at 95°C for 5 min in 30 μL of 5× sample buffer (0.1 M Tris-HCl, pH 6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1.43 M β-mercaptoethanol), and subsequently used for immunoblotting. Additionally, the cell lysates were subjected to immunoprecipitation using the appropriate mouse IgG isotype control that matched the immunoglobulin type of the primary antibody.

Cell fractionation

Nuclear and Cytoplasmic Extraction Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to fractionate cell lysates according to the protocol of the manufacturers as previously described [27, 28].

Wound healing assay

Furthermore, transfected cells were seeded in six-well plates and allowed to reach full confluence. A scratch wound was then created by scraping the cell monolayers with a sterile 200-μL pipette tip. Images of the wounded cell monolayers were captured using a Model IX70 Microscope (Olympus, Tokyo, Japan) at 0 hour and 24 hours post-wounding. The healing rate was determined by measuring the gap size after culturing.

Quantitative real-time (qRT)-polymerase chain reaction (PCR)

Total RNAs were isolated from different cells using TRIzol Reagent (TaKaRa, 9108/9109) according to the instructions of the manufacturer. Subsequently, total RNA (1 μg) was reverse transcribed into cDNA using the GoScript Reverse Transcription Kit (Promega, A5004). Additionally, qRT-PCR amplification was performed for indicated genes using SYBR Green (CFX960 Real-Time PCRT detection system, Bio-Red). The target gene expression was normalized to GAPDH gene expression (Twist1 Forward Primer: GGAGTCCGCAGTCTTACGAG;

Reverse Primer: TCTGGAGGACCTGGTACAGG. GAPDH Forward Primer: GGAGCGAGATCCCTCCAAAAT; Reverse Primer: GGCTGTTGTCATACTTCTCATGG).

Compound screening strategy and molecular docking simulation

For lead compound screening, the 3D model of δ-catenin-Twist1 protein complex was generated using AlphaFold3. Intermolecular forces between the proteins within the complex were calculated using Discovery Studio 2021 (Accelrys Inc, San Diego, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). All data are expressed as mean ± SE. Statistical evaluations were performed using ANOVA with a student's t-test. For comparisons between the two groups, a student's t-test was employed. Additionally, one-way or two-way analysis of variance (ANOVA) with post-Bonferroni corrections was used to compare the effects of different treatments or treatment effects over time. Statistical significance was as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

Twist1 and δ-catenin are significantly unregulated in PCa tissues and indicate a poor prognosis

Twist1 serves as a key inducer of EMT [29]. Aberrant overexpression of Twist1 facilitates EMT, cell motility, and invasive activity [28, 30]. To commence this study, we initially examined the clinicopathological characteristics of Twist1 using data from The Cancer Genome Atlas dataset (TCGA) dateset. TCGA data revealed a significant increase in Twist1 mRNA levels in cancer specimens compared to normal prostate tissue (**Figure 1A**, $P < 0.001$). Box plot also demonstrated a correlation between TWIST1 expression and tumor progression, with patients at adjacent TNM stages exhibiting significant differences in TWIST1 expression (**Figure 1B**, $P < 0.05$). Moreover, higher Twist1 mRNA levels were associated with shorter survival time in patients with PCa (**Figure 1C**). Previous findings from our research indicated that δ-catenin can stimulate the proliferation, migration, and invasion of PCa [28]. Consistent with the observed pattern of Twist1 expression, differential gene expression analysis from the TCGA dataset also revealed significant upregulation of δ-catenin (**Figure 1D**). Additionally, TCGA data demonstrated a positive correlation between Twist1 and δ-catenin expression (R = 0.22, P = 6.2e-07) (**Figure 1E**, **1F**). To further validate this correlation at the protein level, Hek-293T cells were transfected with the respective plasmids, and Western blot analysis confirmed that δ-catenin promotes Twist1 expression in a volume-dependent manner (Supplementary Figure 1D). To assess the metastatic potential of human PCa cell lines, including Rv-1, PC3, DU145, and LNCaP, we conducted cell migration assays (Supplementary Figure 1A, 1B) and colony formation assays (Supplementary Figure 1C). Furthermore, we evaluated Twist1 protein expression in different PCa cell lines, revealing its association with invasive behavior. The expression of Twist1 and the proto-oncogene C-myc showed consistency following treatment with MG132, with volume-dependent changes observed in protein levels (**Figure 1G-I**). Moreover, siRNAs were used to interference the expression of δ-catenin and Twist1 (Supplementary Figure 1E, 1F) and reduces the migratory ability of PC3 (overexpression of δ-catenin and Twist1 showed the opposite effect) (Supplementary Figure 1G). Collectively, these findings suggest that Twist1 and δ-catenin may be potential prognostic markers for PCa metastasis.

δ-catenin increases Twist1 protein level by increasing its stabilization

Consistent with previous studies showing that Twist1 has a relatively short half-life due to its susceptibility to proteasomal degradation [31], detection of protein levels requires high-intensity exposure in PCa cell lines (**Figure 1G**, Supplementary Figure 1F). To explore the expression patterns of Twist1 more efficiently and accurately, we established cell lines transiently expressing Twist1. This approach resulted in significantly higher levels of stable Twist1 expression compared to endogenous production (**Figure 2A**, upper band). Additionally, treatment with MG132 enhanced Twist1 expression, further indicating that proteasomal degradation plays a critical role in regulating Twist1 stability (**Figure 2A**). Differential proto-oncogene expression also indicated the presence of

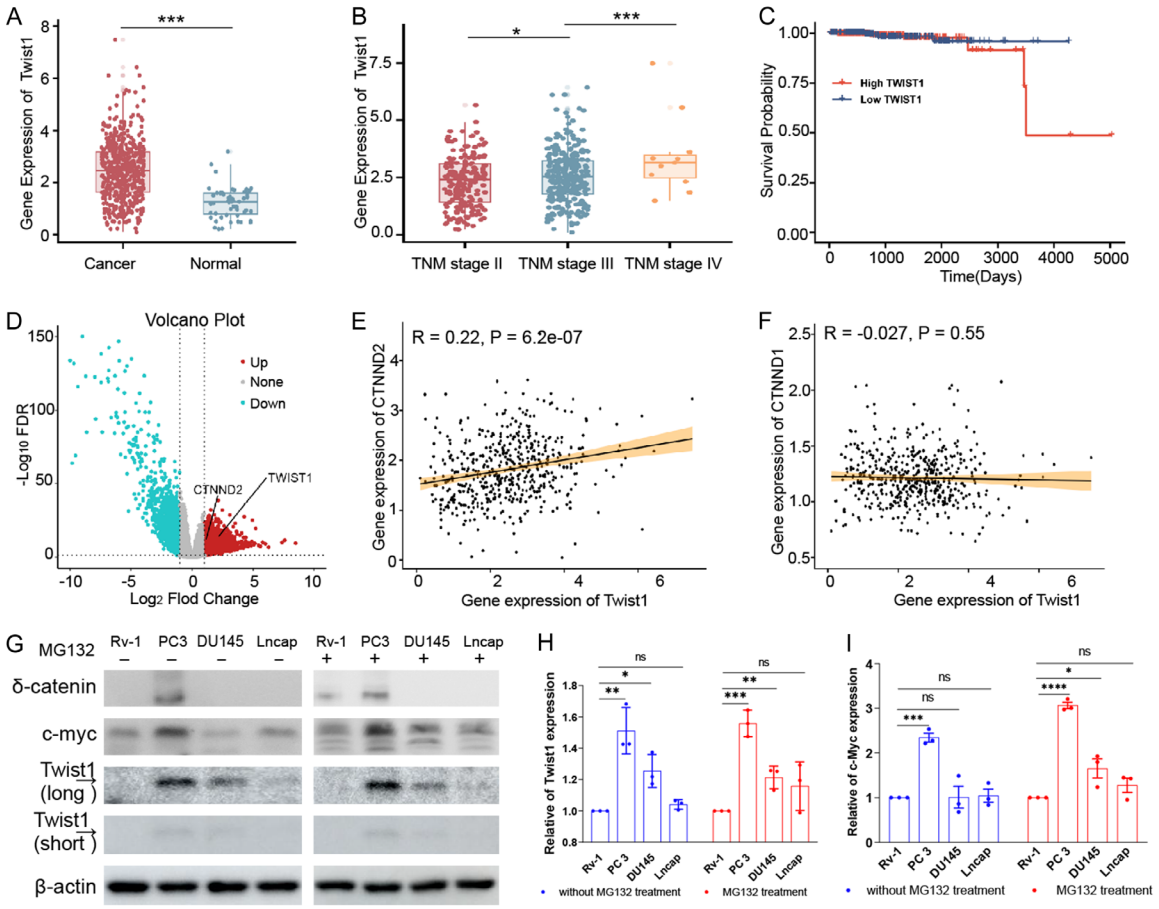

Figure 1. Twist1 and δ-catenin are significantly unregulated in PCa tissues and indicate a poor prognosis. (A) Representative data obtained from TCGA demonstrate a significant difference in TWIST1 expression of between normal prostate tissue and PCa. (B) Correlation between TWIST1 mRNA expression and clinicopathological characteristics in different clinical stages. Box plot showing that TWIST1 expression increases with tumor progression, accompanied by a significant difference in TWIST1 expression between patients of adjacent TNM stages. (C) Kaplar-Meier analysis revealed that patients with PCa and higher TWIST1 expression have poorer overall survival (OS). (D) Volcano plots of differentially expressed genes between normal prostate tissue and PCa. (E, F) Linear correlation analysis between gene expression level of CTNND1, CTNND2, and Twist1. (G) Western blot analysis of Twist1, δ-catenin, and c-Myc in PCa cell lines. (H, I) Western blot analysis in three independent assays. Data are presented as ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the other cell type. ns = not significant. The t-test was used for statistical analysis.

the Twist1/c-Myc axis in PCa cells (**Figure 2B**). A study by Shen and colleagues found that δ-catenin promotes PCa cell deterioration through the EGF/AKT/p21^{Waf} signaling pathway [28]. Beyond its role in cytoskeleton remodeling, δ-catenin plays critical roles in various cell physiology [32]. Given the correlation between δ-catenin and Twist1 expression in PCa (Result1), we hypothesized that δ-catenin could enhance Twist1 expression. Western blot analysis confirmed this hypothesis, showing that δ-catenin increased Twist1 protein levels compared to RV/C (**Figure 2C**, **2D**). In comparing Twist1 expression across different prostate cancer (PC) cell lines (PC3, DU145, and LNCaP), a trace amount of endogenous Twist1 expression was detected (**Figure 2E**). To investigate the regulatory role of δ-catenin on Twist1, we measured Twist1 mRNA levels and observed a significant upregulation following δ-catenin overexpression (**Figure 2F**). Notably, there was a discrepancy between the mRNA and protein levels of Twist1, particularly after treatment with MG132, a proteasome inhibitor. This suggests that the protein accumulation of Twist1 could be attributed to reduced degradation rather than increased translation. The MG132 treatment likely inhibits the proteasome-medi-

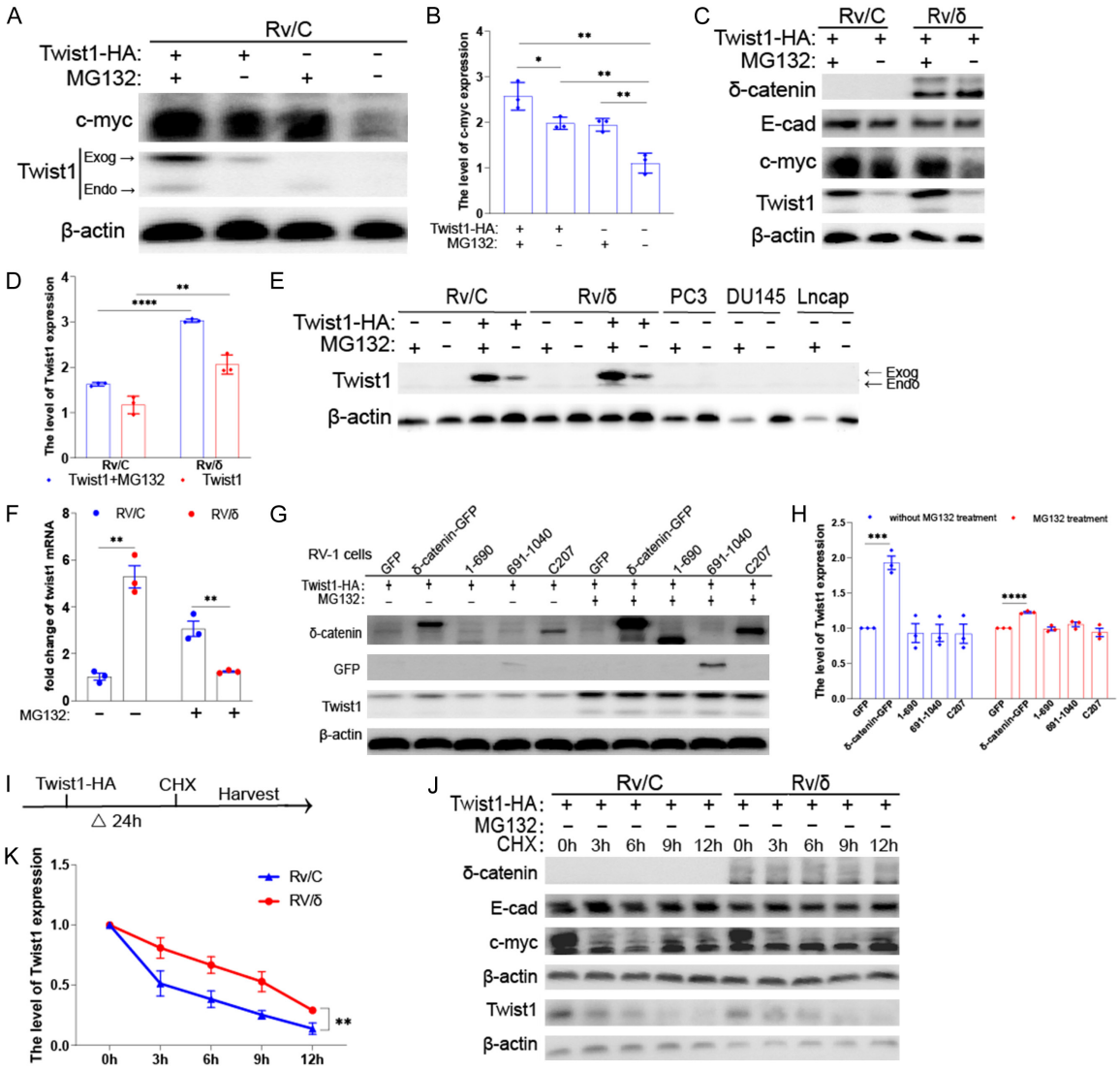

Figure 2. δ-Catenin increases Twist1 protein level by increasing its stabilization. (A) RV/C cells were transfected with Twist-HA and treated with MG132. Cell lysates were immunoblotted with anti-c-myc, anti-twist1, and anti-β-actin. This experiment was independently repeated three times. (B) The quantitation of c-myc is shown in (A). (C) RV/C and RV/δ cells were transfected with Twist-HA and treated with MG132. Subsequently, cell lysates were immunoblotted with anti-c-myc, anti-twist1, and anti-β-actin. (D) Immunoblotted analysis of Twist1 and c-myc is shown in (C). (E) RV/C, RV/δ, and other PCs cells were transfected with Twist-HA and treated with MG132. Cell lysates were immunoblotted with indicated antibodies. This experiment was independently repeated three times. (F) RV/C and RV/δ cells were transfected with the respective plasmid, and Twist1 mRNA level were measured using qRT-PCR. (G) GFP plasmid served as a negative control. Rv/C and RV/δ cells were transfected with GFP, δ-catenin-GFP, or mutant δ-catenin (1-690, 691-1040, C207) plasmids. (H) Immunoblot analysis of Twist1 as shown in (G). (I) RV/C and RV/δ cells were transfected with twist1 plasmid and treated with 100 μg/mL of CHX. (J) Cells were harvested at different time points and cell lysates were immunoblotted with antibodies against Twist1, δ-catenin, c-myc and β-actin. (K) Quantification of Twist1 protein levels in (J). Data are presented as means ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the other cell type. The t-test was used for statistical analysis.

ated degradation pathway, thereby stabilizing Twist1 protein levels despite minimal changes in its mRNA expression. This highlights the complex regulatory mechanisms governing Twist1 expression, involving not only transcriptional but also post-translational modifications.

We hypothesized that δ-catenin could induce Twist1 stabilization. To further understand δ-catenin regulation, we utilized different deletion mutants, including 1-690, 691-1040 (GFP-Target), C207, and a full-length plasmid containing GFP-Target (δ-catenin-GFP). Western

blot analyses verified successful transfection and revealed that full-length δ-catenin significantly stabilized Twist1, especially in comparison to the GFP control group that did not receive MG132 treatment (**Figure 2G**, **2H**). Pursuing our hypothesis that δ-catenin enhances Twist1 stabilization, we treated RV/C and RV/δ cells with cycloheximide (CHX), a protein synthesis inhibitor (**Figure 2I**). Cells were collected at various time points, and those expressing stable δ-catenin showed a markedly reduced exponential decay constant (**Figure 2J**, **2K**). These observations suggest that overexpression of δ-catenin extends the half-life of Twist1, underscoring its role in stabilizing the protein. Additionally, stable δ-catenin expression conferred partial protection against c-myc degradation (**Figure 2J**, upper band). In summary, our findings highlight that δ-catenin stabilizes Twist1, thereby augmenting its protein levels.

Twist1 inhibit the degradation progression of δ-catenin

Typically, δ-catenin binds to the juxtamembrane domain region of E-cadherin [33]. Notably, when cells were treated with both MG132 and CHX (**Figure 3A**), the inhibition of proteasomal degradation resulted in sustained Twist1 expression, while δ-catenin exhibited progressive degradation (**Figure 3B**, **3C**). Concurrent with the increased stability of Twist1, the rate of c-myc degradation also decreased (**Figure 3B**, **3D**), indicating δ-catenin's regulatory effect on the Twist1/c-Myc axis. To investigate the hypothesis that Twist1 contributes to δ-catenin stabilization, Twist1 was overexpressed in RV/δ cells and separately treated with MG132 and CHX. Western blot analysis showed that Twist1 expression alone mitigated δ-catenin degradation (**Figure 3E**, **3G**). The influence of MG132 suggested that δ-catenin stability was compromised by proteasomal activity (**Figure 3F**, **3H**). Moreover, Twist1 expression alone was able to counteract the proteasome-mediated degradation of δ-catenin (**Figure 3E**, **3F**), and stable Twist1 expression increased the non-proteasomal degradation of δ-catenin (**Figure 3F**, **3I**). These results further substantiate the notion that the stabilization of Twist1 and δ-catenin is chiefly governed by proteasomal activity, with Twist1 playing a pivotal role in δ-catenin stability, which is predominantly mediated through the proteasomal pathway.

δ-catenin promotes the ectopic expression of Twsit1

Research on colorectal cancer and other related studies have indicated that TWIST1 is expressed in both the cytoplasm and nucleus. The nuclear localization of TWIST1 has been implicated in the progression and aggressiveness of colorectal cancer and similar diseases [34]. As a tumor inducer, δ-catenin demonstrated elevated level in various cancer, including PCa [35]. Additionally, TWIST1 has been found in endocytic vesicles at the apical surface and interacts with β/δ-catenin during neural tube closure [19]. Given the observation that δ-catenin can stabilize Twist1, it is plausible that δ-catenin may also help retain Twist1 within the nucleus. Western blot analyses revealed a shift in the subcellular localization of Twist1 and c-myc from the membrane and cytoplasm to the nucleus in cells overexpressing δ-catenin (**Figure 4A**, **4C**, **4D**). Considering the crucial role of Twist1 in δ-catenin stability, we further examined Twist1's regulatory influence on the subcellular localization of δ-catenin by stably expressing Twist1. Western blot results indicated that the stable expression of Twist1 altered the subcellular distribution of δ-catenin, which also impacted c-myc levels (**Figure 4B**, **4E**, **4F**).

δ-catenin promotes Twist1 expression by inhibiting GSK-3β

δ-Catenin has been implicated in the EGF/AKT/$p21^{waf}$ signaling pathway, promoting increased proliferation and invasion of PCa cells. As a transcription factor, the regulation of Twist1 phosphorylation can also contribute to the progression and invasion in PC [28, 36]. Through linear correlation analysis and a comparative analysis of transcriptomic data from TCGA, using the cBioPortal and Firebrowse databases, we identified a significant negative correlation between the WNT signaling pathway score and CTNND2 gene expression (**Figure 5A**, **5B**). These findings reveal a potential interaction among Twist1, WNT signaling, and δ-catenin in PC (**Figure 5C**). Furthermore, Gene Set Enrichment Analysis (GSEA) indicated that CTNND2 markedly suppressed the activity of the WNT signaling pathway (**Figure 5D**). Western blot results suggested that δ-catenin inhibited the expression of GSK while promoting the expression of Twist1 (**Figure 5E**, **5H**, **5I**).

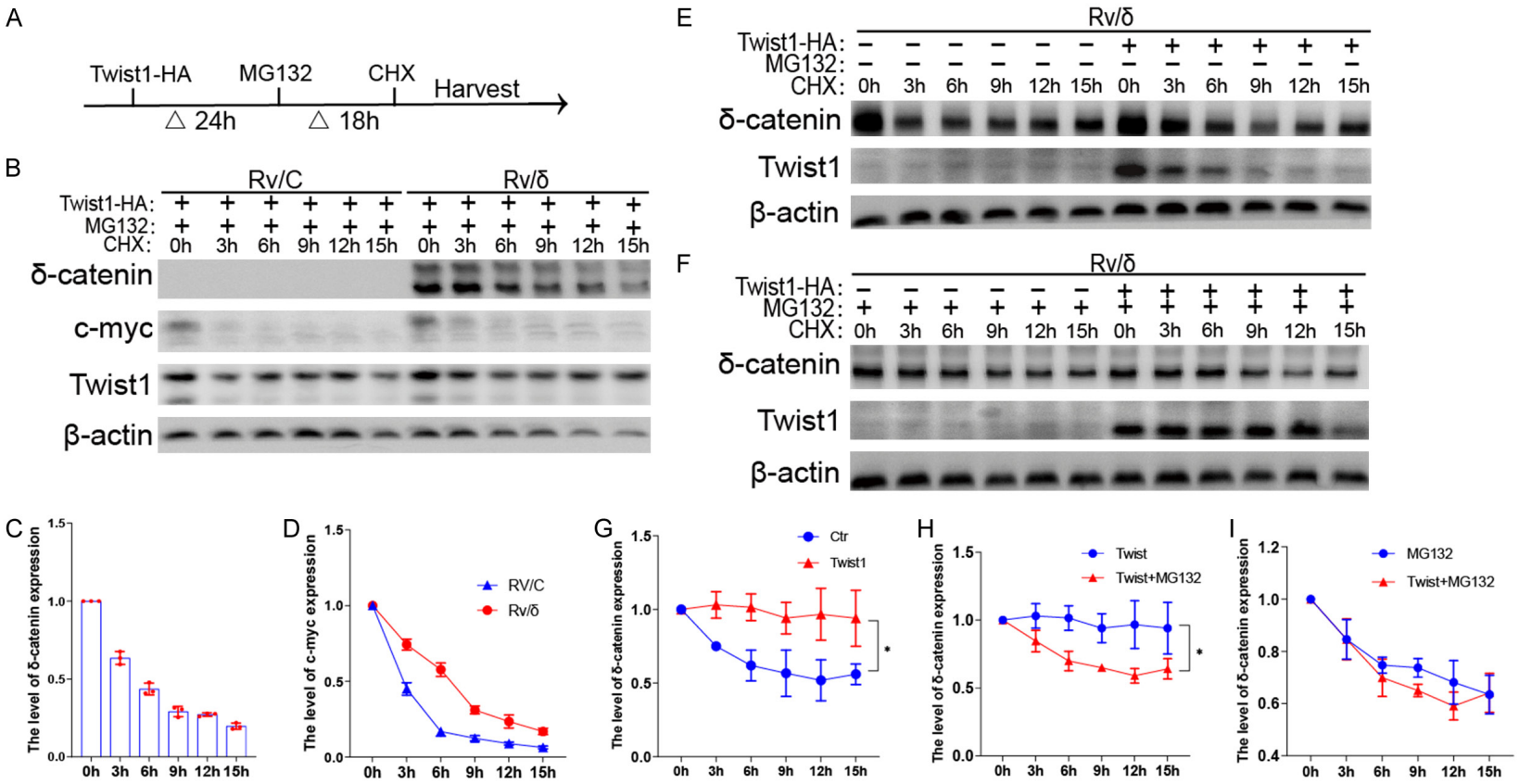

Figure 3. Twist1 inhibit the degradation progression of δ-catenin. (A) RV/C and RV/δ cells were transfected with twist1 plasmid followed by MG132 treatment, then treated with 100 μg/mL of CHX. (B) Cells were harvested at different time points and cell lysates were immunoblotted with antibodies against Twist1, δ-catenin, c-myc and β-actin. (C) Quantitation of δ-catenin/β-actin shown in (B). (D) Quantification of c-myc protein in (B). (E, F) RV/δ cells were transfected with Twist-HA and treated with MG132. Cell lysates were immunoblotted with anti-δ-catenin, anti-twist1, and anti-β-actin. This experiment was independently repeated three times. (G-I) Quantification of δ-catenin protein in (E) and (F). Data are presented as means ± SE. *$P < 0.05$ compared with that of the other cell type. The t-test was used for statistical analysis.

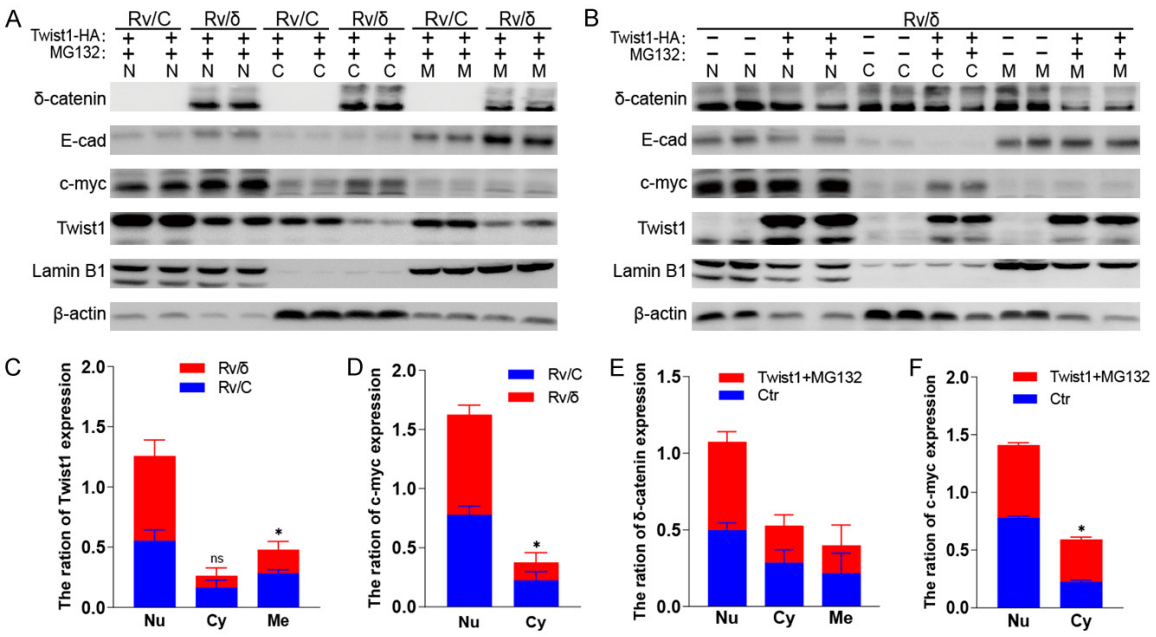

Figure 4. δ-Catenin promotes the ectopic expression of Twsit1. (A, B) RV/C and RV/δ cells were transfected with Twist-HA and treated with MG132 followed by isolation of subcellular structures from cell lysates. (C-F) Denstiometry data of twist1 and c-myc protein in (A, B) using lamin B1 (nuclear), β-actin (cytoplasmic), and E-cad (membrane) densitometry data respectively. Data are presented as means ± SE. *$P < 0.05$ compared with that the other cell type. The t-test was used for statistical analysis.

δ-Catenin exhibited varying degress of inhibition on different subunits of GSK, with a more pronounced inhibition observed on GSK-β (**Figure 5E**, **5F**). A similar pattern was observed for Phosphate-GSK-3β (**Figure 5E**, **5G**). Protein phosphorylation and ubiquitination modification are essential for their stability regulation. Our results also indicated that δ-catenin reduced intracellular ubiquitination levels, with a significant inhibitory effect observed after MG132 treatment (**Figure 5J**). To investigate the hypothesis that GSK-3α/β may influence the regulatory interaction between Twist1 and δ-catenin via the ubiquitination process, we transfected GSK-3α/β and mutant GSK-3α/β KD plasmids into RV/C and RV/δ cells. Western blot analysis revealed that overexpression of GSK-3β inhibited Twist1 expression in RV/C cells post-transfection, but this inhibition was mitigated in the presence of δ-catenin (**Figure 5K**, **5L**). No significant differences were observed after MG132 treatment (**Figure 5M**, **5N**). These findings suggest that, alongside GSK-3β regulation, δ-catenin primarily maintains Twist1 stability through the ubiquitin-proteasome degradation pathway.

δ-catenin competes with Twist1 for binding to Ub

We analyzed the overlap between genes involved in the ubiquitin-mediated proteolysis signaling pathway, retrieved from the hsa04120 KEGG database, and all differentially expressed genes (DEGs) (**Figure 6A**), including up-regulated DEGs (**Figure 6B**), in both cancerous and normal prostate samples from patients with PCa. Venn diagram analysis indicated a significant association between the ubiquitin-mediated proteolysis signaling pathway and PCa development, as confirmed by Fisher's exact test ($P < 0.001$). To assess the effect of δ-catenin on the ubiquitination of Twist1, we treated RV/C and RV/δ cells with Ub-HA plasmids after transfection. The noticeable difference in Twist1 expression levels suggests a pivotal role of ubiquitination in Twist1 degradation (**Figure 6C**). We further examination of ubiquitination in high molecular weight proteins revealed a significant increase at positions corresponding to δ-catenin, indicating a preference for overexpressed ubiquitin to bind with δ-catenin (**Figure 6C**). Considering the regulation of Twist1 stability by GSK-3β, we perform-

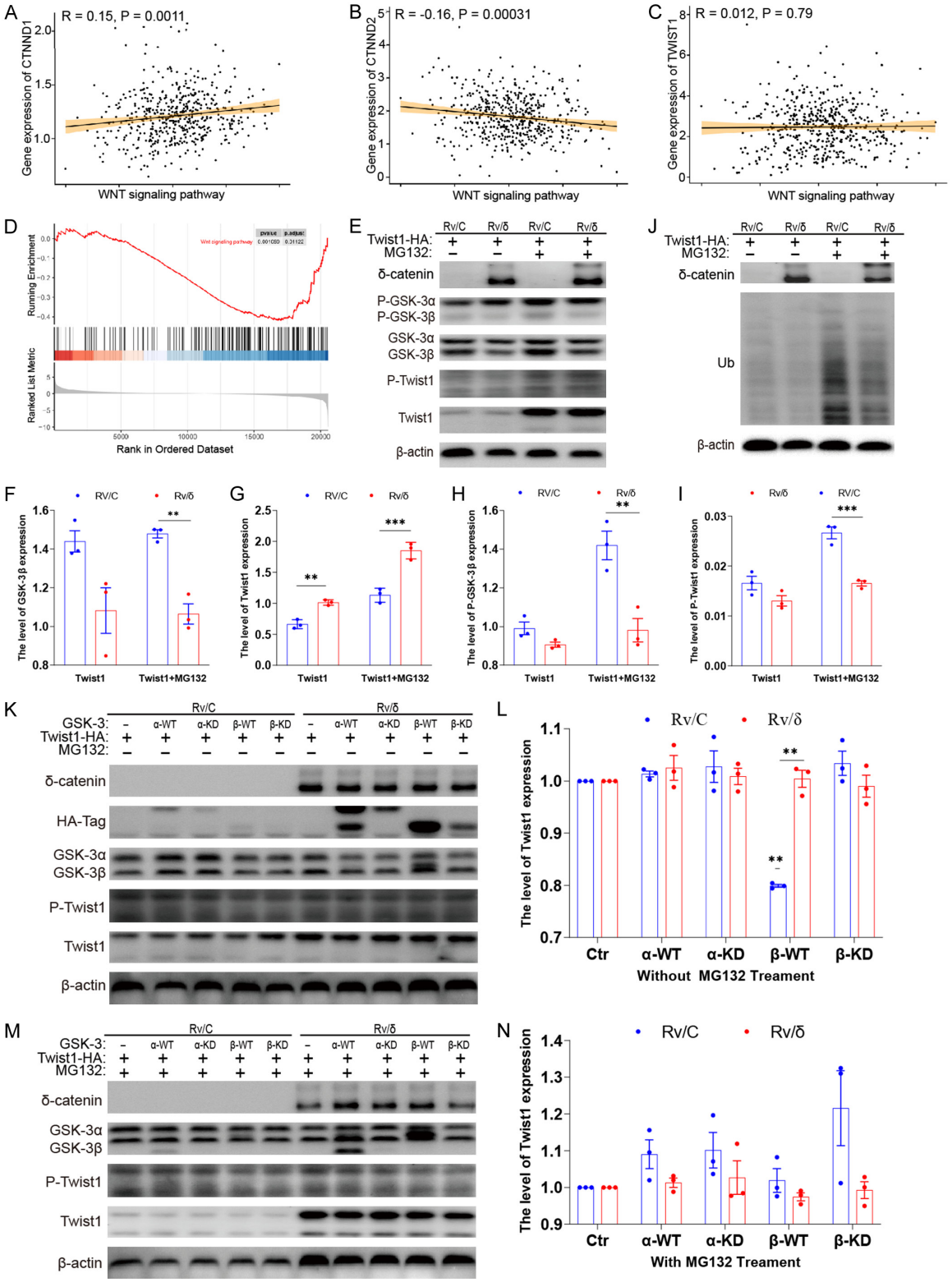

Figure 5. δ-Catenin promotes Twist1 expression by inhibiting GSK-3β. (A, B) Linear correlation analysis between WNT signaling pathway score and gene expression level of CTNND1 and CTNND2. (C) Linear correlation analysis between WNT signaling pathway score and expression level of Twist1. (D) GSEA analysis revealed that CTNND2 significantly attenuated the activity of the WNT signaling pathway. (E) RV/C and RV/δ cells were transfected with Twist-HA and treated with MG132. Cell lysates were immunoblotted with indicated antibodies. This experiment was independently repeated three times. (F-I) Quantification of twist1, GSK-3β, P-twist1, and P-GSK-3β protein in (E). (J) RV/C and

RV/δ cells were transfected with Twist-HA and treated with MG132. Cell lysates were immunoblotted with indicated antibodies. (K) RV/C and RV/δ cells were transfected with Twist-HA, GSK, or GSK-KD and treated without MG132 respectively. Cell lysates were immunoblotted with indicated antibodies. This experiment was independently repeated three times. (L) Quantification of twist1 protein in (K). (M) RV/C and RV/d cells were transfected with Twist-HA, GSK, or GSK-KD and treated with MG132 respectively. Cell lysates were immunoblotted with indicated antibodies. This experiment was independently repeated three times. (N) Quantification of twist1 protein in (M). Data are presented as means ± SE. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to other cell type. The t-test was used for statistical analysis.

ed co-immunoprecipitation experiments. In the absence of MG132 treatment, δ-catenin enhanced the interaction between Twist1 and GSK-3α/β, increasing Twist1's ability to bind ubiquitin. However, this interaction was reduced after MG132 treatment (**Figure 6D**), resulting in decreased ubiquitin pull-down by Twist1. These findings suggest that δ-catenin reduces Twist1 ubiquitination by inhibiting the proteasome. To further elucidate Twist1's role in the binding of Ub to δ-catenin, co-precipitation experiments revealed that δ-catenin expression inhibited the interaction between Twist1 and Ub (**Figure 6E**). To investigate the interaction pattern between CTNND2 and TWST1 proteins, we constructed a model of the CTNND2-TWST1 protein complex using AlphaFold3 and calculated the intermolecular forces between the proteins within the complex using Discovery Studio 2021. The analysis revealed that the CTNND2-TWST1 complex interface possesses a variety of strong interactions, including hydrogen bonds, electrostatic forces, hydrophobic interactions, and pi-stacking interactions. These interactions act synergistically to form a stable protein complex structure (**Figure 6G**, **6H**). Conversely, the co-precipitation of δ-catenin demonstrated a distinct interaction, however the amount of interacting Ub was significantly reduced after Twist1 co-transfection (**Figure 6F**). This suggests that Twist1 can reduce the binding of Ub to δ-catenin, subsequently leading to reduced degradation of δ-catenin. These findings indicate a competitive interaction among δ-catenin, Twist1, and Ub. While the ubiquitination modification of both δ-catenin and Twist1 occurs, the binding energy between δ-catenin and Twist1 undergoes structural changes that conceal the ubiquitination modification site of δ-catenin, thereby reducing its ubiquitination modification. However, the modification site of Twist1 remains partially exposed.

Discussion

Prostate cancer (PCa) is recognized for its considerable heterogeneity, demonstrating a spectrum of clinical behaviors. Some cases progress slowly or remain dormant, while others are highly aggressive and present a substantial mortality risk. This diversity complicates PCa management and contributes to its status as a major cause of cancer-related deaths worldwide [37]. Androgen deprivation therapy (ADT) remains the primary treatment for metastatic PCa [38]. In our previous study, we uncovered a crucial role of basic fibroblast growth factor (bFGF) in stabilizing δ-catenin expression, thereby promoting PCa progression. We identified δ-catenin's involvement in the EGF/AKT/p21Waf signaling pathway, which, when activated, enhances the proliferation and invasion of PCa cells. This highlights δ-catenin's significant role in PCa development and suggests it as a promising target for therapeutic intervention [28, 39]. In the current study, we presented evidence supporting the utilization of Twist1 and δ-catenin as a combined biomarker for PCa. Our investigation into the Twist1/c-myc regulatory axis in tumors reveals δ-catenin's role in regulating proto-oncogenes and pro-EMT transcription factors. These findings deepen our understanding of the molecular mechanisms driving PCa progression and offer insights into potential therapeutic targets for its management [40].

δ-catenin, encoded by the CTNND2 gene, interacts with presenilin-1 (PS-1) in the development of familial Alzheimer's disease [41, 42]. Several studies have shown elevated δ-catenin levels in patients with prostate cancer (PCa) compared to healthy individuals, suggesting its potential as a biomarker for cancer [6, 43]. Our report presents findings from The Cancer Genome Atlas (TCGA) demonstrating that high mRNA levels of Twist1 and δ-catenin are associated with shorter survival times in PCa patients. We examined four human PCa cell lines (Rv-1, PC3, DU145, and LNCap), which exhibit varying metastatic abilities and distinct expression patterns of Twist1 and δ-catenin. Overexpression of δ-catenin in Rv-1 cells resulted in increased Twist1 protein levels, dependent on the presence of the full δ-catenin com-

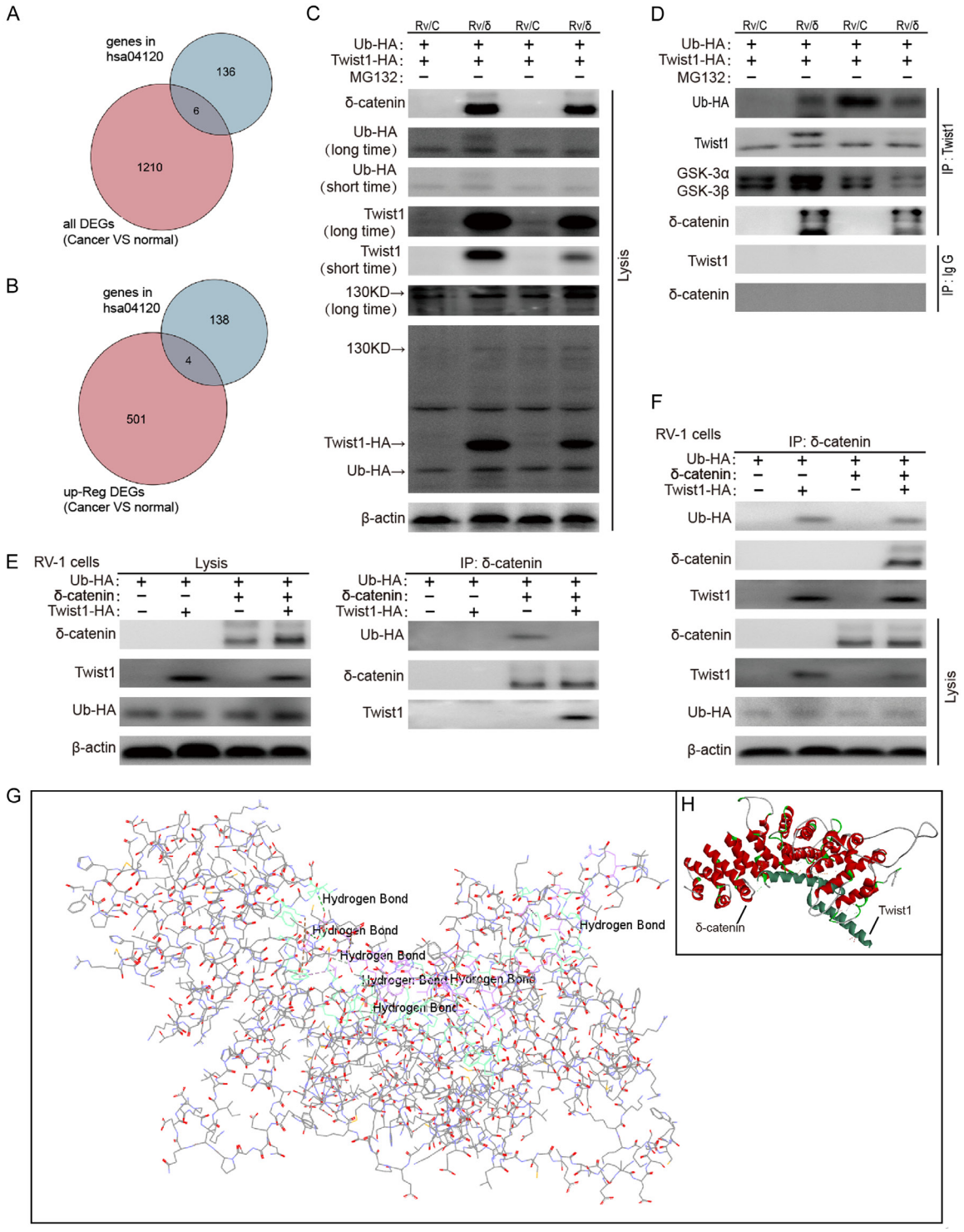

Figure 6. δ-Catenin competes with Twist1 for binding to Ub. (A, B) The Venn diagram illustrates the significant involvement of the ubiquitin-mediated proteolysis-signaling pathway in the development of PCa. (C, D) Twist-HA and Ub-HA were transfected into RV/C and RV/δ cells respectively, and treated with MG132. Subsequently, cell lysates were harvested and subjected to immunoblotting using specific antibodies to analyze the protein expression. (E, F) RV-1 cells were transfected with Ub-HA, Twist1-HA, and δ-catenin. Immunoprecipitation with the δ-catenin or Twist1, followed by immunoblotting with the indicated antibodies. (G) Intermolecular forces between the proteins within the complex. (H) Molecular docking showing the interaction between δ-catenin or Twist1.

ponent (Results 1 and 2). As protein level is a dynamic process involving transcription, translation, and degradation stages, the accumulation of Twist1 may result from decreased degradation even at low translation levels especially after MG132 treatment (**Figure 2F**). These findings suggest a potential mechanism involving the interplay between Twist1 and δ-catenin in PCa progression and metastasis. The stable expression of Twist1 also influenced the degradation rate of δ-catenin. Therefore, monitoring and targeting δ-catenin and Twist1 may represent a novel approach in PCa treatment, offering potential for improving patient outcomes.

δ-Catenin, characterized by its 10 armadillo repeats, plays a crucial role in protein-protein interactions [44]. Twist1's rapid degradation within the cell complicates its detection [31]. In our previous study, we overcame the difficulty of detecting its content by using overexpression techniques. By isolating the different components of Twist1 through cellular substructure separation, we elucidated how δ-catenin controls Twist1 expression. Our research revealed significant findings regarding the subcellular localization of Twist1. We discovered that δ-catenin can induce the ectopic expression of Twist1, prompting its translocation from the cell membrane and cytoplasm to the nucleus. This suggests that δ-catenin plays a crucial role in regulating the nuclear localization of Twist1. Additionally, we verified that the stable expression of Twist1 can influence the subcellular localization of δ-catenin within the nucleus.

In our study, we investigated how metformin affects the bFGF-mediated activation of the AKT/GSK-3β signaling pathway. We observed that metformin effectively suppresses this activation. Additionally, we found that metformin inhibits the interaction between GSK-3β and Twist1, leading to enhanced stability of Twist1. These findings suggest that metformin may influence Twist1 by modulating the AKT/GSK-3β signaling pathway and the interaction between Twist1 and GSK-3β [36]. We also observed that GSK-3β expression is inhibited in the presence of δ-catenin (Result 5). To investigate the specific details of GSK-3α/β, we generated various mutant expression vectors. As anticipated, the overexpression of GSK-3β was found to suppress Twist1 expression. However, this inhibition was compromised in the presence of δ-catenin. Following MG132 treatment, no significant differences were observed in the regulatory mechanism of GSK-3β and Twist1 by δ-catenin. Furthermore, analysis of the expression level of Ub revealed that δ-catenin could inhibit ubiquitination. These findings reveal that δ-catenin predominantly sustains Twist1 stability through the ubiquitin-proteasome degradation pathway, in conjunction with GSK-3β. Recent studies on transcription factors like STAT1 have shown that monoubiquitination can significantly affect the stability of target proteins, although the dependency on proteasome-mediated degradation remains to be fully elucidated [45]. Further investigation is necessary to determine whether δ-catenin plays a role in the ubiquitination of Twist1 during PCa progression. Additionally, it remains to be confirmed whether the ubiquitination of δ-catenin is also affected by the reverse action of Twist1. In this study, we made a significant discovery. We found that the stable expression of δ-catenin results in reduced intracellular ubiquitination levels and increased Twist1 expression. Furthermore, we detected elevated levels of ubiquitination-specific sites corresponding to δ-catenin. Our findings indicate that δ-catenin can compete with Twist1 for binding to ubiquitin (Ub), and that Twist1 can reduce the binding of Ub to δ-catenin. The CTNND2-TWST1 complex interface possesses a variety of strong interactions, including hydrogen bonds, electrostatic forces, hydrophobic interactions, and pi-stacking interactions. These interactions act synergistically to form a stable protein complex structure (**Figure 6G**, **6H**). This interaction consequently reduces the subsequent degradation of δ-catenin. Moreover, besides its role in mediating interactions between specific proteins [44], our study provides further evidence that δ-catenin regulates target proteins and promotes their stable expression. This mechanism contributes to the exacerbation of PCa progression.

Our findings demonstrate that δ-catenin regulates Twist1 expression through ectopic expression and intracellular shuttling. This regulation involves controlling the ubiquitination of Twist1 in cooperation with GSK-3β. Additionally, considering the expression of c-myc, our results contribute to a better understanding of the Twist1/c-myc regulatory axis in tumor development and highlight δ-catenin's involvement in proto-oncogenes and pro-EMT transcription factors [40]. Therefore, our findings underscore

the positive role of δ-catenin in PCa metastasis and emphasize the potential clinical significance of δ-catenin in conjunction with Twist1.

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Disclosure of conflict of interest

None.

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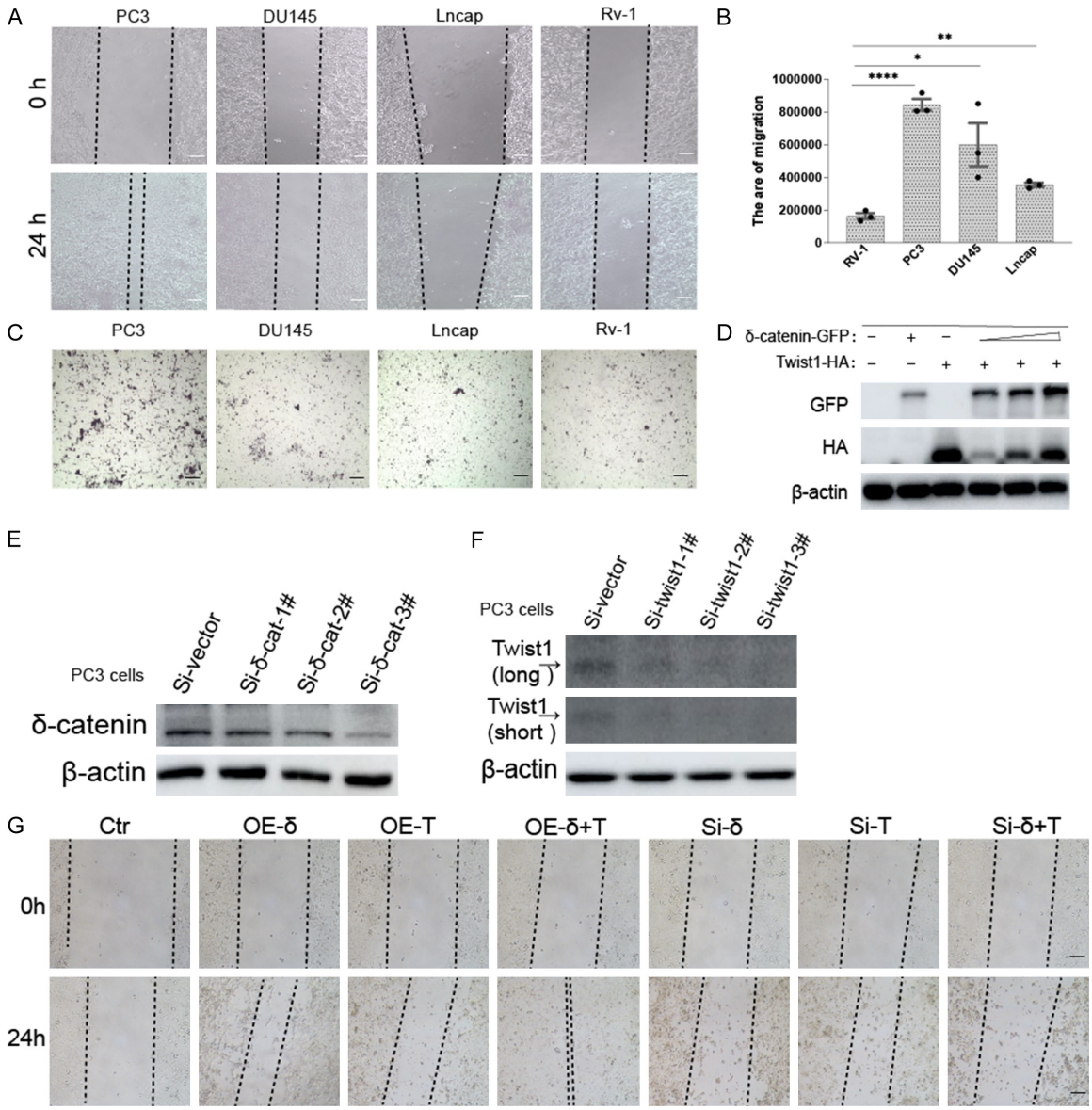

Supplementary Figure 1. Twist1 and δ-catenin are significantly unregulated in PCa tissues and indicate a poor prognosis. A, B. The wound-healing scratch assay was used to detect the cell migration ability of each group. Quantitative analysis of cell migration in three independent experiments (n = 3). Scale bar = 100 μm. C. Images depicting soft agar colony formation by PCs cells. Scale bar = 100 μm. D. Hek-293T cells were transfected with respective plasmids. Lysates were immunoblotted with indicated antibodies. Sample number, n = 3. E, F. PC3 silenced δ-catenin (or Twist1) or vector. Lysates were immunoblotted with indicated antibodies. Sample number, n = 3. G. PC3 that overexpressed δ-catenin (or, and Twist1) and silenced δ-catenin (or, and Twist1) were then used to detect the cell migration ability of each group by wound-healing scratch assay.