

Wilms' Tumor 1-associating Protein Promotes Prostate Cancer Proliferation via Upregulation of CDK4 Transcript

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Abstract

Background: Wilms' tumor 1-associating protein (WTAP) plays an important role in cell physiological function and have attracted increased interest in cancer research recently. Cyclin-dependent protein kinases (CDKs) are known to participate in regulating the cell cycle and often connected to many malignancies in tumor. We aim to explore whether WTAP or CDKs could play an role in the initiation and progression of prostate cancer(PCa) and hope to provide new insights into PCa treatment and prognostic.

Methods: Quantitative real-time PCR, western blotting and immunohistochemistry were performed to explore the expression of WTAP and CDK4 in prostate cancer tissues and cell lines. The survival analysis was used to investigate the association between WTAP expression and the clinical outcomes of prostate cancer. Prostate cancer cell lines were stably transfected with lentivirus approach. CCK-8 assay, colony formation assay, cell invasion and migration assay, cell cycle assay and tumorigenesis in nude mice were performed to study the effect of WTAP in prostate cancer cell lines. RNA immunoprecipitation assay, dual-luciferase reporter assay and siRNA transfection were performed to verify the direct binding sites of WTAP with CDK4 transcript.

Results: In prostate cancer tissues and cell lines, WTAP was significantly up-regulated and high expression of WTAP was connected to poor clinical outcomes. Additionally, cell function test indicated that overexpression of WTAP in prostate cancer cell lines could promote cell proliferation, while knocking down showed an opposite results. Subcutaneous xenograft tumor model revealed that overexpression of WTAP could induce tumorigenesis in vivo. Mechanism study showed that CDK4 expression could regulate the expression level of WTAP. Moreover, WTAP could directly bind to 3'-UTR of CDK4 transcript and enhance its stability. Furthermore, specific inhibitors of CDK4 as well as small interfering RNA (siRNA) of CDK4 reversed the promotion of proliferation induced by WTAP.

Conclusions: These data indicated that WTAP may act as an oncogenic in prostate cancer by directly binding to CDK4 3'-UTR and stabilizing its transcript which might provide new insights into prostate cancer treatment and prognostic.

Introduction

Prostate cancer (PCa) is now the most frequently diagnosed malignance and the second frequent cause of death in males worldwide[1]. Significant progress has been made in the past several years in the treatment of prostate cancer. Nonetheless, clinically significant prostate cancer remains associated with substantial mortality, the estimated number of new diagnosed cases for the US in 2018 is 164,690, with an estimated 29,430 deaths[2]. Surgery and/or radiation therapy can effectively treat clinically localized disease and the endocrine therapy plays a central role in the treatment of the metastatic prostate cancer. However, nearly all patients with advanced prostate cancer progress to castration-resistant prostate cancer (CRPC) which is the lethal form of prostate cancer[3]. Therefore, it is crucial to understand more details about the molecular mechanism of the tumorigenesis and progression of prostate cancer.

Mammalian Wilms' tumor 1-associating protein (WTAP) is first identified as a nuclear protein which interacts with the Wilms' tumor 1 suppressor gene WT1[4]. WTAP is widely expressed in adult tissues and involved in various physiological and pathological processes such as N6-methyladenosine (m6A) RNA modification[5, 6], RNA splicing and stabilization[7, 8], cell cycle regulation[9] and embryonic development[10]. WTAP has also been reported to play an oncogenic role in many malignancies and cause the acceleration of proliferation, promotion of invasion and migration and inhibition of apoptosis in malignant cells[11]. Tang *et al.* revealed that WTAP could promote cell proliferation in renal cell carcinoma by enhancing the stability of CDK2 mRNA[12]. Overexpression of WTAP could also induce many metastasis-associated genes and regulate the migration and invasion of cholangiocarcinoma cells[13]. In colorectal cancer, the interaction between WTAP and carbonic anhydrase IV (CA4) was considered crucial to the mobility abilities of tumor cells[14]. WTAP could promote abnormal proliferation and inhibit the differentiation and act as client protein of Hsp90 in acute myelogenous leukemia[15]. Furthermore, the elevated level of WTAP might have the potential to be an independent prognostic factor in both glioma and pancreatic ductal adenocarcinoma[16, 17]. However, the role of WTAP in prostate cancer is yet to be investigated.

Cyclin dependent kinases (CDKs) are a family containing 20 kinases which participate in regulating the cell cycle. CDKs are traditionally separated into cell-cycle CDKs (CDK1, CDK4 and CDK5) which control cell division and transcriptional CDKs (CDK7, CDK8, CDK9, CDK11 and CDK20) which modulate transcription[18]. In tumor, CDKs are often known to be connected to many malignancies[19]. Recent evidence has revealed that CDKs played an important role in tumor proliferation[20] and the drug response[21, 22]. CDK2 was known to be involved in the renal cell carcinoma proliferation promoting by WTAP[12]. CDK4 and CDK6 kinases are also overexpressed in several malignancies, including sarcoma, glioma, breast tumours, lymphoma and melanoma[23]. Several researches demonstrated that CDK5 might promote prostate cancer growth by regulating various downstream signaling pathways[24, 25].

In the present study, we sought to determine the role of WTAP in prostate cancer and investigated the underlying molecular mechanism. Firstly, we found that WTAP was significantly up-regulated in prostate cancer tissues and related to poor prognosis. Nextly, we revealed that overexpression WTAP could increase the prostate cancer cells proliferation both *in vitro* and *vivo*. Further, we identified that WTAP could promote prostate cancer cells proliferation by regulating CDK4 mRNA stability. Therefore, WTAP might become a novel diagnosis and prognostic bio-marker for prostate cancer.

Materials And Methods

Clinical specimens

All prostate cancer and paired adjacent non-cancerous specimens were collected from patients who underwent radical prostatectomy at the Department of Urology of the First Affiliated Hospital of Nanjing Medical University from 2010 to 2013. No other treatment, such as hormone therapy, chemotherapy or radiotherapy, was used prior to radical prostatectomy. Also the detail information of those patients was

obtained. Appropriate informed consent was provided to the patients and the study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (China). 20 pairs of tissues were used to perform quantitative RT-PCR analyses and 6 pairs were used to perform western blot.

Immunohistochemistry (IHC)

52 pair of prostate cancer tissues and adjacent non-cancerous tissues were stained with anti-WTAP antibody (1:1000, Cell signal technology). Standard staining protocols were used. Positive reactions were defined as showing brown signals in the cell cytoplasm and were scored for scoring staining intensity (SI) and the percentage of positive cells (PP). SI was scored on a scale of 0 to 3. 0 score represents a negative stain; 1 score represents a weak stain; 2 score represents a moderate stain; 3 score represents a strong stain. PP was scored into five categories: 0 (<5% positive cells), 1 (5% to 25%), 2 (26% to 50%), 3 (51% to 80%) or 4 (> 80%). The final score was calculated by multiplying SI and PP score. Each component was scored independently and summed for the results. Two urologists scored the positive level of immunohistochemical staining and patients were divided into two groups by the final score: weak- (0-7) group and strong-staining (8-12) group.

Cell lines and culture conditions

Human prostate cancer cell lines (PC3, DU145 and LNCaP) and human prostatic epithelial cells (RWPE.1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human prostate cancer cell lines were cultured in F-12K, DMEM or RPMI 1640 (Gibco, USA) medium supplemented with 10 % fetal bovine serum (BI, Israel) and 100 U/mL penicillin (Gibco, USA) in humidified air at 37 °C with 5 % CO₂ incubator. Human prostatic epithelial cells (RWPE.1) were cultured in K-SFM with 0.05mg/ml BPE, 5ng/ml EGF and 1% P/S (Gibco, USA).

Plasmid construction and lentivirus packaging

Cells were stably transfected with lentivirus containing the WTAP overexpression (WTAP), WTAP knockdown (shWTAP), a scramble control (SCR) or a negative control (NC) which was an empty lentiviral vector, following the manufacturer's instructions. All plasmids were verified by sequencing (Novobio, Shanghai, China). Cells were plated in 6 wells dishes at 30%~ 50% confluences and infected with the retroviruses. Meanwhile, polybrene (5 µg/ml) was added with the retroviruses to enhance the target cells infection efficiency. Cells were then co-cultured with puromycin(3 µg/ml) for 1-2 weeks and stable transfected cells were selected. CDK4 siRNA (5'-GCAUGUAGACCAGGACCUATTUAGGUCCUGGUCUACAUGCTT-3') and negative control were obtained from GenePharma (GenePharma, China). Lipofectamine 3000 kit (Invitrogen, USA) was used for transfection according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR analyses (qRT-PCR)

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (TaKaRa,) and cDNA was synthesized using Primescript RT Reagent (TaKaRa) according to the standard manufacturer's protocols. Quantitative RT-PCR analyses were performed using a protocol from fluorescent SYBR Green I methodology (Takara) on an ABI StepOne Plus instrument (Applied Biosystems, Carlsbad, CA, USA) and in a total reaction volume of 10 μ l which includes 5 μ l of SYBR Premix (2x), 3 μ l of DEPC water, 1 μ l of cDNA solution, 0.4 μ l of PCR forward primer solution (10 μ M), 0.4 μ l of PCR reverse primer solution (10 μ M) and 0.2 μ l of ROX Reference Dye I (50x). The relative quantitative value was expressed by the $2^{-\Delta\Delta C_t}$ method. Each experiment was performed in triplicates and repeated three times. The PCR primers were showed in Supplementary Table S1.

Cell proliferation assays

Cell proliferation was assessed by CCK-8 assay (Dojindo, Japan) according to the manufacturer's instructions. Cells were diluted to 2000 cells per wells with medium and seeded in a 96-well plate. After cultured for 24, 48, 72, 96 and 120 h at 37°C, spent medium was replaced with 100 μ l fresh medium containing 10% CCK-8 and incubated at 37°C for 1 h. The absorbance was finally determined at 450 nm using a microplate reader (Thermo Scientific, USA).

Colony formation assays

Cells were trypsinized into single-cell suspension. 600 cells were plated into 6-well plate and maintained in media containing 10% fetal bovine serum (FBS) for 10-20 days. Then colonies were fixed with methanol and stained with crystal violet (Beyotime, Beijing, China) after washed by phosphate-buffered saline (PBS), Visible colonies were manually counted after dried at room temperature. Three parallel plates were measured in each treatment group.

Cell migration/invasion assay

Cells ($2.0 \sim 4.0 \times 10^4$) were suspended in 200 μ l with serum-free medium and seeded in the top chamber of the transwell (Millicell, US) which was coated with or without Matrigel (BD Biosciences, USA) for the invasion and migration assays. Culture medium containing 10 % FBS was placed in the bottom chamber. Cells were incubated for 24, 48 or 72 h at 37 °C. The cells that did not migrate through the membrane were manually wiped out with a cotton swab. Cells which passed through the filter were stained using 0.1 % crystal violet after being fixed with methanol. Images of five random fields (200 \times) were captured from each membrane. And then the number of migratory or invasive cells was counted. Each experiment was performed in triplicate.

Western blotting analysis

Cells were lysed by using the mammalian protein extraction reagent RIPA (Beyotime, Beijing, China). Protein was separated by 10 % sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF, Milipore, USA). After blocked with 5%

skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at room temperature, the membranes were incubated for one night at 4°C with β -actin (1:1000, Cell signal technology, USA), GAPDH(1:1000, Cell signal technology, USA), β -catenin (1:1000, Cell signal technology, USA), CDK4 (1:1000, Cell signal technology, USA), CDK6 (1:1000, Cell signal technology, USA), CDK2 (1:1000, Cell signal technology, USA), MMP2 (1:1000, Cell signal technology, USA), MMP9 (1:1000, Cell signal technology, USA) or WTAP (1:1000, Abcam USA) antibodies. The secondary antibody was horseradish peroxidase- conjugated goat anti-mouse-IgG (1:1000, Cell signal technology, USA) or anti-rabbit-IgG (1:1000, Cell signal technology, USA). Enhanced chemiluminescence chromogenic substrate was used to visualize the bands, and the intensity of the bands was quantified by densitometry (Quantity One software; Bio-Rad, USA).

RNA stability

PC3 or LNCaP cells lines were treated with 4ug/ml actinomycin D (Act D) for 0, 1, 2, 4, 6 or 8 h. Total RNAs were extracted and used to generate cDNA. And then qRT-PCR was performed. The level of CDK4 transcript was normalized to β -actin and the relative half-life of CDK4 was calculated.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed according to manufacturer's protocol(Promega, USA). The full length wild type CDK4 3'-UTR (wt) and mutant CDK4 3'-UTR (mut) was inserted into the pLenti-UTR-Luc vector (Promega, USA) and Renilla luciferase vector (pRLCMV; Promega, USA) was used as an internal control. The reporters were then transfected into PC3 and LNCaP WTAP overexpression cells (WTAP) and the control cells (NC) using Lipofectamine 3000 reagent (Invitrogen USA). After 48 hours of transfection, the luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega, USA). Renilla luciferase activity was normalized against Firefly luciferase activity.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation was performed by using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to a standard protocol. Prostate cancer cells were lysed by RIP lysis buffer. Cell lysates were immunoprecipitated with anti-WTAP antibody or non-immunized IgG at 4 °C overnight, followed by RNA purification. Then RT-PCR and qRT-PCR were used to measure the expression level of CDK4 transcript in the WTAP or IgG immunocomplexes.

Tumorigenesis in nude mice

BALB/C male nude mice (4-6-weeks old, 18–22 g) were randomly divided into two groups and each containing 4 mice. Stabled WTAP-knockdown PC3 cells or scramble control cells (1×10^6 cells in 0.15 ml PBS) was subcutaneously injected into the mice and the growth of tumors was followed up for 4 weeks. Tumor volume was measured using a caliper per 1 week. After 4 weeks, mice were sacrificed and measured for final tumor size. Mouse studies were conducted according to the Guide for the Care and

Use of Laboratory Animals and approved by the Animal Care and Use Committee of Nanjing Medical University.

Statistically analysis

All data were expressed as mean \pm SD (standard deviation), and analysis were performed using SPSS version 17.0 software (IBM). A Student's t test or a chi-square test was used as appropriate. Patient survival was evaluated by the Kaplan-Meier method and compared by the log-rank test. Values of $P < 0.05$ were considered statistically significant.

Results

WTAP was significantly up-regulated in prostate cancer and related with prostate cancer patient prognosis.

To identify the role of WTAP in prostate cancer, we firstly collected 20 pairs of prostate cancer specimens and paired-adjacent non-cancerous specimens to perform qRT-PCR and 6 pairs of tissues to perform western blot to analyze the expression of WTAP. The results showed that the mRNA and protein level of WTAP were remarkably up-regulated in the prostate cancer specimens compared with the paired adjacent non-cancerous specimens (Figure 1a and b). We then compared the protein level of WTAP in four different prostate cancer cell lines with a normal human prostatic epithelial cell line RWPE.1 and discovered that WTAP was also up-regulated (Figure 1c).

To explore whether the WTAP expression level was connect with the outcome of prostate cancer patients we performed IHC on 52 prostate cancer patient tissue samples (Figure 1e) and divided them into two groups. We found that patient with high WTAP expression showed trend of worse distant metastasis while no significant difference was found in age, Gleason scores, tumor stage or lymphatic metastasis (Table 1). Kaplan-Meier analysis demonstrated that prostate cancer patients with high WTAP expression appeared a significantly shorter biochemical recurrence-free survival time than those with low WTAP expression ($P < 0.05$) (Figure 1d). All date above indicated that a high WTAP expression level might be an independent risk factor for prostate cancer patients.

WTAP promoted proliferation, migration and invasion of prostate cancer cell lines *in vitro* and *in vivo*

To determine whether WTAP was critical to prostate cancer cell proliferation and migration *in vivo*, we firstly conducted the stably transferred cell lines. The efficiency of the transfection was tested by qRT-PCR and western blot (Supplementary Figure S1).

CCK-8 assay indicated that overexpression of WTAP could increase the cell proliferation in prostate cancer cell lines compared with the negative control (NC) group (Figure 2b), while knockdown of WTAP lead to decreasing compared with the a scramble control (SCR) group (Figure 2a). In colony formation assays, overexpression of WTAP could significantly promote cell colony formation (Figure 2d) meanwhile the colony formation rate was decreased in WTAP knocking-down cells (Figure 2c). We also used a flow

cytometry to perform cell cycle analysis and found an increased percentage of cells in the G1 phase in WTAP knockdown cells and a decreased percentage of cells in G1 phase cells in WTAP overexpression cells (Figure 2e and f). We also established subcutaneous xenograft tumor model with WTAP knockdown or the scramble control cells. Results showed that tumors grew slower in the WTAP knockdown group (shWTAP) (Figure 3a, b and c). These above information indicated that WTAP could promote the proliferation in prostate cancer cells lines. The migration and invasion capacity was tested by migration and invasion assays. The results showed that the number of migrating and invasion cells in three cell lines were increased in the up-regulated groups while reduced in the down-regulated groups (Supplementary Figure S2). These data suggested that WTAP may be also involved in migration and invasion ability of prostate cancer cell lines *in vivo* and act as a positive regulator.

WTAP enhanced the stability of CDK4 mRNA by directly binding to the 3'-UTR of CDK4 transcript

To investigate the molecular mechanism of WTAP regulation in cell proliferation, we first performed series of western blot to identify expression change of several cell proliferation related proteins. We found that the knockdown of WTAP could remarkably decrease CDK4 expression and the WTAP overexpression showed an opposite result in PC3 and LNCaP cells (Figure 4a). We didn't find any meaningful changes of CDK6 expression. It indicated that WTAP might regulate the cell cycle via CDK4 in prostate cancer cells. To explore how WTAP could regulate CDK4, we co-cultured the WTAP knockdown or overexpression prostate cancer cells lines with actinomycin D (Act D) for several hours. The results illustrated that the relative half-life of CDK4 transcripts was significantly decreased by knocking down WTAP and overexpressed cell lines showed the opposite results (Figure 4b). After that, RNA immunoprecipitation (RIP) assay was used to investigate whether WTAP could directly bind to CDK4 transcript. Two different prostate cancer cell lines were treated with WTAP antibody or control rabbit IgG. Results showed that a higher CDK4 transcript expression was detected in the WTAP immunocomplexes compared to the control IgG immunocomplexes and the β -actin immunocomplexes which was a negative control was hardly detected (Figure 4c). These all demonstrated that, in prostate cancer cell lines, WTAP could physically bind to CDK4 transcript.

Finally, we constructed a dual-luciferase reporter assay containing a wild type CDK4 3'-UTR (wt) and a mutant type (mut) to verify whether 3'-UTR was required for WTAP binding to the CDK4 transcript. We found that overexpression of WTAP increased the relative luciferase activities of the wild type reporter in both PC3 and LNCaP cell lines while no significant differences were detected in the mutant group (Figure 4d). Putting those all together, we considered that WTAP could improve the stability of CDK4 mRNA due to a direct bind to the 3'-UTR of CDK4 transcript.

CDK4 interference decreased the cell proliferation induced by WTAP

To further confirm the role of CDK4 in the WTAP related cell function, we then transfected WTAP overexpressed cell and control cells with CDK4 small interference RNA (siCDK4) or a control vector (CTRi). We found siCDK4 reverse the proliferation driven by the overexpression of WTAP and inhibited cell proliferation in both CCK-8 assay and colony formation assays (Figure 5a and b). We then selected two

distinguish CDK4 inhibitors, palbociclib and abemaciclib. The WTAP overexpressed cell (WTAP) and control cell (NC) were treated with palbociclib, abemaciclib or DMSO. We found that the optical density(OD) value fold (WTAP/NC) caused by the overexpression of WTAP was reversed when treated with palbociclib or abemaciclib in CCK-8 assay (Figure 5c), and the colony fold (WTAP/NC) showed a similar change in colony formation assays (Figure 5d). These together suggested that the up-regulation of proliferation caused by WTAP could be reversed by the inhibiting CDK4.

Discussion

In our study, we firstly demonstrated that WTAP could promoting cell proliferation in prostate cancer by regulating the stability of CDK4 transcript and might be a potential prognostic indicator for prostate cancer patients.

Recently studies showed that WTAP was overexpressed in many malignancies including renal cell carcinoma, cholangiocarcinoma, gliomas and acute myeloid leukemia and the WTAP was thought to be critical in oncogenesis and development progress in these malignancies[11, 12, 15, 16]. We found WTAP was also up-regulated in prostate cancer specimens and IHC analysis showed the overexpression of WTAP was significantly associated with poor clinical outcomes. At the same time, high WTAP expression was related to shorter biochemical recurrence-free survival time in prostate cancer patients. Then, we investigated the physiological function of WTAP *in vitro* and *in vivo*. By knocking-down the expression of WTAP in prostate cancer cell lines, we found cell proliferation was significantly decreased in CCK8 and colony formation assays while over-expression of WTAP showed the opposite outcomes. Transwell migration and invasion assays revealed that the up-regulation of WTAP could promote cell migration and invasion. *In vivo*, WTAP-knockdown PC3 cells grew slower and smaller in nude mice and. All the above results suggested that WTAP could play an oncogenic role in prostate cancer.

The view of cell division cycle is that it is an autonomous mechanism regulated by a small number of enzymes, mainly the CDKs. Recent studies showed that WTAP could promote the cell cycle by enhancing the stabilization of cyclin A2 mRNA in HUVECs[9] as well as CDK2 in RCC cells[12]. However, Small *et al.* reported that cell cycle was inhibited when WTAP was overexpressed in VSMCs[26]. This indicated that the function of WTAP in cell cycle might be cell-type specific. In our study, we observed that percentage of cells in the G1 phase was increased when WTAP was knocked down, and decreased when WTAP was overexpressed. Also, we found WTAP could regulate the protein expression of CDK4. Aberrations in cell cycle control are often considered as one of the hallmarks in cancer and CDK4 is frequently found to be abnormally expressed in cancer[27]. The activity of CDK4 is known restricted to the G1-S phase and CDK4, together with CDK6 and D-type cyclins (D1, D2, or D3), can form a protein kinase complex that is important for G1 phase progression[28]. This data was consisted with what we found in the cell cycle analysis. Consequently, we thought to exam the role of CDK4 in prostate cancer.

CDK4/CDK6 inhibitors are now used for hormone receptor (HR)-positive and human epidermal growth factor receptor 2 (HER2)-negative breast cancer[29]. The palbociclib and abemaciclib were known to

target the ATP binding site of CDK4 and CDK6 inhibit the phosphorylation of the retinoblastoma protein (RB)[30]. We found that inhibit CDK4 by small interference RNA or specific CDK4 inhibitors could reverse the cell proliferation induced by WTAP in prostate cancer cell lines. Furthermore, CDK4 mRNA stability was found enhanced in the WTAP overexpressed cell lines after co-cultured with actinomycin D and decreased in the WTAP knocked down cell lines. RNA stability is critical to the regulation of the gene expression and contribution to many cell function[31]. Wang *et al.* reported that CCI-779 could inhibit G2-M progression and invasion of castration-resistant prostate cancer via attenuation of UBE2C mRNA stability[32]. Therefore, we further investigate how the WTAP regulation the stability of CDK4 mRNA. RIP assays revealed that a direct bind between WTAP and CDK4 transcript was existed. In dual-luciferase assay, cell lines overexpression of WTAP showed an increased relative luciferase activity after being transfected with CDK4 3'-UTR reporter vector. All these data suggested that, in prostate cancer cell lines, WTAP could enhance the stability of CDK4 transcripts by binding to its 3'-UTR.

Declarations

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Authors' contributions

LZ, JW and YRG carried out the experiments and LZ drafted the manuscript; ZJW, LHL, YFC, FQ helped to collect prostate cancer tissue; LXH and GC managed the

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

The collection and use of the prostate cancer tissue samples were reviewed and approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

RIP:RNA Immunoprecipitation

DLR: Dual-luciferase Reporter assay

3'-UTR:3'- Untranslated Regions

CDK: Cyclin Dependent Kinase

WTAP: Wilms' Tumor 1-associating Protein

IHC:Immunohistochemistry

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Tables

Table 1 Association between WTAP expression and clinical outcomes of the prostate cancer patients

Parameters	Total (%)	WTAP expression		P value
		Strong (%)	Weak (%)	
Age(years)				0.868
≤65	19(36.5)	14 (73.7)	5(26.3)	
≥65	33(63.5)	25 (75.8)	8(24.2)	
Gleason Score				0.172
≤6	7 (13.5)	6(85.7)	1 (14.3)	
7	32 (61.5)	21(65.6)	11(34.4)	
≥8	13 (25.0)	12(92.7)	1(7.3)	
Histological grade				0.149
II	18(34.6))	14(77.8)	4(22.2)	
III	5(9.6)	2(40.0)	3(60.0)	
IV	29(55.8)	23(79.3)	6(20.7)	
Tumor stage				0.878
T2	35(72.8)	25(71.4)	10(28.6)	
T3	12(16.0)	10(83.3)	2(16.7)	
T4	5(11.2)	4(80.0)	1(20.0)	
Lymph node metastasis				0.815
Absence	45(86.5)	34(75.6)	11(24.4)	
Presence	7(13.5)	5(71.4)	2(28.6)	
Distant metastasis				< 0.001*
Absence	31(59.6)	29(93.55)	2(6.45)	
Presence	21(40.4)	10(47.62)	11(52.38)	

*Statistically significant

Figures

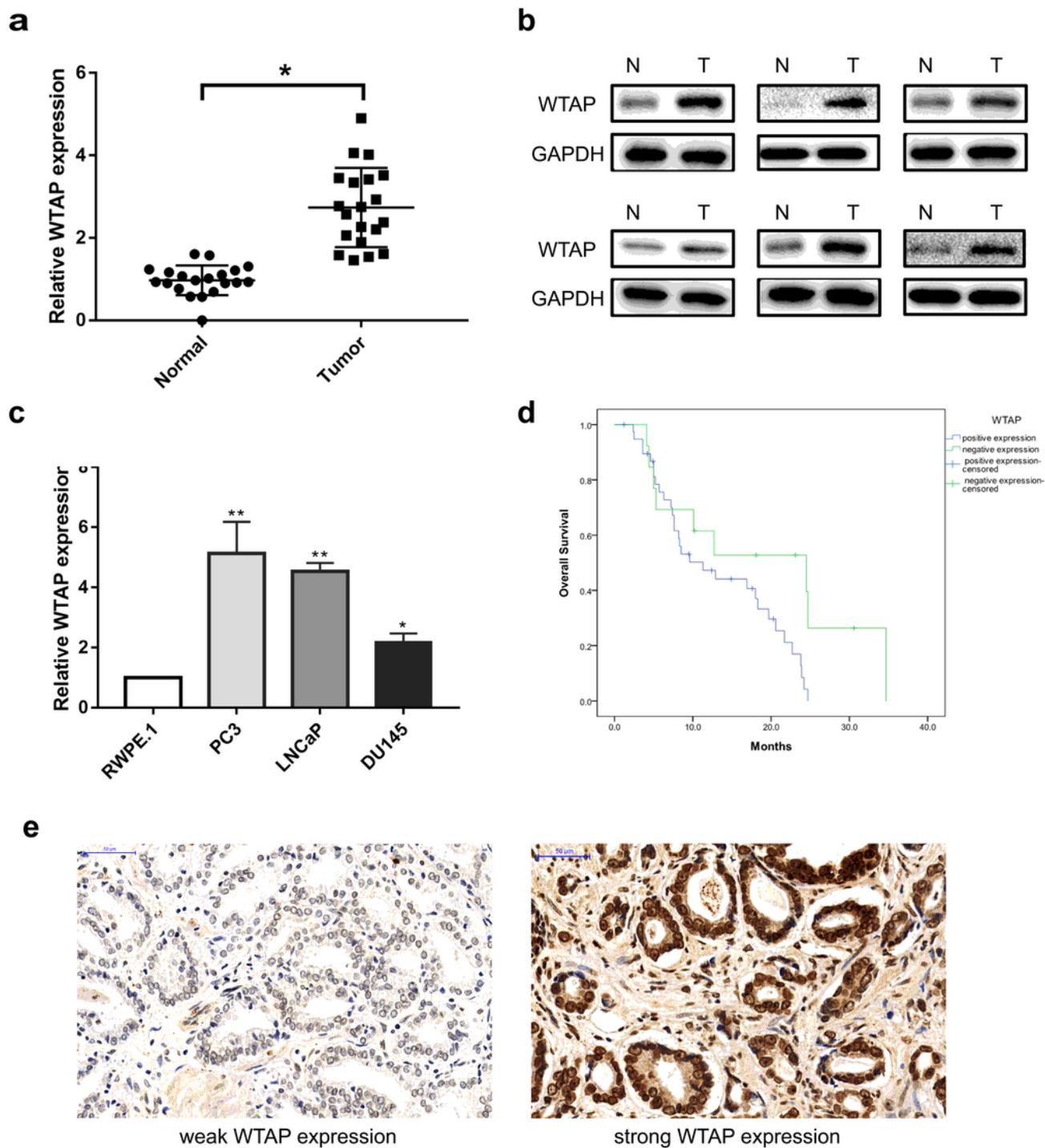


Figure 1

The expression levels of WTAP are negatively connected with the progression of prostate cancer. a,b) The mRNA and protein levels of prostate cancer tissues and paired paracancer tissues. c) mRNA level of WTAP in human prostate cancer cell lines (PC3, DU145 and LNCaP) and human prostatic epithelial cells (RWPE.1). d) Kaplan-Meier analysis of biochemical recurrence-free survival time in 52 pairs of prostate cancer patients. The log-rank test was used to compare significance between two groups. e) IHC on

radical prostatectomy specimens (200 \times). Data represents the mean \pm SD from three independent experiments. *P<0.05

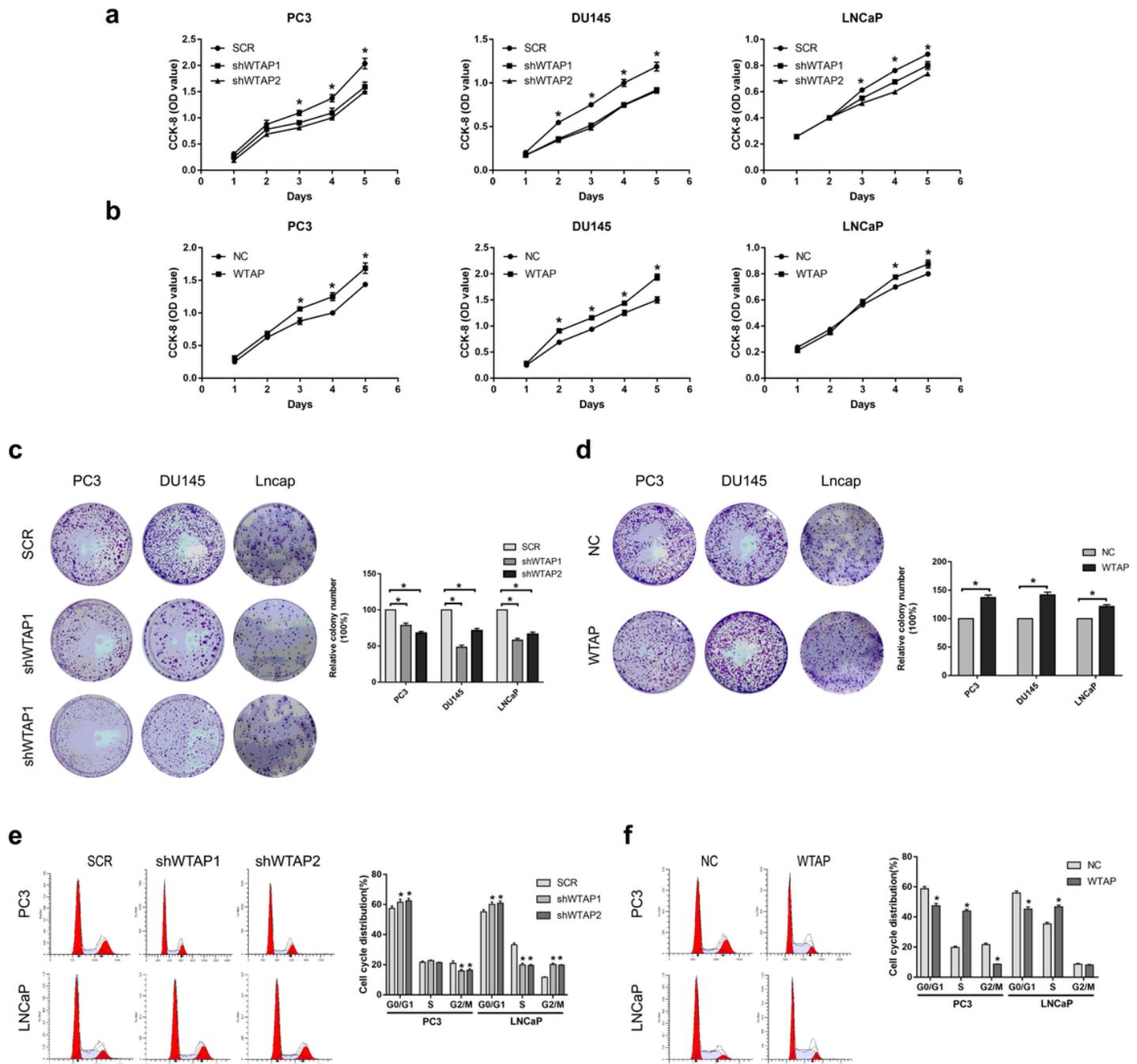


Figure 2

WTAP induced cell proliferation in vitro. a,b) Cell proliferation was measured by CCK-8 assays of PC3 or LNCaP cell lines after knocking-down or overexpression WTAP. c,d) Photographs of colony formation assays. Cell colony number was counted and presented as histogram. e,f) Cell cycle analysis performed by flow cytometry of PC3 or LNCaP cell lines after knocking down or overexpressing WTAP. The

histogram presents the percentage of cells in G0/G1, S and G2/M. Date represents the mean \pm SD from three independent experiments. *P<0.05



Figure 3

WTAP promoted proliferation in vivo. a Subcutaneous xenograft tumor model with WTAP knockdown (shWTAP) or scrambled negative control cells (SCR). b Tumor volume was measured at the indicated weeks after transplanting. c Tumor weight was measured after 4 weeks from transplanting. Date represents the mean \pm SD from three independent experiments. *P<0.05

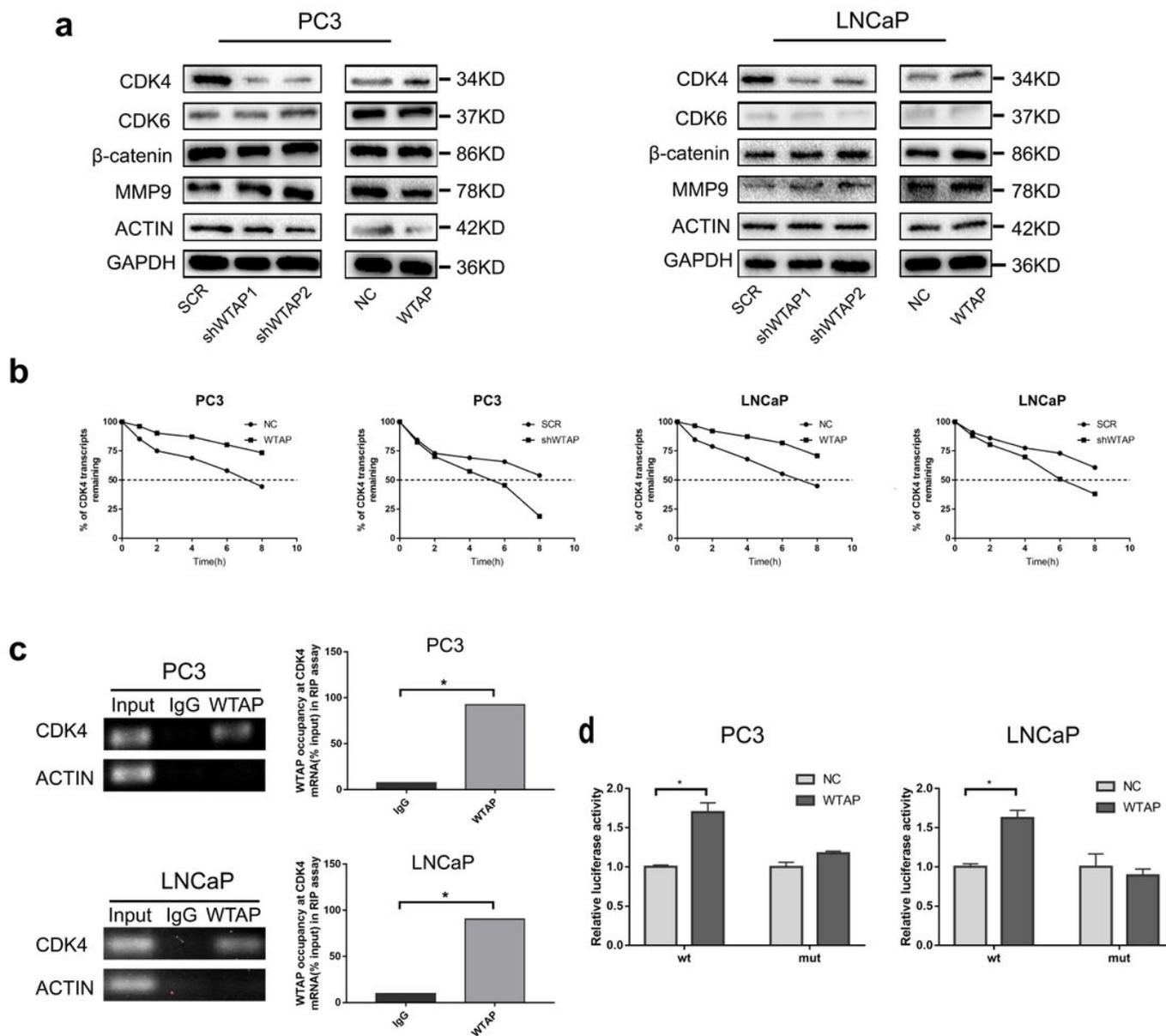


Figure 4

WTAP regulated CDK4 mRNA stability by binding to its transcript. a Western blot analysis of CDK4, CDK6, β -catenin and MMP9 in PC3 and LNCaP cells with WTAP knockdown or overexpression. b In PC3 or LNCaP cells, overexpression of WTAP could increase the half-life of CDK4 transcript and knockdown WTAP could shorten the half-life of CDK4 transcript. c Prostate cancer cell lysates were immunoprecipitation with WTAP antibody or control IgG followed by RT-PCR and qRT-PCR. d The effect of WTAP on a wild type pLenti-UTR-Luc vector (wt) or a mutant pLenti-UTR-Luc vector (mut) were measured by luciferase reporter gene assays in PC3 and LNCaP cells. Data represents the mean \pm SD from three independent experiments. *P<0.05

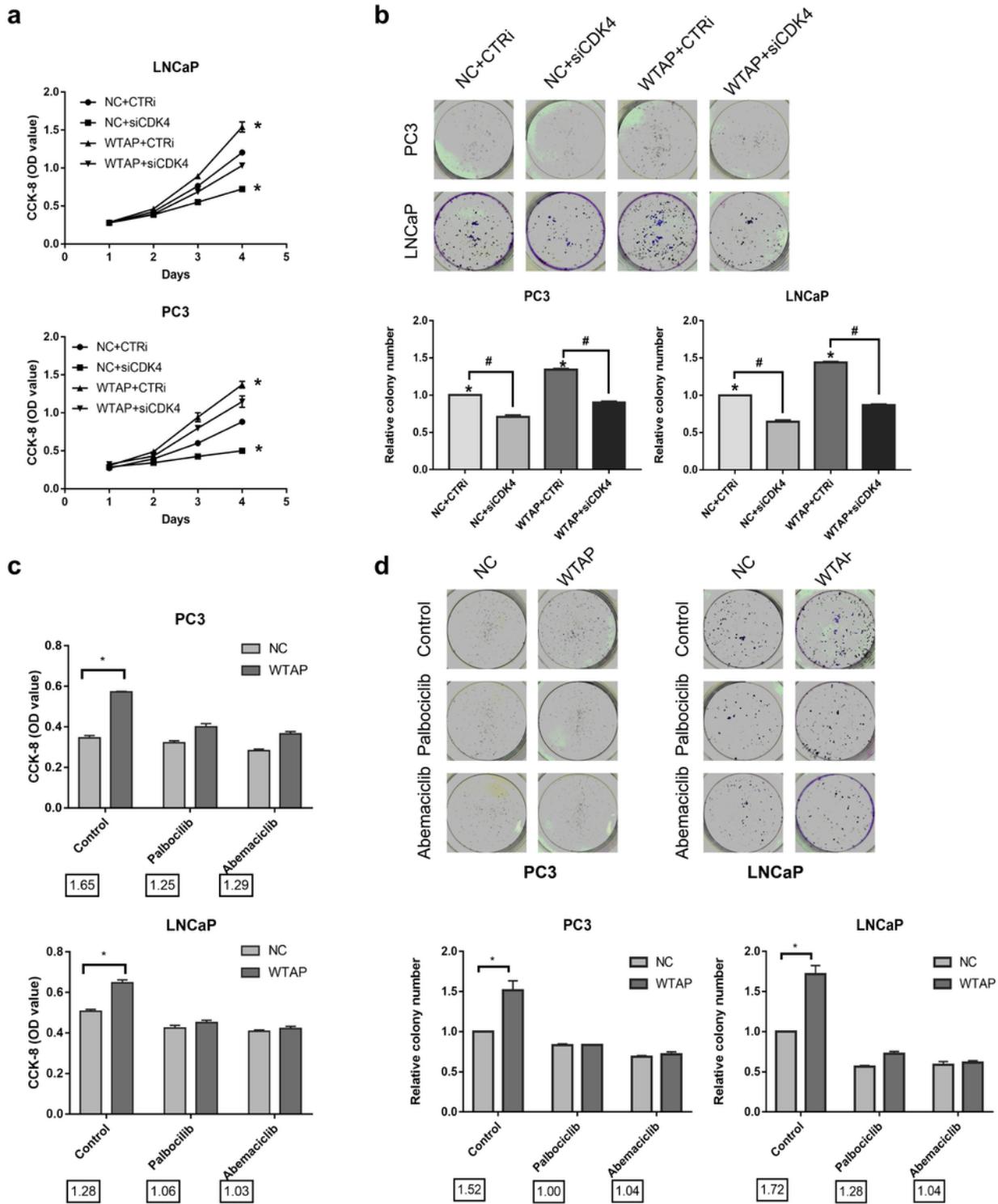


Figure 5

Reduced of CDK4 expression could reverse the proliferation induced by WATP. a,b) The effect of CDK4 small interference RNA (siCDK4) on PC3 and LNCaP cells with WTAP overexpression were tested by CCK-8 and colony formation assays. Cell colony number was counted and presented as histogram. c,d) CCK8 and colony formation assays were used to test the effect of CDK4 inhibitors (palbociclib and abemaciclib) on PC3 and LNCaP cells with WTAP overexpression. The fold change (WTAP/NC) was

presented under the histogram. Data represents the mean \pm SD from three independent experiments.
*P<0.05

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