ORIGINAL RESEARCH

ZNF433 positively regulates the beta-catenin/TCF pathway in prostate cancer and enhances the tumorigenicity of cancer cells

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Background: Prostate cancer often shows the over-activation of beta-catenin/t-cell factor (TCF) signaling. It remains largely unknown how the beta-catenin/TCF transcriptional machinery is tightly controlled.

Methods: The ZNF433 mRNA and protein levels in the clinical tissues were examined using q-PCR, Western blot and immunohistochemistry. The phenotypes of prostate cancer cells were examined using MTT assay, Boyden chamber assay and anchorage-independent assay. The interaction between ZNF433 and beta-catenin was evaluated by immunoprecipitation.

Results: In the present study, ZNF433 was upregulated in prostate cancer samples, and promoted the growth and migration of prostate cancer cells. Furthermore, ZNF433 was the binding partner of beta-catenin and activated beta-catenin/TCF signaling in prostate cancer. Moreover, ZNF433 enhanced the binding between beta-catenin and TCF4. In addition, NC043, small antagonist for beta-catenin/TCF complex, inhibited the malignant behaviors of prostate cancer cells driven by ZNF433.

Conclusion: In summary, these studies demonstrate the tumor-promoting roles of ZNF433 in prostate cancer, and suggesting that ZNF433 was a potential target for the treatment.

Keywords: ZNF433, beta-catenin, prostate cancer, motility

Introduction

Prostate cancer is a common malignancy for male.[1] Recently, great advance has been made for the diagnosis and treatment of prostate cancer.[2] However, the outcome for this malignancy remains poor.[1] Therefore, investigating the mechanism for prostate cancer and identifying potential targets would help the treatment.

Beta-catenin/TCF signaling is over-activated in most of the cancer types,[3] which is one of the clinical features of prostate cancer.[4] Beta-catenin is the key effector for this signaling.[5] In the absence of Wnt ligand, the protein level of beta-catenin is downregulated by the cytoplasmic destruction complex.[6,7] Upon treatment of the Wnt ligand, the destruction complex is disassociated, which leads to the accumulation of beta-catenin and its translocation to the nucleus. In the nucleus, beta-catenin activates the expression of *c-Myc*, *cyclinD1*, *MMP7*, *Snail*, and other target genes.[8–10] The clinical prostate cancer samples show the over-activation of beta-catenin.[11,12] Furthermore, the activation of beta-catenin/TCF signaling promotes prostate cancer cell proliferation and motility,[13–15] suggesting the important role of beta-catenin/TCF signaling in the development of prostate cancer.

ZNF433 belongs to transcriptional factors with the zinc finger motif. At present, the roles of ZNF433 remain poorly understood. A recent study indicated that ZNF433 was

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a susceptibility gene for multiple sclerosis.[16] Here, the expression status and functions of ZNF433 in prostate cancer were examined, and its molecular mechanism was investigated.

Materials and methods
Cell lines
PC3, DU145, LNCap, and RWPE-1 (normal prostate epithelial cells) were purchased from the Cell Bank of Shanghai Institutes for Biological Science. DMEM (containing 10% fetal bovine serum [Thermo Fisher Scientific, Waltham, MA, USA], 100 units/mL of penicillin, and 100 g/mL of streptomycin) was used to cultivate these cell lines. Cells were maintained in an incubator with 5% CO_2 at 37°C.

Clinical samples
All procedures performed in studies that involved human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments, or comparable ethical standards.

The clinical samples were collected from patients who underwent surgery at Nanjing Medical University. Written informed consent was obtained from the patients. Tissues were kept in liquid nitrogen. The present study was approved by Ethics Committee of the Huai'an First People's Hospital.

Q-PCR
The complementary DNA (cDNA) was prepared using the kit purchased from Promega Corporation (Fitchburg, WI, USA) after the total RNA was extracted using Trizol (Thermo Fisher Scientific). The mRNA level of ZNF433 in the cancerous tissues and paired non-cancerous tissues was examined by quantitative real-time PCR using SYBR Green Realtime PCR Master Mix (TOYOBO) following the instructions of the manufacturer. Sequences of quantitative real-time PCR primers are listed as follows:

18S forward primer: 5′-TAAATCAGTTATGGT TCCTT-3′

18S reverse primer: 5′-CGACTACCATCGAAA GTTGA-3′

ZNF433 forward primer: 5′-gaacatatat gtagagtacg-3′

ZNF433 reverse primer: 5′-cctacttctc catacacact-3′.

Western blot
Cells were harvested using the RIPA buffer. After centrifugation at 12,000 *g* for 15 minutes, the supernatant was collected and the protein quantification was determined using Bradford. Equal amount of protein was loaded to each line and separated by SDS-PAGE. After transferred onto a polyvinylidene fluoride (PVDF) membrane, the protein was blocked with 5% BSA solution for 1 hour at room temperature. Then, the primary antibodies were incubated with the membrane overnight. On the next day, tris buffered saline tween solution was used to wash the membranes. The secondary antibody was incubated with the membranes for 1 hour at 25°C. The membranes were examined by enhanced chemiluminescence enhanced chemiluminescence (ECL) kit.

Immunohistochemistry (IHC)
Tissues were embedded with paraffin and cut into 5 μm sections. The sections were subjected to deparaffinization and rehydration using xylene and ethanol. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 solution. Sodium citrate solution (pH 6.0) was used to retrieve the antigens, and 5% BSA solution was used to block the non-specific binding. ZNF433 antibody was incubated with the tissues overnight, and the tissues were visualized with the secondary antibody (Envision, Gene Technology, Shanghai, China). Then, DAB was used to develop the sections and the tissues were counterstained with hematoxylin.

GST pull-down
The beta-catenin CDS was inserted into the pGEX-4T-1 vector. The fusion protein GST-ZNF433 was purified. The lysis buffer (50 mM of Tris-Cl [pH 7.5], 150 mM of NaCl, 0.1% NP40, and a protease inhibitor cocktail) was used to prepare LNCap cell lysate. Then, GST-ZNF433 fusion protein and LNCap cell lysates were incubated overnight at 4°C. Then, 50 μL of Glutathione Sepharose 4B beads were added to the supernatant and incubated at 4°C for 1 hour. After washing, the proteins were harvested using Laemmli buffer, and SDS-PAGE was performed.

Immunoprecipitation assay
Cells were harvested using RIPA buffer, centrifugated at 4°C for 20 minutes (12,000 *g*). The supernatant was incubated with the primary antibody overnight at 4°C. The next day, the protein A beads were added to the supernatant and incubated for another 4 hours. After extensive wash, the immunoprecipitants were harvested with loading buffer and examined by SDS-PAGE.

Vector construction
The ZNF433 CDS was inserted into expression vector pcDNA3.1 to obtain the myc tag. The beta-catenin CDS was inserted into expression vector pCMVTag2B to obtain the Flag tag. The TCF4 CDS was inserted into expression vector pCMV-HA to obtain the HA tag.

RNA interference

The lentivirus to knock down the expression of ZNF433 and control virus were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). The same MOI lentivirus was used to infect the cells for 24 hours, then the cells were selected with puromycin for 7 days.

Luciferase assay

DU145 cells were seeded in 24-well plates. After 16 hours, 0.1 μg of Topflash, 0.5 μg of expression vector, and 0.05 μg of TK Renilla (internal control for transfection efficiency) were transfected. After 48 hours, Wnt3a (100 ng/mL) was used to treat the cells for 8 hours. Then, the reporter activity was measured according to the instruction (Promega Corporation).

Migration assay

The upper chamber contained 2×10^5 cells suspended in 0.05 mL of medium with 1% FBS. About 0.15 mL of medium with 10% FBS was put in the lower chamber as the chemoattractant. After 12 hours, the migrated cells were detected with hematoxylin and eosin staining. Cells were counted under the microscope.

Cell growth assay

Cells were seeded in 96-well plates (1,000 cells/well). Every other day, cell growth was determined by incubation with MTT solution (50 μg/well) for four hours. DMSO (0.2 mL) was used to resolve the cellular MTT. The OD was measured at 540 nm. All experiments were repeated three times.

Soft agar assay

The plates were first coated with bottom layer containing 0.5% agarose and 10% FBS in DMEM. The upper layer (0.35% agarose and 10% FBS in DMEM) contained 2×10^3 cells/well in 12-well plates. After 14 days, the colonies were counted. The experiments were repeated for three times.

Animal experiments

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. About 1×10^6 cells (pcDNA3.1 transfected DU145 cells and ZNF433 transfected DU145 cells) were subcutaneously injected into the one point of 6-week old nude mice. Each group contained four mice. NC043 (10 μm) were used to treat the mice three times per week. Tumor volume was monitored.

Statistical analyses

The Prism GraphPad software was used for statistical analysis. $P < 0.05$ was considered statistically significant. Data were presented as mean ± standard error of the mean (SEM).

Results

ZNF433 expression was elevated in prostate cancer

First, the mRNA levels of ZNF433 in 30 prostate cancer tissues were examined and paired with normal prostate tissues. It was observed that ZNF433 mRNA levels were upregulated in prostate cancer tissues (Figure 1A). Furthermore, ZNF433 protein levels were found to be elevated in cancer tissues by Western blot and IHC (Figure 1B and C). Consistent with these observations, data obtained from the GEPIA database revealed that higher ZNF433 protein levels correlated with the shorter survival of patients (Figure 1D). In addition, ZNF433 expression in normal prostate epithelial cells (RWPE-1) and cancer cells were examined. Higher ZNF433 protein levels were observed in prostate cancer cells (Figure 1E). Moreover, the expressions of ZNF433 were gradually increased in the primary tumor and metastatic tissues when compared with the adjacent normal tissues (Figure 1F). Collectively, these results revealed the upregulation of ZNF433 in prostate cancer.

The growth and migration advantages of prostate cancer cells overexpressing ZNF433

To understand the roles of ZNF433 in prostate cancer, ZNF433 (myc-ZNF433) was forced to express in LNCap and DU145 (Figure 2A). Overexpressing ZNF433 facilitated the growth of cancer cells in the MTT assay (Figure 2B). In addition, ZNF433 enhanced the motility of LNCap and DU145 cells in the migration assay (Figure 2C). Moreover, the upregulation of ZNF433 enhanced the anchorage independence of LNCap and DU145 cells (Figure 2D). In order to further analyze the roles of endogenous ZNF433, ZNF433 expression was downregulated in LNCap and DU145 cells by sh RNAs (Figure 3A). Downregulation of ZNF433 in LNCap and DU145 cells inhibited cell growth (Figure 3B) and migration (Figure 3C). Furthermore, the downregulation of ZNF433 inhibited the colony formation of cancer cells on the soft agar (Figure 3D). These results demonstrated that ZNF433 promoted the progression of prostate cancer.

ZNF433 activated the beta-catenin/TCF pathway

Reporter assay was used to screen pathways influenced by ZNF433 in DU145 cells. As shown in Figure 4A, the knockdown of the expression of ZNF433 inhibited the TOPflash
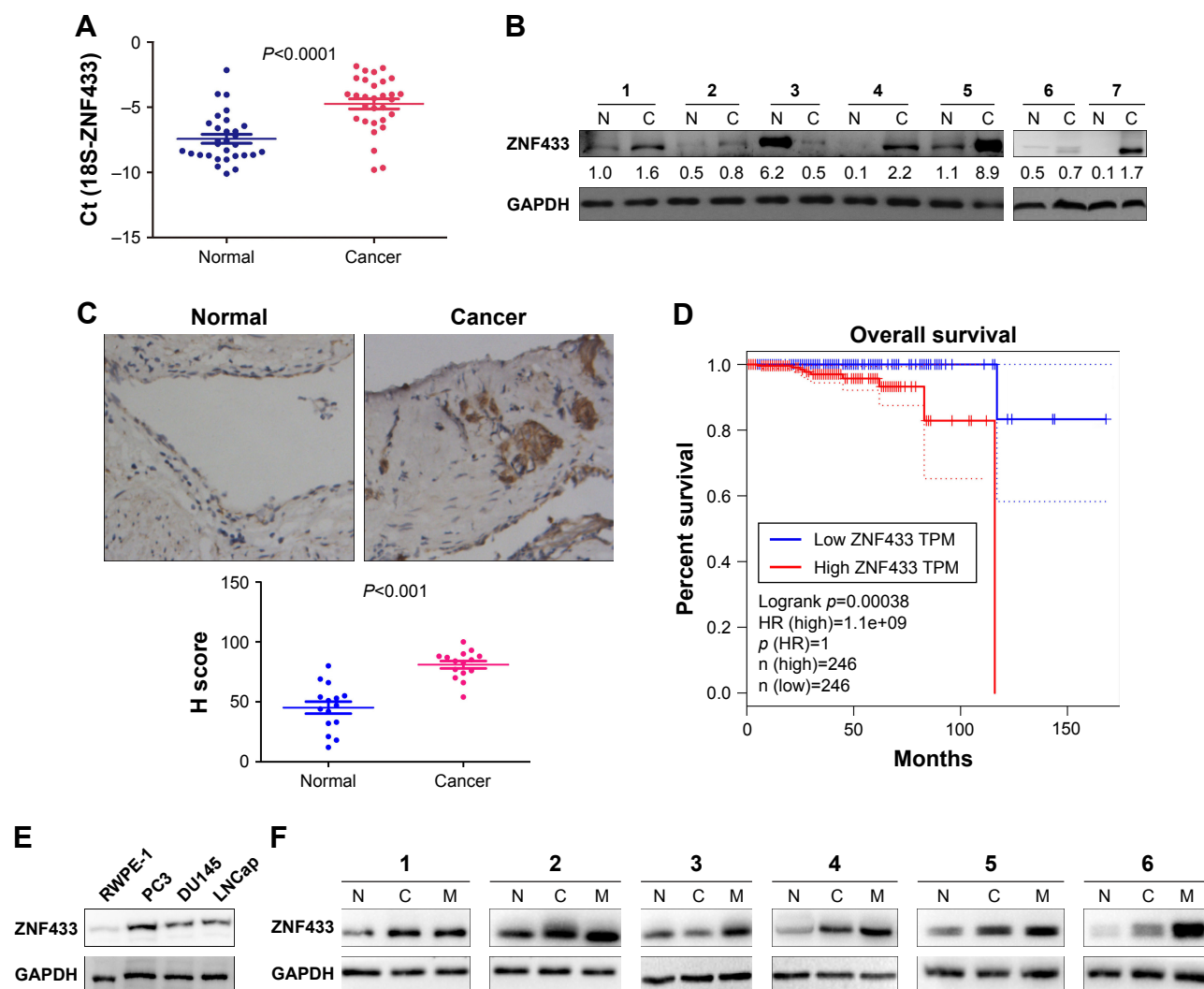
Figure 1 The mRNA and protein levels of ZNF433 were elevated in prostate cancer cells.

Notes: (**A**) The mRNA levels of ZNF433 in 30 prostate cancer tissues and paired normal tissues were examined using q-PCR, and 18S was used as an internal control. (**B**) The protein levels of ZNF433 in seven prostate cancer tissues and paired normal tissues were examined using immunohistochemistry. (**C**) The protein level of ZNF433 in prostate cancer tissues and paired normal tissues was examined using immunohistochemistry. Fifteen normal tissues and 15 cancerous tissues were examined, and the representative images were shown. (**D**) The GEPIA database was mined for the correlation between ZNF433 expression and survival. (**E**) The protein levels of ZNF433 in normal prostate epithelia cell line (RWPE-1) and cancer cell lines were examined using Western blot. (**F**) The expressions of ZNF433 in the primary tumor and metastatic tissues were examined.

Abbreviations: C, cancer; N, normal; M, metastasis.

reporter activity. In addition, the knockdown ZNF433 expression in DU145 and LNCap cells decreased the protein levels of c-Myc, CyclinD1, and N-cadherin (three targets of the beta-catenin/TCF complex, Figure 4B). Furthermore, knocking down beta-catenin abolished migration and colony formation advantages of ZNF433-overexpressed DU145 cells (Figure 4C and D). Collectively, these results suggest that ZNF433 enhanced the malignant behaviors of prostate cancer through beta-catenin/TCF pathway.

ZNF433 strengthened the binding of beta-catenin to TCF4

The detailed mechanism for ZNF433 to activate beta-catenin/TCF signaling was studied. The binding between

ZNF433 and the key effectors of this pathway was first tested. As shown in Figure 5A, the GST pull-down assay demonstrated the binding between endogenous beta-catenin and fusion protein GST-ZNF433. Consistent with this observation, exogenously expressed myc-ZNF433 and Flag-beta-catenin bound to each other in LNCap cells (Figure 5B). Furthermore, endogenous ZNF433 and beta-catenin bound to each other in LNCap cells (Figure 5C). Most interestingly, ZNF433 overexpression in LNCap cells potentiated the binding of beta-catenin to TCF4 (Figure 5D), which was attenuated by NC043, an inhibitor for beta-catenin and TCF4 (Figure 5E). In summary, these results suggests that ZNF433 activated the beta-catenin/TCF pathway by strengthening the binding of beta-catenin to TCF4.
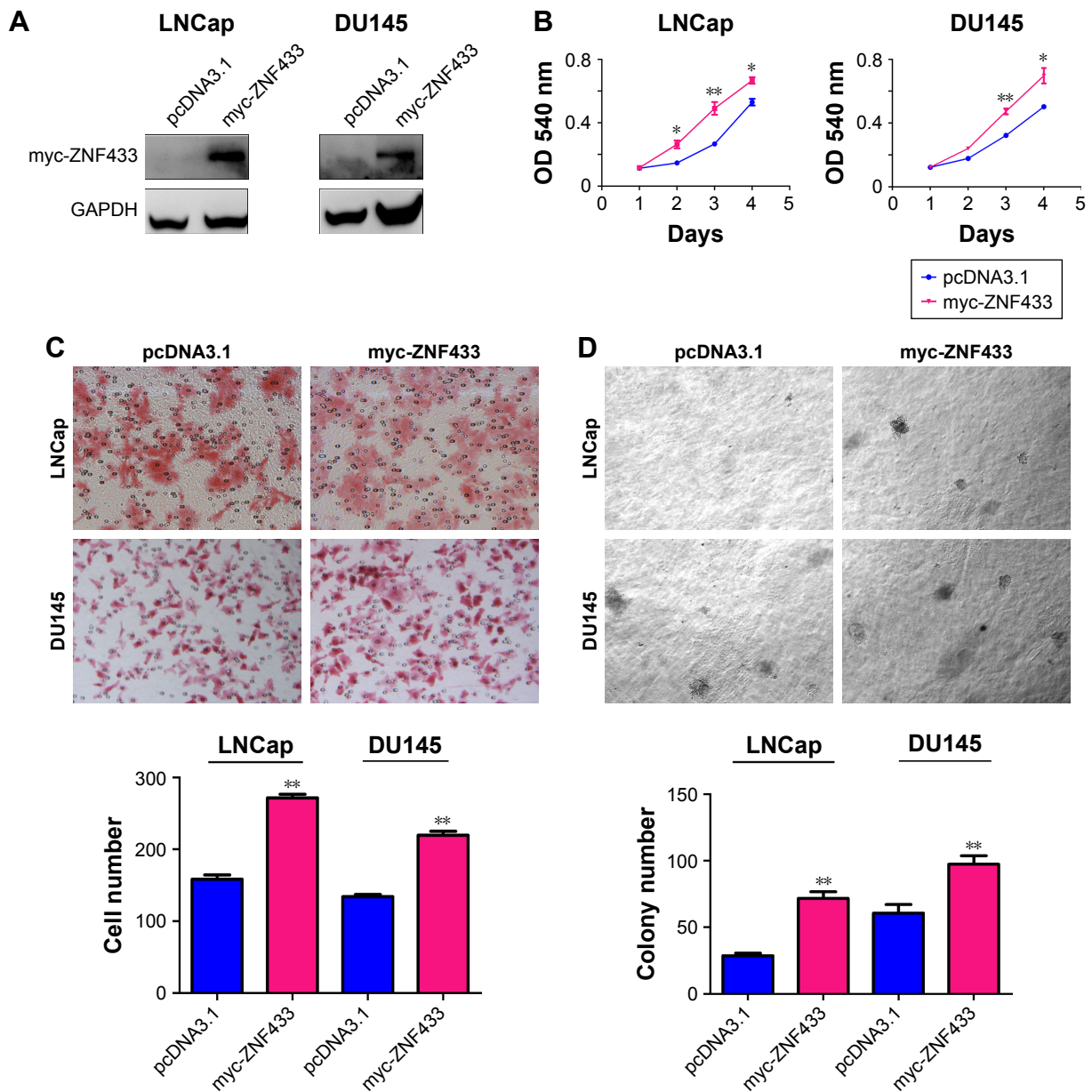
Figure 2 ZNF433 promoted the growth and migration of prostate cancer cells.
Notes: (**A**) ZNF433 was overexpressed in LNCap and DU145 cells. LNCap and DU145 cells were transfected with ZNF433 expression vectors and control plasmids. Cells were selected with G418. (**B**) MTT assay was performed to evaluate the roles of ZNF433 on the growth of LNCap and DU145 cells. (**C**) Boyden chamber assay was performed to evaluate the roles of ZNF433 on the migration of LNCap and DU145 cells. Four fields were photographed for each group, and the representative images were shown. (**D**) Soft agar assay was performed to evaluate the roles of ZNF433 on the anchorage-independent growth of LNCap and DU145 cells. Four fields were photographed for each group, and the representative images were shown. *$P<0.05$; **$P<0.01$.

NC043 inhibited the oncogenic functions of ZNF433 in prostate cancer cells

NC043 has been reported as an inhibitor for beta-catenin and the TCF4 complex.[17] It was speculated that NC043 could attenuate the oncogenic functions of ZNF433 when ZNF433 activates the beta-catenin/TCF signaling pathway in prostate cancer cells. Indeed, NC043 attenuated the migration of DU145 and LNCap cells induced by ZNF433 overexpression (Figure 6A and B). Similarly, NC043 attenuated the colony formation of DU145 cells induced by ZNF433

overexpression (Figure 6C). In the in vivo tumorigenesis assay, ZNF433 promoted the tumorigenicity of DU145 cells. However, NC043 treatment impaired the tumorigenesis of DU145 cells driven by ZNF433 (Figure 6D). These observations suggest that NC03 exhibit therapeutic effects for the overexpression of ZNF433 in tumors.

Discussion

Targeting the beta-catenin/TCF signaling is recognized as the promising strategies for cancer treatment.[18] Although the
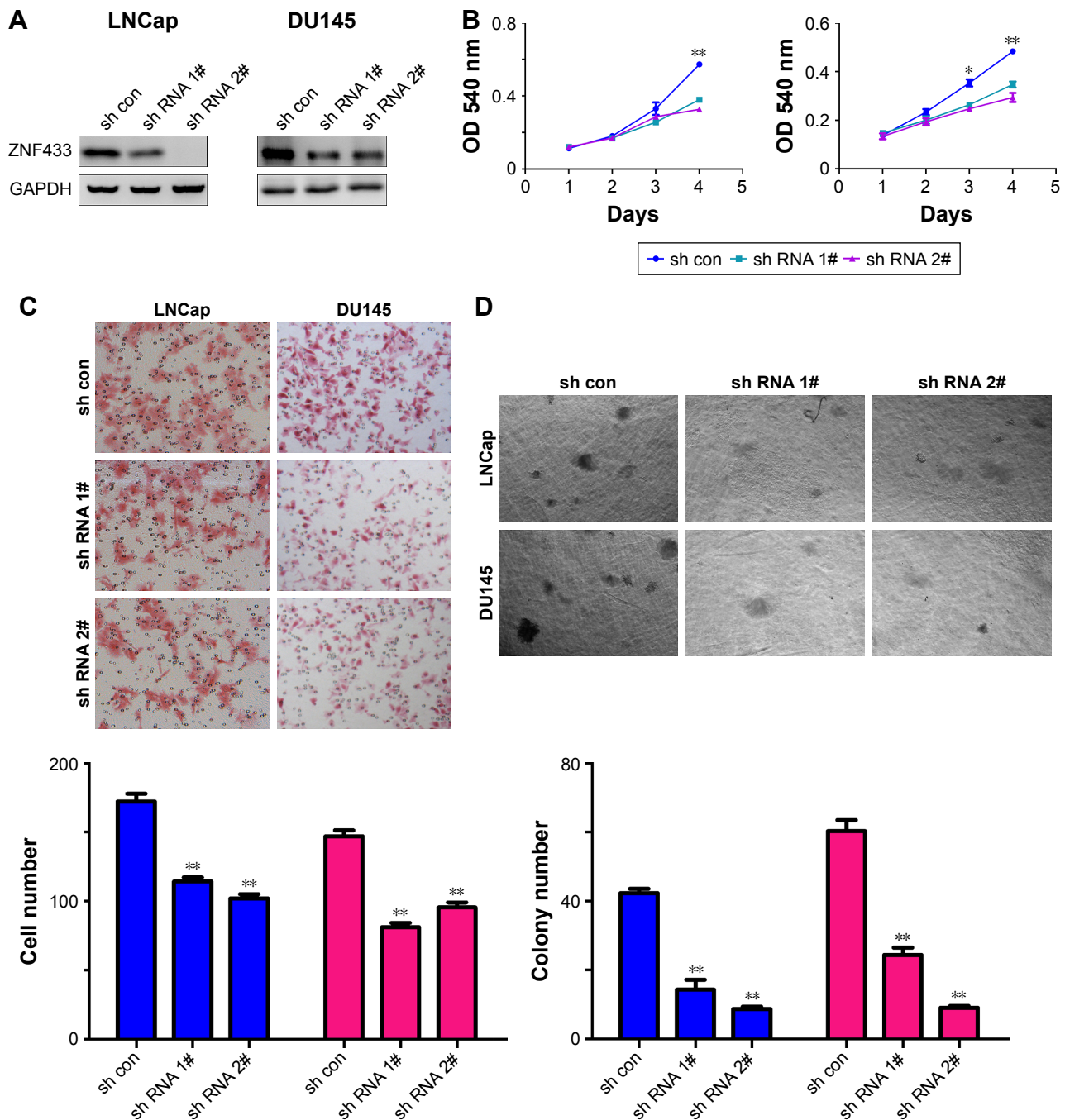
Figure 3 The knockdown of the expression of ZNF433 inhibited the growth and migration of prostate cancer cells.
Notes: (**A**) ZNF433 was knocked down in LNCap and DU145 cells. LNCap and DU145 cells were infected with the ZNF433 lentivirus. (**B**) MTT assay was performed to evaluate the role of ZNF433 knockdown on LNCap and DU145 cell growth. (**C**) Boyden chamber assay was performed to evaluate the role of ZNF433 knockdown on LNCap and DU145 cell migration. Four fields were photographed for each group, and the representative images were shown. (**D**) Soft agar assay was performed to evaluate the role of ZNF433 knockdown on the anchorage-independent growth of LNCap and DU145 cells. Four fields were photographed for each group, and the representative images were shown. *$P<0.05$; **$P<0.01$.

frame of the beta-catenin/TCF signaling pathway has been established, the fine tune of the beta-catenin/TCF complex, the nuclear transcriptional machinery, remains poorly understood. In this study, zinc finger protein ZNF433 was found to regulate the beta-catenin/TCF complex by enhancing its interaction.

With regard to the biological functions, ZNF433 potentiated the malignant behaviors of prostate cancer cells. Consistently, the expression of ZNF433 was upregulated in prostate cancer. In summary, these observations suggest the tumor-promoting effects of ZNF433 in the development of prostate cancer.
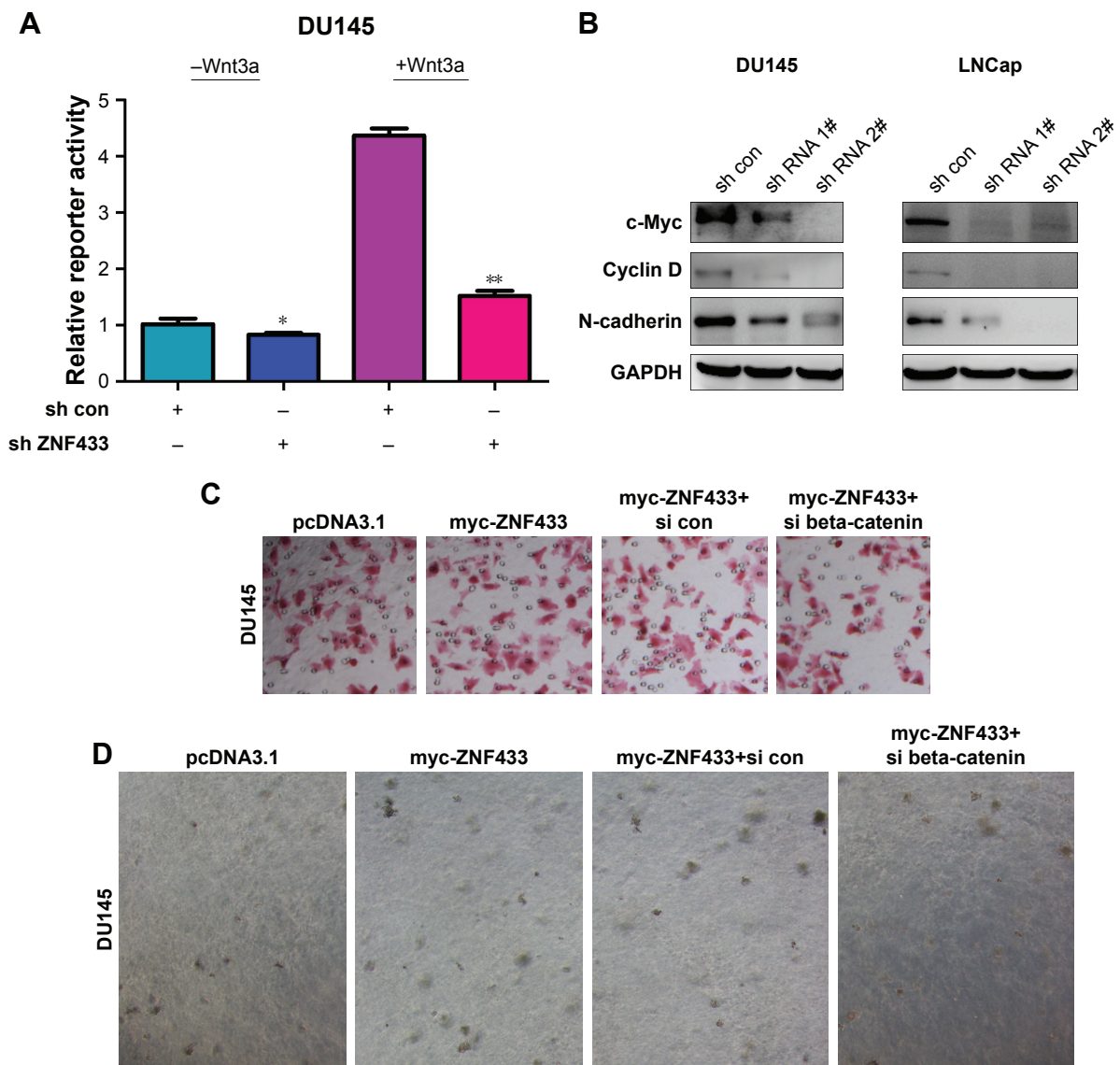
Figure 4 ZNF433 promoted the anchorage-independent growth and migration of prostate cancer cells by activating the beta-catenin/TCF signaling pathway.
Notes: (**A**) Luciferase assay was performed to examine the effects of ZNF433 on the reporter activity of TOPflash. Cells were treated with or without the Wnt3a protein. (**B**) The effects of ZNF433 knockdown on the expression of the beta-catenin/TCF target gene were examined. (**C**) The knockdown of beta-catenin abolished the promoting effects of ZNF433 on the migration of DU145 cells. Four fields were photographed for each group, and the representative images were shown. (**D**) The knockdown of beta-catenin abolished the promoting effects of ZNF433 on the anchorage-independent growth of DU145 cells. Four fields were photographed for each group, and the representative images were shown. *$P<0.05$; **$P<0.01$.

One interesting finding of the present study was the identification of the novel functions of ZNF433. Although the study conducted by Nischwitz et al provided evidence that ZNF433 was a susceptibility gene for multiple sclerosis,[16] the functions of ZNF433, especially its function in cancer, remain unknown. Here, we first demonstrated the functions of ZNF433 in prostate cancer and further examined the functions of ZNF433 in other cancer types, as needed.

Another interesting finding was the activation of beta-catenin/TCF pathway through ZNF433. ZNF433 bound with beta-catenin. Although the domains in beta-catenin and

ZNF433 were not mapped, which were responsible for the interaction, ZNF433 was able to enhance the binding between TCF4 and beta-catenin. To date, several proteins are found to modify the interaction between beta-catenin and TCF4 and activate this pathway.[19] These data suggest that ZNF433 might be a target for the cancer treatment.

In this study, we have shown the inhibitory effects of NC043 on the migration and tumorigenesis of prostate cancer cells. NC043 has been reported to interfere with the interaction between beta-catenin and TCF4.[17] Our observations further demonstrated that ZNF433 activated beta-catenin/
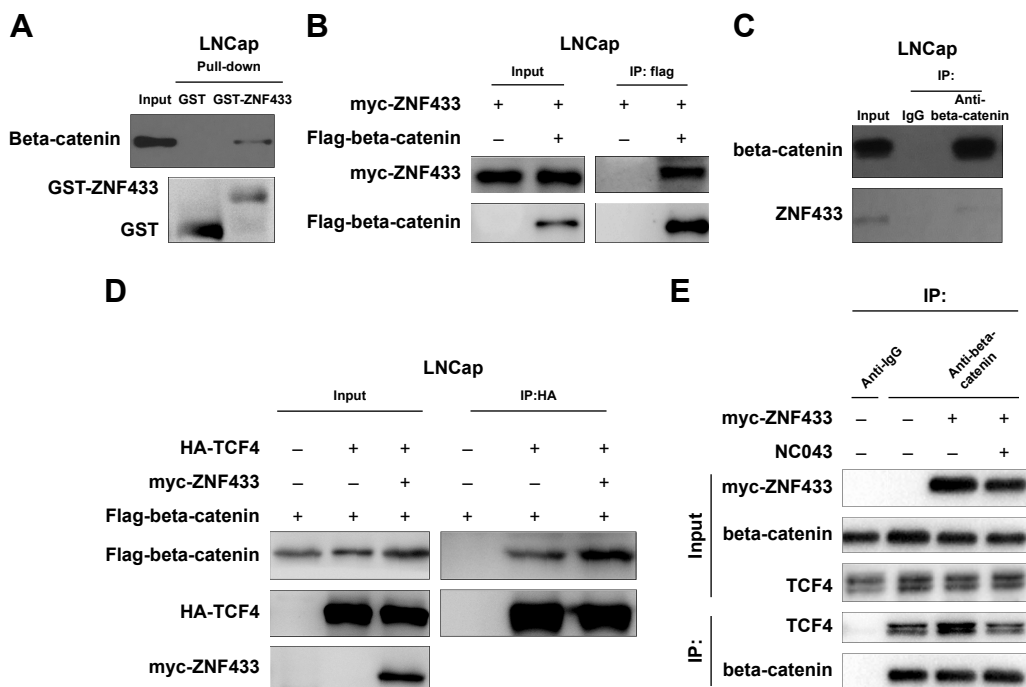
Figure 5 ZNF433 interacted with beta-catenin in prostate cancer cells.

Notes: (**A**) GST pull-down was performed to evaluate the interaction between beta-catenin and ZNF433. (**B**) The interaction between exogenously expressed beta-catenin and ZNF433 was examined using immunoprecipitation. (**C**) The interaction between endogenously expressed beta-catenin and ZNF433 was examined by immunoprecipitation. (**D**) The effects of ZNF433 expression on the interaction between beta-catenin and TCF4 were examined. (**E**) NC043 impaired the interaction between beta-catenin and TCF4 induced by ZNF433.
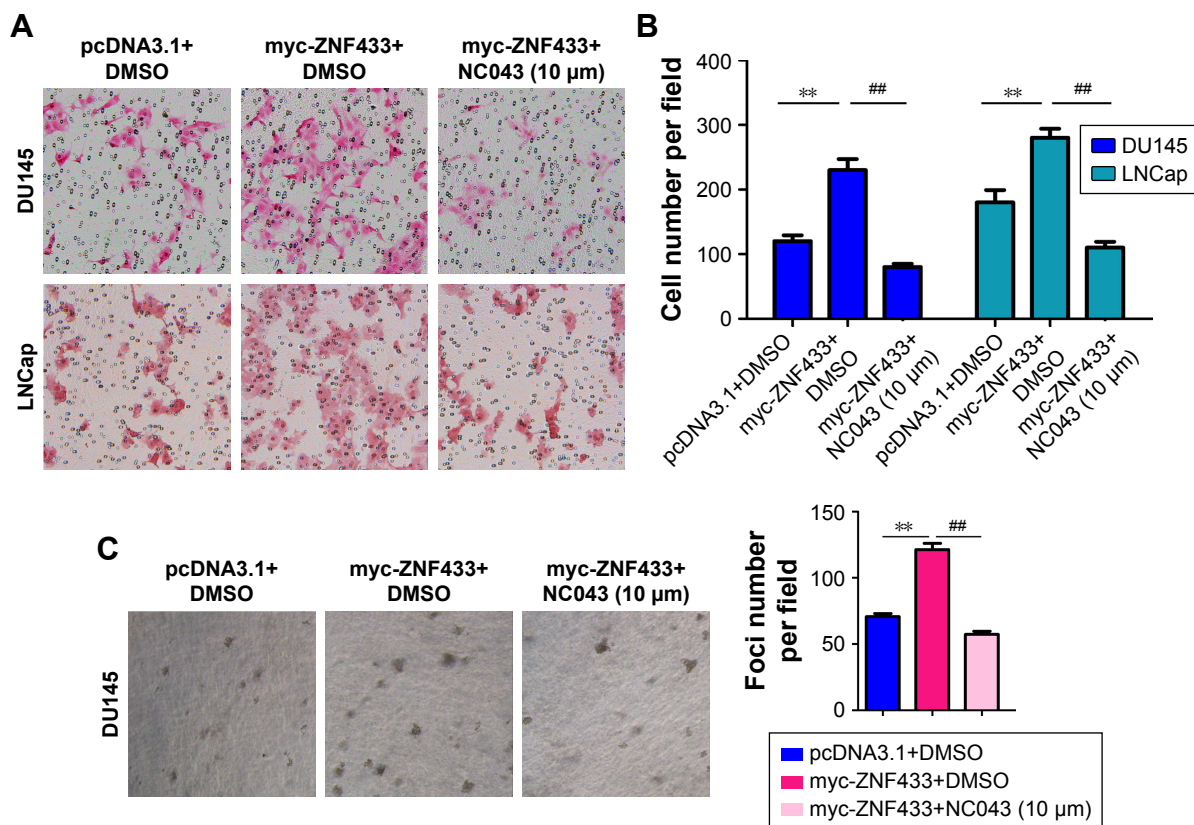
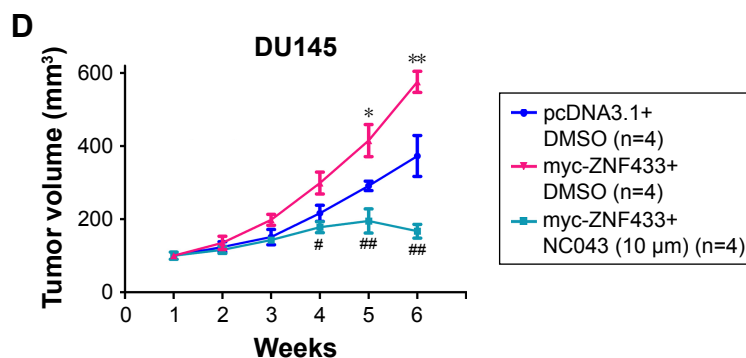**Figure 6** (*Continued*)

D

Figure 6 The therapeutic effects of NC043 on prostate cancer cells.

Notes: (**A**) The effects of NC043 on the migration of DU145 and LNCap cells were examined using the Boyden chamber. (**B**) The statistical analysis for (**A**). (**C**) The effects of NC043 on the colony formation of DU145 cells were examined using the anchorage-independent assay. (**D**) The effects of NC043 on the tumorigenesis of DU145 cells were examined in vivo. $*P<0.05$; $**P<0.01$; $^\#P<0.05$; $^{\#\#}P<0.01$.

TCF signaling by enhancing the interaction between beta-catenin and TCF4.

Conclusion

We demonstrated the tumor-promoting functions of ZNF433 in prostate cancer and indicated ZNF433 as a promising target for the treatment.

Author contributions

Zongyuan Xu and Shuo Gu designed the present study. Peijin Hou, Kun Liu, Xiaobing Niu, Bingjian Wei, and Fei Mao collected the results. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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