

RETRACTED ARTICLE: CCNB1 Expedites the Progression of Cervical Squamous Cell Carcinoma via the Regulation by FOXM1

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Background: Cervical squamous cell carcinoma (CSCC) is responsible for 80–85% of cervical cancer. Cyclin B1 (CCNB1) represents a hub gene during the development of cervical cancer. However, the oncogenic role of CCNB1 in CSCC remains unclear. Our study aims to explore the mechanism underlying CCNB1 regulation on cell cycle progression in CSCC cells.

Methods: First, we analyzed differentially expressed genes from CSCC dataset GSE63678 and conducted gene function enrichment analysis. Subsequently, CCNB1 expression was knocked down in CSCC cell lines to assess cell proliferation, apoptosis, and cell cycle distribution. After the validation of the binding relationship between forkhead box protein M1 (FOXM1) and the promoter of CCNB1, the effect of FOXM1 on CCNB1 expression and on CSCC cell growth and apoptosis was verified. We further analyzed the histone ChIP-Seq data of CCNB1 in CSCC cells and measured the acetylation levels of the CCNB1 promoter histones.

Results: CCNB1 was overexpressed in CSCC tissues and cells, and CCNB1 silencing inhibited the growth of CSCC cells, and promoted cell cycle arrest and apoptosis. FOXM1 potentiated CCNB1 transcription by binding to its promoter and recruiting CBP/P300, a histone acetyltransferase. Further increasing FOXM1 expression or increasing P300 activity in CSCC cells with CCNB1 knockdown elevated CCNB1 expression and proliferation and cell cycle progression of CSCC cells. Knockdown of CCNB1 activated the p53 pathway in CSCC cells.

Conclusion: FOXM1 inhibited the activation of the p53 pathway by recruiting CBP/P300, which promoted the transcription of CCNB1, resulting in the growth and cell cycle progression of CSCC cells.

Keywords: cervical squamous cell carcinoma, CCNB1, FOXM1, CBP/P300, p53 pathway, cell cycle

Introduction

Cervical cancer remains to be the second leading reason of cancer-related death in female aged 20 to 39 years, leading to 10 premature fatalities per week in this population.¹ The chronic infection induced by a sexually delivered virus called human papillomavirus is a main contributor for the progression of cervical cancer.² Cervical squamous cell carcinoma (CSCC) is the major subtype of cervical cancer, accounting for 80–85% of all cervical cancer diagnoses.³ Major progresses have been witnessed in the management of locally advanced and high-risk early stage patients during the past decade with the combination of cisplatin with radiation and

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gemcitabine added to cisplatin chemoradiation at advanced stage.⁴ Nevertheless, the 3-year to 5-year survival of patients in many developing countries is still under 50% for all stages combined.⁵ Consequently, much more efforts are needed to further elucidate the molecular mechanisms that involved in initiation and progression of CSCC, which might be helpful for developing better targets for the treatment of CSCC.

Defective cell cycle regulation represents the hallmark of malignancies supporting the cancer development, and normal cell cycle is governed by the synchronized and consecutive regulation of cyclin-dependent kinases activity.⁶ Cyclins A, D and E mediate the transition from G1 to S stage, while cyclins A and B the passage from G2 to M stage.⁷ Intriguingly, cyclin B1 (CCNB1), an important component of the cell cycle pathway, was identified as one of the hub genes to exert a substantial influence on the development of cervical cancer.⁸ Therefore, targeting CCNB1 may be a promising strategy for the treatment of papillomavirus-related malignancy by reactivating p53.⁹ A microarray screen was performed in the present work, which revealed that CCNB1 was significantly overexpressed in CSCC tissues and cell lines. Moreover, a transcriptional factor, forkhead box protein M1 (FOXM1), located in 12p13.33, was identified as an upstream modulator of differentially expressed genes in cervical cancer.¹⁰ In addition, overexpression of FOXM1 results in malignant phenotypes by directly upregulating CCNB1.¹¹ Nevertheless, the molecular mechanisms behind remain unknown up to now. In the current study, we determined the connection between FOXM1 and CCNB1 expression in the context of CSCC. By gain- and loss-of-function studies, we explored the biological function of CCNB1 and FOXM1 in cell growth, apoptosis and cell cycle progression, and elucidated the possible underlying mechanisms *in vitro*.

Materials and Methods

Reagents, Plasmids, Antibodies and Primers

CREB binding protein (CBP)/E1A binding protein P300-specific activator N-(4-chloro-3-trifluoromethyl-phenyl)

-2-ethoxy-6-pentadecyl-benzamide (CTPB, #CAS No: 586976-24-1, MedChemExpress, Monmouth Junction, NJ, USA) was configured into a solution using dimethylsulfoxide (DMSO). Three small interfering RNAs (siRNAs) targeting CCNB1 (CAT#: SR311714) were synthesized and purchased from OriGene Technologies (Beijing, China). The overexpression vector and empty vector of FOXM1 were synthesized at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Antibodies used in Western blot analysis including: CCNB1 (ab32053, Abcam, Cambridge, MA, USA, KO validation), FOXM1 (#GTX102170, GeneTex, Inc., Alton Pkwy Irvine, CA, USA, KO validation), proliferating cell nuclear antigen (PCNA, #60097-1-1, ProteinTech Group, Chicago, IL, USA, KO validation), B-cell CLL/lymphoma 2 (Bcl-2, #MA5-11757, Invitrogen, KD validation), BCL-associated X (Bax, ab32503, Abcam, KO validation), p53 up-regulated modulator of apoptosis (PUMA, #GTX29643, Genetex, KO/KD validation), p53 (#13-4000, Invitrogen, IP-MS validation), p53 (#33-7000, Invitrogen Inc., Carlsbad, CA, USA, KD validation), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab8245, Abcam), acetylation of histone H3 lysine 27 (H3K27ac, #MA5-24671, Invitrogen, cell treatment validation). The primers used for quantitative real-time polymerase chain reaction (qPCR) were designed using Primer Premier 5.0 (Premier, Canada) and synthesized by Sangon. The primer sequence list is shown in Table 1.

Bioinformatics Analysis

Firstly, the expression microarray GSE63678 for CSCC was downloaded from Gene Expression Omnibus (GEO) database website (<https://www.ncbi.nlm.nih.gov/geo/>) with the GPL platform: [HG-U133A_2] Affymetrix Human Genome U133A 2.0 Array. Differentially expressed genes were analyzed with the help of the Limma R package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>). The pHeatmap R package (<https://cran.r-project.org/web/packages/pheatmap/index.html>) was utilized to create a heatmap. Functional analysis of differentially expressed

Table 1 Primer Sequences Used for RT-qPCR

Targets	Forward (5'-3')	Reverse (5'-3')	Accession No
CCNB1	GACCTGTGTCAGGCTTCTCTG	GGTATTTTGGTCTGACTGCTTGC	NM_031966
FOXM1	TCTGCCAATGGCAAGGTCTCCT	CTGGATTCCGGTCGTTTCTGCTG	NM_001243088
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA	NM_001256799

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative real-time polymerase chain reaction.

genes was subsequently conducted using Gene Set Enrichment Analysis (GSEA) software (<https://www.gseamsigdb.org/gsea/index.jsp>). Expression of cyclin D1 (CCND1), cell division cycle 7-related protein kinase (CDC7), CCNB1, and cyclin E2 (CCNE2) in gynecologically associated malignancies, including endocervical adenocarcinoma (CESC), breast carcinoma (BRCA), ovarian cancer (OV), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS), in addition to the correlation between CCNB1 and FOXM1 in The Cancer Genome Atlas (TCGA)-CESC were predicted using the GEPIA website (<http://gepia.cancer-pku.cn/index.html>). The transcription factor binding to CCNB1 promoter was predicted using the TRRUST website (<https://www.grnpedia.org/trrust/>). JASPAR website (<http://jaspar.genereg.net/>) was then applied to predict the binding sites between FOXM1 and CCNB1 promoter. The level of promoter histone acetylation of CCNB1 was predicted using the ENCODE website (<https://www.encodeproject.org/>).

Cell Culture and Transfection

CSCC cells (CaSki, ME-180, C33A, and SiHa) derived from Otwo Biotech (<http://www.otwobiotech.com/>, Shenzhen, Guangdong, China) as well as normal immortalized epithelial cells (HaCaT) derived from CLS Cell Lines Service GmbH (https://www.cls-gmbh.de/en/800_HaCaT.html, Eppelheim, Germany) were included. After confirmation of the absence of mycoplasma contamination using short sequence tandem repeat region analysis, cells were grown in Dulbecco's modified Eagle's medium (Noble Ryder Technology Co., Beijing, China) supplemented with penicillin-streptomycin (100 $\mu\text{g}/\text{mL}$) and 10% FBS at 37°C in a 5% CO₂ incubator.

SiHa and CaSki cells were plated into 6-well plates at a concentration of 1×10^6 cells/well and cultured till their confluency reached 70%–80%. Cells were then transfected with siRNA or overexpression plasmids (50 nM) using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) as per the kit instructions. At 48 h post-transfection, the transfection efficiency was assessed by RT-qPCR and Western blot.

5'-Ethynyl-2'-Deoxyuridine (EdU) Assay

EdU staining was carried out using a Click-iTEdU imaging kit (Invitrogen) as per the protocol. In brief, the cells were exposed to 50 μM EdU for 2 h and fixed with 4% formaldehyde. Cells were then treated with 2 mg/mL glycine to neutralize formaldehyde and permeabilized

with 0.5% Triton X-100. Finally, the cells were reacted with 100 μL 1X Apollo reaction solution for 0.5 h and incubated with 100 μL Hoechst 33342 (5 $\mu\text{g}/\text{mL}$). Images were acquired using an Olympus IX-71 inverted microscope (Tokyo, Japan). The percentage of EdU-positive cells was determined by dividing the number of EdU-positive cells by the number of Hoechst-stained cells.

CellTiter Glow

A total of 2×10^3 cells was plated in 96-well plates in quadruplicate. At the indicated time points, cell viability was assessed by Celltiter Glo assay (Promega, Madison, WI, USA), and bioluminescence signals were measured with a TECAN Infinite 2000 plate reader (TECAN, Maennedorf, Zürich, Switzerland).

Clonogenic Assays

A total of 1×10^2 cells were plated into 6-well plates. After 2 weeks of cell growth, 4% paraformaldehyde fixing and a 0.1%–0.4% crystal violet (Beyotime, Shanghai, China) staining, clonality was measured under the microscope.

RT-qPCR

Cells were harvested after transfection, and RNA was isolated using TRIzol reagent (Sigma-Aldrich). Then, 10 ng RNA was applied for reverse transcription using the TaqMan MicroRNA RT kit (Applied Biosystems, Life Technologies, Madison, WI, USA). Briefly, 5 μL RNA was added to 10 μL stock solution containing 0.15 μL dNTP (100 nM), 1 μL multiscribe enzyme (50 U/ μL), 1.5 μL 10 \times RT-buffer, 0.19 μL RNase inhibitor (20 U/ μL), 4.16 μL RNase-free H₂O and 3 μL primers. Quantitative real-time PCR of each miRNA was performed in a total volume of 10 μL containing 5 μL TaqMan stock solution, 3.17 μL RNase-free H₂O, 0.5 μL TaqMan primer, and 1.33 μL cDNA. PCR was performed in a quadruple reaction in Rotor-Gene Corbett Q 6000 PCR. Data were collected and analyzed using Rotor-Gene quantitative software.

Western Blot

Cells were lysed with radio immunoprecipitation assay lysis buffer containing proteinase inhibitors (Roche, Basel, Switzerland) and phosphatase inhibitors (Sangon Biotech, Shanghai, China) on a shaker at 4°C overnight. After quantification using the Pierce bicinchoninic acid assay protein assay kit (Thermo Fisher Scientific), equal amounts of protein were boiled and loaded for Western blot analysis. Polyvinylidene fluoride membranes were

then probed with primary antibodies (1:1000) at 4°C for one night and then with horseradish peroxidase-conjugated anti-rabbit/mouse IgG secondary antibody (CW Biotechnology Co., Ltd., Beijing, China) for 60 min at ambient temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific) and a ChemiDoc Touch imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Flow Cytometry

Apoptosis rate and cell cycle distribution of CSCC cells were assessed by flow cytometric analysis. Cells were fixed in cold citric acid buffer (No. orb-EHJ024527, BIOHJSW, USA) for 10 min at 4°C to disperse the cells into a cell density of 2×10^5 cells/200 mL citric acid buffer, which were transferred into Falcon tubes. PE Annexin V Apoptosis Assay Kit (No. 640934-1, BioLegend, San Diego, CA, US) was used to determine the relative number of AnnexinV-fluorescein isothiocyanate (FITC)-positive, propidium iodide (PI)-negative cells. Cells were stained with PI using the PI Cell Cycle Kit (No. CSK-0112, Nexcelom Bioscience, Lawrence, MA, USA). Cells were treated as indicated and then FAScan was run to detect apoptosis rate and cell cycle distribution.

Chromatin Immunoprecipitation (ChIP)-qPCR

ChIP-qPCR was performed using the EZ-Magna ChIP™ Kit (Millipore Corp, Billerica, MA, USA) in strict accordance with the protocol. Antibodies against H3K27ac (5 µg, Abcam, Cambridge, MA, USA), FOXM1 (8 µg; Abcam, Cambridge, MA, USA) or IgG (5 µg; Sigma-Aldrich Chemical Company, St. Louis, MO, USA) were used to precipitate the chromatin. Primers used are listed below: CCNB1-promoter ChIP P1: forward, CCTATACAGGGGACCGGTTC, reverse, GCGGGACTTAGAAACCAGG; CCNB1-promoter ChIP P2: forward, GGTGGCTTACATATAGGGAGAG, reverse GCGACA TGGGGCTGCTTTAA; CCNB1-promoter ChIP P3: forward, AAGCCATCTGCCAAGAGCAG, reverse CTCTTTTACTGACGCTGCC.

Co-Immunoprecipitation (Co-IP)

Cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (P0013D, Beyotime, Shanghai, China) containing a mixture of protease inhibitors (469313200, Roche Diagnostics, Co., Ltd., Rotkreuz, Switzerland).

Immunoprecipitation and Western blot analyses were performed using anti-p300 antibody (ab54984, Abcam) and FOXM1 antibody (ab207298, Abcam), or control anti-immunoglobulin G antibody (X0936, DAKO, Santa Clara, CA, US).

Luciferase Assay

A total of 5×10^4 cells were seeded in 24-well plates the day prior to the transfection. Cells were then transfected with Lipofectamine 2000 (Invitrogen, USA) as per the manufacturer's instructions. At 48 h post-transfection, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Statistical Analysis

The values were displayed as the mean \pm SD of three replicates. Unpaired *t*-test (for comparison between two samples) and one-way or two-way analysis of variance followed by Tukey's test (for comparison among multiple samples) were applied for statistical analysis using SPSS 21.0 software (IBM, Chicago, IL, USA). $p < 0.05$ was indicative of a statistically significant difference.

Results

CCNB1 is Significantly Overexpressed in CSCC Tissues and Cells

We first downloaded the GSE63678 expression microarray from the GEO database, which contained five CSCC tissues and five normal tissues. A total of 584 upregulated genes and 519 downregulated genes (Figure 1A) were screened out in the CSCC tissues. The heatmap in Figure 1B shows the top 50 differentially expressed genes. We subsequently observed that the cell cycle pathway was significantly positively correlated in CSCC tissues by GSEA software (Figure 1C). Moreover, we further determined the expression of CCND1, CDC7, CCNB1, and CCNE2 in gynecological malignancies (CESC, BRCA, OV, UCEC, and UCS) by GEPIA. The expression of CCNB1 in gynecological malignancies was much higher than in the corresponding normal tissues (Figure 1D–G). In a study by Xiao et al, it was noted that MNX1 promotes the proliferation of SCC by promoting the expression of CCNE1 and CCNE2.¹² Zhen et al proposed that Toona Sinensis and Moschus Decoction promoted cell cycle arrest in CC cells by suppressing CDC7 expression.¹³ The occurrence and metastasis of CC caused by CCNB1 have not been thoroughly studied, so we chose CCNB1 as our study subject. Thus, we speculated that CCNB1 has a relevance in cervical

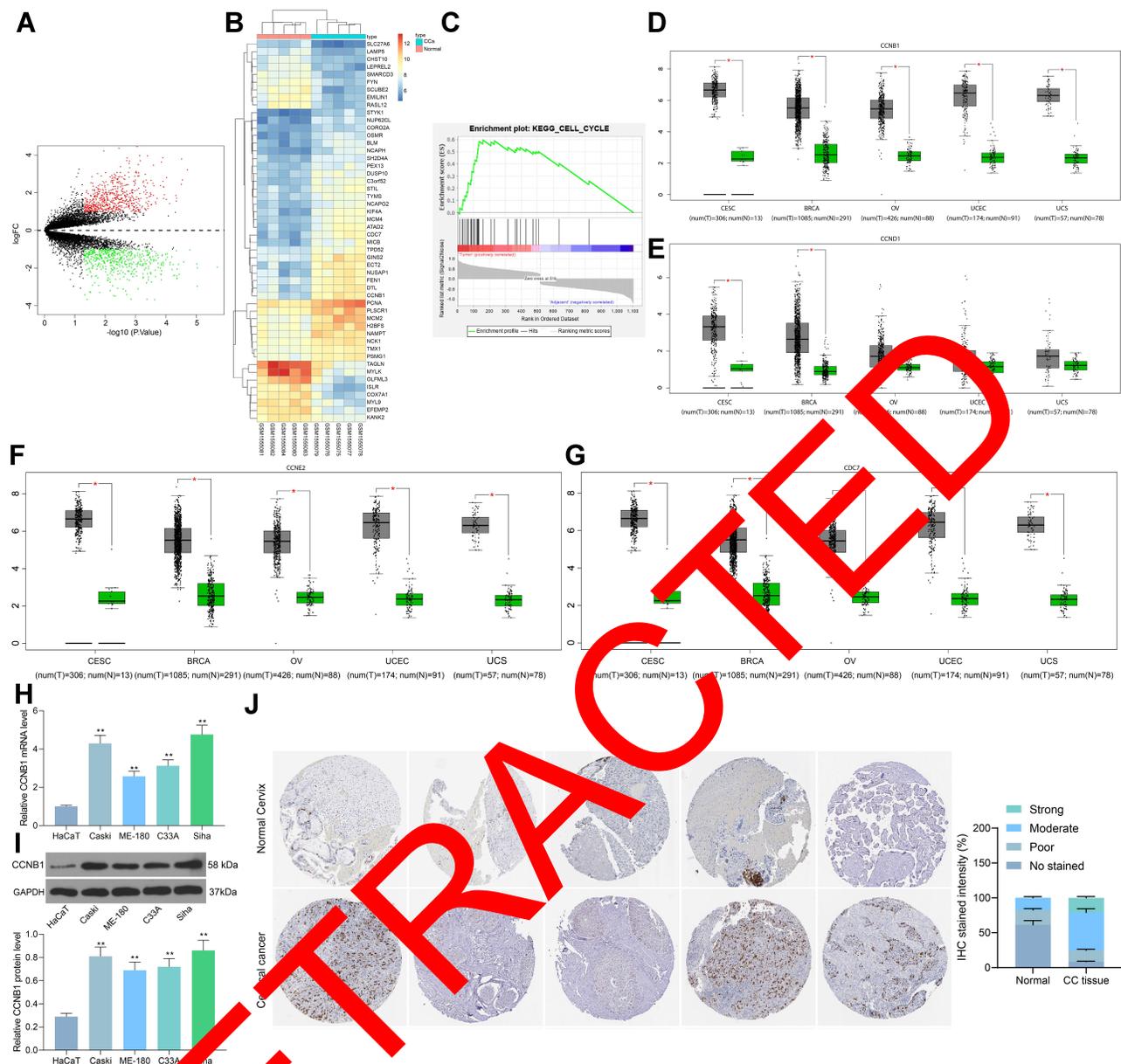


Figure 1 CCNB1 is overexpressed in CSCC tissues and cells. **(A)** Volcano diagram of differentially expressed genes in CSCC microarray GSE63678; **(B)** heatmap of the top 50 differentially expressed genes in CSCC microarray GSE63678; **(C)** cell cycle pathway positively correlates with cancer tissue in GSEA; **(D–G)** the expression of CCNB1 **(D)**, CCND1 **(E)**, CCNE1 **(F)**, and CDC7 **(G)** in gynecological malignancies, including CESC, BRCA, OV, UCEC, and UCS; **(H)** the mRNA expression of CCNB1 in normal cervical epithelial cells HaCaT and in CSCC cells measured by RT-qPCR; **(I)** the protein expression of CCNB1 in normal cervical epithelial cells HaCaT and in CSCC cells measured by Western blotting; **(J)** the intensity of staining in normal cervix and in cervical cancer tissues by IHC database and statistical analysis. The experiments were performed in triplicate and the results were expressed as mean ± SD. One-way analysis of variance followed by Tukey's test were applied for statistical analysis **(H and I)**. **p* < 0.05 vs normal tissues; ***p* < 0.01 vs HaCaT cells.

Abbreviations: CESC, cervical squamous cell carcinoma; CCNB1, cyclin B1; qPCR, quantitative real-time polymerase chain reaction; CCND1, cyclin D1; CDC7, cell division cycle 7-related protein kinase; CCNE2, cyclin E2.

carcinogenesis, and we detected the CCNB1 expression in normal cervical epithelial cells as well as in CSCC cells and noted that the CCNB1 expression was remarkably enhanced in CSCC cell lines (Figure 1H and I). Furthermore, we further queried from the human protein atlas (HPA) database that the

staining intensity of CCNB1 in normal cervical tissues was either poorly- or not-stained, whereas that in cervical cancer tissues was mostly moderately- or strongly stained (Figure 1J). All in all, we conjectured that CCNB1 plays an important part in the CSCC development.

CCNB1 Knockdown Hampers Growth and Cell Cycle Progression of Caski and Siha Cells

To clarify the role of CCNB1 in CSCC growth, we transfected three siRNAs targeting CCNB1 into Caski and Siha cells with the relative high level of CCNB1. We performed RT-qPCR and Western blot by detecting the CCNB1 expression in cells to confirm the success of transfection and to exclude off-target effects, and we found the highest efficiency of CCNB1-siRNA-#2 (Figure 2A and B). We assayed cell activity by CellTiter Glow kit and we noted that CCNB1-siRNA significantly inhibited CSCC cell activity

(Figure 2C). Moreover, we further observed that the proliferative capacity of Caski and Siha cells was greatly reduced in CSCC cells with poor expression of CCNB1 (Figure 2D and E). Furthermore, we tested the proportion of apoptosis in the cells, and the results of flow cytometry showed an augment in the proportion of apoptosis after diminishing the CCNB1 expression in the cells (Figure 2F). Subsequently, we used flow cytometry to examine cell cycle distribution in Caski and Siha cells, which revealed significantly more S-phase cell cycle arrest in poorly-expressing CCNB1 cells (Figure 2G). Later, Western blot was utilized to measure the expression of proliferation- and apoptosis-related proteins. After CCNB1 knockdown, the expression patterns of the

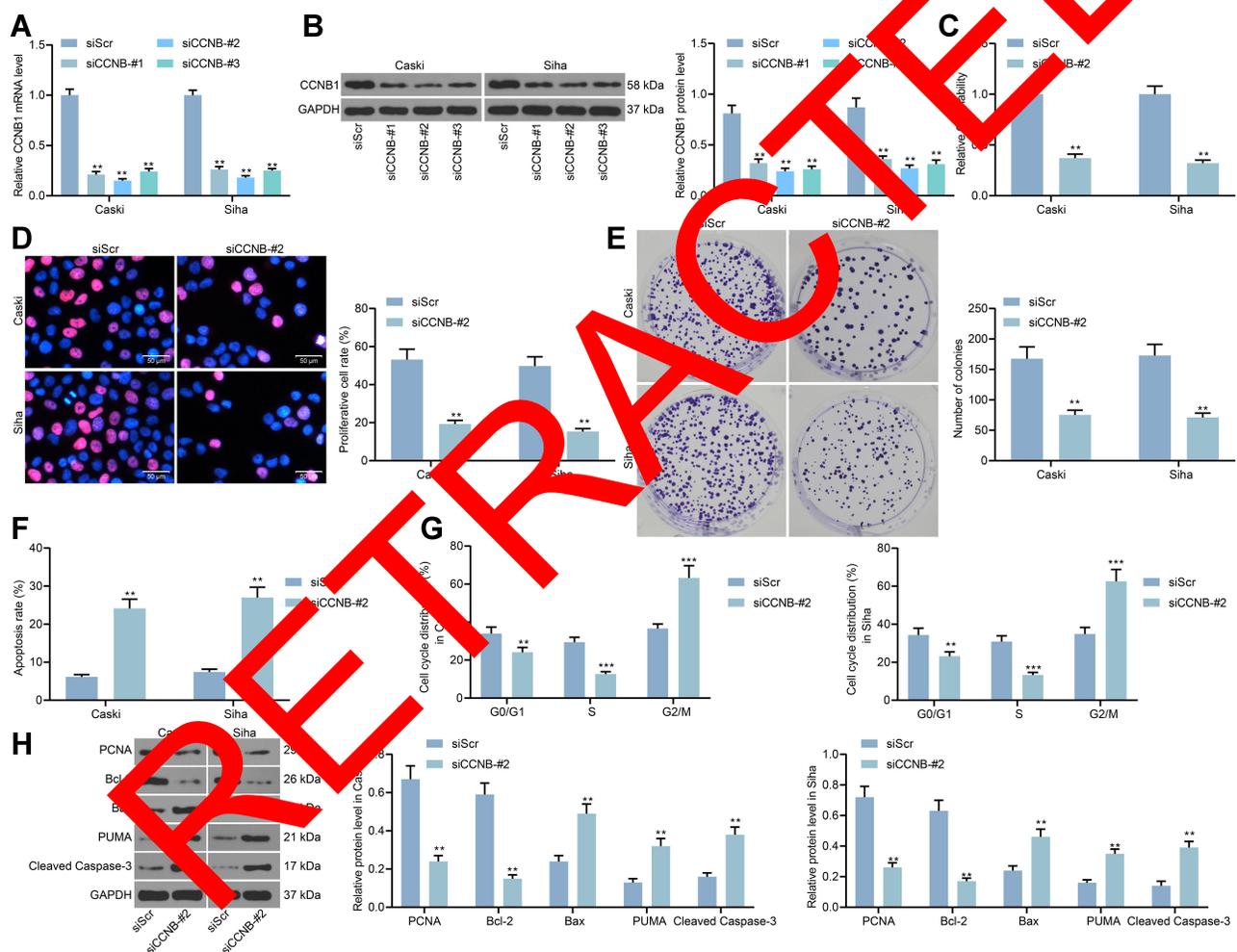


Figure 2 Knockdown of CCNB1 inhibits the growth of Caski and Siha cells and cell cycle progression in vitro. Three siRNAs targeting CCNB1 were delivered into Caski and Siha cells, which have the relative high expression of CCNB1. (A) The mRNA expression of CCNB1 in cells measured by RT-qPCR; (B) the protein expression of CCNB1 in cells measured by Western blot; (C) cell growth activity in Caski and Siha cells evaluated by Cell Titer Glow kit; (D) the proliferative activity of Caski and Siha cells measured by EdU staining; (E) the number of colonies formed evaluated by colony formation assay; (F) cell apoptosis in Caski and Siha cells by flow cytometry; (G) cell cycle distribution in Caski and Siha cells by flow cytometry; (H) the expression of PCNA, Bcl-2, Bax, PUMA and Cleaved Caspase-3 in Caski and Siha cells measured by Western blot. The experiments were performed in triplicate and expressed as mean \pm SD. Two-way analysis of variance followed by Tukey's test were applied for statistical analysis. ** $p < 0.01$, *** $p < 0.001$ vs siScr.

Abbreviations: CCNB1, cyclin B1; PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell CLL/lymphoma 2; Bax, BCL-associated X; PUMA, p53 up-regulated modulator of apoptosis; qPCR, quantitative real-time polymerase chain reaction; siRNAs, small interfering RNA; EdU, 5'-Ethynyl-2'-deoxyuridine.

proliferation-related factors PCNA and Bcl-2 decreased remarkably, whereas the expression patterns of the apoptosis-related proteins Bax, PUMA and Cleaved Caspase-3 increased significantly in Siha and Caski cells (Figure 2H).

FOXM1 Activates CCNB1 Expression Transcriptionally

To further clarify the upstream regulatory mechanism of CCNB1, we used the TRRUST website to predict the transcription factors (Figure 3A) of CCNB1. We focused on FOXM1 and noticed a moderate positive correlation between FOXM1 and CCNB1 expression in TCGA-CESC (Figure 3B). We then predicted FOXM1 binding sites around 1000 bp upstream of CCNB1 via the JASPAR website. FOXM1 was found to be able to bind to the

promoter sequence upstream of CCNB1 (Figure 3C and D). Thus, we tested the binding relationship between FOXM1 and CCNB1 in Caski and Siha cells by CHIP-qPCR experiments, which displayed that the enrichment level of the CCNB1 promoter fragment pulled down using anti-FOXM1 antibody was much higher than that of IgG (Figure 3E). To further determine the binding relationship between FOXM1 and the CCNB1 promoter, we constructed a luciferase reporter vector containing the CCNB1 promoter and an overexpression plasmid of FOXM1. We co-transfected the overexpression plasmid of FOXM1 with the luciferase reporter vector into 293T cells, and we found that the luciferase activity in 293T cells was significantly increased (Figure 3F). We also found that FOXM1 was expressed at significantly higher

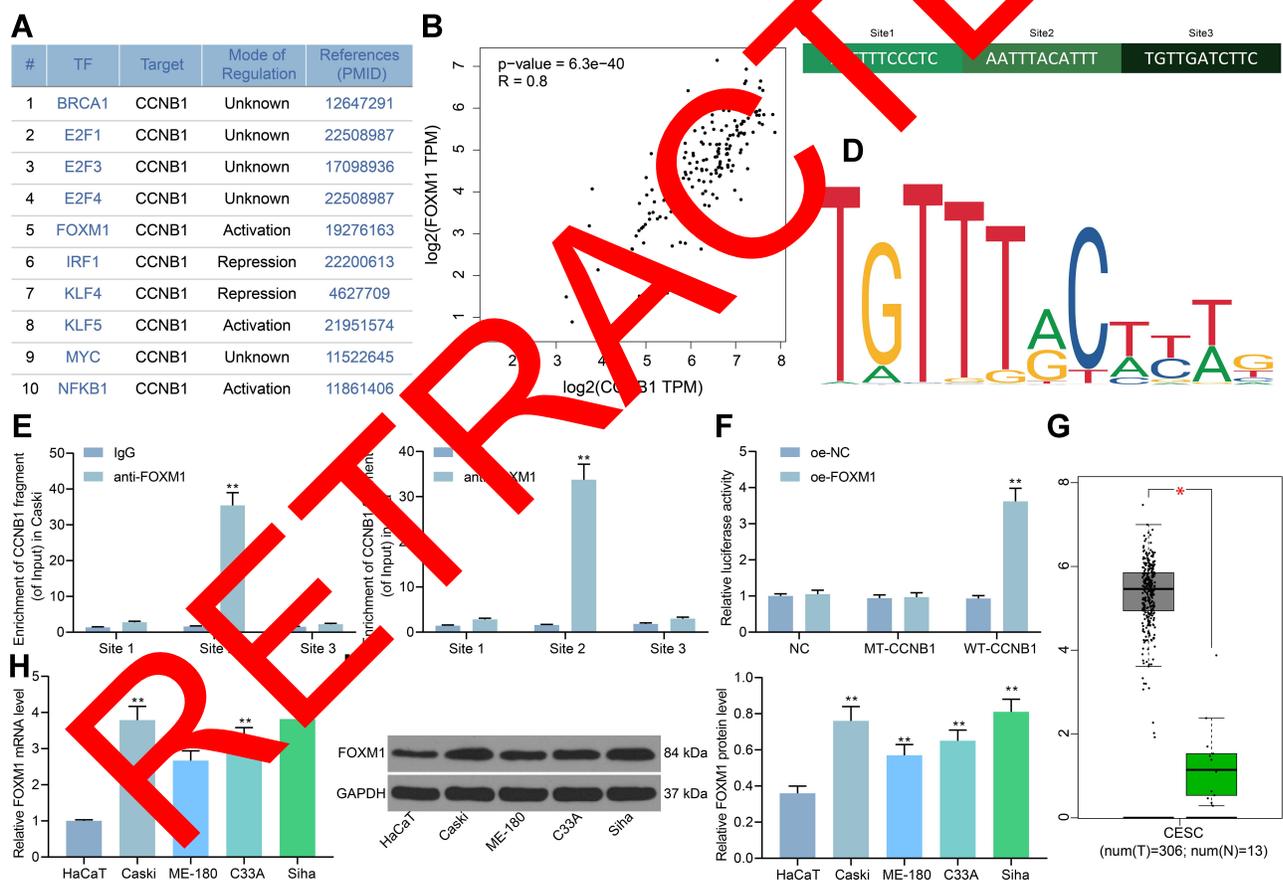


Figure 3 CCNB1 is transcriptionally activated by FOXM1. **(A)** The transcription factors for CCNB1 predicted by the TRRUST website; **(B)** correlation between FOXM1 and CCNB1 expression in TCGA-CESC dataset analyzed by Spearman's rho correlation; **(C)** the binding relationship of FOXM1 to the CCNB1 promoter predicted by the JASPAR website; **(D)** the conservative binding sequence of FOXM1; **(E)** binding of FOXM1 to the CCNB1 promoter in Caski and Siha cells detected by CHIP-qPCR experiments; **(F)** luciferase activity in 293T cells co-transfected with the FOXM1 overexpression plasmid and the luciferase reporter vector assessed by luciferase reporter assay; **(G)** the expression of FOXM1 in normal cervical tissue and CESC predicted by GEPIA website; **(H)** the mRNA expression of FOXM1 in normal cervical epithelial cells HaCaT and in CSCC cells measured by RT-qPCR; **(I)** the expression of FOXM1 in normal cervical epithelial cells HaCaT and in CSCC cells measured by Western blot. The experiments were performed in triplicate and expressed as mean \pm SD. One-way analysis of variance followed by Tukey's test were applied for statistical analysis (**E**, **F**, **H** and **I**). * $p < 0.05$ vs normal tissues; ** $p < 0.01$ vs HaCaT cells, IgG or oe-NC treatment.

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; CSCC, cervical squamous cell carcinoma; CESC, endocervical adenocarcinoma; CHIP, chromatin immunoprecipitation.

levels in CESC than in normal cervical tissues (Figure 3G), and the same trend was noted in CSCC cell lines (Figure 3H and I).

Overexpression of FOXM1 Promotes Growth of CSCCs

To determine the role of FOXM1 in regulating CCNB1 expression in CSCC cell growth as well as apoptosis, we further transfected FOXM1 overexpression plasmids into Caski and Siha cells with poor expression of CCNB1. There was a significant elevation in CCNB1 expression in Caski and Siha cells after overexpression

of FOXM1 (Figure 4A and B). We found that after upregulation of CCNB1 in Caski and Siha cells by the FOXM1 overexpression plasmid, the activity as well as the proliferative capacity of CSCC cells was enhanced as well (Figure 4C and D). Furthermore, we found that S-phase cell arrest due to CCNB1-siRNA was significantly mitigated by the overexpression of FOXM1 (Figure 4E), and even the level of apoptosis was significantly reduced (Figure 4F). The application of Western blot to detect apoptosis- and proliferation-related proteins in cells has confirmed experimental results (Figure 4G).

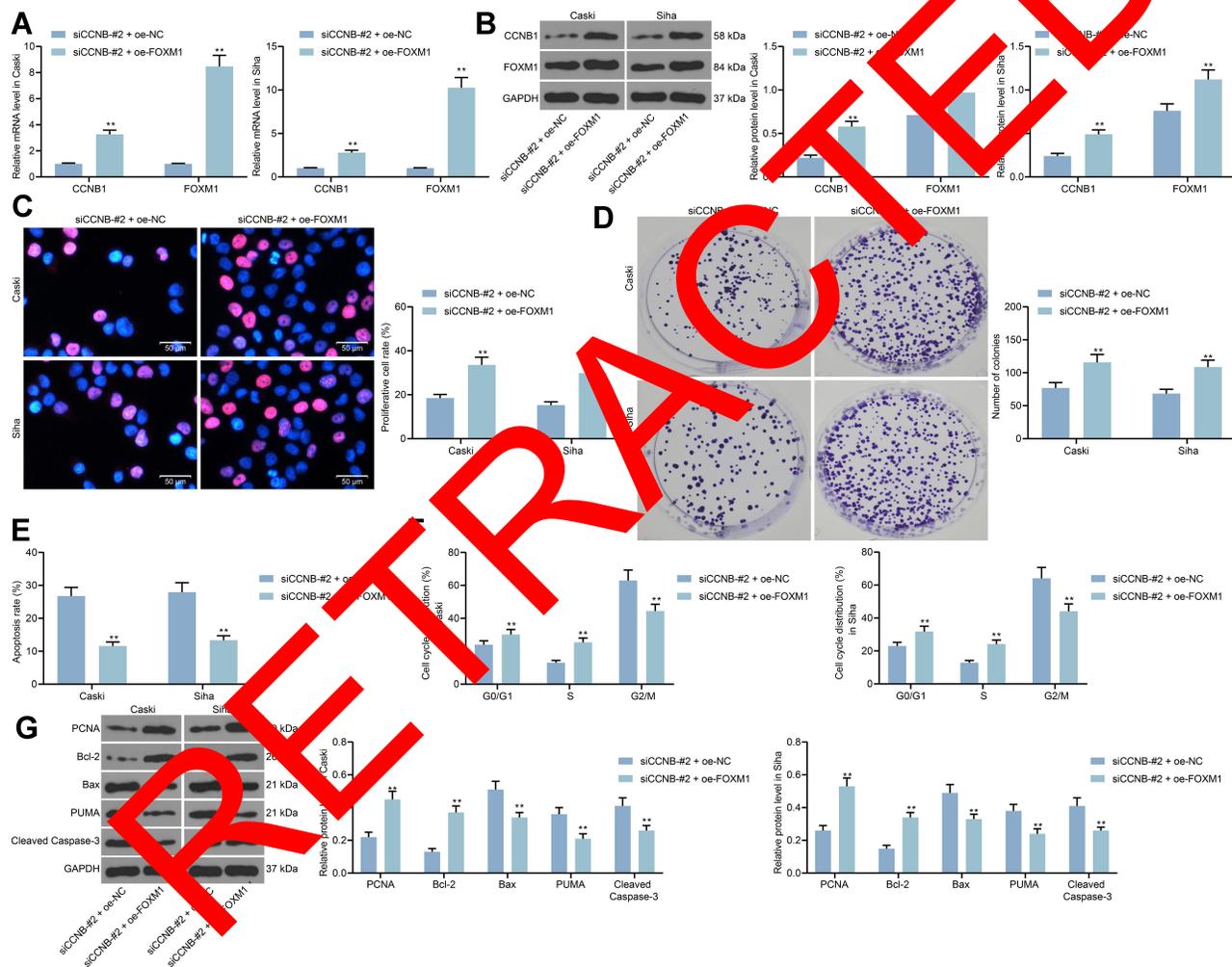


Figure 4 Overexpression of FOXM1 promotes the growth of CSCC cells. The overexpression plasmid of FOXM1 was further delivered into Caski and Siha cells with poor expression of CCNB1. (A) The expression of CCNB1 and FOXM1 in CSCC cells after co-transfection at mRNA level measured by RT-qPCR; (B) the expression of CCNB1 and FOXM1 in CSCC cells after co-transfection at protein level measured by Western blot; (C) the proliferative activity of Caski and Siha cells measured by EdU staining; (D) the number of colonies formed assessed by colony formation assay; (E) cell apoptosis in Caski and Siha cells by flow cytometry; (F) cell cycle distribution in Caski and Siha cells by flow cytometry; (G) the protein expression of PCNA, Bcl-2, Bax, PUMA and Cleaved Caspase-3 in Caski and Siha cells measured by Western blot. The experiments were performed in triplicate and expressed as mean \pm SD. Two-way analysis of variance followed by Tukey's test were applied for statistical analysis. ** $p < 0.01$ vs siCCNB1 + oe-NC.

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; CSCC, cervical squamous cell carcinoma; CHIP, chromatin immunoprecipitation; PCNA, proliferating cell nuclear antigen; Bcl-2, B-cell CLL/lymphoma 2; Bax, BCL-associated X; PUMA, p53 up-regulated modulator of apoptosis; qPCR, quantitative real-time polymerase chain reaction; EdU, 5'-Ethylnyl-2'-deoxyuridine; oe, overexpression; NC, negative control.

FOXMI Promotes H3K27ac to Activate CCNB1 Expression by Recruiting CBP/P300

We speculated that there could be a super enhancer in the CCNB1 promoter sequence to promote the CCNB1 transcription. We first predicted the H3K27ac level of the promoter of CCNB1 in Hela cells via the ENCODE website, and we found that the promoter sequence of CCNB1 had a significant H3K27ac level (Figure 5A). We first examined the acetylation levels of histone H3K27 in normal cervical epithelial cells as well as in CSCC cells, and we found that the levels of H3K27ac were significantly higher in the CSCC cell lines than those of HaCaT cells (Figure 5B). ChIP experiments were then conducted using

H3K27ac antibodies to detect the acetylation modification of H3K27 in the promoter histone of CCNB1, we found that the promoter of CCNB1 has a higher level of acetylation modification (Figure 5C) in CSCC cells. In a study by Mansour et al, it was noted that CBP/P300 activated the TAL1 promoter H3K27ac, thereby enhancing TAL1 expression.¹⁴ Thus, we hypothesized that CBP/P300 promoted acetylation modification of the CCNB1 promoter to facilitate its transcription. We first used the CBP/P300-specific inhibitor P300/CBP-IN-5 in the CSCC cell lines and found that the histone H3K27 acetylation level of the CCNB1 promoter was significantly reduced in CSCC cells (Figure 5D) and that the cells had significantly lower levels of CCNB1 mRNA and protein (Figure 5E and F). To further determine that FOXM1 enhances CCNB1

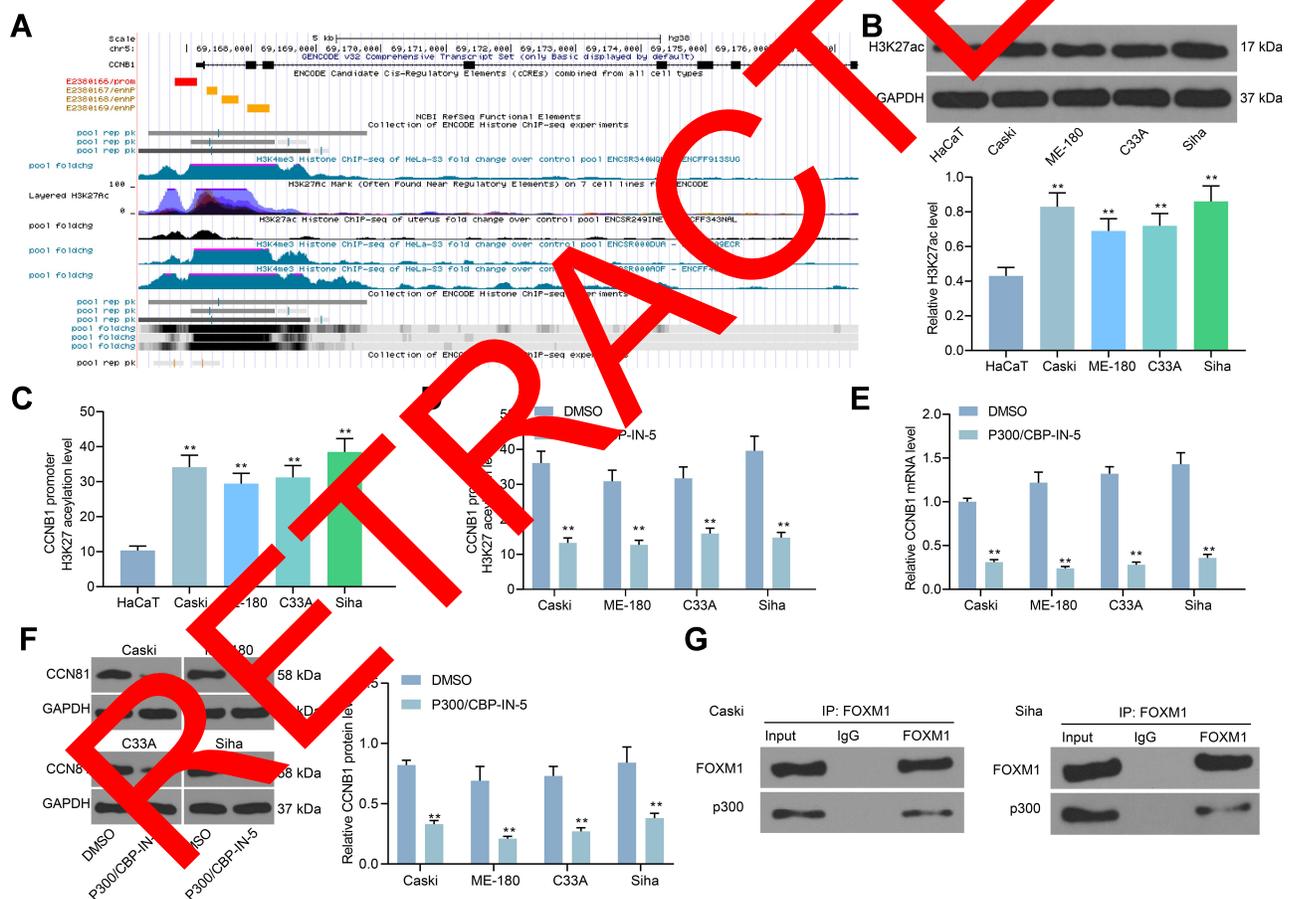


Figure 5 FOXM1 promotes H3K27ac levels by recruiting CBP/P300. (A) H3K27ac levels of the promoter of CCNB1 in Hela cells predicted by ENCODE website; (B) H3K27ac levels in HaCaT and CSCC cells determined by Western blot; (C) histone H3K27 acetylation levels of the CCNB1 promoter sequence in HaCaT and CSCC cells measured by ChIP experiments; (D) histone H3K27 acetylation levels of the CCNB1 promoter sequence in CSCC cells in response to the CBP/P300 specific inhibitor measured by ChIP experiments; (E) the mRNA expression of CCNB1 in CSCC cells in response to the CBP/P300 specific inhibitor measured by RT-qPCR; (F) the protein expression of CCNB1 in CSCC cells in response to the CBP/P300 specific inhibitor measured by Western blot; (G) Co-IP detection of FOXM1 binding to p300 in Caski and SiHa cells. The experiments were performed in triplicate and expressed as mean \pm SD. Two-way analysis of variance followed by Tukey's test were applied for statistical analysis (B–F). ** $p < 0.01$ vs HaCaT cell or CSCC cells treated with DMSO.

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; CSCC, cervical squamous cell carcinoma; ChIP, chromatin immunoprecipitation; qPCR, quantitative real-time polymerase chain reaction; CBP, CREB binding protein; CTPB, N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide; siRNAs, small interfering RNA.

transcription by recruiting p300 and thereby enhancing CCND1 transcription, we used a Co-IP assay to examine the binding relationship of FOXM1 to p300 in SiHa cells and Caski cells. We found FOXM1 expression in the complexes precipitated with the anti-p300 antibody and p300 expression in the complexes precipitated with the anti-FOXM1 antibody. The above results suggest that FOXM1 activated the expression of CCNB1 by recruiting p300 (Figure 5G). The above data signposted that the transcription factor FOXM1 modifies histone H3K27ac by recruiting CBP/P300 to promote CCNB1 expression.

CTPB Treatment Activates CCNB1 in CSCC Cells to Promote Their Growth

To further explore the effect of H3K27ac modification of the CCNB1 promoter on CSCC growth and apoptosis, we added the CBP/P300-specific activator CTPB to Caski and SiHa cells with poor expression of CCNB1. Significant augments in the level of H3K27 acetylation of the CCNB1 promoter histone and the expression of CCNB1 were observed after CTPB supplement (Figure 6A–C). Additionally, the proportion of EdU-positive cells was

drastically increased in Caski and SiHa cells (Figure 6D), and the proportion of apoptosis in Caski and SiHa cells was considerably downregulated (Figure 6E), and the cell cycle arrest was remarkably inhibited (Figure 6F). It showed that enhancing the H3K27ac modification of the CCNB1 promoter histone by CBP/P300 in cells significantly promoted the expression of CCNB1 in CSCC cells, as well as cell growth and cell progression.

Silencing of CCNB1 Activates p53 Pathway in CSCC Cells

GSEA of differentially expressed genes revealed that the p53 pathway was significantly negatively regulated in cancer tissues (Figure 7A). It was noted by Fan et al that CCNB1 silencing significantly promoted the activation of the p53 pathway in hepatocellular carcinoma cells.¹⁵ Thus, we first examined the expression of CDK1, p53 and p21 in the p53 pathway in cells and found that after silencing of CCNB1 the expression of CDK1 was significantly decreased, while the expression of p53 and p21 was drastically boosted. However, upon further CCNB1 expression promoted by overexpression of FOXM1 or enhancement

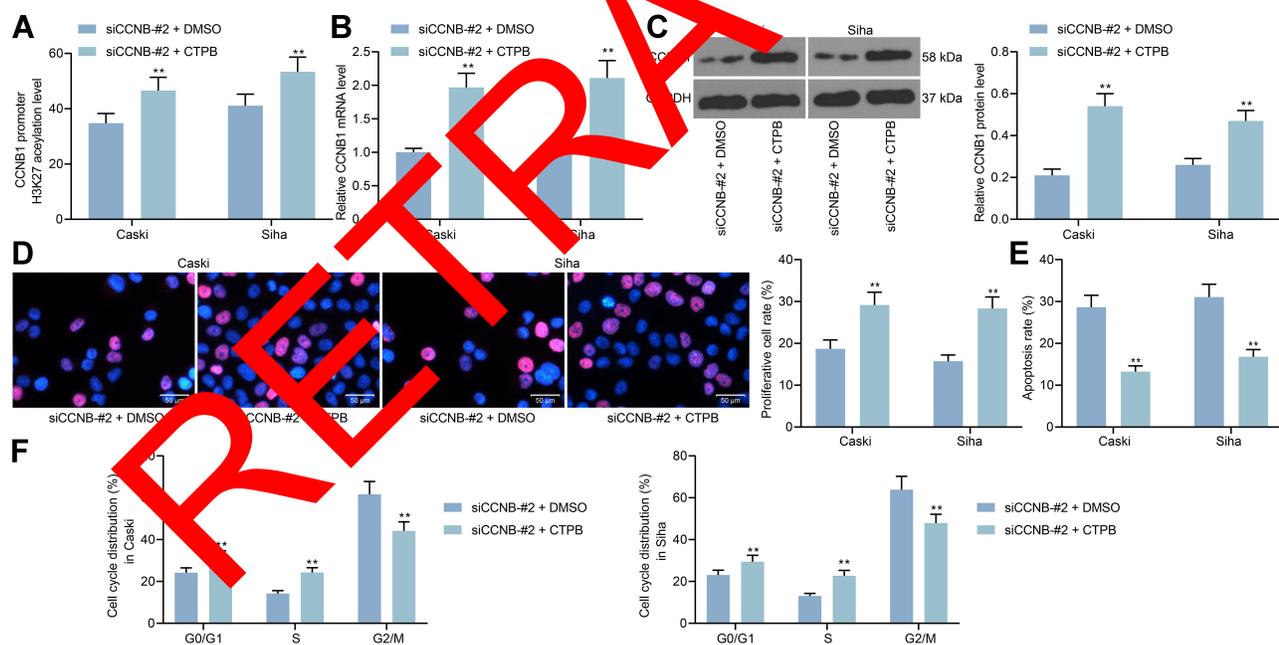


Figure 6 CTPB treatment activates the expression of CCNB1 to promote the growth of CSCC cells. P300-specific activator CTPB was added to Caski and SiHa cells with stable poor expression of CCNB1. (A) Histone H3K27 acetylation levels of the CCNB1 promoter sequence in CSCC cells measured by ChIP experiments; (B) the mRNA expression of CCNB1 in CSCC cells in response to the CBP/P300 specific inhibitor measured by RT-qPCR; (C) the protein expression of CCNB1 in CSCC cells in response to the CBP/P300 specific inhibitor measured by Western blot; (D) the proliferative activity of Caski and SiHa cells measured by EdU staining; (E) cell apoptosis in Caski and SiHa cells by flow cytometry; (F) cell cycle distribution in Caski and SiHa cells by flow cytometry. The experiments were performed in triplicate and expressed as mean \pm SD. Two-way analysis of variance followed by Tukey's test were applied for statistical analysis. ** $p < 0.01$ vs siCCNB1 + DMSO.

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; CSCC, cervical squamous cell carcinoma; EdU, 5'-Ethylnyl-2'-deoxyuridine; qPCR, quantitative real-time polymerase chain reaction; CBP, CREB binding protein; CTPB, N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide; Co-IP, co-immunoprecipitation.

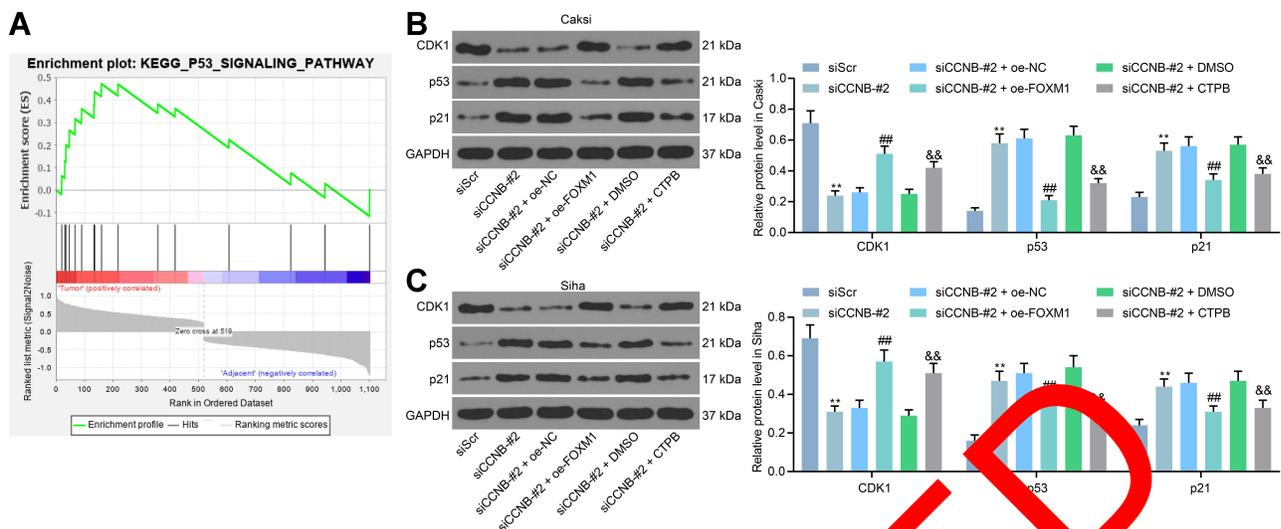


Figure 7 Silencing of CCNB1 activates the p53 pathway in CSCC cells. CSCC cells were transfected with siCCNB1 alone or in the presence of oe-FOXM1 or CTPB. (A) The p53 pathway negatively correlates with cancer tissue in GSEA; (B and C) the protein expression of p53, p21, and CDK1 in Caski (B) and Siha (C) cells. The experiments were performed in triplicate and expressed as mean \pm SD. Two-way analysis of variance followed by Tukey's test were applied for statistical analysis. ** $p < 0.01$ vs siCCNB1 + oe-NC, && $p < 0.01$ vs siCCNB1 + DMSO.

Abbreviations: CCNB1, cyclin B1; FOXM1, forkhead box protein M1; CSCC, cervical squamous cell carcinoma; CTPB, N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide; siRNAs, small interfering RNA; oe, overexpression; NC, negative control.

of H3K27ac modification of the CCNB1 promoter, p53 and p21 expression in cells was notably inhibited and that of CDK1 was increased (Figure 7B and C).

Discussion

Each year, 530,000 new CC diagnoses and 275,000 deaths are reported in a world range, contributing to a mortality-to-incidence ratio of about 50%. Besides, human papillomavirus E6 and E7 interact with a broad spectrum of signaling pathways, including the mediation of cell cycle and the governance of apoptosis, which are of great importance in sustaining normal cell functions.¹⁷ In the present study, we established that impairment of the CCNB1 function may offer a promising therapeutic option for treating CSCC by halting cell cycle progression and proliferation. Moreover, we proposed that FOXM1 directly bound to CCNB1 and recruited CBP/P300 to enhance the H3K27ac level.

Previous studies identified the oncogenic role of CCNB1 in different cancers, including hepatocellular carcinoma¹⁵ and cervical cancer.¹⁸ Also, CCNB1 represents a prognostic factor for overall survival and metastasis-free survival in breast cancer.¹⁹ Meanwhile, our bioinformatics website prediction verified its overexpression in CSCC clinical samples and cell lines. Therefore, we conducted loss-of-function assays to substantiate the inhibitory role of siCCNB1 in vitro. As expected, siCCNB1

contributed to repressed CSCC cell activities, as manifested by reduced PCNA and Bcl-2, whilst restored Bax, PUMA and Cleaved Caspase-3 expression. In order to expound its upstream factor, we applied a bioinformatics website TRRUST to predict the transcription factors for CCNB1. FOXM1 was found as one of the possible transcription factors that work. Given the potential of the multifarious oncogene, FOXM1 has emerged as an imperative biomolecule implicated in the initiation and development of cancers.²⁰ Moreover, FOXM1 is a typical proliferation-associated transcription factor, which provokes cell proliferation and shows a proliferation-specific expression pattern.²¹ For instance, microRNA-374b reduced cervical cancer cell proliferation and invasion through targeting FOXM1.²² Our subsequent JASPAR website prediction, CHIP and luciferase report assay revealed that there is a binding relationship between CCNB1 and FOXM1. Lee et al provided that suppression of FOXM1 diminished hepatocellular carcinoma cell viability and the expression of CCNB1,²³ which was largely in line with our observations in the following rescue experiments where overexpression of FOXM1 abrogated the suppressive effect of siCCNB1 on cell viability and cell cycle progression.

Sengupta and George reported that transcription factors binding to enhancers contribute to the recruitment of the mediator complex, which expedites enhancer connection

with the basal transcription machinery and RNA polymerase II at promoters.²⁴ More specifically, the expression of vital oncogenes is controlled by large regulatory elements, known as super-enhancers, which recruit transcriptional apparatus and are characterized by acetylation of H3K27.¹⁴ ENCODE website exhibited that the promoter sequence of CCNB1 has a distinct H3K27ac level. Similarly, we found that the acetylated levels of histone H3K27ac were significantly higher in CSCC cell lines than in HaCaT cells by ChIP and that FOXM1 bound to p300 by Co-IP. Furthermore, the transcriptional activity of FOXM1 involves binding of CDK-cyclin complexes to modulate CDK phosphorylation of the FOXM1B Thr 596 residue, which is crucial for recruitment of p300/CBP coactivator proteins.²⁵ By applying CBP/P300-specific inhibitor P300/CBP-IN-5, we observed that the levels of H3K27ac and the expression of CCNB1 at mRNA and protein levels were remarkably reduced. Therefore, we established the involvement of CBP/P300 in the mediation of FOXM1 on the expression of CCNB1. Kim et al proposed that suppression of p300 acetyltransferase activity with the help of a catalytic p300/CBP inhibitor exhibited effective growth inhibitory effects in melanoma cells.²⁶ Functional rescue experiments in our study further validated that the CBP/P300-specific activator CTPB promoted EdU-positive cells and reduced apoptosis in the presence of siCCNB1. Lastly, ChIP-seq in the present study disclosed that p53 pathway was significantly conversely regulated in CSCC. Knockdown of FOXM1 in malignant meningioma cells led to increased cell proliferation, angiogenesis and invasion by regulating cyclin D1 and p21.²⁷ While our Western blot results exhibited that siCCNB1 expedited the p53 pathway, while further overexpression of FOXM1 or CTPB contributed to the p53 pathway deficit.

In conclusion, the above results indicated that the anti-tumor effect of siCCNB1 in CSCC are likely caused by cell cycle arrest via transcriptional regulation by FOXM1 and/or acetylation modification of its histone H3K27ac by p300/CBP. CCNB1 inhibition may offer a possibly effective therapeutic strategy by blocking the cell cycle progression in CSCC. Nonetheless, in vivo studies are warrant to establish CCNB1 inhibition as a therapeutic option for the CSCC treatment and control.

Data Sharing Statement

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

Disclosure

The authors declare no conflicts of interest in this work.

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