RNA BIOLOGY
2025, VOL. 22, NO. 1, 1–13
https://doi.org/10.1080/15476286.2025.2478539

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RESEARCH PAPER

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Circular RNA Circ_0002762 promotes cell migration and invasion in cervical squamous cell carcinoma via activating RelA/nuclear factor kappa B (Nf-kB) signalling pathway

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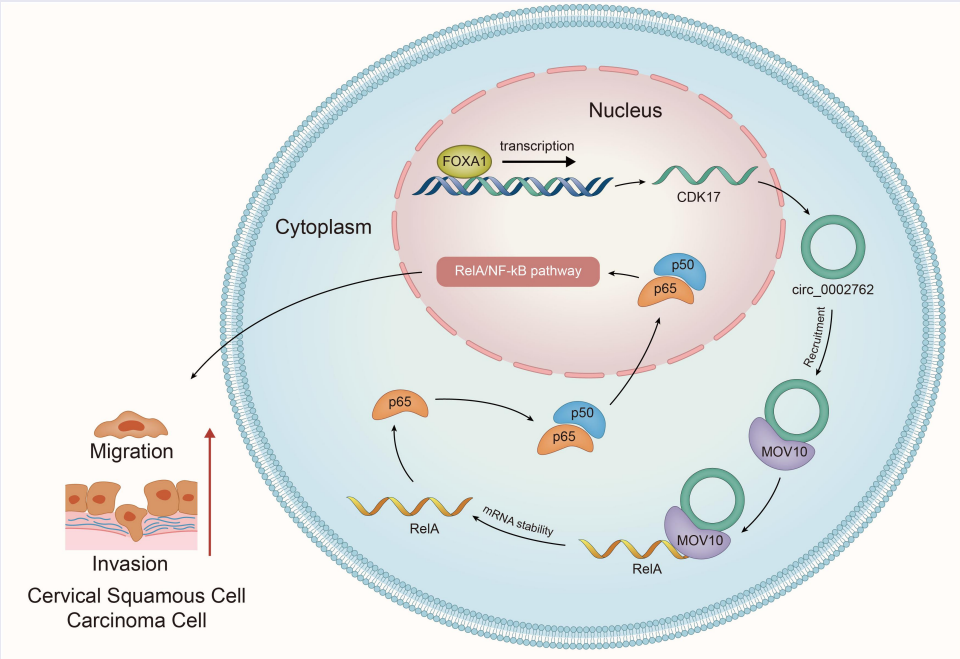
ABSTRACT

Cervical cancer is a leading cause of cancer-related deaths, with cervical squamous cell carcinoma (CSCC) accounting for a majority of cases. Circular RNAs (circRNAs) have been repeatedly suggested as crucial effectors in modulating the development of multiple malignancies. The expression of circ_0002762 was predicted to be high in CSCC tissues in GEO dataset, but the functional role and underlying regulatory mechanism of circ_0002762 in CSCC was unclear. By series of functional assays and mechanism assays, supported by bioinformatics analysis, reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis and western blot assays, we identified that circ_0002762 aberrantly up-regulated in CSCC, promoting CSCC cell migration and invasion. Mechanically, circ_0002762 was transcriptionally activated by Fork head box A1 (FOXA1). Moreover, the involvement of nuclear factor kappa B (NF-kB) signalling in circ_0002762 regulation mechanism in CSCC cells was ascertained. Additionally, circ_0002762, predominantly accumulated in cell cytoplasm, was proved to recruit Mov10 RISC complex RNA helicase (MOV10) to enhance RelA mRNA stability, thus affecting CSCC cell migration and invasion. In summary, FOXA1-mediated circ_0002762 up-regulation could enhance the migratory and invasive abilities of CSCC cells via the MOV10/RelA/NF-kB pathway.



ARTICLE HISTORY
Revised 18 February 2025
Accepted 7 March 2025

KEYWORDS
Cervical squamous cell carcinoma; circ_0002762; MOV10; RelA/NF-kB; FOXA1

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/15476286.2025.2478539

Introduction

Cervical cancer (CC) is one of the most prevalent malignancies affecting women health worldwide. Cervical squamous cell carcinoma (CSCC), as the major subtype, accounts for nearly 90% deaths caused by cervical cancer in developing countries. HPV infection is widely recognized as a major pathogenic factor for CC [1,2]. Treatment options for CC often involve radiotherapy, neoadjuvant chemotherapy, pharmacotherapy, and surgical interventions, among other approaches [3–6]. While significant achievements in early-stage screening and human papillomavirus vaccination have contributed to the decrease in the incidence rate of CSCC [7], the survival of patients with advanced CSCC remains poor [8]. Therefore, it is imperative to explore more efficient biomarkers for prognosis improvement of CSCC patients.

Circular RNA (circRNA) is a novel class of non-coding RNA named for its unique circular structure. Unlike linear RNA, circRNA has its 5' and 3' ends connected to form a closed loop, which confers greater stability [9–11]. CcircRNAs have attracted much attention for mounting studies that have reported their potential role as effectors in carcinogenesis and development of cancers. As an illustration, hsa_circ_0000117 has been confirmed as an oncogenic factor in gastric cancer [12], and circPTN has been claimed to exert a promoting influence on glioma cell proliferation and stemness [13]. Hsa_circ_0007364 contributes to the accelerated progression of CC via modulation of methionine adenosyltransferase II alpha (MAT2A)/microRNA-101-5p [14]. In CSCC, circ_POLA2 has been reported as an oncogene [7], while circRNA_0000140 contributes to repressed tumour growth and lung metastasis [15]. It has been reported that circ_0002762 expression was high in CC tissues [16]. In this study, our main purpose was to identify the specific role and regulatory mechanism concerning circ_0002762 in CSCC.

Transcription factors are key cellular components controlling gene expression by recognizing specific DNA sequence to determine transcription [17]. The aberrant expression of circRNAs in various diseases has also been related to the abnormal activity of transcription factors. For instance, E2F transcription factor 1 (E2F1) and eukaryotic translation initiation factor 4A3 (EIF4A3) have been ascertained to activate hsa_circ_0005320 transcription, contributing to progression of triple-negative breast cancer [18]. The molecular mechanisms involving circRNAs and transcription factors in CSCC are still largely unexplored. Herein, we also intended to fathom out the upstream transcription factor leading to the abnormally high expression of circ_0002762 in CSCC.

The most commonly studied downstream regulatory networks of circRNAs include competing endogenous RNA (ceRNA) mechanism and RNA binding protein (RBP)-mediated regulation. For example, circRNA-104718 sponges miRNA-218-5p to modulate thioredoxin domain-containing protein 5 (TXNDC5) expression, thus promoting hepatocellular carcinoma development [19], and circMRPS35 recruits lysine acetyltransferase 7 (KAT7) to affect histone modification, thereby inhibiting gastric cancer progression [20]. This study was also targeted to ravel out the underlying regulatory mechanism through which circ_0002762 exerts its influence on CSCC cell behaviours. In addition, previous study reports revealed the mechanism of action of circ_0002762 in CC, which tends to affect CC through the ceRNA network [21,22].

Accumulating studies have unveiled that dysregulated nuclear factor kappa B (NF-kB) activity could induce inflammation-related diseases including cancers, and NF-kB has also been suggested as the putative targets for developing therapeutic strategies [23]. NF-kB family members function as dimers in two separate but interconnected arms of the NF-kB pathway (canonical or non-canonical) [24]. Based on previous publications, NF-kB pathway has often been verified as a crucial participant in circRNAs-networks in cancer cells. Specifically, hsa_circ_0006852 activates NF-kB pathway to influence hepatocellular carcinoma cell malignant behaviours in vitro and tumour growth in vivo [25]. In addition, it has also been reported that the NF-kB pathway plays a role in the development of CC and drug resistance [26,27].

In our study, we investigated that circ_0002762, activated by FOXA1 transcription, activates the NF-kB pathway by recruiting MOV10 stabilized RelA mRNA, and ultimately promotes CSCC cell migration and invasion.

Methods

Cell culture

Cervical epithelial cell line H8 and three cervical cancer cell lines including HeLa, CaSki and C33A were procured from the American Type Culture Collection (ATCC). Human embryonic kidney 293T cells were purchased from the National Institutes for Food and Drug Control. H8 and C33A were cultivated in H-DMEM (MEM-CD148/05), while HeLa and CaSki were, respectively, cultured in MEM (A4192201) and RPMI (A4192301) 1640. Ten percent foetal bovine serum (FBS; 16140071) and 1% penicillin–streptomycin (Pen/Strep; 15140122) solution were added to each medium. For cultivation of 293T cells, the medium (RPMI 1640) was supplemented with 10% FBS. The medium, FBS and Pen/Strep were all procured from Thermo Fisher Scientific (Rockford, IL, USA). All cells were incubated in a humid atmosphere at 37°C.

Cell transfection

Small interference RNAs (siRNAs) targeting FOXA1 (si-FOXA1–1; 5'-UGAGUUCAUGUUGCUGACCGG-3', si-FOXA1–2; 5'-AUGUUGAAGGACGCCGGGGUC-3', si-FOXA1–3; 5'-UCAUGCUGUUCAUGGCGCCCG-3'), CTCF (si-CTCF-1; 5'-UCCUUUAAUAAAAGUUUCGGA-3', si-CTCF-2; 5'-UGAACAAGCUGAAGUUCUCCU-3', si-CTCF-3; 5'-AUAUCAUGGGUUCACUUUCCG-3'), EP300 (si-EP300–1; 5'-AAUAGAGAGCCAAAAUCUGUG-3', si-EP300–2; 5'-AGCUGUUUAUGUUUAGAAGCU-3', si-EP300–3; 5'-UAUUUAUCAAACCUAAUCCAG-3'), circ_0002762 (si-circ_0002762–1; 5'-UUUUCGAAUUCUAUGAACCAC-3', si-circ_0002762–2; 5'-UACUAAUCUCUGUUACAACUU-3', si-circ_0002762–3; 5'-AGUAUUUUUUCAGAGAAACUA-3') and MOV10 were designed for knockdown of respective genes, with si-NC as the negative control. Full sequence of FOXA1 or RelA was

sub-cloned into pcDNA3.1 vectors to construct pcDNA3.1-FOXA1 or pcDNA3.1-RelA for overexpression of each gene, and the empty pcDNA3.1 plasmids were used as negative control. The cells (8.3×10^4) were transfected indicated plasmids using the Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA), and then were harvested after 48 h later.

Reverse transcription quantitative real-time polymerase chain reaction (rt-qPCR)

RT-qPCR was done as described in the previous literature for the measurement of RNAs [28]. Total RNA was extracted from cell samples (1.3×10^5) using TRIzol reagent (15596018/15596026, Invitrogen). Subsequently, isolated RNA was converted into cDNA using reverse transcription kit (RR037Q/RR037A, Takara, Tokyo, Japan). Quantitative real-time PCR was performed using SYBR Green (4309155, Applied Biosystems, Foster City, CA) methods. The relative gene expression was measured and normalized to β-actin by the $2^{-\Delta\Delta Ct}$ method.

Luciferase reporter assay

Luciferase reporter assay was carried out as previously described [29]. Cignal Finder Reporter Array Kit (336841, QIAGEN, Dusseldorf, Germany) was procured to detect the luciferase activity of various signalling pathways in transfected cells. For detecting interaction between FOXA1 and circ_0081672 promoter, the sequences of circ_0081672 promoter with wild or mutated FOXA1 binding sites were sub-cloned into pGL3 vectors (1073930010, Sigma-Aldrich, St. Louis, MO, USA). Then, the constructed vectors were co-transfected with pcDNA3.1-FOXA1 or pcDNA3.1 into 293T cells (1.2×10^5). After 48-h transfection, the luciferase activities were detected by the Dual-luciferase reporter assay system (E1910, Promega, Madison, WI, USA) following the instruction and the results were normalized to the renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay procedures were in line with previous study [29]. ChIP Assay Kit (26157, Thermo Fisher Scientific) was used according to manufacturer's instructions. In brief, the cells (1×10^7) were cross-linked in 1% formaldehyde (E672002, Sangon Biotech, Shanghai, China) and sonicated on ice for obtaining 200–500 bp fragments. Then, the fragmented chromatin was cultured overnight with FOXA1 antibody (1/1000; WH0003169M1, Sigma-Aldrich). Anti-IgG (1/40000; MABE-253, Sigma-Aldrich) was used as negative control. DNAs precipitated was purified and analysed by RT-qPCR.

DNA pull down or RNA pull down assay

This assay was performed as previously described [30]. Pierce™ Magnetic RNA-Protein Pull-Down Kit (20164, Thermo Fisher Scientific) was utilized for implementation of RNA pull down assay, while Pierce™ Biotinylated Protein Interaction Pull-Down Kit (21115, Thermo Fisher Scientific) was for DNA pull down assay. At first, biotinylated circ_0002762 promoter probes with wild type (WT) or mutant (MUT) predicted binding site of FOXA1, biotinylated RelA 3'UTR sense WT/MUT, as well as negative control probes (Bio-NC) or RelA 3'UTR antisense were procured. Then, the procured probes were incubated with lysates of 1×10^7 cells for 2 h at 25°C. Afterwards, the RNA/protein or DNA/protein duplexes were captured by streptavidin-coupled dynabeads (H9914, Bedford, MA, USA) for 1 h at 25°C. The proteins were then purified and analysed by western blot.

CCK-8 assay

The cells were subjected to various treatments and incubated for 48 h. Following this, the cells were evenly distributed into 96-well plates at a density of 2×10^3 cells per well. The cells were then cultured for 0, 1, 2, and 3 days. Afterward, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) solution was added to each well, and the cells were incubated for an additional hour. The medium was subsequently removed, and cell proliferation was assessed by measuring the absorbance at 450 nm.

Transwell assay

Transwell assay was carried out in line with previous report [30]. Cell migration and invasion were analysed using the transwell chamber (pore size: 8 μm; 3428/CLS3396-2EA, Corning, NY, USA). In brief, for detecting cell migratory capabilities, 8000–36000 cells (48 h after transfection) in 200 μL serum-free DMEM (D5030, Thermo Fisher Scientific) were seeded into the upper chamber, and the lower chamber was filled with 600 μL complete DMEM medium. After incubation for 48 h, the cells in the upper chamber were removed. Cells in the basal chamber were fixed and stained with 1% crystal violet (V5265, Sigma-Aldrich). Cell number was counted using a light microscope (DMI8, Leica, Wetzlar, Germany). To detect cell invasion, the procedures were similarly carried out except that the chambers were pre-coated with Matrigel matrix (A1413302, Thermo Fisher Scientific).

Wound healing assay

Wound healing assay was implemented in line with the procedures described in previous study [31. 21600] cells (48 h after transfection) were cultured and transfected in 6-well plates and the cell density reached 100% before wounds were made by 200 μL pipette tips (0 h). Then, the cells were cultured with serum-free RPMI 1640 medium for 24 h. The scratched lines were photographed at 0 h and 24 h to estimate the cell migration ability.

Western blot

Western blot analysis was done as per the procedures described in a previous publication [32]. Total protein was extracted from 1×10^7 cells (48 h after transfection) using RIPA buffer (R0278, Sigma-Aldrich) added with proteinase (P4032, Sigma-Aldrich) and phosphatase inhibitor cocktail (P2850, Sigma-Aldrich).

Protein extraction was loaded to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 1610174, Bio-Rad Laboratories, Shanghai, China) and then transferred onto the polyvinylidene fluoride membranes (PVDF; IPVH00010, Millipore). Primary antibodies were incubated with the membranes, including Anti-FOXA1 (1/1000–1/10000; F1555), Anti-p65 (1/1000–1/10000; AB3375), Anti-Histone H3 (1/1000–1/5000; H0164), Anti-IL-8 (1/1000; SAB1409244), Anti-MOV10 (1/2000–1/10000; PLA0195) procured from Sigma-Aldrich and Anti-β-actin (1/100–1/2000; PA1–46296) purchased from Invitrogen overnight at 4°C. After that, secondary antibodies (1/2000–1/100000; AP510, Sigma-Aldrich) were added for cultivation. Eventually, the proteins were visualized via an enhanced chemiluminescence (ECL) detection system (35055, Thermo Fisher Scientific) and the densitometry was analysed with Protein Thermal Pierce™ (4466038, 4466038, Thermo Fisher Scientific).

RNA binding protein immunoprecipitation (RIP) assay

The Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (638970, Merck, Darmstadt, Germany) was procured to conduct RIP assay following the manufacturer's instructions. The procedures were in accordance with previous assay [33]. Briefly, indicated cells (1×10^7) at 90% confluency were harvested using RIP lysis buffer (20–188, Millipore). The cell lysates were incubated with RIP buffer (SAB1404621, Sigma-Aldrich) containing magnetic beads (HY-K0208, MedChemExpress, NJ, USA) conjugated with Anti-MOV10 (1/50–1/100; PLA0195, Sigma-Aldrich), Anti-Argonaute-2 (Anti-AGO2, 1/30–1/50; TS-10X10ML-U, Millipore) or negative control Anti-immunoglobulin G (Anti-IgG, 1/40000) at 4°C overnight. Then, proteinase K (4333793, Invitrogen) was added to digest the protein, and the supernatants containing RNA were extracted and subjected to RT-qPCR analysis.

Statistical analysis

All statistical analyses were performed using the SPSS 21.0 software. Comparisons between two groups were analysed by the Student's t-test. Data among three groups were analysed by analysis of variance (ANOVA). Each experiment was performed for three times. All data were demonstrated as the mean ± standard deviation (SD). $p < 0.05$ was considered to be statistically significant.

Results

The primary objective of this study was to elucidate the functional role of circ_0002762 in CSCC. Based on bioinformatics analysis and RT-qPCR results, circ_0002762 expression was high in CSCC tissues on CSCC cells. Therefore, it was hypothesized that circ_0002762 up-regulation might be relevant to CSCC cell malignant behaviours. After the circular structure of circ_0002762 was identified, functional assays were designed and carried out to determine the changes in CSCC cell behaviours under the influence of gene overexpression or depletion. Additionally, mechanistic assays were performed to explore the potential interactions between circ_0002762 and various molecular factors within the cells.

Circ_0002762 is aberrantly overexpressed in CSCC

For determination of our research target, aberrantly expressed circRNAs in CSCC tissues were predicted in GSE102686 database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102686). Among the five most highly expressed genes, only circ_0002762 and circ_0081672 had not been studied in cervical cancer (Figure 1A). For further screening, GEO dataset was again applied. Both genes were found to be overexpressed in CSCC tissues compared with paired non-tumour tissues (Figure 1B). Then, the expression patterns of the two candidates in CSCC cells and cervical epithelial cells were measured through RT-qPCR. The results indicated that circ_0002762 expression in all CSCC cells was more than four times that of cervical epithelial cells, while the expression of circ_0081672 was significantly high only in HeLa cells ($p < 0.01$, Figure 1C). Given that circ_0002762 expression was highest in HeLa and CaSki cells, these two cell lines were utilized in subsequent assays. Next, the structure formation diagram of circ_0002762 was demonstrated in Figure 1D. For examination of the circular structure of circ_0002762, we treated cells with actinomycin D (ActD) and RNase R. After HeLa and CaSki cells were treated with ActD, the RT-qPCR results showed circ_0002762 degraded much more slowly than cyclin-dependent kinase 17 (CDK17) mRNA, which is the host gene of circ_0002762 ($p < 0.01$, Figure 1E). This suggested that circ_0002762 had greater stability than linear RNA. Additionally, in comparison with CDK17 mRNA, circ_0002762 was hardly digested by RNase R ($p < 0.01$, Figure 1F). The results in Figure 1E–F jointly evidenced that circ_0002762 was a bona fide circRNA. To conclude, circ_0002762 is up-regulated in CSCC.

Circ_0002762 enhances CSCC cell migration and invasion

Subsequently, the functional role of circ_0002762 in CSCC cells was evaluated via loss-of-function assays. Initially, the knockdown efficiency of si-circ_0002762–1/2/3 was checked through RT-qPCR. The results demonstrated that circ_0002762 expression was decreased by more than 60% after transfection of si-circ_0002762–1/2/3 ($p < 0.01$, Figure 2A). Si-circ_0002762–1/2, which exhibited higher knockdown efficiency, was chosen for further experiments. CCK-8 results indicated that circ_0002762 knockdown inhibited cell proliferation (Figure 2B). Subsequent transwell assays revealed a significant reduction in the number of migrated and invaded cells upon circ_0002762 knockdown ($p < 0.01$, Figure 2C). Wound healing assay results indicted wound healing ability of cells was obviously weakened, reflecting inhibited cell migration after circ_0002762 was knocked down ($p < 0.01$, Figure 2D). In summary, depletion of circ_0002762 leads to a decrease in the migratory and invasive capabilities of CSCC cells.

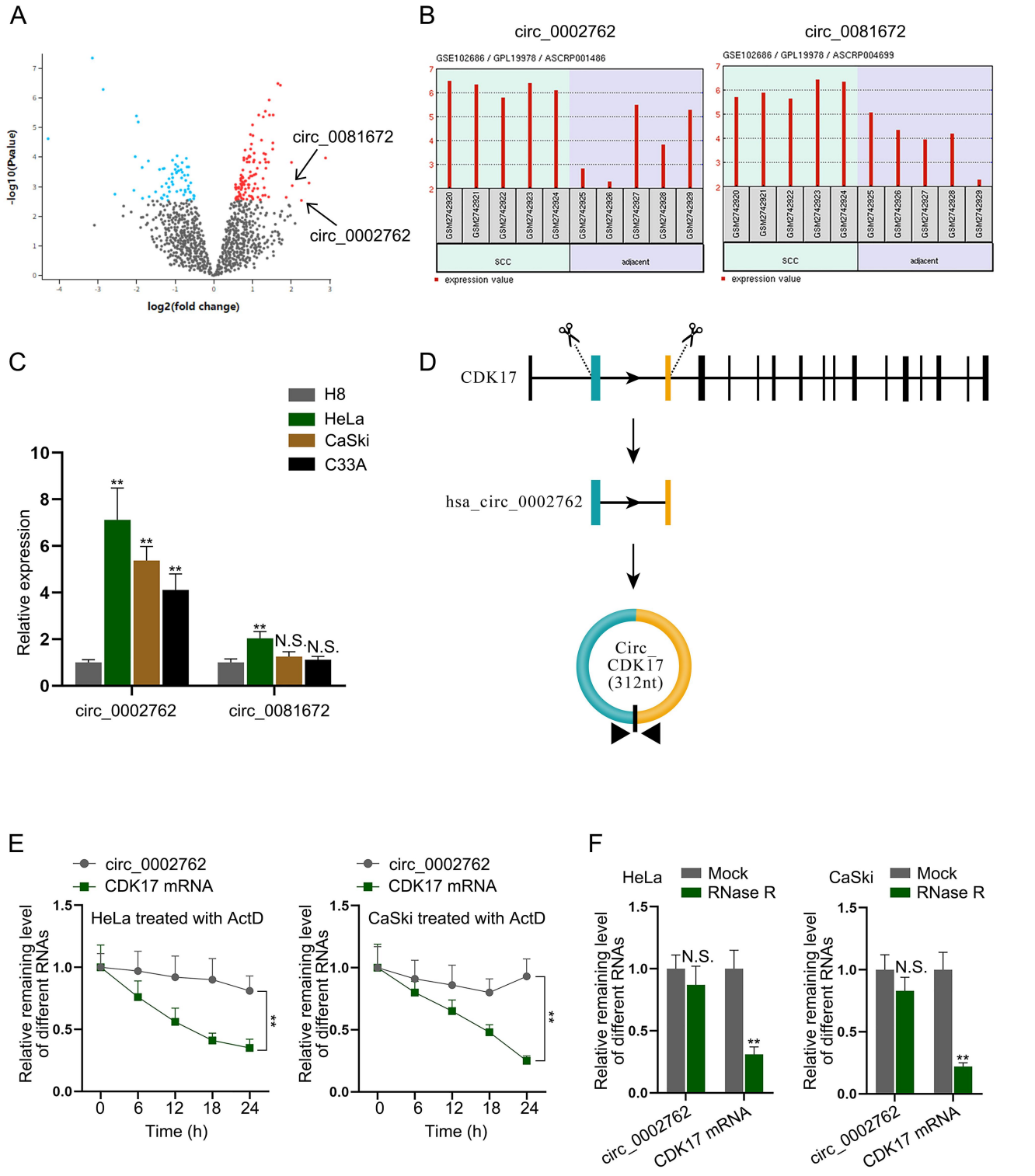

Figure 1. Circ_0002762 is up-regulated in CSCC cells (A) GSE102686 was analysed to screen for circRNAs aberrantly expressed in CSCC, leading to the identification of circ_0081672 and circ_002762 as candidate circRnas. (B) Expression profiles of circ_0002762 and circ_0081672 in CSCC tissues and paired para-cancerous cervical tissues were obtained from GSE102686. (C) Rt-qPCR was performed to examine expression levels of circ_0002762 and circ_0081672 in the three CSCC cell lines and cervical epithelial cell line (H8), revealing that circ_0002762 is highly expressed in CSCC cells. (D) Schematic diagram was presented to illustrate the splicing pattern of circ_0002762 (312nt). (E) Following ActD treatment, Rt-qPCR analysis showed that the mRNA stability of circ_0002762 is stronger than that of CDK17 mRNA. (F) RNA extractions were treated with RNase R and remaining levels were quantified with Rt-qPCR, indicating a significant level of circ_0002762. $^{**}p < 0.01$; N.S.: no significance.

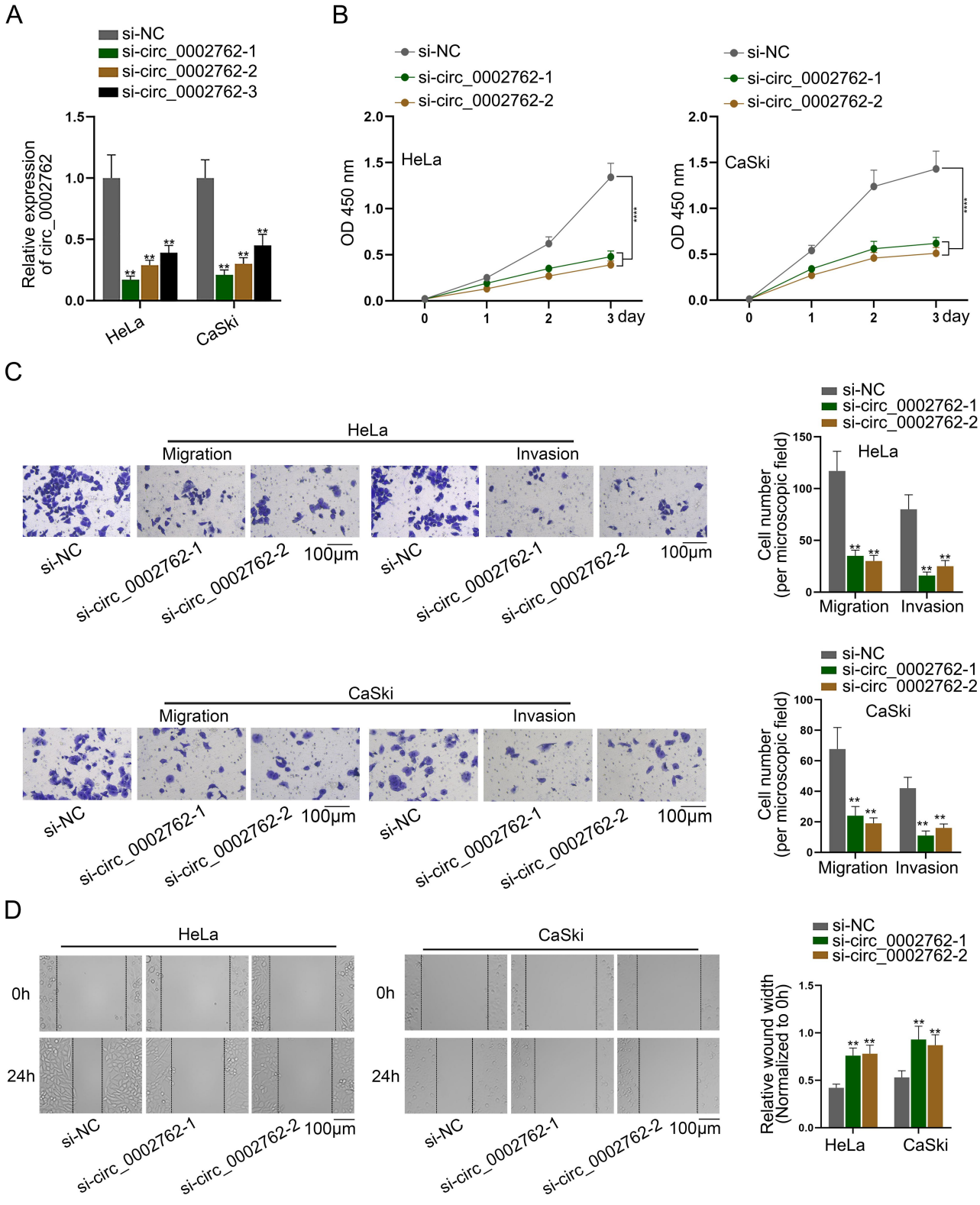

Figure 2. Circ_0002762 facilitates CSCC cell migration and invasion (A) Rt-qPCR analysis was done to examine si-circ_0002762 efficiency. (B). CCK-8 results indicated that knockdown of circ_0002762 inhibited cell proliferation. (C) The inhibitory effect of circ_0002762 silence on CSCC cell migration and invasion was evaluated by transwell assays. (D) Wound healing assay showed that the migration ability of CSCC cells weakened after circ_0002762 knockdown. $^{**}p < 0.01$.

Circ_0002762 is transcriptionally activated by FOXA1

Afterwards, we searched on UCSC (http://genome.ucsc.edu/) for prediction of the transcription factor contributing to the high expression of circ_0002762 in CSCC cells. FOXA1, CTCF and EP300 with the highest cluster scores were screened out preliminarily ($p < 0.01$, Figure S1A). Then, we knocked down the three candidates in cells, respectively. Si-FOXA1–1/2, si-CTCF-1/2, and si-EP300–1/2 were chosen based on RT-qPCR results, demonstrating a knockdown efficiency of up to 75%

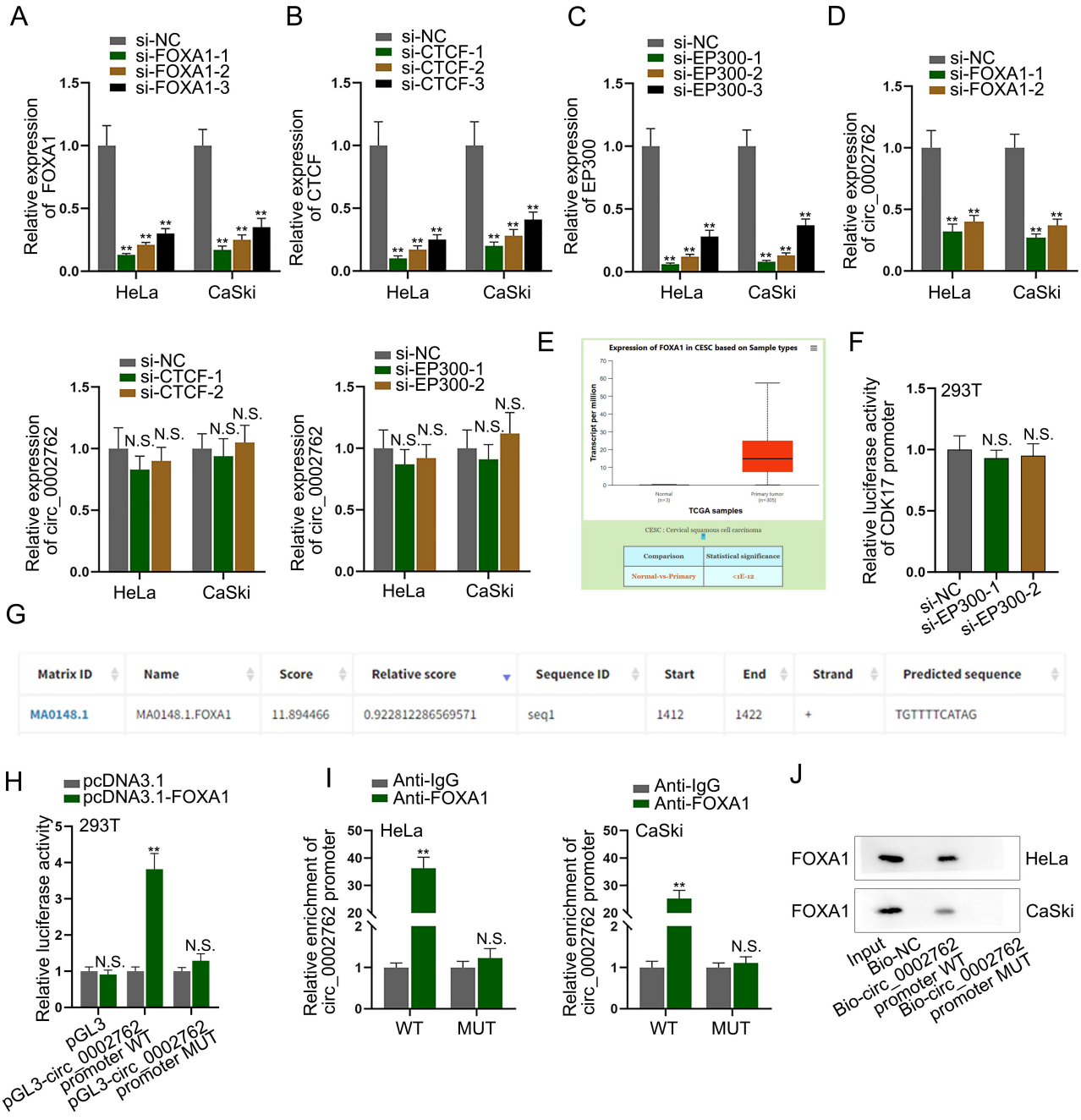

Figure 3. Circ_0002762 is transcriptionally modulated by FOXA1 (A–C) Rt-qPCR was done to assess the knockdown efficiency of si-FOXA1, si-CTCF and si-EP300. (D) Rt-qPCR results indicated that circ_0002762 expression reduced in cells with knockdown of FOXA1, CTCF or EP300. (E) UALCAN database results showed that FOXA1 was highly expressed in CSCC. (F) Results of luciferase reporter gene assay indicated that FOXA1 did not affect CDK17 promoter activity. (G) the binding sites of FOXA1 on CDK17 promoter predicted on JASPAR were demonstrated. (H) The interaction between FOXA1 and CDK17 promoter at predicted sites were evaluated via luciferase reporter assay. (I-J) The affinity of FOXA1 and CDK17 promoter was further confirmed via ChIP and DNA pull down assays. $^{**}p < 0.01$; N.S.: no significance.

($p < 0.01$, Figure 3A–C). RT-qPCR results also demonstrated that circ_0002762 expression decreased with FOXA1 silence, while that was hardly changed in response to CTCF or EP300 silence ($p < 0.01$, Figure 3D). Therefore, we identified FOXA1 as a transcription factor that plays a regulatory role in CSCC. According to the analysis from UALCAN (https://ualcan.path.uab.edu/), FOXA1 is highly expressed in CSCC (Figure 3E). Additionally, we found that the knockdown of FOXA1 did not significantly affect the promoter activity of the host gene CDK17 for circ_0002762 (Figure 3F). This indicates that FOXA1 does not regulate circ_0002762 by influencing its host gene. For further validation, we continued to carry out luciferase reporter assay after obtaining potential binding site of FOXA1 on circ_0002762 promoter from JASPAR (http://jaspar.genereg.net/) (Figure 3G). After detection, we noticed that after transfection of pcDNA3.1-FOXA1, the luciferase activity of pGL3-circ_0002762 promoter WT in 293T cells was strengthen by more than three times, but that of pGL3-circ_0002762 promoter MUT showed no variation ($p < 0.01$, Figure 3H). In ChIP assay, enrichment of circ_0002762 promoter WT by Anti-FOXA1 was nearly 35 times higher than that by Anti-IgG, reflecting the strong binding between FOXA1 and

circ_0002762 promoter ($p < 0.01$, Figure 3I). DNA pull down assay further confirmed the interaction between FOXA1 and circ_0002762 promoter ($p < 0.01$, Figure 3J). Thereby, it could be confirmed that FOXA1 could enhance circ_0002762 transcription.

Circ_0002762 modulates Nf-kB signaling pathway through RelA

Next, the regulatory mechanism by which circ_0002762 exerted influences on CSCC cell migration and invasion was investigated. Since signalling pathways have been widely reported to affect various biological processes of cells, we subsequently detected the luciferase activity of different signalling pathways before and after depletion of circ_0002762. As a result, we discovered that NF-kB signalling could be inhibited when circ_0002762 was depleted, implying circ_0002762 could regulate NF-kB signalling pathway in CSCC cells (Figure 4A). Then, the influence of circ_0002762 on expression of key factors (IKKα and RelA) and downstream (IL-8) of NF-kB pathway was detected through RT-qPCR. It was found that the RelA and IL-8 expressions decreased by more than 50% after circ_0002762 down-regulation ($p < 0.01$, Figure 4B). In addition, western blot analysis revealed that IL-8 expression in cells was decreased, and nuclear expression of RelA/p65 was also inhibited in circ_0002762 depleted cells (Figure 4C). Next, subcellular fractionation assay was carried out to determine the subcellular location of circ_0002762. From the results, it could be seen that circ_0002762 mostly existed in cytoplasm (Figure 4D). Considering the major existence of circ_0002762 in cytoplasm, we analysed the possibility that whether circ_0002762 could act as ceRNA to regulate RelA in CSCC cells. Through RIP assay, it was shown that little RelA could be precipitated in Anti-AGO2, indicating circ_0002762 could not regulate RelA via sequestering miRNAs ($p < 0.01$, Figure 4E). Herein, it is confirmed that circ_0002762 could positively affect RelA expression to modulate NF-kB signalling activation.

Circ_0002762 regulates RelA expression via recruiting MOV10

After ruling out the possibility that circ_0002762 regulated RelA through a ceRNA mechanism, we hypothesized that a specific RBP might be involved in the regulation of circ_0002762 on RelA in CSCC cells. Therefore, we predicted RBPs that have the potential to bind with both circ_0002762 and RelA through catRAPID (http://service.tartaglialab.com/page/catrapid_group) and starBase (http://starbase.sysu.edu.cn/index.php). We analysed the predicted top 100 candidates with highest scores from both websites with Venn diagram. Among these candidates, UPF1, MOV10, NUMA1, DGCR8, and SND1 were identified (Figure 5A). The expression patterns of the candidates in CSCC were acquired from UALCAN (http://ualcan.path.uab.edu/index.html). It was shown that only MOV10 was high expressed in tumour tissues (Figure S1B). Subsequently, RIP assay was performed and the binding between MOV10 and circ_0002762 or between MOV10 and RelA was ascertained, as enrichment of circ_0002762 and RelA in Anti-MOV10 was 30–60 times more than Anti-IgG ($p < 0.01$, Figure 5B–C). RNA pull down assay results also unveiled that MOV10 could be pulled down by RelA 3'UTR in HeLa and CaSki cells (Figure 5D). Thereafter, the knockdown efficiency of si-MOV10–1/2 was analyzed to be high up to 75%, so the two plasmids were selected ($p < 0.01$, Figure 5E). It was confirmed that MOV10 could positively regulate RelA expression, as MOV10 knockdown could result in obvious decrease of RelA/p65 at both RNA and protein levels ($p < 0.01$, Figure 5F–G). Considering that 3'UTR was determinative to mRNA stability [34], we detected RelA stability after knocking down MOV10 or circ_0002762. The result showed that RelA stability was notably abated after silencing MOV10 or circ_0002762 ($p < 0.01$, Figure 5H). In a word, circ_0002762 recruited MOV10 to modulate the mRNA stability of RelA in CSCC cells.

Circ_0002762 facilitates CSCC cell migration and invasion through up-regulating RelA

Next, functional experiments were designed in a rescue way to validate whether circ_0002762 affected CSCC cell migration and invasion via RelA/NF-kB pathway. HeLa cell with highest circ_0002762 expression was selected to conduct rescue assays. Preliminarily, it was validated that pcDNA3.1-RelA transfection could elevate the expression of RelA in HeLa cells by more than six times ($p < 0.01$, Figure 6A). CCK-8 results showed that the inhibitory effect of transfection of si-circ_0002762 on cell proliferation was reversed after cotransfection of pcDNA3.1-RelA (Figure 6B). Then, in transwell assay results, we could observe that co-transfection of pcDNA3.1-RelA restored the inhibition of cell migration and invasion ability of si-circ_0002762–1 ($p < 0.01$, Figure 6C). Wound healing assay also validated that wound healing ability impeded by circ_0002762 silence was recovered after RelA up-regulation ($p < 0.01$, Figure 6D). To be concluded, circ_0002762 facilitates CSCC cell migration and invasion through up-regulating RelA.

Discussion

In this study, we focused on the function of circRNA circ_0002762 in CSCC and its potential regulatory mechanisms. CC is a leading cause of cancer-related mortality, with CSCC accounting for the majority of cases. CircRNAs, which are covalently closed and endogenous biomolecules, have attracted great attention in recent decades. The crucial correlation between aberrantly expressed circRNAs and the progression of multiple cancers, including metastasis, has been elucidated in several studies [35–37]. Specifically, circ_0000467, aberrantly up-regulated in colorectal cancer, has been validated to facilitate colorectal cancer via miR-382-5p/EN2 axis [38]. In addition, circRUNX1 has been suggested to facilitate papillary thyroid cancer metastasis via modulation on miR-296-3p/DDHD2 [39]. As to the identification of the functional roles of circRNA in CSCC, circ_POLA2, highly expressed in CSCC tissues and cells, has been confirmed to exacerbate malignant behaviours of

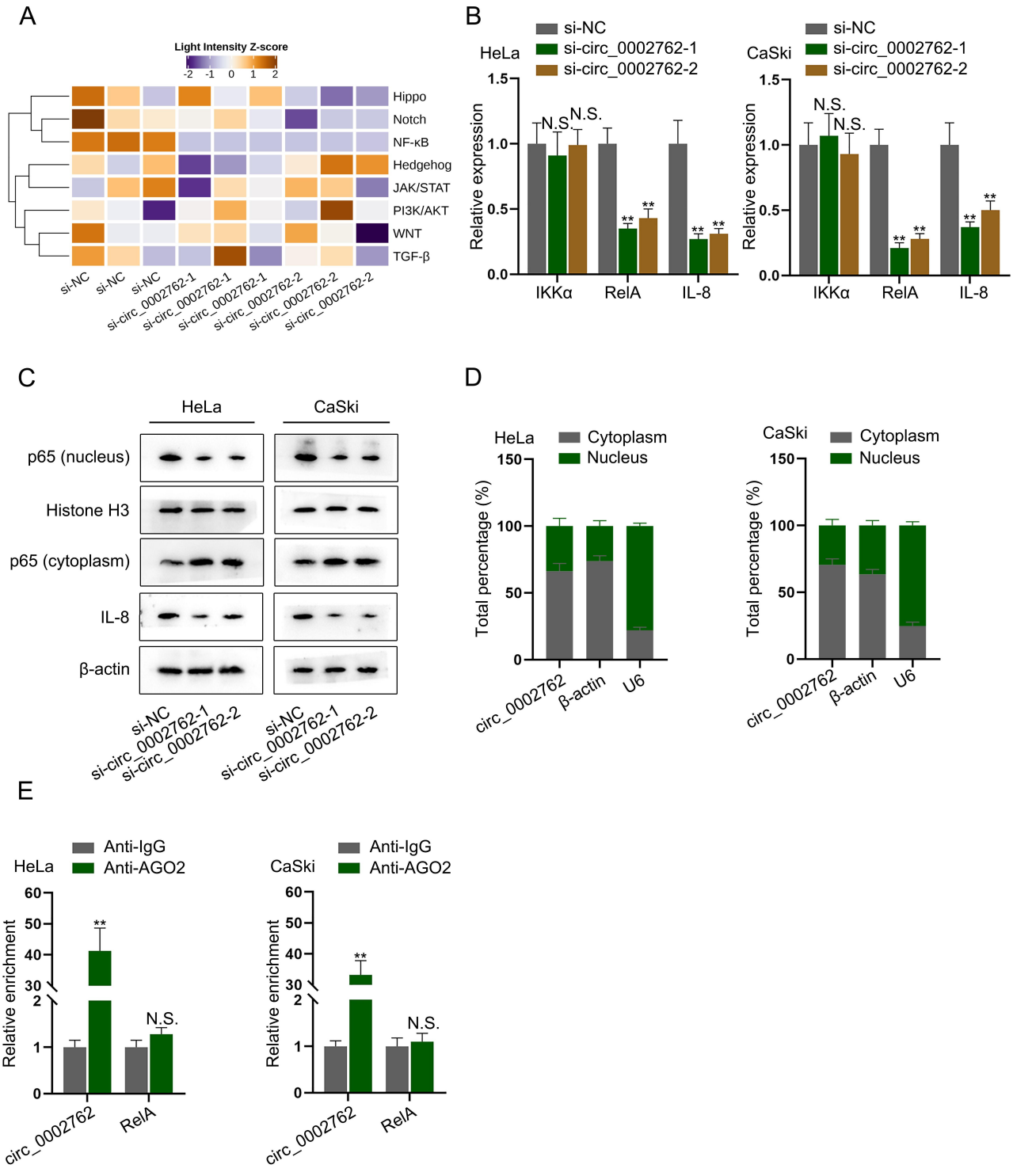

Figure 4. Circ_0002762 regulates RelA/NF-kB pathway (A) The activation of different pathways before and after circ_0002762 depletion was assessed with cignal finder reporter array kit. (B) Rt-qPCR was employed to investigate the levels of IKKα and RelA (key genes of nf-kB signalling) and IL-8 (downstream gene of nf-kB signalling) in si-circ_0002762-transfected cells, revealing a significant decrease in the expression of RelA and IL-8. (C) Subcellular fractionation assay and western blot were conducted to detect the protein levels of RelA/p65 (in cell nucleus and cytoplasm) and IL-8 following circ_0002762 knockdown, showing reduced expression of nuclear RelA/p65 and IL-8. (D) Results from subcellular fractionation indicated that circ_0002762 is primarily located in the cytoplasm. (E) In RIP assay, enrichment of circ_0002762 and RelA in Anti-AGO2 and Anti-IgG (negative control) was measured via Rt-qPCR, with results showing that RelA was almost not enriched. $^{**}p < 0.01$; N.S.: no significance.

cervical cancer cells [7]. This sparked our interest in the role of circRNAs in CSCC. In this study, the focus was to explore the potential involvement of circ_0002762 in CSCC. Initially, we confirmed the circular structure of circ_0005273, followed by the verification of its overexpression in CSCC tissues and cell lines through bioinformatics analysis and RT-qPCR. To further investigate the function of this circRNA, we conducted a series of functional experiments and mechanistic studies. Functional assay results explicitly illustrated that circ_0002762 depletion

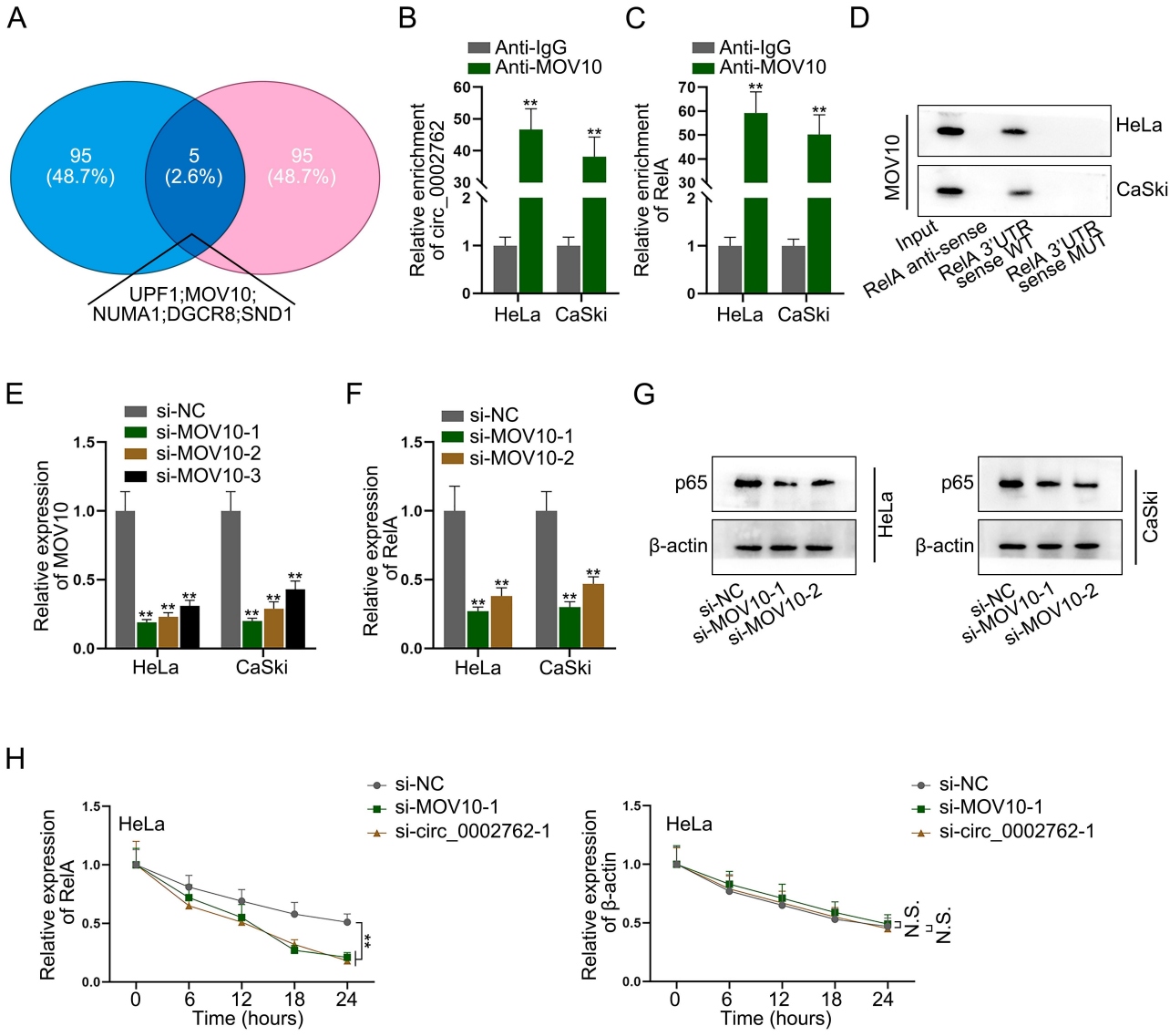

Figure 5. Circ_0002762 recruits MOV10 to stabilize RelA (A) Bioinformatics tools (catRAPID and starBase) were applied for prediction of RBPs which could bind to circ_0002762 and RelA. (B-C) in RIP assay, the enrichment of circ_0002762 and RelA in Anti-MOV10 was detected via Rt-qPCR. (D) after RNA pull down, western blot was performed to detect the protein level of MOV10 pulled down by RelA 3'UTR anti-sense or RelA 3'UTR sense WT/MUT, revealing that MOV10 was not detected in the MUT group. (E) Rt-qPCR analysis examined the knockdown efficiency of si-MOV10. (F) Rt-qPCR analysis showed that the expression of RelA mRNA decreased after MOV10 silencing. (G) the decreased expression of RelA/p65 after MOV10 gene knockdown was detected by Western blot. (H) RelA mRNA stability was assessed by measuring RelA mRNA levels in cells with MOV10 depletion or circ_0002762 silence after α-amanitin addition. $^{**}p < 0.01$; N.S.: no significance.

could impede CSCC cell migration and invasion. This determined the promoting influence of circ_0002762 on CSCC cell migration and invasion. Moreover, hinted by previous study claiming that the aberrantly high expression of circMTO1 was induced by transcriptional regulation of ZNF460, a transcription factor [40], we also explored the transcription factor leading to the aberrantly expression of circ_0002762 in CSCC cells. Through bioinformatics analysis and mechanistic assays, we identified FOXA1 as the transcriptional activator of circ_0002762. This further underscores the importance of transcription factors in regulating circRNA expression.

The regulatory mechanism of molecular factors was highly related to the subcellular distribution [41]. Therefore, we carried out subcellular fractionation assay and confirmed circ_0002762 mainly existed in CSCC cell cytoplasm. Emerging evidence has highlighted the crucial roles of signalling pathways in governing biological behaviours of cancer cells and also elucidated the participation of different signalling pathways in circRNA modulation mechanisms in malignancies [42,43]. Our study demonstrates that circ_0002762 can activate the NF-kB signalling pathway and positively regulate the expression of RelA, a key factor in the NF-kB signalling pathway.

Considering the cytoplasmic accumulation of circ_0002762, we checked whether circRNAs functioned as a miRNA sponge or protein recruiter to regulate RelA in CSCC cells. RIP assay ruled out the possibility that circ_103829 sponged miRNAs to regulate RelA. Afterwards, we screened out MOV10 as the potential RBP recruited by circ_0002762 to influence RelA.

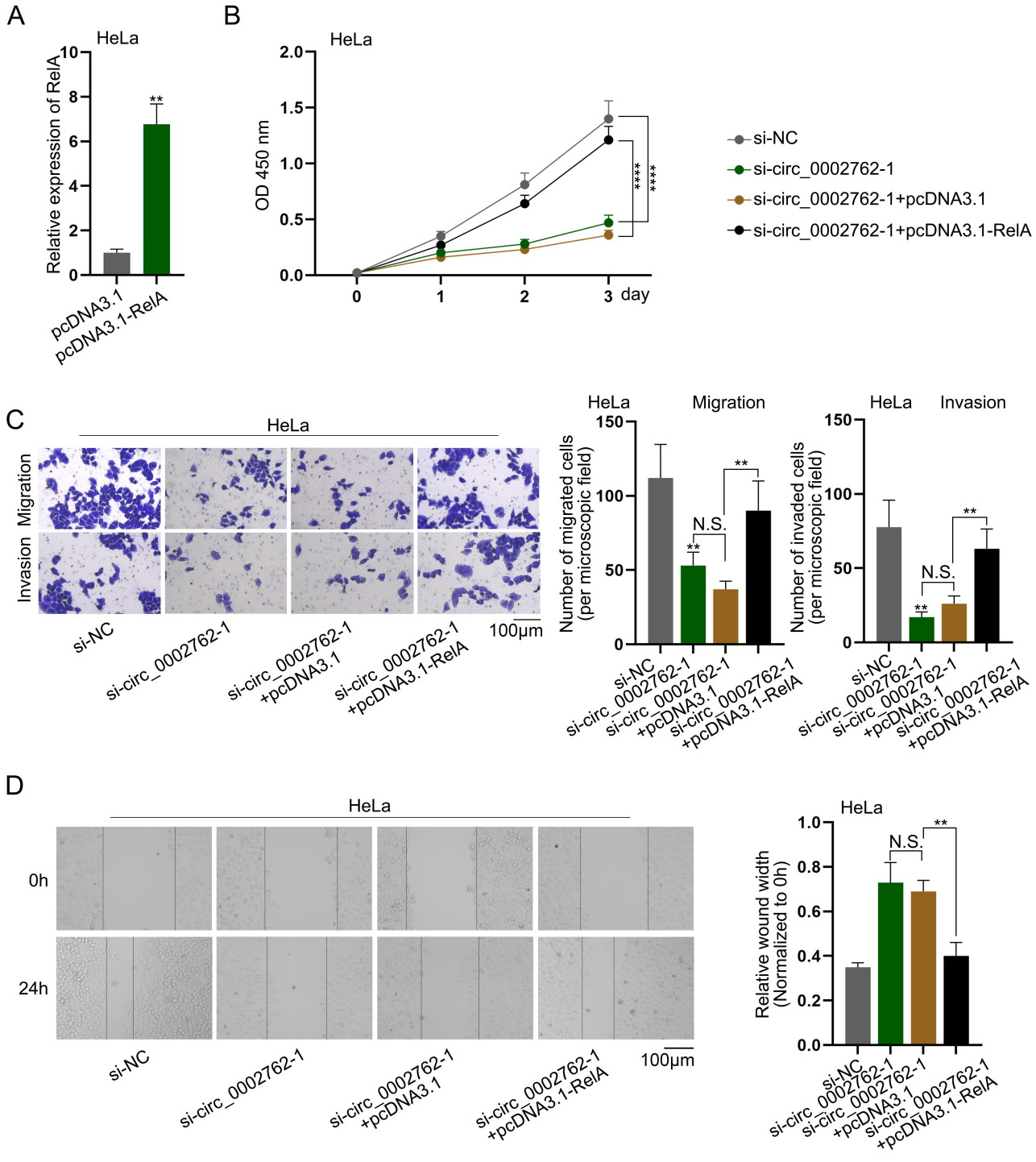

Figure 6. Circ_0002762 promotes CSCC migration and invasion via RelA (A) Rt-qPCR examined pcDNA3.1-RelA overexpression efficiency. Rescue experiments were conducted with HeLa cells transfected with indicated plasmids, including si-nc, si-circ_0002762–1, si-circ_0002762–1+pcDNA3.1, and si-circ_0002762–1 +pcDNA3.1-RelA. (B). CCK-8 results indicated that the inhibitory effect of knockdown of circ_0002762 on cell proliferation was reversed by overexpression of RelA. (C-D) HeLa cell migration and invasion were evaluated via transwell assays and wound healing assays after transfection of indicated plasmids, revealing that the inhibitory effect of circ_0002762 knockdown on migration and invasion was reversed by RelA overexpression. $^{**}p < 0.01$; N.S.: no significance.

Referring to existing study, circRHOBTB3 recruited HuR to regulate the stability of PTBP1 mRNA [44]. Consistently, we also confirmed that circ_0002762 affected the expression of RelA via MOV10-mediated mRNA stability maintenance. Ultimately, we carried out rescue experiments and validated that circ_0002762 affected migration and invasion of CSCC cells via modulation on RelA.

However, there was a lack of animal models and clinical samples in this study, which were necessary for confirming the clinical values of the molecular axis involving circ_0002762/MOV10/RelA in CSCC. Nevertheless, the results of this study provide strong evidence for the functional role of circ_0002762 in CSCC cells, suggesting that FOXA1 May act as a promoter of circ_0002762 transcriptional

activation, participating in the regulation of CSCC cell migration and invasion. By recruiting MOV10, circ_0002762 plays a role in the regulation of RelA mRNA stability, thereby influencing the activation of the RelA/NF-kB signalling pathway. This finding offers new insights into the role of circ_0002762 in CSCC and may have significant implications for future therapeutic strategies.

Acknowledgments

Sincerely, thanks for all supports.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

CRediT: **Lei Ji:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration; **Youguo Chen:** Resources, Software, Visualization, Writing – original draft, Writing – review & editing; **Xiaoping Chen:** Data curation, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) reported that there is no funding associated with the work featured in this article.

Data availability statement

The data will be available from the corresponding author on reasonable request.

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