β-catenin/LEF-1 Transcription Complex is Responsible for the Transcriptional Activation of LINC01278

Shaojian Lin
Sun Yat-Sen Memorial Hospital
Weiwei Zhang
Guangdong Provincial Hospital of Traditional Chinese Medicine
Ziwen Shi
Sun Yat-Sen Memorial Hospital
Langping Tan
Sun Yat-Sen Memorial Hospital
Yue Zhu
Sun Yat-Sen Memorial Hospital
Honghao Li (lihonghao63@163.com)
Sun Yat-Sen Memorial Hospital https://orcid.org/0000-0003-2553-9056
Xinzhi Peng
Sun Yat-Sen Memorial Hospital

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Abstract

Background: Our previous study shows that LINC01278 inhibits the development of papillary thyroid carcinoma (PTC) by regulating miR-376c-3p/DNM3 axis. However, the regulation mechanism of LINC01278 expression in PTC cells is still unclear.

Methods: The luciferase reporter and ChIP assays were used to confirme the binding of LEF-1 to the putative promoter site of LINC01278. The RNA immunoprecipitation was used the enrichment of LINC01278 in β-catenin protein. Western blot was used to detected the expression of target proteins.

Results: Firstly, the online PROMO algorithm determined a putative LEF-1 binding site on LINC01278 promoter. Then, the luciferase reporter and ChIP assays confirmed the binding of LEF-1 to the putative promoter site of LINC01278. Furthermore, the overexpression of β-catenin increased the binding of LEF-1 to the LINC01278 promoter, and the knockdown or overexpression of LEF-1 or β-catenin can affect the expression level of LINC01278. In addition, RNA immunoprecipitation showed that LINC01278 was enriched in β-catenin protein. RNA pulldown and western blot also confirmed that LINC01278 precipitated β-catenin in TPC-1 and BCPAP cells. Furthermore, the knockdown or overexpression of LINC01278 significantly affected the expression of β-catenin and targets of Wnt/β-catenin signaling pathway (CCND2, CyclinD1, MYC, and SOX4).

Conclusion: In summary, we found the transcriptional activation of LINC01278 by the β-catenin/LEF-1 transcription factor, and the negative feedback regulation of LINC01278 on Wnt/β-catenin signaling pathway activation.

Background

Thyroid cancer is the most common malignant tumor of the endocrine system[1, 2]. In the past 20 years, its incidence has increased rapidly worldwide[3]. Although it is unclear whether the increase in thyroid cancer incidence reflects an increase in true incidence or an improvement of diagnostic technology[4, 5]. Papillary thyroid carcinoma (PTC) is the most common subtype of thyroid malignant tumors, accounting for more than 80% of thyroid cancer, which originates from thyroid follicular epithelial cells[6]. A large proportion of thyroid cancers, especially PTCs, usually exhibit indolent biological behavior and have a good prognosis[7]. However, some patients show aggressive clinical manifestations, such as distant metastasis and recurrence[8]. In the case of high morbidity and low mortality, the pathogenesis of PTC should be further studied.

Long-chain no-coding RNAs (lncRNAs) are a type of transcripts that are longer than 200 bp and lack protein coding ability[9]. Numerous studies have shown that lncRNAs play a vital role in a variety of important physiological and pathological processes, including cancer[10]. Our previous study showed that LINC01278 was significantly down-regulated in PTC tissues and cell lines[11]. The lower expression of LINC01278 was related to tumor size, lymph node metastasis and higher pathological grade and

clinical stage. In addition, LINC01278 inhibited the development of PTC by regulating miR-376c-3p/DNM3 axis. However, the regulation mechanism of LINC01278 expression in PTC cells is still unclear.

Typical Wnt signaling is a conservative signal transduction pathway, which plays a key role in normal development and cancer progression. In the absence of Wnt ligands, the central protein β-catenin of this signaling pathway binds to the GSK3β/Axin/APC complex. The latter phosphorylates β-catenin, and phosphorylated β-catenin is degraded by the ubiquitination-proteasomal system[12]. In the presence of Wnt ligands, Wnt ligands bind to Wnt receptors (Frizzled and LRP5/6) and subsequently activate Dsh/Dvl protein. The Dsh/Dvl protein interacts with the GSK3β/Axin/APC complex, causing the latter to release β-catenin. β-catenin accumulates in the cytoplasm and merges into the nucleus. After entering the nucleus, β-catenin binds to the TCF/LEF-1 transcriptional regulators to activate the transcription of target genes[13].

In this study, we explored the transcriptional regulation of the β-catenin/LEF-1 transcription complex on *LINC01278* gene, and the negative feedback mechanism of LINC01278 on the post-transcriptional regulation of β-catenin.

Methods

Cells culture

Human thyroid cancer cell lines (TPC-1 and BCPAP) were obtained from ATCC (American Type Culture Collection) (Manassas, VA, USA), and cultured in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell transfection

LEF-1, β-catenin and LINC01278 overexpression vectors were bought from Kang-cheng Biotechnology Co (Guangzhou, China). Small interfering RNA (siRNA) against LEF-1, β-catenin and LINC01278 were designed and purchased from RiboBio Co., Ltd. (Guangzhou, China). All vectors and siRNAs were transfected into TPC-1 and BCPAP cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Analysis of online database

The online PROMO algorithm analysis (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to predict the binding of transcription factors to the LINC01278 promoter sequence[14, 15].

Luciferase reporter assay

The wild promoter sequence of LINC01278 (WT) and mutant promoter sequence of LINC01278 (MUT) in which the LEF-1-binding site was mutated were cloned into a pGL3-Basic vector (Promega), respectively. The recombinant pGL3-Basic vector was co-transfected into TPC-1 and BCPAP cells with LEF-1 overexpression (LEF-1) or negative control (NC) plasmid. After 48 h, the cells were lysed, and the luciferase activity was analyzed using Dual-Luciferase Reporter Kit (Promega) and Varioskan Lux detection system (Thermo Scientific), which was standardized to Renilla.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed and crosslinked in 1% formaldehyde for 10 min at 37 °C and incubated with protease inhibitors. Chromatin was isolated and enzymatically fragmented using an EZ-Zyme Chromatin Prep Kit (17375, Merck). Rabbit anti-LEF-1 antibody (EPR2029Y, 1:50, Abcam) or nonspecific IgG (1:200, Sigma) was used to precipitate DNA crosslinked with the LEF-1. The immunoprecipitated promoter fragment containing the LEF-1 response element was probed by PCR using primers targeting the regulatory region of *LINC01278* gene and visualized by agarose gel electrophoresis.

Western blot

Cells were lysed using RIPA buffer. The lysate was separated by 10 − 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked with 5% nonfat milk and incubated with anti-LEF-1 (1:1000, ab137827, Abcam), anti-β-catenin (ab16051, 1:1000, Abcam), anti-CCND2(ab230883, 1:1000, Abcam), anti-CyclinD1 (ab226977, 1:1000, Abcam), anti-MYC (ab32072, 1:1500, Abcam), anti-SOX4 (ab86809, 1:1000, Abcam), and anti-GAPDH (ab8245, 1:1000, Abcam) antibodies overnight at 4°C. Then the membrane was washed with TBST three times and incubated with the secondary antibody (Abcam, Cambridge, MA) for 1.5 h at room temperature. The ECL chromogenic solution was used to display the chemiluminescence of the bands. The Quantity One 4.4.0 software was used for densitometry determination.

Quantitative real-time PCR

Total cellular RNAs were isolated and purified using TRIzol reagents (Invitrogen) and cDNA synthesis was performed using PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq TM (TaKaRa) in the ABI 7900HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The comparative cycle threshold (CT) ($2^{-C}T$) method was used to measure the LINC01278 level, and GAPDH was used as an internal control. The sequence of primers was as follows: LINC01278, F: 5'-

CTGTTGCCCTCCTTCACCTA-3', R: 5'-TGGTCTACAGGGAGTGCAAG-3'; GAPDH, F: 5'-TGTTCGTCATGGGTGTGAAC-3', R: 5'-ATGGCATGGACTGTGGTCAT-3'.

RNA immunoprecipitation (RNA IP)

Cells were fixed and crosslinked in 1% formaldehyde, and lysed using RIPA buffer supplemented with protease-inhibitor cocktail and RNase inhibitor. The cell lysates were incubated with magnetic beads conjugated with anti-β-catenin antibody (Millipore). Mouse IgG (Millipore) was used as the negative control. The immunoprecipitated RNA was extracted from the eluate, and the enrichment of LINC01278 or control ACTB was analyzed by quantitative RT-PCR.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (SPSS, Armonk, NY, USA). The data came from at least three independent experiments, evaluated by Student's t test, and presented as mean ± SD. $P < 0.05$ was considered as statistically significant.

Results

LEF-1 binds to the promoter region of LINC01278 gene

Firstly, the online PROMO algorithm was used to analyze the promoter sequence of LINC01278, and determined a putative LEF-1 binding site (Fig. 1A). Then, luciferase reporter assays further showed that the luciferase activity of the TPC-1 and BCPAP cells co-transfected with LINC01278-WT and LEF-1 was significantly increased, compared with the NC group, whereas the luciferase activity of cells co-transfected with LEF-1 and LINC01278-MUT showed no significant change, compared with the NC group (Fig. 1B). ChIP assays further demonstrated the binding of LEF-1 to the LINC01278 promoter (Fig. 1C). In addition, when LEF-1 was knocked down, LINC01278 levels were significantly reduced in TPC-1 and BCPAP cells (Fig. 1D-F). Moreover, when LEF-1 was over-expressed, the level of LINC01278 increased significantly in TPC-1 and BCPAP cells (Fig. 1D-F). These data indicated that LEF-1 could bind to the promoter region of *LINC01278* gene, activating its transcription.

Overexpression of β-catenin increases the binding of LEF-1 to the LINC01278 promoter

Based on β-catenin as a cofactor of TCF/LEF-1 transcription activation, we studied the effect of β-catenin on LEF-1activation of LINC01278 transcription. As shown in Fig. 1C, the overexpression of β-catenin increased the binding of LEF-1 to the LINC01278 promoter. Furthermore, down-regulation of β-catenin reduced the LINC01278 expression, while up-regulation of β-catenin increased the LINC01278 expression (Fig. 2).

LINC01278 interacts with β-catenin

Our previous research indicated that LINC01278 inhibited the epithelial-to- mesenchymal transition (EMT) [11]. As we all know, Wnt/β-catenin signal pathway activation regulates EMT[16]. Therefore, we speculated that LINC01278 regulated β-catenin levels in some way. As shown in Fig. 3A, we performed RNA immunoprecipitation, detected the enrichment of LINC01278 by using qRT-PCR, and found that LINC01278 was enriched in β-catenin protein. Then, RNA pulldown and western blot also confirmed that LINC01278 precipitated β-catenin in TPC-1 and BCPAP cells (Fig. 3B). These results demonstrated that LINC01278 associated with β-catenin directly in PTC cells.

LINC01278 suppressed the Wnt/β-catenin activation

Furthermore, we evaluated the effect of LINC01278 on activation of Wnt/β-catenin signaling pathway. As shown in Fig. 3C and D, the knockdown of LINC01278 significantly increased the expression of β-catenin and targets of Wnt/β-catenin signaling pathway (CCND2, CyclinD1, MYC, and SOX4), while the overexpression of LINC01278 suppressed the expression of β-catenin and targets. These data indicated that LINC01278 suppressed the Wnt/β-catenin activation.

Discussion

In recent years, the important role of lncRNAs has continued to appear in a variety of physiological and pathological processes, including tumorigenesis. Our previous research have found that LINC01278 was significantly low expressed in PTC tissues and cells, and exerted a tumor suppressor function in tumor cells[11]. However, Huang et al. show that LINC01278 promotes the metastasis of hepatocellular carcinoma (HCC) by targeting miR-1258-Smad2/3[17]. In addition, Qi et al. find that the expression of LINC01278 in osteosarcoma tissues was enhanced, and it is related to the clinical staging, distant metastasis and poor prognosis of patients[18]. LINC01278 promotes osteosarcoma cell proliferation and inhibits cell apoptosis through miR-133a-3p/PTHR1 signal. These two latest studies suggest that LINC01278 plays a cancer-promoting function in HCC and osteosarcoma, as opposed to its function in thyroid cancer. The role of LINC01278 in tumor cells may depend on specific tumor types or downstream targets.

In this study, we revealed the transcriptional activation of LINC01278 by the β-catenin/TCF/LEF-1 transcription factor. We cloned the LINC01278 promoter sequence containing the putative LEF-1 binding site or the putative LEF-1 binding site mutation into the firefly luciferase reporter system vector. The combination of transcription factor LEF-1 and false site of promoter can regulate the expression of downstream firefly luciferase. This experiment confirmed the binding of transcription factor LEF-1 to the putative promoter site. ChIP experiments also confirmed the endogenous binding of transcription factor LEF-1 to the LINC01278 promoter, and showed that β-catenin can enhance the binding of LEF-1 to the LINC01278 promoter. The results of western blot showed that knockdown or overexpression of LEF-1 or

β-catenin can affect the expression level of LINC01278. Our results discovered for the first time the regulatory mechanism of LINC01278 transcriptional activation.

In addition, we discovered the negative feedback regulation of LINC01278 on Wnt/β-catenin signaling pathway activation. The results of RNA IP and RNA pulldown indicated the direct binding of LINC01278 to β-catenin. Subsequently, the results of western blot showed that LINC01278 blocked the accumulation of β-catenin in the cytoplasm, and finally inhibited the expression of Wnt/β-catenin signal targets. β-catenin has a variety of regulatory mechanisms, such as APC degradation complex and CTNNBIP1. Here, we reported a new regulatory mechanism of Wnt/β-catenin activation. In addition, the relationship between LINC01278 and other Wnt/β-catenin modulators needs further study.

Conclusion

In conclusion, we found the transcriptional activation of LINC01278 by the β-catenin/TCF/LEF-1 transcription factor, and the negative feedback regulation of LINC01278 on Wnt/β-catenin signaling pathway activation. This is the first report on the regulation mechanism of LINC01278 expression, as well as a new mechanism report on Wnt/β-catenin signaling pathway activation. Although, the specific interaction between them needs more research.

Declarations

Authors' contributions

1) Conception and design, acquisition of data, or analysis and interpretation of data: All authors.

2) Drafting the article or revising it critically for important intellectual content: All authors.

3) Final approval of the version to be published: All authors.

4) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: Xinzhi Peng.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

All the authors agree to the publication clause.

Ethics approval and consent to participate

All research complied with the principles of the Declaration of Helsinki, and was approved by the Medical Ethics Committee of the Jinan Central Hospital.

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Figures

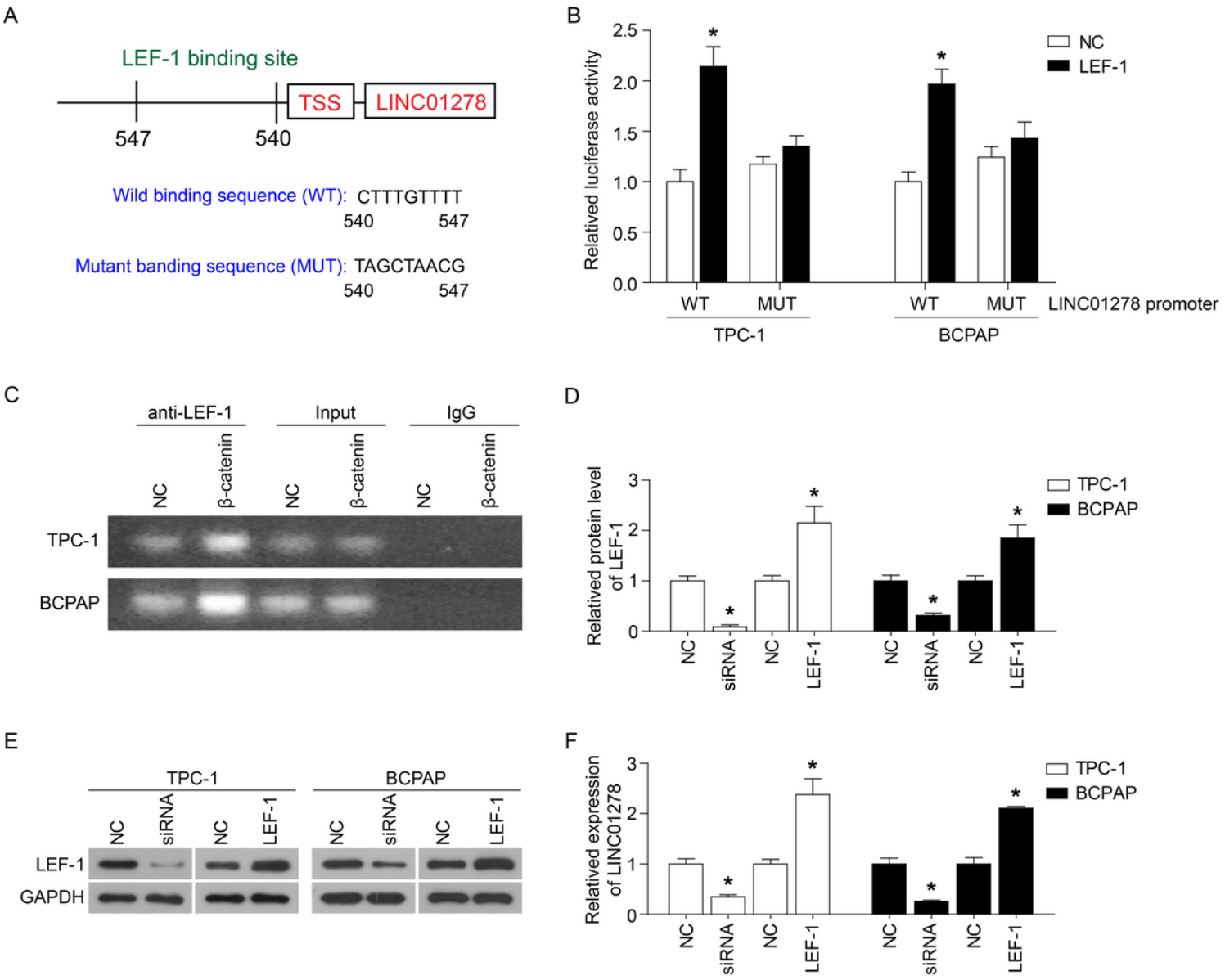
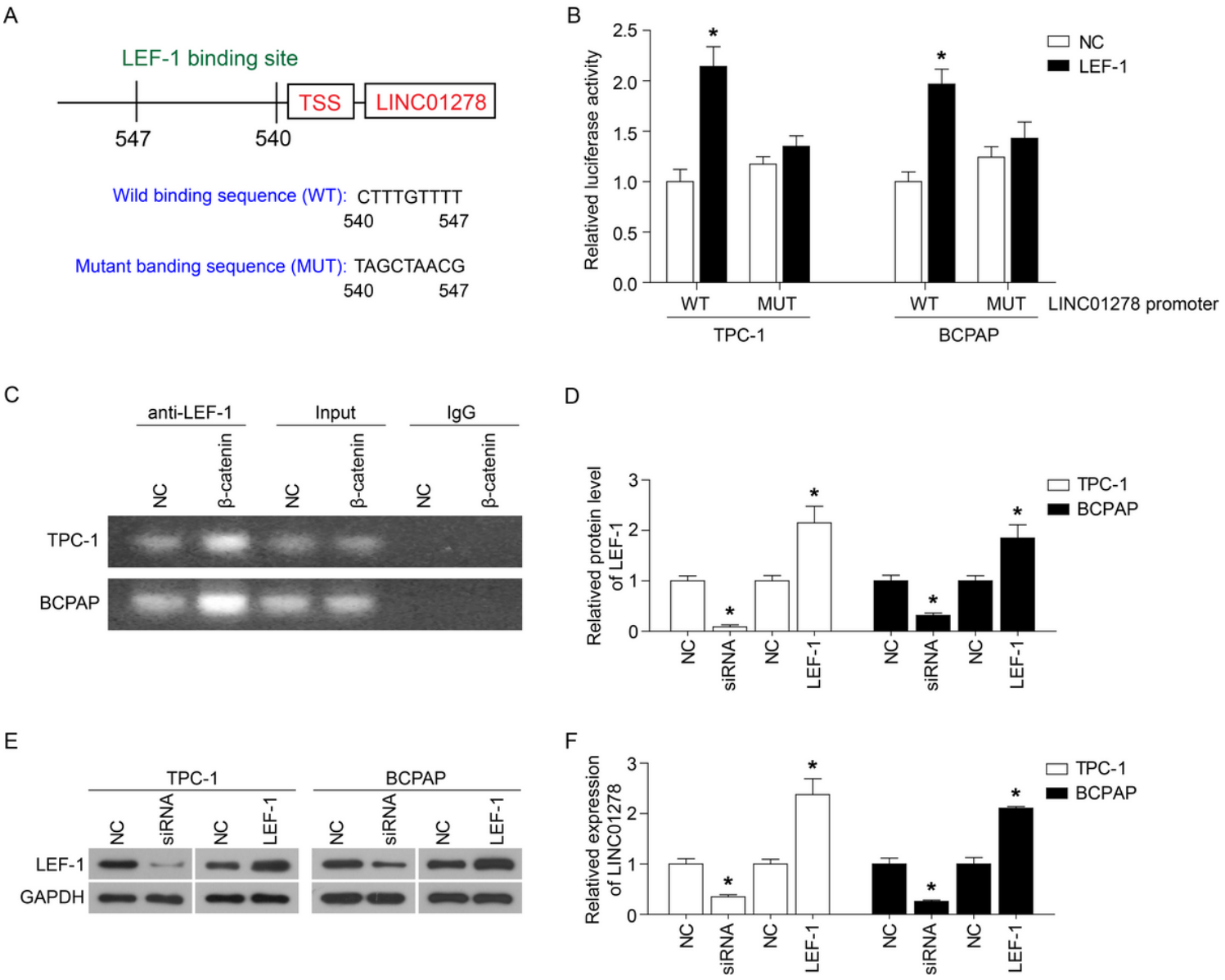


Figure 1

LEF-1 binds to the promoter region of LINC01278 gene. (A) The putative LEF-1 binding site on LINC01278 promoter sequence determined by online PROMO algorithm. (B) The luciferase activity of TPC-1 and BCPAP cells which were co-transfected with LINC01278-WT and LEF-1, or LEF-1 and LINC01278-MUT detected by luciferase reporter assays. (C) The immunoprecipitated promoter fragment containing the LEF-1 response element was probed by PCR using primers targeting the regulatory region of LINC01278 gene and visualized by agarose gel electrophoresis. (D and E) The protein expression of LEF-1 in TPC-1 and BCPAP cells which were transfected with LEF-1 siRNA (siRNA) or overexpression plasmid (LEF-1). (F) The relative expression of LINC01278 in TPC-1 and BCPAP cells which were transfected with LEF-1 siRNA (siRNA) or overexpression plasmid (LEF-1). *P<0.05.



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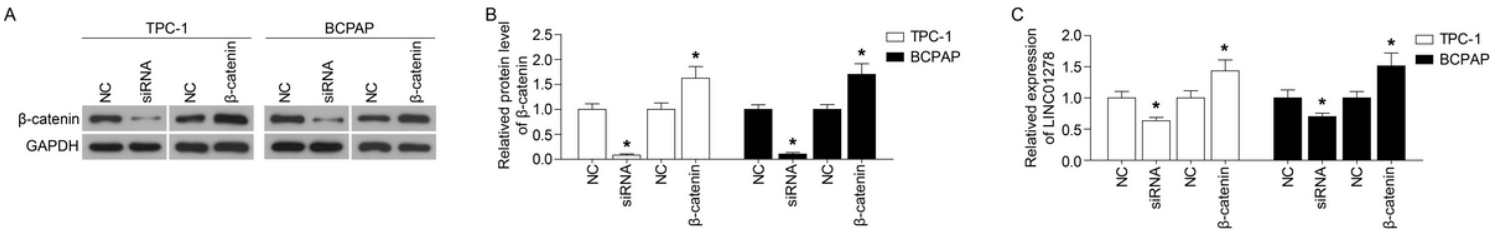
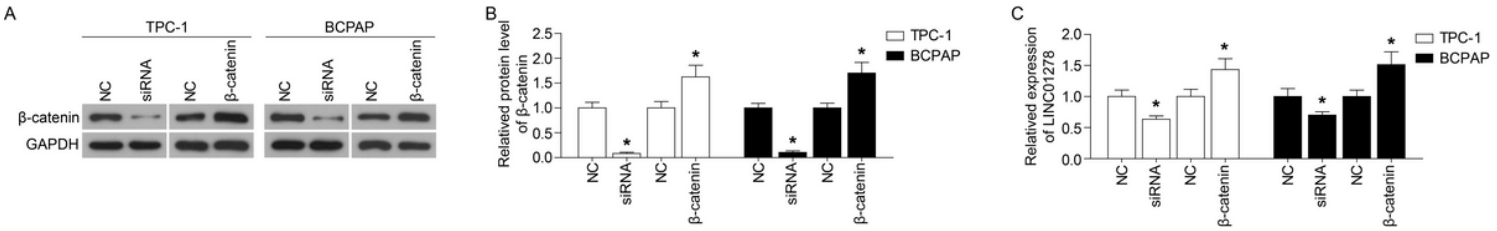


Figure 2

Overexpression of β-catenin increases the binding of LEF-1 to the LINC01278 promoter. (A and B) The protein expression of β-catenin in TPC-1 and BCPAP cells which were transfected with β-catenin siRNA (siRNA) or overexpression plasmid (β-catenin). (C) The relative expression of LINC01278 in TPC-1 and BCPAP cells which were transfected with β-catenin siRNA (siRNA) or overexpression plasmid (β-catenin). *P<0.05.



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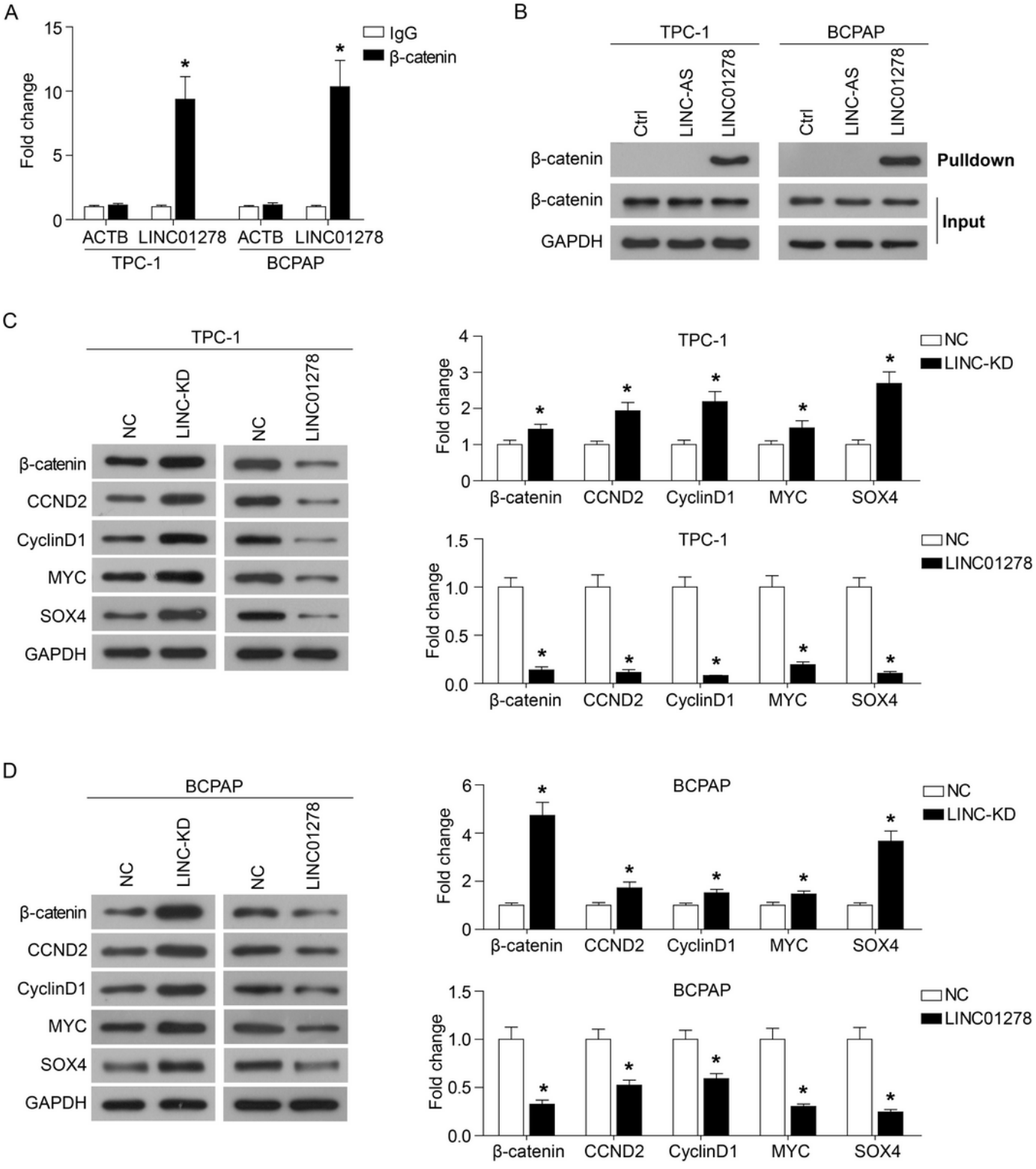

Figure 3

LINC01278 interacts with β-catenin. (A) The enrichment of LINC01278 in β-catenin protein by using RNA immunoprecipitation and qRT-PCR. ACTB was used as the control. (B) LINC01278 precipitated β-catenin in TPC-1 and BCPAP cells detected by RNA pulldown and western blot. The expression of β-catenin and targets of Wnt/β-catenin signaling pathway (CCND2, CyclinD1, MYC, and SOX4) in TPC-1 (C) and BCPAP

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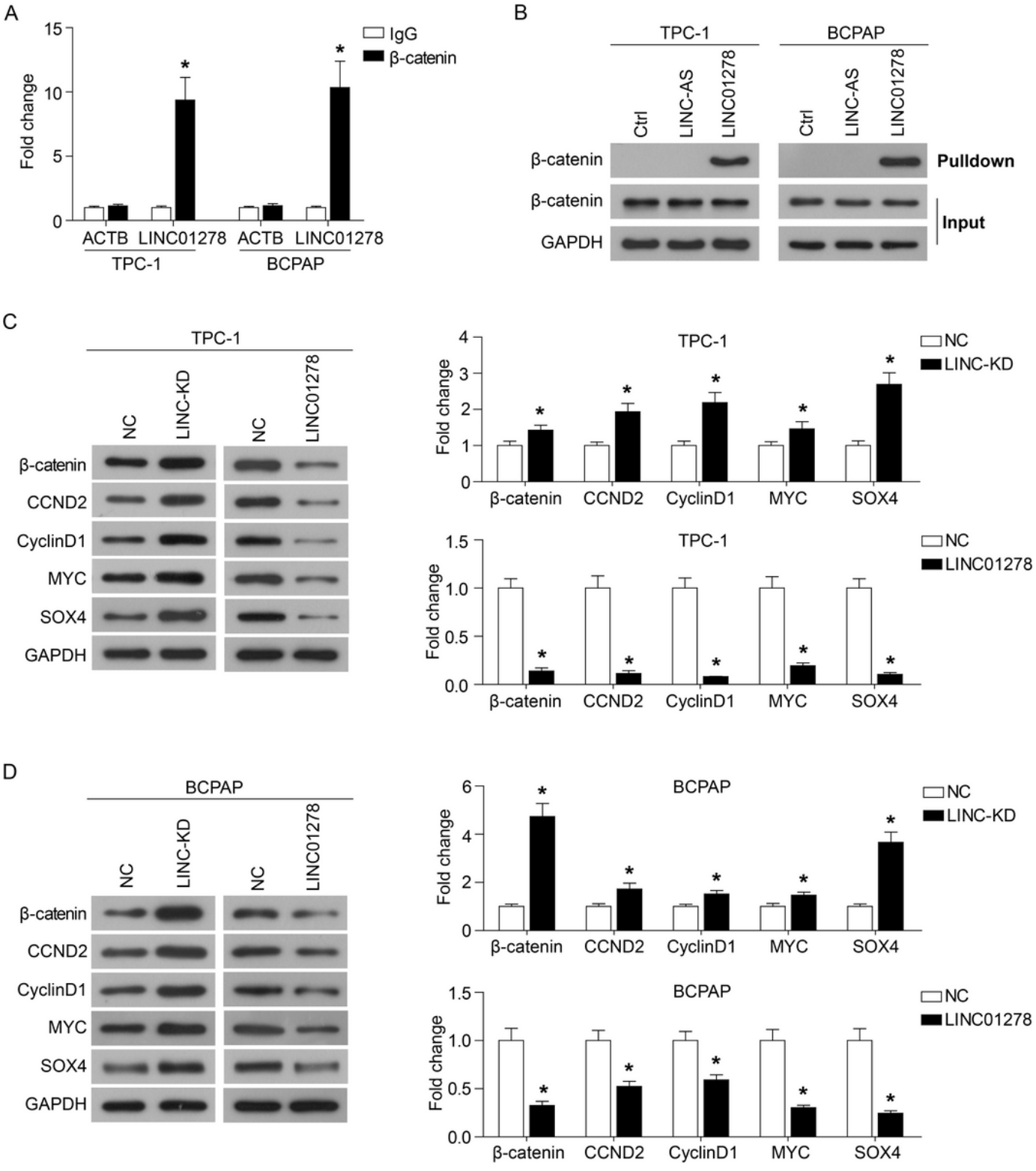

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