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Original Article

Effects of the carcinogen TCDD on δ-catenin stability and tumorigenic potential in prostate cancer cells

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Abstract: δ-Catenin is a member of the p120-catenin subfamily of armadillo proteins and is known to be upregulated in prostate cancer, promoting tumorigenesis. Unfortunately, the molecular mechanism underlying this effect remains unclear. The carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been linked to an increased risk of prostate cancer. In this study, we explored the effect of TCDD on δ-catenin in prostate cancer cells. TCDD increased the protein levels of δ-catenin in a dose-dependent manner by enhancing its stability. Moreover, TCDD led to an increase in β-catenin expression but a decrease in E-cadherin levels. Further experiments revealed that TCDD stabilized the expression of δ-catenin by inhibiting its ubiquitination-mediated degradation. Finally, TCDD enhanced the motility and migration ability of prostate cancer cells through δ-catenin. These findings suggest that TCDD plays a role in stabilizing δ-catenin in prostate cancer cells, offering a new perspective on preventing δ-catenin degradation and potentially increasing the predictive value of δ-catenin for prostate cancer.

Keywords: δ-catenin, prostate cancer, TCDD, ubiquitination sites

Introduction

Prostate cancer (PCa) is a hormone-related tumor dependent on androgen, which acts as a "prostate fuel" to stimulate the growth of prostate cancer cells and disease progression. Currently, "castration therapy" is used in clinical practice to regulate androgen levels and prevent prostate cancer. Worse, "androgen resistance" often occurs in patients with PCa at later stages of development [1]. According to data released by the World Health Organization (WHO) in 2020, prostate cancer is the third most common malignant tumor diagnosed after lung cancer and colorectal cancer. The number of new cases worldwide is up to 1.4 million, and the global burden of prostate cancer is expected to increase to nearly 2.3 million confirmed cases and 740,000 deaths by 2040 [2]. With the extension of life expectancy, a high-fat westernized diet [3], and the popularization of early screening methods for prostate cancer, its incidence and detection rate are increasing year by year, present a major threat to the health of adult males.

δ-Catenin, encoded by the CTNND2 gene, also known as NPRAP (neural plakophilin related armadillo protein), is a member of the catenin family of cell adhesion molecules, mainly functioning as a catenin in intracellular or cell connection-related processes. It belongs to the $p120^{ctn}$ subfamily and contains 10 armadillo repeats [4] (a motif consisting of 42 amino acids), indicating its ability to interact with many proteins. Previous studies have confirmed that δ-catenin interacts with E-cadherin [5], p0071 [6], Kaiso [7], Densin-180 [8], and other proteins. Initially discovered to bind to the loop region of presenilin-1 [9], which is highly expressed in brain tissue and promotes the growth of axons and dendrites of nerve cells [10], δ-catenin has been found to exhibit abnormal expression in various tumors including breast cancer [11], non-small cell lung cancer [12], ovarian cancer [13], colorectal cancer

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[14], esophageal squamous cell carcinoma [15], and liver cancer [16]. Human EST databases have also revealed δ-catenin mRNA expression in kidney, ovary, breast, and esophageal tumors [17]. It is noteworthy that δ-catenin expression is upregulated in 80% of prostate tumors and is positively correlated with the Gleason score [18]. It has been proposed as a potential biomarker for prostate cancer [19] and been related to tumor development.

The state and function of δ-catenin in PCa have been studied extensively in recent years. The overexpression of δ-catenin in prostate cancer cells has been fully demonstrated, and numerous studies have confirmed significantly increased levels of δ-catenin mRNA and protein in prostate cancer cells [20]. In cancer patients, δ-catenin has been found to accumulate in the interstitium, and significantly elevated levels of δ-catenin have been reported in urine [21]. These findings strongly support the use of δ-catenin as a diagnostic marker for PCa. For example, it has been suggested that glycogen synthase kinase 3 (GSK3) phosphorylates δ-catenin, negatively regulating its stability through ubiquitin-proteasome-mediated proteolysis [22]. Other studies have identified δ-catenin as a new member of the GSK3β signaling pathway, involved in β-catenin turnover in neurons [23]. The E3 ligase β-TrCP-1 has been found to mediate δ-catenin ubiquitination [24], and subsequent studies have reported that δ-catenin can degrade through lysosome-dependent pathways. However, protein degradation is inefficient when δ-catenin is overexpressed. Similar to p120^{ctn} [25], δ-catenin is phosphorylated by Src kinase at multiple tyrosine residues. Src-mediated phosphorylation enhances the ability of δ-catenin to induce the translocation of β-catenin to the nucleus [26]. In addition, Src kinase activity is essential for the E3 ligase Hakai to stabilize δ-catenin. Hakai inhibits δ-catenin ubiquitin-proteasome degradation by stabilizing Src and inhibiting the binding of δ-catenin to GSK3β [27]. Gene loci rearrangement of δ-catenin (CTNND2) is common in prostate cancer and may be the cause of δ-catenin upregulation [28]. Further research has demonstrated that δ-catenin promotes PCa cell growth and activates the β-catenin signaling pathway by altering the cell cycle and gene expression profile [29]. In addition, δ-catenin has been reported to promote angiogenesis by stabilizing HIF-1α and activating VEGF in CWR22Rv-1 (RV1) cell lines [30]. It is well known that δ-catenin activates the classical Wnt/β-catenin/LEF1 signaling pathway to mediate downstream target gene transcription [31], showing exon mutations and gene profiles that promote cancer cell survival and metabolism. In addition to the interaction between δ-catenin and p120^{ctn} and the classical Wnt-GSK3-β-catenin-LEF1 signaling pathway [32], the non-classical Wnt signaling pathway regulated by Kaiso transcription inhibitors is also a downstream target of δ-catenin and p120^{ctn} [33]. Taken together, these findings provide strong evidence supporting the important role of δ-catenin in the progression of PCa. Unfortunately, the specific mechanism through which δ-catenin promotes the progression of PCa and its main physiological functions in PCa are not yet clear.

The chemical 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) activate aryl hydrocarbon receptors (AhR), leading to toxicity. After TCDD binds to AhR, AhR is activated and dissociates from the Hsp90/XAP2/p23 chaperone protein complex. Subsequently AhR binds to a ligand and translocate to the nucleus to form an active heterodimer with ARNT (aromatic hydrocarbon receptor nuclear translocation protein) [34]. The TCDD/AhR/Arnt complex then acts as a transcription factor and binds to response elements (DRE or XRE) [35], leading to changes in gene transcription and resulting in toxic effects. Recently, AhR has been identified as a target gene of the putative Wnt/β-catenin classical pathway in prostate cancer cells [36]. Some studies have shown that TCDD-mediated activation of AhR may increase the risk of prostate cancer, especially in human and animal models of prostate cancer [37]. In addition, TCDD treatment can reduce the weight of the ventral prostate, seminal vesicle, and epididymis, as well as daily sperm production, in rats during uterine and lactation periods [38]. TCDD can also cause ventral prostate hypoplasia and dorsolateral prostate dysplasia in mice [39]. TCDD has been reported to mediate prostate cancer migration. However, it has also been found that treating androgen-sensitive and castration-resistant PCa cells with TCDD may inhibit AR signaling and prostate cancer progression [40]. Most *in vivo* and *in vitro* experiments indicate that TCDD can affect androgen receptor function and promote the develop-

ment of prostate cancer. For example, in animal experiments, long-term exposure to TCDD has been linked to tumors in the liver, thyroid, lung, prostate, skin, mouth, and ovary [41]. In conclusion, TCDD plays a crucial role in prostate cancer, and further research is needed to elucidate its biological function in tumorigenesis.

This study utilized the overexpression of δ-catenin in PCa cells as the *in vitro* cell culture model to explore whether TCDD could stabilize δ-catenin expression by regulating the ubiquitination of Lys1049 and Lys1158 to reduce its degradation. The findings revealed that TCDD regulated the ubiquitination of δ-catenin to antagonize the proteasome degradation, increase δ-catenin stability, and promote its nuclear localization. These observations have important implications for understanding the biological processes of prostate cancer.

Materials and methods

Plasmids

The construction of green fluorescent protein (GFP)-δ-Catenin was previously described by Kim *et al.* [42]. The HA-tagged Ub was kindly provided by Professor Kwang Youl Lee from Chonnam National University.

Antibodies and reagents

The following antibodies were used in this study: anti-δ-Catenin (#611537; BD Bioscience, San Diego, CA, USA), anti-GFP (#G1544; Sigma-Aldrich, St. Louis, MO, USA), anti-LaminB (#SC-374015, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-HA-Tag (#SC-7392, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-p-GSK3 α/β (#9331, Cell Signaling Technology, Danvers, MA, USA), anti-Ubiquitin (#SC-47721, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-β-actin (#A5441; Sigma-Aldrich, St. Louis, MO, USA), and anti-GSK3 α/β (#5676, Cell Signaling Technology, Danvers, MA, USA). The HA epitope was detected using media from 12CA5 hybridomas provided by Professor Kwang Youl Lee from Chonnam National University. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (#1746-01-6) was obtained from Sigma-Aldrich.

Cell culture, transfection and TCDD treatment

CWR22Rv-1 (human prostate cancer cell line, RV1) and PC3 cells were cultured in Roswell Park Memorial Institute medium (RPMI), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO_2. RV/δ and RV/C cells, which overexpress mouse GFP-δ-Catenin and GFP, respectively, were derived from RV1 cells and grown in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, and 125 μg/mL G418 (Sigma, St Louis, MO) at 37°C with 5% CO_2. RV1 cells were transfected using PEI (#24765-2; PolyScience Inc; Warrington, PA, USA) according to the manufacturer's instructions. After 24 h, the cells were treated with 100 nM TCDD for the indicated time periods.

Western blot and immunoprecipitation

Cell lysate preparation and western blot analyses were performed as described by Shrestha *et al.* [24]. Briefly, cells were harvested using lysis buffer (MLB) (10% glycerol, 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture). Protein concentration was determined using a BCA kit (Pierce, Rockford, IL, USA). The lysates were separated on a sodium dodecyl sulfate (SDS) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk and incubated with the appropriate primary antibodies, followed by a secondary antibody. Finally, protein bands were visualized using an enhanced chemiluminescence assay (Millipore; Billerica, MA, USA).

For immunoprecipitation, the cell lysates were obtained as described above and incubated with a primary antibody according to the manufacturer's protocol for 16 hours at 4°C and purified using protein G Sepharose beads (GE Healthcare, Uppsala, Sweden). The resulting immune complexes were washed three times and denatured at 95°C for 2 minutes with 15 μL of 2x 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). Subsequently, the samples were loaded onto an SDS gel for immunoblotting.

CHX treatment

We treated prostate cancer cells with the protein synthesis inhibitor cycloheximide (CHX; 100 μg/mL) and collected cells at various time

Table 1. Primers used for qRT-PCR analysis of RV/C and RV/δ cells

Gene	Primer sequences (5' to 3')
β-Actin	Forward CACTGCAAACGGGGAAATGG
	Reverse TGAGATGGACTGTCGGATGG
δ-catenin	Forward GGTGCATGTTTGCGAGGAAGC
	Reverse ATGGGCGAGCTGGTGCTGTAGGAC

points (0, 3, 6, 9, and 12 h) to investigate the half-life of the δ-catenin protein in RV/δ cells after TCDD treatment.

Wound healing scratch assay

The wound healing scratch test was used to determine cell migration in RV1 cells treated with 100 nM TCDD for 24 h. Briefly, the cells were plated in 6-well plates and incubated in medium with 2.5% FBS until reaching full confluency. Subsequently, the cells were scratched using a sterile 100-μL pipette tip. The medium was replaced with phosphate-buffered saline (PBS), and the wound gap was photographed using a microscope (Nikon, Japan) at 0 h and 24 h. Quantitative analysis of cell migration was repeated in 3 independent experiments (n = 3).

CCK8 assay

RV1 and RV/δ cells treated with 0, 50, 100, and 200 nM TCDD for 24 h were digested using trypsin. The cell concentration was adjusted to 5000 cells/mL. Then, 200 μL of this cell suspension was transferred into 96-well plates. Each group had three parallel control wells. After the addition of the CCK8 reagent (Thermo Fisher Scientific, Waltham, MA, USA), the cells were incubated at 37°C for 4 h. The plates were shaken for an additional 10 min. Absorbance values at 490 nm were measured using a Microplate Reader (Bio-Rad Co., Singapore).

Cell fractionation

Nuclear and cytoplasmic proteins from RV/δ cells treated with 100 nM TCDD for 24 h were separated using the Nuclear and Cytoplasmic Extraction Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, the cells were harvested with trypsin-EDTA and centrifuged at 500 × g for 5 min. The supernatant was then removed carefully, and the cell pellet was washed with PBS, followed by centrifugation at 500 × g for 3 min. The supernatant was discarded carefully again to leave the cell pellet as dry as possible. Next, specific buffers provided by the kit were added into tubes containing the cell pellets to obtain cytoplasmic and nuclear proteins separately. Protein extracts from different cell compartments were subjected to immunoblotting. Lamin B was used as a nuclear protein marker, while β-actin was used as a cytoplasmic protein marker.

RNA isolation and qRT-PCR

RV/C and RV/δ cells treated with 100 nM TCDD for 24 h were used for qRT-PCR analysis. RNA isolation, semi-quantitative Reverse Transcriptase PCR (sqRT-PCR), and quantitative Reverse Transcription PCR (qRT-PCR) were performed. Total RNA was isolated from cells using TRIzol Reagent (15596018; Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using a high-capacity cDNA synthesis kit (4374967; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, sqRT-PCR was conducted in a thermal cycler (T100, Bio-Rad, Hercules, CA, USA). The PCR signal intensity of each gene was visualized through electrophoresis in agarose gels and EtBr Imaging (GelDoc XR+; Bio-Rad, Hercules, CA, USA). In addition, qRT-PCR was performed for indicated genes using SYBR Green (04887352001, Roche, Basel, Switzerland). The PCR thermal cycling included a denaturing step at 95°C for 10 min, followed by 45 cycles of incubations at 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. The target gene expression was normalized to β-actin gene expression. Gene-specific primer sequences used for qRT-PCR are listed in **Table 1**.

Clonogenic assay

RV1 cells treated with 100 nM TCDD for 24 h (5×10^3 cells) were seeded on a 6-well soft agar plate, maintained at 37°C for 10 days, washed with PBS, and stained with Giemsa solution. The number of colonies containing more than 50 cells was used to determine the colony formation efficiency as follows: colony formation efficiency = numbers of colonies/numbers of cells seeded.

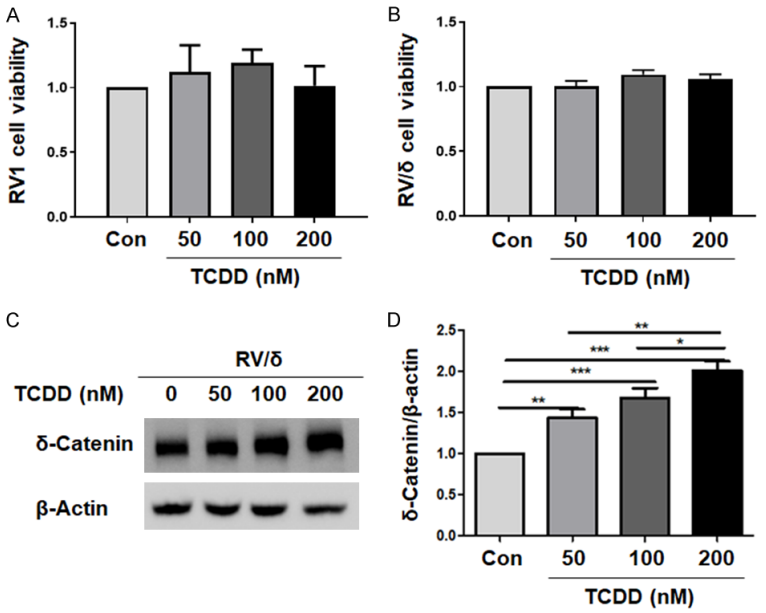


Figure 1. TCDD increased δ-catenin expression in a dose-dependent manner. (A, B) The survival rates of RV1 (A) and RV/δ (B) cells treated with TCDD at different concentrations (0, 50, 100, and 200 nM) were determined using the CCK-8 assay. There was no significant difference. (C) Representative blots of RV/δ cells treated with different TCDD concentrations (0, 50, 100, and 200 nM) for 24 h, showing δ-catenin and β-actin levels. (D) Quantitative analysis of δ-catenin expression levels in RV/δ cells treated with different TCDD concentrations (0, 50, 100, and 200 nM) for 24 h. Data are expressed as the mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). The data are expressed as the mean ± SE. ANOVA with a student's t-test was used for data analysis. Comparisons between two groups were performed using a student's t-test. One-way or two-way analyses of variance (ANOVA) with post-Bonferroni corrections were used to compare the effect of various treatments or to assess the effect of treatment over time. *P* values below 0.01 were considered statistically significant.

In the Single Exponential Decay Model, blot data were quantified using Quantity One Software (BioRad). A linear model was used for statistical analysis. The protein degradation rate was determined using a one-phase exponential decay model with the equation: $Y(t) = Y_0 \times \exp(-K \times t)$, where K represents the exponential decay constant, and span is the difference between the initial protein data and the plateau of protein levels.

Results

TCDD increased δ-catenin expression in RV/δ cells in a dose-dependent manner

To study the effects of TCDD on the survival of RV1 and RV/δ cells, we treated these cells with varying concentrations of TCDD (0, 50, 100, and 200 nM) for 24 h and assessed their survival using CCK-8. The results revealed that the viability of RV1 and RV/δ cells were largely unaffected after TCDD treatment (**Figure 1A**, **1B**). A previous study by Kim *et al.* [43] has reported low levels of the δ-catenin protein expression in RV1 cells. Therefore, RV/δ cells with a stable δ-catenin expression level were examined to explore the effect of TCDD concentration on δ-catenin expression levels. The results showed that RV/δ cells treated with varying concentrations of TCDD (0, 50, 100, and 200 nM) for 24 h exhibited upregulated δ-catenin expression in a dose-dependent manner (**Figure 1C**, **1D**). These results indicated that TCDD treatment had no significant effect on prostate cancer cell viability but upregulated δ-catenin expression in RV/δ cells in a dose-dependent manner.

TCDD increased the expression of both exogenous and endogenous δ-catenin

To investigate the effects of TCDD on endogenous and exogenous δ-catenin expression, two types of prostate cancer cell lines were utilized: RV1 and RV/δ (RV1 cells that overexpress mouse δ-catenin-GFP). Following treatment with the same concentration of TCDD for 24 h, δ-catenin levels were evaluated through western blot analysis. TCDD treatment increased δ-catenin expression levels in both RV1 and RV/δ cells (**Figure 2A**, **2B**). Quantitative analysis of endogenous δ-catenin levels in RV1 and exogenous δ-catenin levels in RV/δ revealed that TCDD significantly upregulated both forms

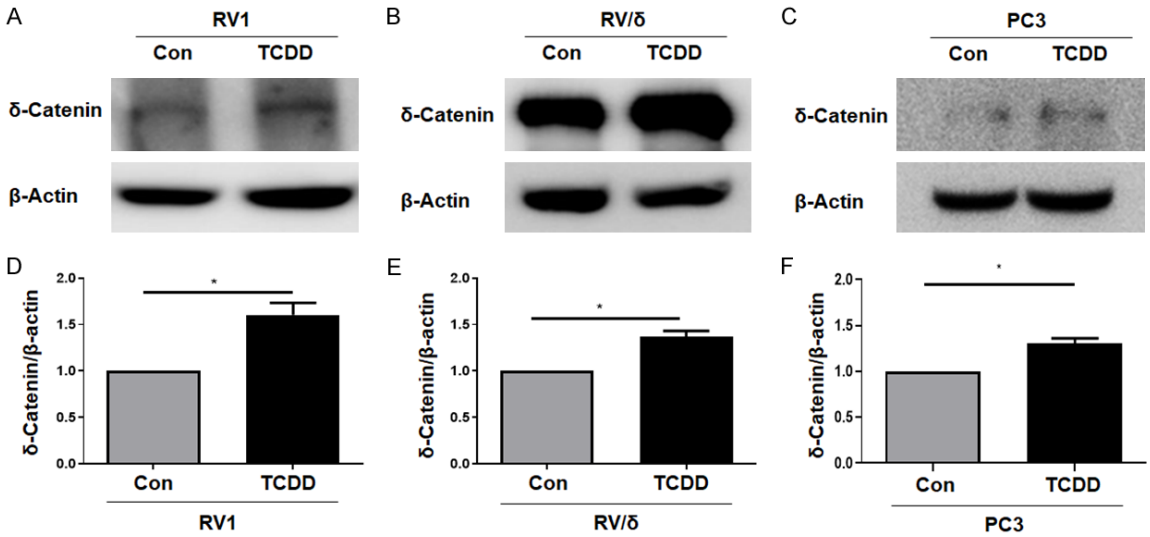


Figure 2. TCDD increased both the exogenous and endogenous expression of δ-catenin. (A-C) The representative blots of δ-catenin and β-actin in RV1 (A), RV/δ (B), and PC3 (C) cells that were either untreated or treated with 100 nM TCDD for 24 h. (D-F) Quantitative analysis of exogenous δ-catenin expression levels in RV1 (D), RV/δ (E) and PC3 (F) cells that were either untreated or treated with 100 nM TCDD for 24 h. Data are expressed as the mean ± SD (n = 3). *P < 0.05.

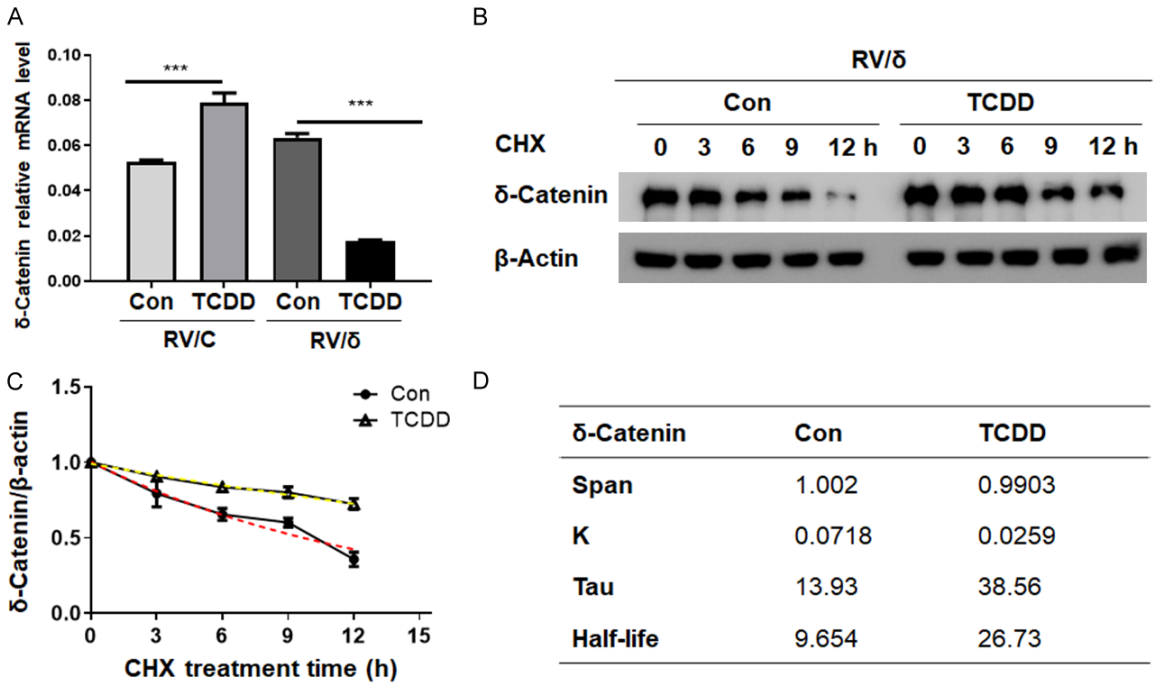
of δ-catenin expression in prostate cancer cells (**Figure 2D**, **2E**). Moreover, another prostate cancer cell line, PC3 cells, showed similar results to RV1 cells (**Figure 2C**, **2F**). These findings indicated that TCDD increased the exogenous and endogenous δ-catenin expression levels in prostate cancer cells.

TCDD increased the protein levels of δ-catenin by enhancing its stability

Previous results indicated that TCDD elevated the protein levels of δ-catenin in RV/δ cells. We determined the effect of TCDD on δ-catenin protein expression, but its effect on the mRNA levels of δ-catenin is not clear. To address this, we conducted Real-Time Quantitative Reverse Transcription (qRT-PCR) to assess the δ-catenin mRNA levels in RV/C and RV/δ cells. We found that δ-catenin mRNA levels in RV/δ cells significantly decreased, while those in RV/C cells significantly increased after TCDD treatment (**Figure 3A**). These results suggested that TCDD promoted the upregulation of δ-catenin protein expression through posttranslational modification rather than transcription. Moreover, these results suggested that TCDD enhanced the δ-catenin protein stability. To verify that TCDD regulates δ-catenin levels through post-transcriptional modification in RV/δ cells, cells were treated with 100 nM TCDD for 24 h and then exposed to 100 μg/mL CHX, an inhibitor of protein-synthesis, at different time points (0, 3, 6, 9, and 12 h). Compared with the control group, the half-life of δ-catenin in the TCDD group was significantly elevated (**Figure 3B-D**), confirming the role of TCDD in stabilizing the δ-catenin protein. In conclusion, TCDD stabilized δ-catenin through post-translational modification, reducing its degradation and upregulating its protein expression.

TCDD induced the translocation of δ-catenin from the cytoplasm to the nucleus

Considering the nuclear localization of stable β-catenin, which shares a homologous structure with δ-catenin, we investigated the subcellular localization of δ-catenin. Protein separation kits were used to separate and extract proteins from the cell membrane, cytoplasm, and nucleus. Western blot analysis was conducted to evaluate δ-catenin protein expression in each compartment (**Figure 4A**). TCDD significantly enhanced the expression of δ-catenin in the nucleus while decreasing its expression in the cytoplasm (**Figure 4B**). These observations indicated that TCDD treatment promoted δ-catenin translocation from cytoplasm to the nucleus, increasing the nuclear levels of δ-catenin.



δ-Catenin	Con	TCDD
Span	1.002	0.9903
K	0.0718	0.0259
Tau	13.93	38.56
Half-life	9.654	26.73

Figure 3. TCDD increased δ-catenin protein level by enhancing its stability. A. mRNA levels of δ-catenin were measured using qRT-PCR in RV/C and RV/δ cells that were either untreated or treated with 100 nM TCDD for 24 h. B. Representative blots of δ-catenin and β-actin in RV/δ cells that were either untreated or treated with 100 nM TCDD for 24 h, followed by treatment with 100 μg/mL CHX for different durations (0, 3, 6, 9, and 12 h). C. One Phase Exponential Decay Model was used to analyze δ-catenin protein expression using paired T-tests. D. The formula $Y(t) = Y_0 \times \exp(-K \times t)$ was used to analyze the half-life of δ-catenin protein in the control group and the TCDD group. K is the exponential decay constant, and span is the difference between the initial protein data and the plateau of protein levels. Data are expressed as the mean ± SD (n = 3). ***P < 0.001.

TCDD increased β-catenin levels but decreased E-cadherin levels in prostate cancer cells

E-cadherin is an important cell adhesion molecule, and its reduced expression or inactivation is often associated with tumor progression. E-cadherin downregulation is a hallmark of epithelial-mesenchymal transition (EMT), a process linked to cell metastasis and invasion. β-catenin, a key component of the classical Wnt signaling pathway, promotes androgen signaling by binding to AR proteins and enhances AR-mediated activation of androgen to regulate gene transcription. β-catenin also plays an important role in the Wnt signaling pathway, cell adhesion, and cancer. In order to explore β-catenin and E-cadherin expression in prostate cancer cells after δ-catenin entry into the nucleus, RV/δ cells were treated with different concentrations of TCDD (0, 50, 100, and 200 nM) for 24 h, and the protein levels of β-catenin and E-cadherin were determined using western blot analysis (**Figure 5A**). TCDD induced a significant, dose-dependent upregulation in β-catenin levels in RV/δ cells (**Figure 5B**). E-cadherin expression, on the other hand, was significantly downregulated in a dose-dependent manner (**Figure 5C**). These observations suggested that β-catenin expression was upregulated after δ-catenin entry into the nucleus, while E-cadherin expression was downregulated.

TCDD stabilized δ-catenin expression in a manner independent of GSK3α/β phosphorylation

δ-Catenin can be phosphorylated by GSK3α/β, and its expression is negatively regulated through a ubiquitin-proteasome-mediated protein degradation pathway [22]. Therefore, we hypothesized that TCDD could improve δ-catenin protein stability by inhibiting GSK3α/β phosphorylation. RV/δ and RV/C cells were treated with 100 nM TCDD for 24 h, and GSK3α/β phosphorylation and δ-catenin pro-

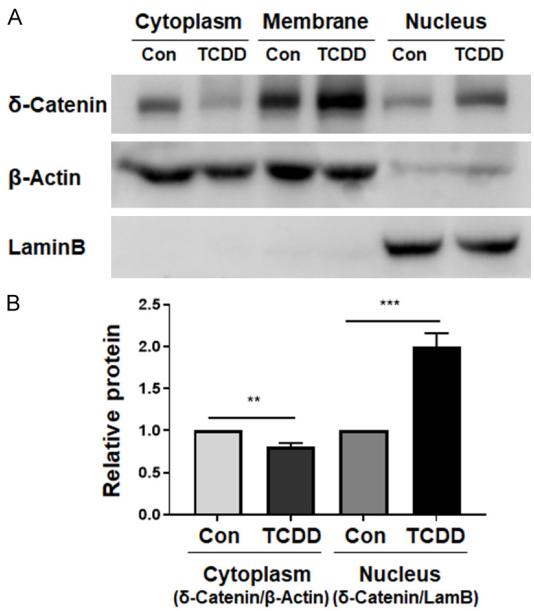


Figure 4. TCDD induced the translocation of δ-catenin from the cytoplasm to the nucleus. A. Representative blots of δ-catenin, β-actin, and Lamin B, illustrating δ-catenin protein expression in the cell membrane, cytoplasm, and nucleus of RV/δ cells after TCDD treatment. B. Quantitative analysis of δ-catenin expression level in the cytoplasm and nucleus of RV/δ cells after 100 nM TCDD treatment for 24 h. Data are expressed as the mean ± SD (n = 3). $^{**}P < 0.01$; $^{***}P < 0.0001$.

tein expression were detected using western blot analysis (**Figure 6A**). Quantitative analysis of δ-catenin protein expression in RV/δ cells revealed that TCDD significantly upregulated δ-catenin protein expression (**Figure 6B**). However, no significant changes in GSK3α/β phosphorylation levels were observed after TCDD treatment (**Figure 6C**, **6D**). These results have excluded the possibility that TCDD may stabilize δ-catenin protein expression through a GSK3α/β phosphorylation-dependent pathway. Therefore, the hypothesis that TCDD improves the stability of δ-catenin protein by inhibiting GSK3α/β phosphorylation is not supported.

TCDD stabilized δ-catenin expression by inhibiting its degradation through the ubiquitin-proteasome pathway

As the hypothesis that TCDD enhances δ-catenin protein stability by inhibiting GSK3α/β phosphorylation was not supported by our results, we considered the possibility that TCDD might stabilize δ-catenin expression by inhibiting its degradation through the ubiquitin-proteasome pathway. To investigate the relationship between TCDD and δ-catenin ubiquitination, RV/δ cells were treated with 10 μM MG132 and 100 nM TCDD. First, δ-catenin ubiquitination in RV/δ cells was assessed through an IP assay (**Figure 7A**). In addition, δ-catenin ubiquitination was confirmed through an IP assay with HA-Ub plasmid transfection (**Figure 7C**). We found that RV/δ cells treated with 100 nM TCDD exhibited a significant reduction in δ-catenin-bound ubiquitin molecules (**Figure 7B**). Moreover, RV/δ cells transfected with HA-Ub plasmid also showed a significant decrease in ubiquitin-bound δ-catenin (**Figure 7D**). Therefore, IP experiments from both positive and reverse perspectives confirmed that TCDD stabilized δ-catenin expression by inhibiting the ubiquitin-proteasome degradation pathway. These observations indicated that TCDD inhibited the ubiquitin-proteasome-mediated degradation of δ-catenin in RV/δ cells and stabilized its expression. However, the specific ubiquitination sites targeted by TCDD remains unclear and need to be further explored.

TCDD acted on the δ-catenin ubiquitination sites Lys1049/1158 to stabilize it

The δ-catenin ubiquitination site is located between the residues 1040 and 1070, and the main ubiquitination sites are Lys1049 and Lys1158 [24]. To determine the specific ubiquitination sites targeted by TCDD, RV1 cells were transfected with full-length δ-catenin FL-GFP-δ-catenin or one of the δ-catenin mutants FL-KK1049/1050RR and D1070-1140 KKK1049/1050/1158RRR, and then treated with 100 nM TCDD for 24 h. The expression of δ-catenin protein after transfection with different plasmids was detected through western blot analysis (**Figure 8A**, **8C**). Interestingly, we found that TCDD significantly stabilized δ-catenin in cells transfected with full-length GFP-δ-catenin. However, in cells transfected with the δ-catenin mutants FL-KK1049/1050RR and D1070-1140 KKK1049/1050/1158RRR, the stabilizing effect of TCDD on δ-catenin was reversed (**Figure 8B**, **8D**). After transfection with the δ-catenin mutants FL-KK1049/1050RR and D1070-1140 KKK1049/1050/1158RRR, TCDD was not able to stabilize δ-catenin protein expression by targeting the ubiquitination

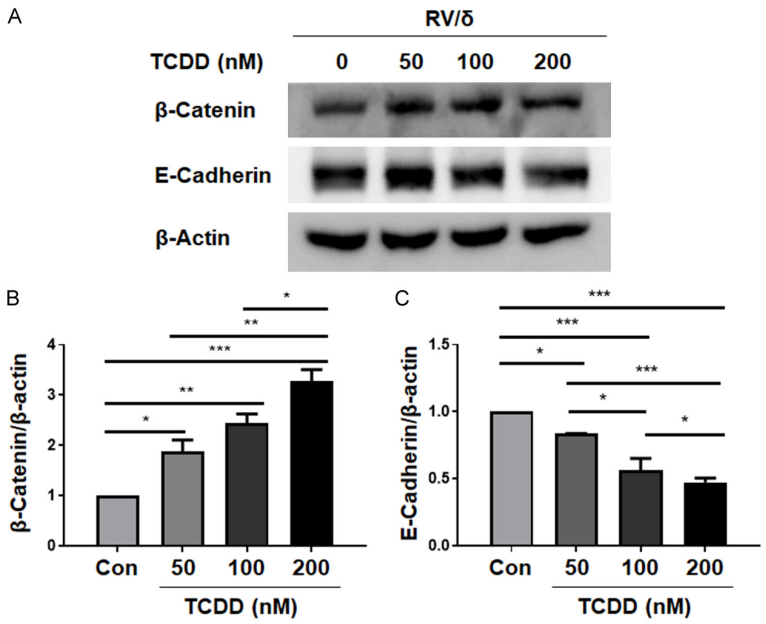


Figure 5. TCDD increased β-catenin levels and decreased E-cadherin levels in prostate cancer cells. (A) Representative blots of β-catenin, E-cadherin, and β-actin in RV/δ cells treated with 0, 50, 100, and 200 nM TCDD for 24 h. (B, C) Quantitative analysis of β-catenin (B) and E-cadherin (C) expression levels in RV/δ cells treated with 0, 50, 100, and 200 nM TCDD for 24 h. Data are expressed as the mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

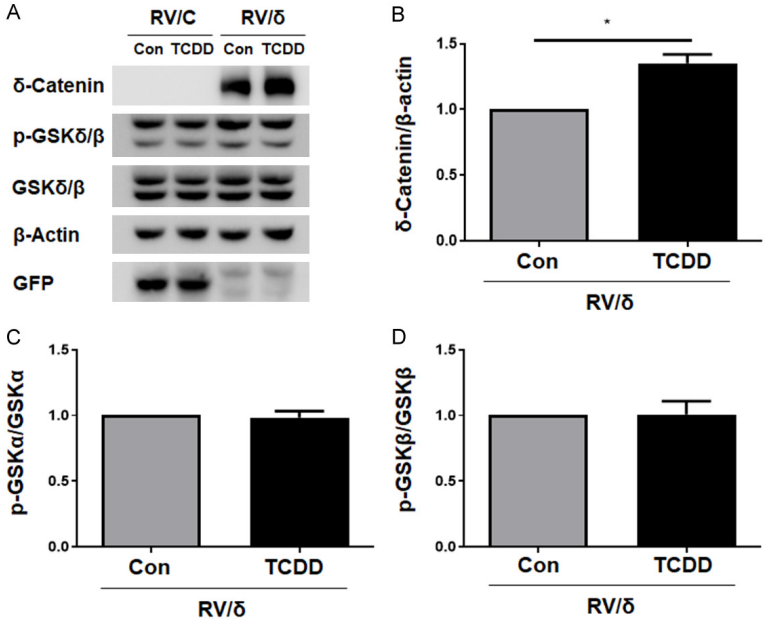


Figure 6. TCDD stabilized δ-catenin expression in a GSK3α/β phosphorylation-independent manner. (A) Representative blots of δ-catenin, GSK3α/β, p-GSK3α/β, β-actin, and GFP. RV/C and RV/δ cells were treated with 100 nM TCDD for 24 h. (B-D) Quantitative analysis of δ-catenin (B), p-GSK3α (C), and p-GSK3β (D) expression levels. The p-GSK3α and p-GSK3β expression levels revealed no statistically significant difference. Data are expressed as the mean ± SD (n = 3). *P < 0.05.

sites Lys1049 and Lys1158. Previous studies have shown that δ-catenin in prostate cancer cells can undergo degradation by two degradation pathways: ubiquitin-proteasome degradation and lysosomal degradation [24]. It can be speculated that δ-catenin protein expression in prostate cancer cells decreases due to degradation through these two pathways [24]. Our findings demonstrated, for the first time, that TCDD acted on the major δ-catenin ubiquitination sites, Lys1049 and Lys1158, inhibiting their ubiquitination and reducing degradation to stabilize the expression of the δ-catenin protein. However, we cannot rule out the possibility of other potential ubiquitination sites affected by TCDD.

TCDD increased the motility of prostate cancer cells

In order to examine whether TCDD promotes the migration of prostate cancer cells *in vitro*, we performed a scratch experiment after treating RV1 cells with 100 nM TCDD for 24 h (**Figure 9A**). The results showed that TCDD significantly increased the rate of wound healing in RV1 cells compared with the control group (**Figure 9B**), indicating that TCDD promoted the migration of RV1 cells *in vitro*.

TCDD increased the proliferation of prostate cancer cells

To investigate the effect of TCDD on the proliferation of prostate cancer cells *in vitro*, clonogenic assays were performed on RV1 cells treated with 100 nM TCDD for 24 h (**Figure 10A**). The results showed that TCDD significant-

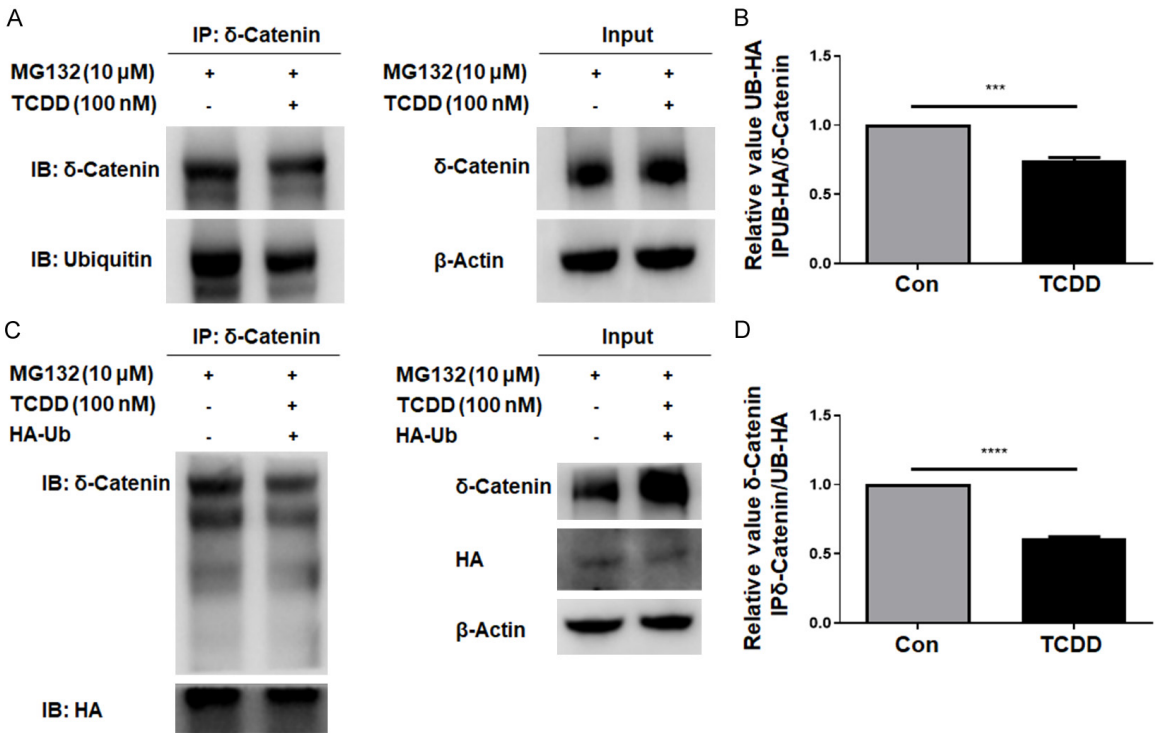


Figure 7. TCDD stabilized δ-catenin expression by inhibiting its ubiquitin-proteasome-mediated degradation. A. Representative δ-catenin-bound ubiquitin IP assay in RV/δ cells treated with 10 μM MG132 and 100 nM TCDD. B. Quantitative analysis of δ-catenin-bound ubiquitin IP assay. C. Representative IP assay in RV/δ cells transfected with HA-Ub plasmid and treated with 10 μM MG132 and 100 nM TCDD. D. Quantitative analysis of ubiquitin-bound δ-catenin IP assay. Data are expressed as the mean ± SD (n = 3). ***P < 0.001; ****P < 0.0001.

ly increased the proliferation rate of RV1 cells compared with the control group (**Figure 10B**), indicating that TCDD promoted the proliferation ability of RV1 cells.

A proposed model illustrating how TCDD enhances δ-catenin stability

Based on these findings, we proposed a model of TCDD-induced δ-catenin stabilization (**Figure 11**). As shown in the figure, TCDD acted on the δ-catenin ubiquitination sites Lys1049/1158, inhibiting its ubiquitination and reducing its degradation, thus stabilizing δ-catenin. We ruled out the possibility that TCDD stabilizes δ-catenin through the GSK3α/β phosphorylation-dependent pathway. TCDD induced the translocation of overexpressed δ-catenin from the cytoplasm to the nucleus. It also upregulated β-catenin expression while downregulating E-cadherin expression. Finally, TCDD significantly promoted the proliferation and migration of prostate cancer cells, exhibiting a protumor effect. In summary, TCDD regulated the δ-catenin ubiquitination sites in prostate cancer cells, inhibited ubiquitin-proteasome-mediated δ-catenin degradation, stabilized δ-catenin expression, and promoted cancer progression.

Discussion

Prostate cancer is one of the most common malignant tumors among elderly men. Therefore, it is crucial to understand its pathogenesis and develop new early treatment methods. δ-catenin is a protein specific to nerve cells [17]. Previous studies have shown that the absence of δ-catenin is associated with the severity of psychiatric impairments in individuals with Cri-du-Chat syndrome (CDCS) [44].

Recent studies have shown that δ-catenin protein expression is upregulated and positively correlated with the Gleason score in 80% of prostate tumors. In particular, the expression of δ-catenin in prostate cancer is closely associated with tumor progression, malignancy, mortality, and recurrence. This makes δ-catenin a promising target for the treatment of prostate cancer and underscores its importance as

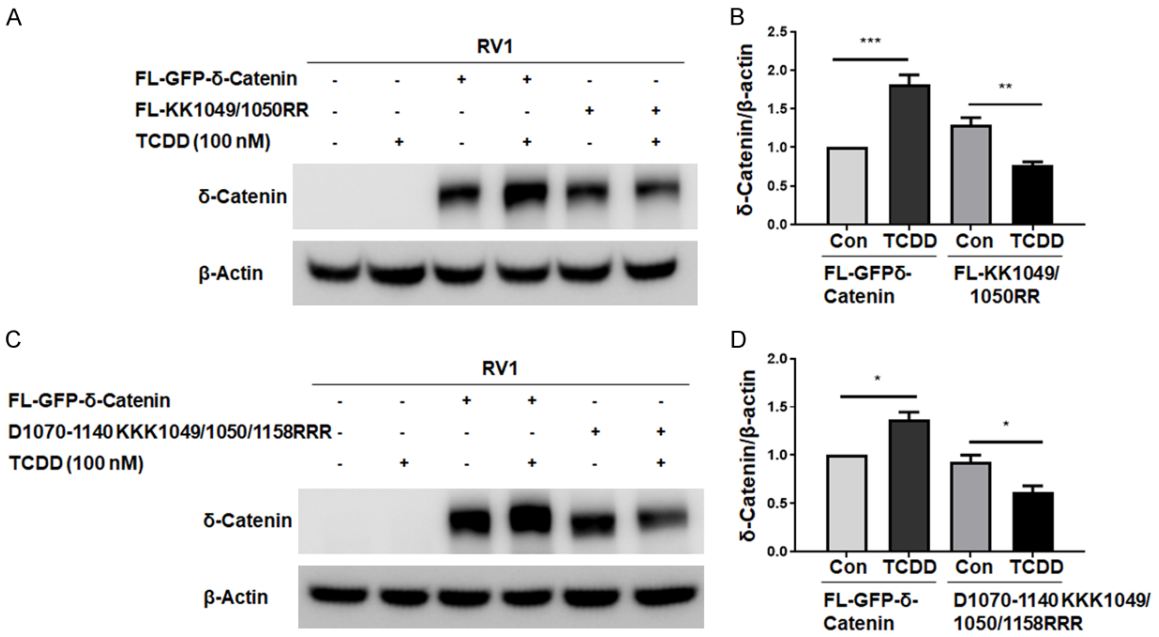


Figure 8. TCDD acted on the δ-catenin ubiquitination sites Lys1049/1158 to stabilize δ-catenin. (A) Representative blots of δ-catenin and β-actin in RV1 cells transfected with FL-GFP-δ-catenin or FL-KK1049/1050RR (FL-GFP-δ-catenin mutant) and treated with 100 nM TCDD for 24 h. (B) Quantitative analysis of δ-catenin expression levels shown in (A). (C) Representative blots of δ-catenin and β-actin in RV1 cells transfected with FL-GFP-δ-catenin or D1070-1140 KKK1049/1050/1158RRR (FL-GFP-δ-catenin mutant) and treated with 100 nM TCDD for 24 h. (D) Quantitative analysis of δ-catenin expression levels shown in (C). Data are expressed as the mean ± SD (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

a marker for prostate cancer [20]. Our previous studies have shown that δ-catenin transcription and protein expression are significantly upregulated in prostate cancer. δ-catenin can promote the growth and progression of prostate cancer cells through various mechanisms. In addition, previous studies identified a strong negative regulator that modulates δ-catenin expression in prostate cancer cells, resulting in low endogenous expression of delta-catenin. Therefore, prostate cancer cell lines, including the human prostate cancer-derived cell line CWR22Rv-1 (RV1), RV1 cells overexpressing GFP-δ-catenin (RV/δ), and RV1 cells overexpressing GFP (RV/C), were cultured *in vitro* to establish cell models of prostate cancer in this study.

The compound TCDD has been implicated in cancer [45]. Studies have shown that exposure to TCDD reduces sperm count, gonad weight, and serum testosterone levels, interfering with prostate development in rats. It is proposed that TCDD, herbicides, and other lipophilic substances can mediate the progression of prostate cancer. Several studies have indicated a strong correlation between TCDD and prostate cancer, highlighting the need for further research to clarify its biological function in prostate cancer progression.

Previous research has indicated that δ-catenin degradation depends on the ubiquitin-proteasome degradation pathway. Therefore, this study investigated whether TCDD affects δ-catenin degradation by interfering with its ubiquitination. Ubiquitination is a prevalent post-translational modification, playing a crucial role in various cellular processes such as proteolysis and signal transduction. The process of ubiquitination, which regulates protein degradation, is a three-step cascade reaction that requires the participation of multiple enzymes. UB binding to target proteins is catalyzed by ubiquitin-activating enzyme E1, ubiquitin-binding enzyme E2, and ubiquitin-ligase E3 [46]. While the ubiquitin-proteasome pathway is a common mechanism for endogenous protein degradation, not all ubiquitination modifications lead to degradation. Certain ubiquitination modifications can change protein activity and lead to other biological effects, such as

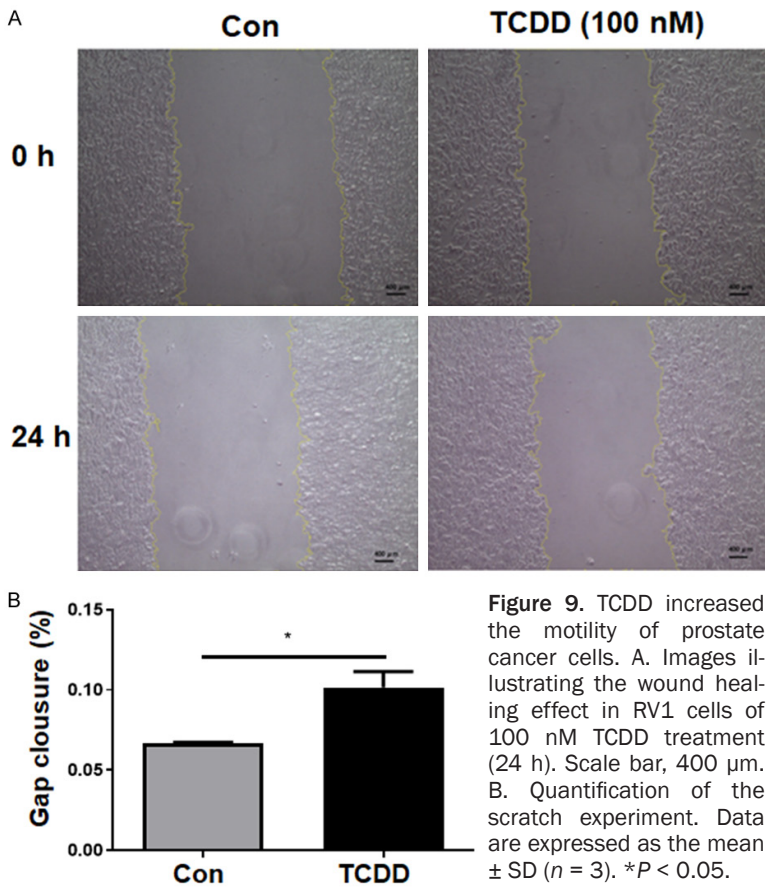


Figure 9. TCDD increased the motility of prostate cancer cells. A. Images illustrating the wound healing effect in RV1 cells of 100 nM TCDD treatment (24 h). Scale bar, 400 μm. B. Quantification of the scratch experiment. Data are expressed as the mean ± SD (n = 3). *P < 0.05.

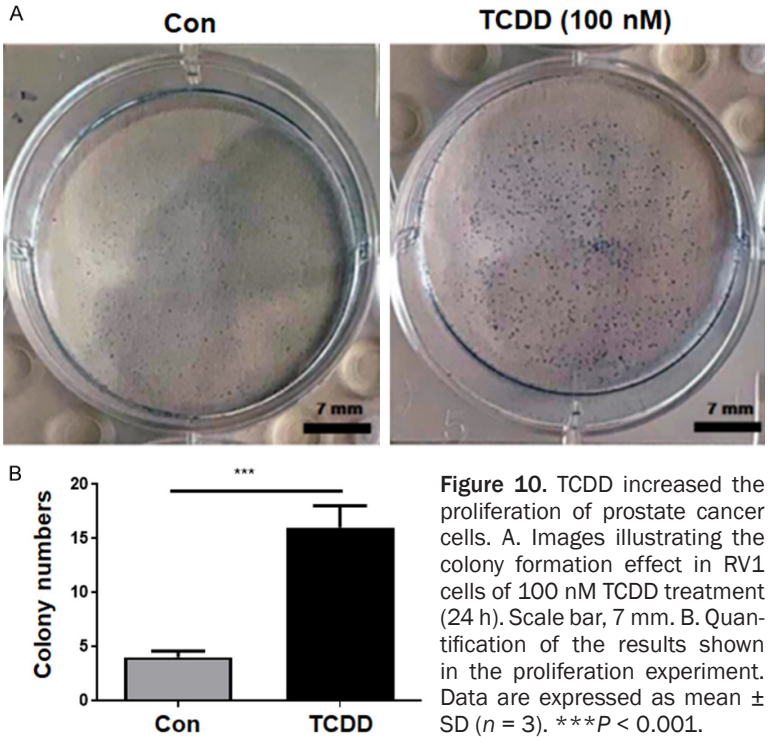


Figure 10. TCDD increased the proliferation of prostate cancer cells. A. Images illustrating the colony formation effect in RV1 cells of 100 nM TCDD treatment (24 h). Scale bar, 7 mm. B. Quantification of the results shown in the proliferation experiment. Data are expressed as mean ± SD (n = 3). ***P < 0.001.

DNA damage repair and immune response [47].

This study first found that TCDD treatment had no significant effect on the viability of prostate cancer cells. However, after TCDD treatment, the levels of δ-catenin protein, expressed both exogenously and endogenously, increased in prostate cancer cells in a dose-dependent manner. Interestingly, δ-catenin mRNA levels were not affected, indicating that TCDD regulated δ-catenin levels post-translationally rather than acting at the transcriptional level. These results suggest that TCDD increases the protein level of δ-catenin by enhancing its stability. A discrepancy arises when comparing the δ-catenin expression levels in the western blot (**Figure 2**) with the δ-catenin mRNA levels in qRT-PCR (**Figure 3A**). However, the mechanism underlying the difference is not fully understood. It is plausible to speculate that TCDD may increase δ-catenin protein levels by enhancing its stability through an unknown mechanism. Therefore, future research on this unknown mechanism is considered necessary.

Furthermore, TCDD enhanced the stability of δ-catenin and significantly increased δ-catenin levels in the nucleus while decreasing its levels in the cytoplasm, indicating the translocation of δ-catenin to the nucleus. In addition, TCDD treatment upregulated β-catenin expression but downregulated E-cadherin expression. Previous studies have revealed that δ-catenin can be

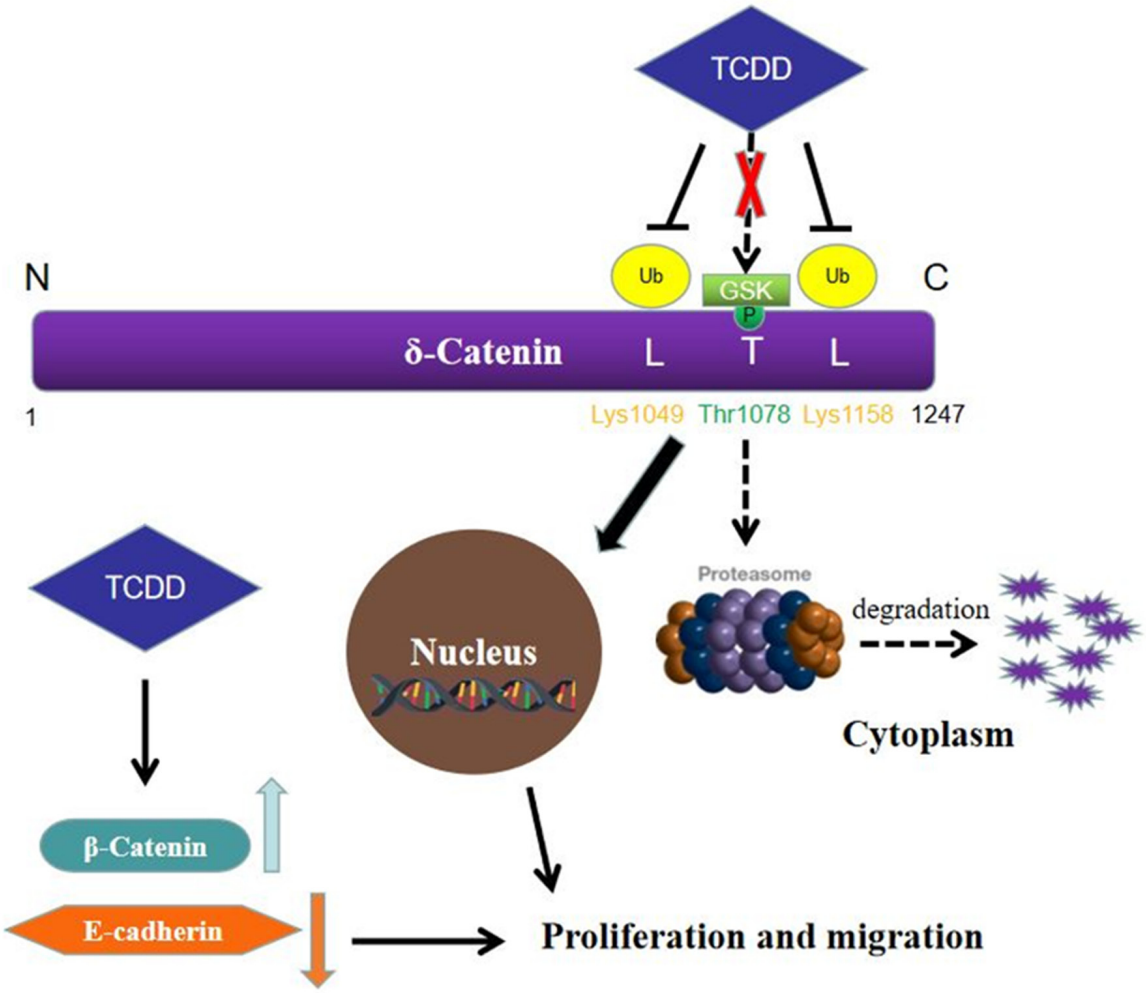


Figure 11. Potential mechanism of TCDD induced δ-catenin stabilization.

phosphorylated by GSK-3α/β and its expression is negatively regulated through the ubiquitin-proteasome-mediated protein degradation pathway. Therefore, this study hypothesized that TCDD inhibits GSK-3α/β phosphorylation and thus improves the stability of δ-catenin. However, no significant changes in GSK-3α/β phosphorylation levels were observed after TCDD treatment. Prostate cancer cells transfected with δ-catenin mutant plasmids were utilized to evaluate the effect of TCDD on δ-catenin ubiquitination sites. Interestingly, TCDD-induced stabilization of δ-catenin was significantly reversed in cells expressing the δ-catenin mutants. This finding demonstrated for the first time that TCDD acted on the δ-catenin ubiquitination sites Lys1049 and Lys1158, inhibiting the ubiquitination and degradation of δ-catenin, thereby stabilizing its protein levels. However, it cannot be ruled out that TCDD may also regulate δ-catenin expression through other potential ubiquitination sites. Moreover, this study found that δ-catenin was stabilized by TCDD detectably in the nucleus fraction but not in the cytoplasm fraction. Finally, the results of scratch assay and clonogenic assay indicated that TCDD treatment significantly increased the migration and proliferation of prostate cancer cells.

In conclusion, as shown in **Figure 11**, TCDD regulated the δ-catenin ubiquitination site in prostate cancer cells, inhibited the ubiquitin proteasome-mediated δ-catenin degradation, stabilized its expression, and promoted cancer progression, indicating TCDD as a potential inducer of prostate cancer. This study provides new insights into how posttranslational modifi-

cation of δ-catenin affects its stability and function in prostate cancer, enhancing the potential predictive value of δ-catenin in prostate cancer.

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Disclosure of conflict of interest

None.

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