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CDCA4 interacts with IGF2BP1 to regulate lung adenocarcinoma proliferation via the PI3K/AKT pathway

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Abstract

Background: Lung adenocarcinomas (LUAD) remain the leading cause of death in many countries. In this study, we investigated the role of division cycle-associated 4 (CDCA4) in the carcinogenesis of LUADs.

Methods: Real-time fluorescent quantitative polymerase chain reaction and western blot were performed to detect the messenger RNA and protein levels of CDCA4 in cells. Cell counting kit 8, real-time cell analysis, clone formation, EdU assays, and cell-cycle assays were used to preliminarily investigate the proliferation and cell-cycle–related functions of CDCA4 in lung adenocarcinoma. Immunoprecipitation assays were used to identify possible targets of CDCA4. A xenograft model was used to examine how CDCA4 knockdown affects LUAD cells growth in vivo.

Results: We found that the expression of CDCA4 was upregulated in LUAD cell lines. When CDCA4 was knocked out, the ability of LUAD cells to proliferate was dramatically reduced, and the cell cycle was stalled in the S phase. Meanwhile, boosting the CDCA4 expression had the opposite effect. The critical protein levels of phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway were subsequently examined. The findings demonstrated that elevated CDCA4 lowered the phosphate and tensin homolog expression and increased the p-PI3K and p-AKT levels. Moreover, we demonstrated that CDCA4 favorably regulated IGF2BP1, a downstream target. The downregulation of the IGF2BP1 expression could reverse the proliferation promotion effect induced by the CDCA4 overexpression.

Conclusions: CDCA4 can operate as an oncogenic factor to control the growth of lung adenocarcinoma via the PI3K/AKT pathway.

KEYWORDS
CDCA4, IGF2BP1, lung adenocarcinoma, proliferation

INTRODUCTION

Lung cancer is the most prevalent respiratory cancer worldwide, with significant morbidity and mortality. In fact, lung cancer is the leading cause of cancer deaths across the world.[1,2] Non-small cell lung cancer (NSCLC) accounts for 85% of all pathological types of lung cancer.[3] Lung adenocarcinoma (LUAD) is the most prevalent malignancy among NSCLC. More than half of the lung cancer patients are at the advanced stage at their initial visit because of the lack of effective screening and diagnosis methods.[4] Although the treatment of LUAD is gradually advancing, the treatment effect of advanced LUAD patients is not ideal owing to the recurrence rate, metastasis rate, drug resistance, and individualized problems.[5] Therefore, a more comprehensive and in-depth analysis of the incidence and development mechanism of LUAD has important clinical significance.

Sitong Feng and Haixia Cao are both the first authors.

The cell cycle is a process in cell life activities, and the loss of control over any link can cause cancer.[6] Cell division cycle-associated 4 (CDCA4), also known as SEI-3/hematopoietic progenitor protein (HEPP), is a member of the gene family related to the cell division cycle.[7] Cell division cycle-related gene families are cell proliferation regulators that function at different stages in the cell cycle. The loss of control of these genes affects switching of the control points during the cell cycle, homeostasis maintenance of complexes, spindle assembly, and proper segregation of chromosomes.[8–11] Past studies have demonstrated that CDCA4 promotes the growth of different cancers. The loss of CDCA4 in osteosarcoma significantly reduces the growth, invasion, and migration of osteosarcoma cancer cells.[12] Similarly, knocking down CDCA4 in Wilms tumor cells decreased the cell viability and promotes cell apoptosis.[13] CDCA4 plays a distinct role as a downstream target in cancer cell proliferation, invasion, and drug resistance pathways.[14–16] Correlational analyses suggested that the CDCA4 expression was upregulated in the LUAD tissues when compared to that in non-cancerous lung tissues, and it was found to be correlated with the LUAD stage.[17] However, the biological relevance of CDCA4 in lung cancer has received little attention and the underlying mechanism remains unclear. Here, we investigated the expression of CDCA4 in LUAD cell lines and the effect of CDCA4 on the proliferation of LUAD both in vitro and in vivo. In addition, we identified the interaction between CDCA4 and IGF2BP1 to preliminarily explore the mechanism through which CDCA4 affects the LUAD proliferation. Our results suggested that the overexpression of CDCA4 elevated the protein level of IGF2BP1, which in turn promoted the proliferation of LUAD cells through the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway.

METHODS

Cell culture

Human LAD cell lines (A549, H1299, PC9, and H460) were sourced from the Shanghai Cell Bank Chinese Academy of Sciences. H1299, A549, and H460 cells were cultured in RPMI-1640 medium (Gibco). The PC9 cells were cultured in the Dulbecco's Modified Eagle Medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS) (WISENT) and 1% penicillin/streptomycin (Gibco). All cells were cultured at 37°C in a constant temperature incubator with 5% CO_2. The changes in the cell morphology and density were monitored daily under a microscope. When the cell confluence reached approximately 80%, the cells were digested with trypsin for passage, after which the medium was changed every 3 days.

Lentivirus transfection

Lentiviral vectors expressing CDCA4-Cas9/single guide RNA (sgRNA) plasmid and CDCA4 were obtained from Vigene Biosciences. The day before transfection, we inoculated 6-well plates with appropriate concentrations of the cells and ensured that the cell confluence had reached ~60% after 24 hours. The resultant amount of virus was calculated according to the information provided by the manufacturer. The solution was changed at the 24th hour of lentivirus addition. After 3 days of culture, the cells were screened in a medium supplemented with 4 mg/mL puromycin, and the overexpression and knockdown-stable transfectants were obtained after 1 week.

RNA sample extraction and real-time quantitative polymerase chain reaction

The RNA was extracted from LAD cells by using the Takara Min-iBEST Universal RNA Extraction Kit (Takara Biotechnology). RNA was reverse transcribed into DNA with the PrimeScrip RT Master Mix (Takara Biotech) according to the results of RNA sample concentration determined by measuring absorbance at 260/280 nm. The ABI 7300 PCR system (Applied Biosystems) was used to identify any gene expression. The reaction system was processed as per the manufacturer's requirement of 20 μL. Using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression as the reference, the 2-ΔΔCT method was performed to examine the gene transcript levels.

Protein extraction and western blotting

The lung tissue samples obtained from the Jiangsu Cancer Hospital were stored at −80°C. A pestle was used to grind the lung tissues, and tissue protein extraction reagents were used to extract the total protein. The cells were washed twice with phosphate buffered saline (PBS) before lysing them at 4°C for 20 minutes. The lysis solution used included the radioimmunoprecipitation assay (RIPA) buffer from Beyotime Biotechnology containing the enzyme inhibitors. The lysates were centrifuged at 12 000 × *g* for 20 minutes in a 4°C pre-cooled centrifuge. The supernatant was collected as a protein sample. The concentration of the protein samples was determined using the BCA Protein Assay Kit (Beyotime Biotechnology), and the proteins were denatured in an LDS buffer. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% bovine serum albumin for 30 minutes. Next, the protein bands were incubated with GAPDH (5174 S, CST), CDCA4 (11625-AP, Proteintech), IGF2BP1 (22803-1-AP, Proteintech), P53 (2527 S, CST), $P21^{Waf1/Cip1}$ (2947 S, CST), phosphatase and tensin homolog (PTEN) (ab267787, Abcam), PI3K (ab191606, Abcam), P-PI3K (ab182651, Abcam), AKT (ab179463, Abcam), P-AKT (ab192623, Abcam), CDK2 (10122-1-AP, Proteintech), and cyclinA2 (18202-1-AP, Proteintech) overnight at 4°C. The next day, tris-buffered saline-Tween (TBS-T) was used to wash the protein bands before treating them with secondary antibodies for 1 hour at room temperature. The protein bands were washed again with TBS-T to ensure a clear

background. Chemiluminescent agents (Millipore) were used to measure the protein bands.

Cell proliferation assays

Cell proliferation was detected by using the cell counting kit 8 (CCK8). The cells were seeded into a 96-well plate (4×10^3 cells/well) for cell adhesion. A diluted CCK8 solution was added to the cells, and the cells were incubated in the incubator for 1 hour. Next, the absorbance was measured at 450 nm with a microplate reader (Bio-Rad) at 24, 48, and 72 hours.

The proliferative activity of lung cancer cells was assessed using the real-time cell analysis (RTCA) DP Instrument (Roche Diagnostics). At the density of 5×10^3 cells/well, the cells were seeded into a 16-well microplate (E-plates) (Roche Diagnostics). The RTCA DP Instrument was used to automatically record the impedance every 15 minutes.

The EdU Kit (C0071s, Beyotime) was used to detect cell proliferation. The cells were seeded into a 96-well plate (2×10^4 cells/well) and incubated with the EdU reagent for 2 hours the next day. After the cells are immobilized in 4% paraformaldehyde, the fluorescent dye was added.

Colony formation assay

By using a plate colony formation experiment, the cells' capacity to form colonies was determined. The cells were seeded into a 6-well plate (500 cells/well) and cultured at 37°C in a 5% CO_2 atmosphere, with the medium refreshed every 3 days. The culture was terminated when macroclones (>50 cells/colony) formed under the microscope. The 6-well plates were then dyed with 0.5% crystal violet solution at room temperature after fixing them with 4% paraformaldehyde for 30 minutes. The number of clonal colonies was manually enumerated.

Flow cytometry

The experimental cells were digested with trypsin, washed twice with PBS, and fixed overnight at 4°C with 75% cold ethanol. The samples were then analyzed with the Cell Cycle Detection Kit (keyGEN) by flow cytometry (BD Biosciences), followed by data analysis with FlowJo V10.

Xenograft experiment

We created a nude mouse A549 cell xenograft model and assessed how CDCA4 knockdown affects the in vivo cell growth. All animal-related protocols were approved by the Animal Experiment Ethics Committee of Nanjing Medical University. Female BALB/C nude mice ($n = 10$; weighing: 17–24 g at 4 weeks of age) were bought from the Shanghai Animal Experimental Center. The mice were randomly assigned to two groups of five mice each. A 200-μL cell suspension (1×10^7 cells/200 μL) was subcutaneously inoculated into the nude mice (experimental mice were subcutaneously injected with CDCA4 knocks down A549 cells in the right axilla, and control mice were subcutaneously injected with the same volume of negative control group A549 cells). The mouse weight and volume (volume = [length × width 2]/2) were measured at 3-day intervals with vernier calipers. After 30 days, the mice were sacrificed and their tumor weights were determined using an electronic balance. The tumor tissues were immobilized in 4% formaldehyde and analyzed by immunohistochemistry.

Immunoprecipitation

Immunoprecipitation (IP) analysis was performed using the Pierce Classic IP Kit (Thermo Science). Briefly, A549 cells overexpressing CDCA4 were lysed in the IP lysis buffer supplemented with protease and phosphatase inhibitors. The lysate was centrifuged at 12 000 $\times g$ for 20 minutes in a 4°C precooled centrifuge. The supernatants were incubated overnight at 4°C with the protein G beads-bound antibodies against igG (sc-2025, Santa Cruz Biotechnology), antibodies against CDCA4 (11625-AP, Proteintech), and antibodies against IGF2BP1 (55246-1-AP, Proteintech). After washing in accordance with the manufacturer's instructions, the proteins were denatured in a 2× LDS buffer (Beyotime Biotechnology). Finally, anti-CDCA4 antibody and anti-GF2BP1 antibody were used for the immunoblotting analyses.

Statistical analysis

The experimental data were processed with GraphPad Prism 6.0 software. Two groups of data were compared by Student's *t*-test, whereas multiple groups were compared by one-way analysis of variance. All experimental data met the requirements of three independent replicates and were expressed as the mean ± standard deviation. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

The overexpression of CDCA4 in the LUAD tissues and cells is associated with a poor prognosis

We reviewed the relevant data from The Cancer Genome Atlas (TCGA) to investigate the CDCA4 expression in LUAD, and the findings indicated that LUAD expressed

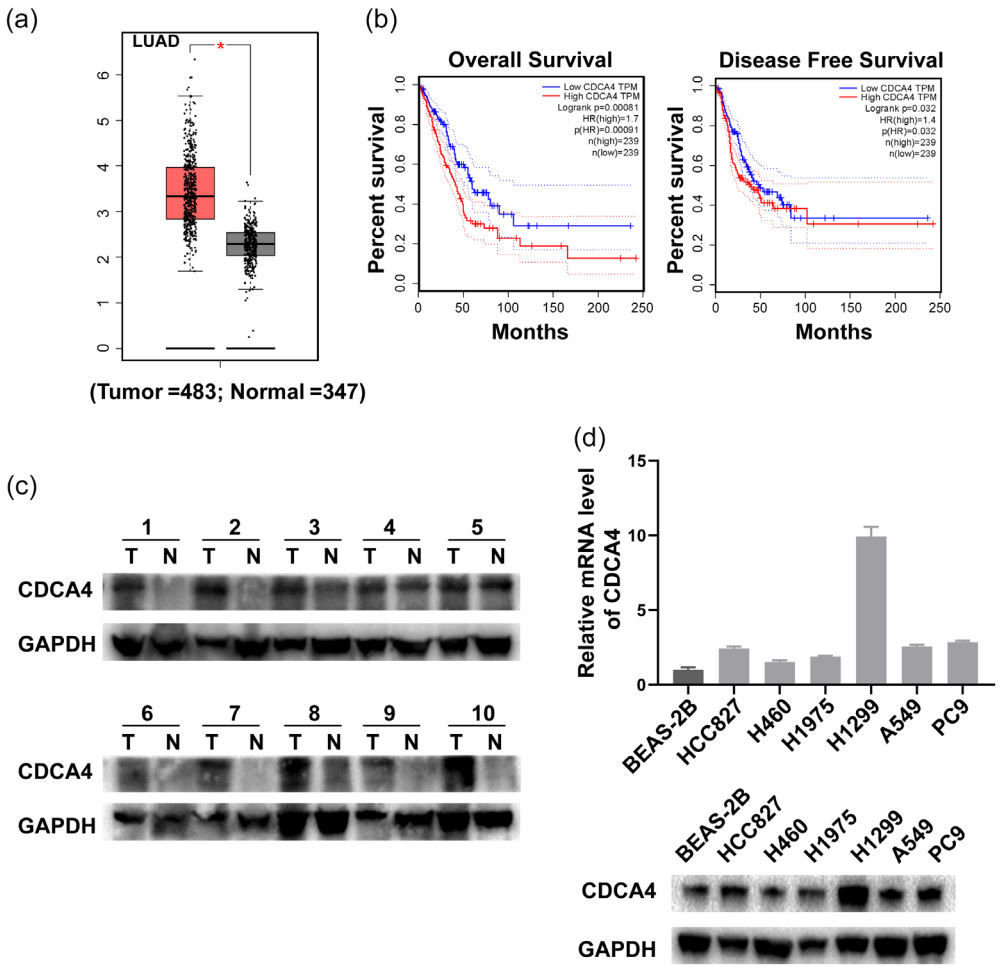


FIGURE 1 The cell division cycle-associated 4 (CDCA4) expression was upregulated in the lung adenocarcinomas (LUAD) tissues and cell lines. (a) The CDCA4 expression was higher in the 483 LUAD tissues than in the 347 normal lung tissues, with reference to the The Cancer Genome Atlas (TCGA) database ($p < 0.05$). (b) Kaplan–Meier overall survival curves and disease-free survival curves in 478 LUAD patients. (c) Comparison of the CDCA4 protein levels in the non-small cell lung cancer (NSCLC) tissues and adjacent non-tumor tissues. (d) The messenger (mRNA) and protein expression of CDCA4 in LUAD cell lines

CDCA4 at a higher level than other normal lung tissues (Figure 1(a)). In addition, Kaplan–Meier survival curves revealed that patients with a high CDCA4 expression outlived those with a low CDCA4 expression (Figure 1(b)). Real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) and western blotting were used to evaluate the levels of CDCA4 mRNA and proteins in the lung cancer cell lines and tissues. As shown in Figure 1(c),(d), lung cancer cell lines exhibited much higher levels of the CDCA4 expression than healthy human bronchial epithelial cells BEAS-2B. Similarly, the level of CDCA4 protein in the NSCLC tissues was higher than those in the adjacent non-tumor tissues. These results suggested that CDCA4 may play a pro-carcinogenic role in LUAD. To further reveal the critical role of CDCA4 in the development of LUAD, four cell lines were selected for the next step of the experiment.

Knockout of CDCA4 inhibits LUAD cells proliferation and causes S-phase arrest

We established stable models of the CDCA4 overexpression and deletion in lung cancer cell lines A549, H460, H1299, and PC9, respectively, based on the degree of CDCA4 expression. Lentiviral infection was used to upregulate and knockdown the CDCA4 expression in LUAD cells. RT-qPCR and western blotting assays validated the outcomes and efficiency of the lentiviral intervention (Figure 2(a)). The CCK-8 assay revealed that the proliferation rate of A549 and H460 cells increased after the upregulation of CDCA4. After CDCA4 silencing, the proliferation rate of cells in the H1299 and PC9 knockout groups decreased in comparison to that in the control group (Figure 2(c)). RTCA verified the effect of CDCA4 on the proliferation of LUAD cells (Figure 2(d)). In addition, the EdU assay implied that CDCA4 silencing inhabited LUAD cell proliferation (Figure 2(e)). Furthermore, boosting the CDCA4 expression increased the number of clones in A549 and H460 cells more than in the control group. Moreover, as predicted, knocking down CDCA4 in H1299 and PC9 cells reduced the number of clones when compared to that in the control group (Figure 2(b)). Finally, flow cytometry was performed to assess the effect of CDCA4 on the cycle distribution of LUAD cells. As shown in Figure 3, CDCA4 knockdown resulted in an increased percentage of cells in the S-phase when compared to that in the controls. Moreover, upregulating the CDCA4 expression led to a statistically significant reduction in the proportion of S-phase

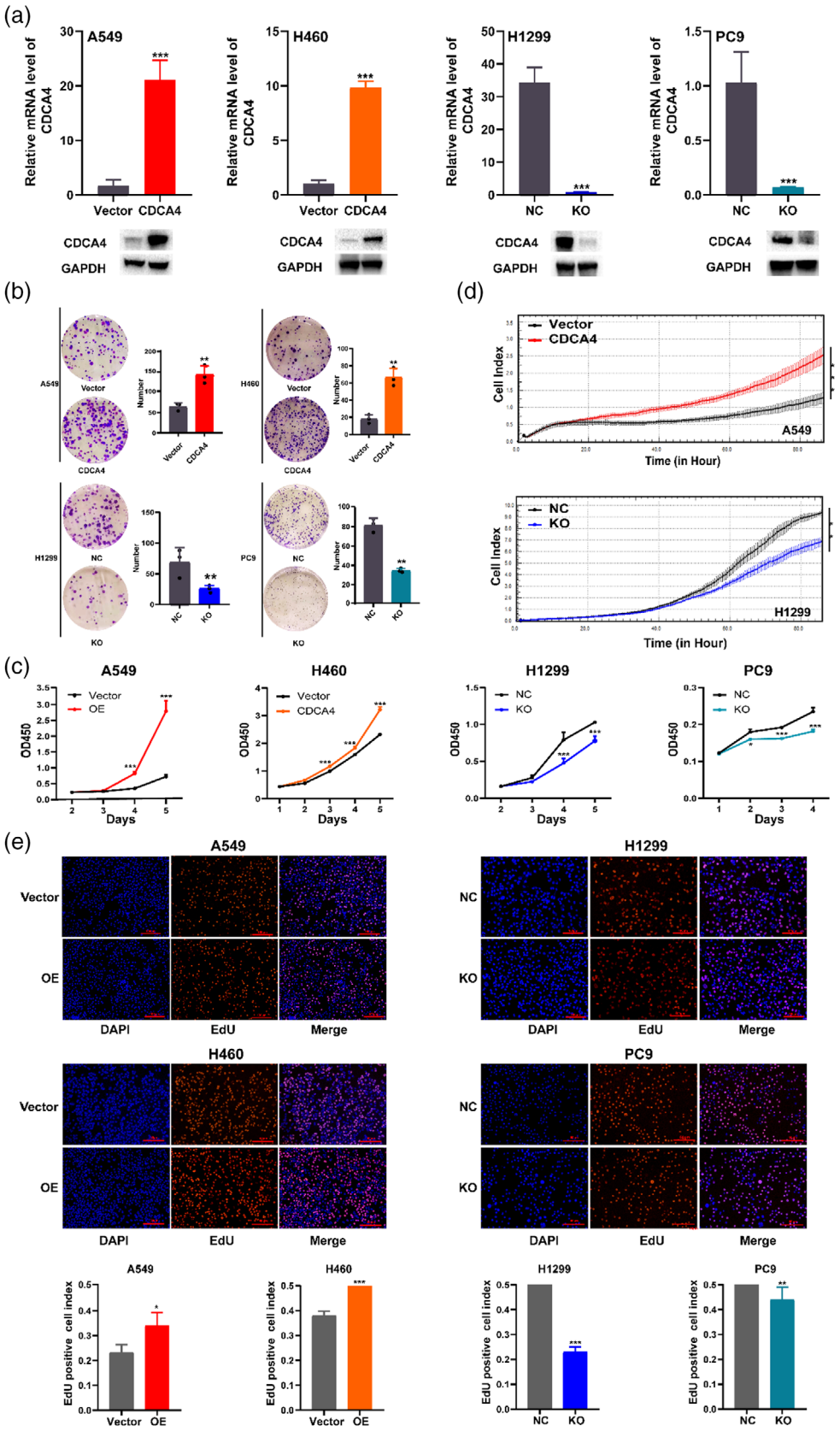


FIGURE 2 Knockout of cell division cycle-associated 4 (CDCA4) inhibits lung adenocarcinomas (LUAD) cell proliferation. (a) Validation of the messenger (mRNA) and protein level efficiency of CDCA4 overexpression cells A549, H460, and CDCA4 knockdown cells H1299, PC9 (mean ± standard deviation [SD], Student's *t*-test, $^{***}p < 0.005$). (b) CDCA4 overexpression in A549 and H460 promotes the ability of colony formation and CDCA4 knockdown in H1299 and PC9 inhibits the cellular clonogenic ability (mean ± SD, Student's *t*-test, $^{*}p < 0.05$, $^{***}p < 0.005$). (c) The knockdown of CDCA4 expression in H1299 and PC9 inhibited proliferation, whereas the CDCA4 overexpression in A549 and H460 promoted growth of cells (mean ± SD, Student's *t*-test, $^{*}p < 0.05$, $^{***}p < 0.005$). (d) Differences in the cell proliferation capacity reflected by real-time cell analysis (RTCA) results in A549 and H1299 (mean ± SD, Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.005$). (e) The EdU assay was used to detect the changes in the proliferation ability after CDCA4 knockout and overexpression in LUAD cells (mean ± SD, Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.005$)

cells in A549 cells ($p < 0.05$). These results suggest that CDCA4 promoted lung cancer cell growth and altered the cell-cycle progression. Moreover, knocking down CDCA4 halted the S-phase of the LUAD cell cycle.

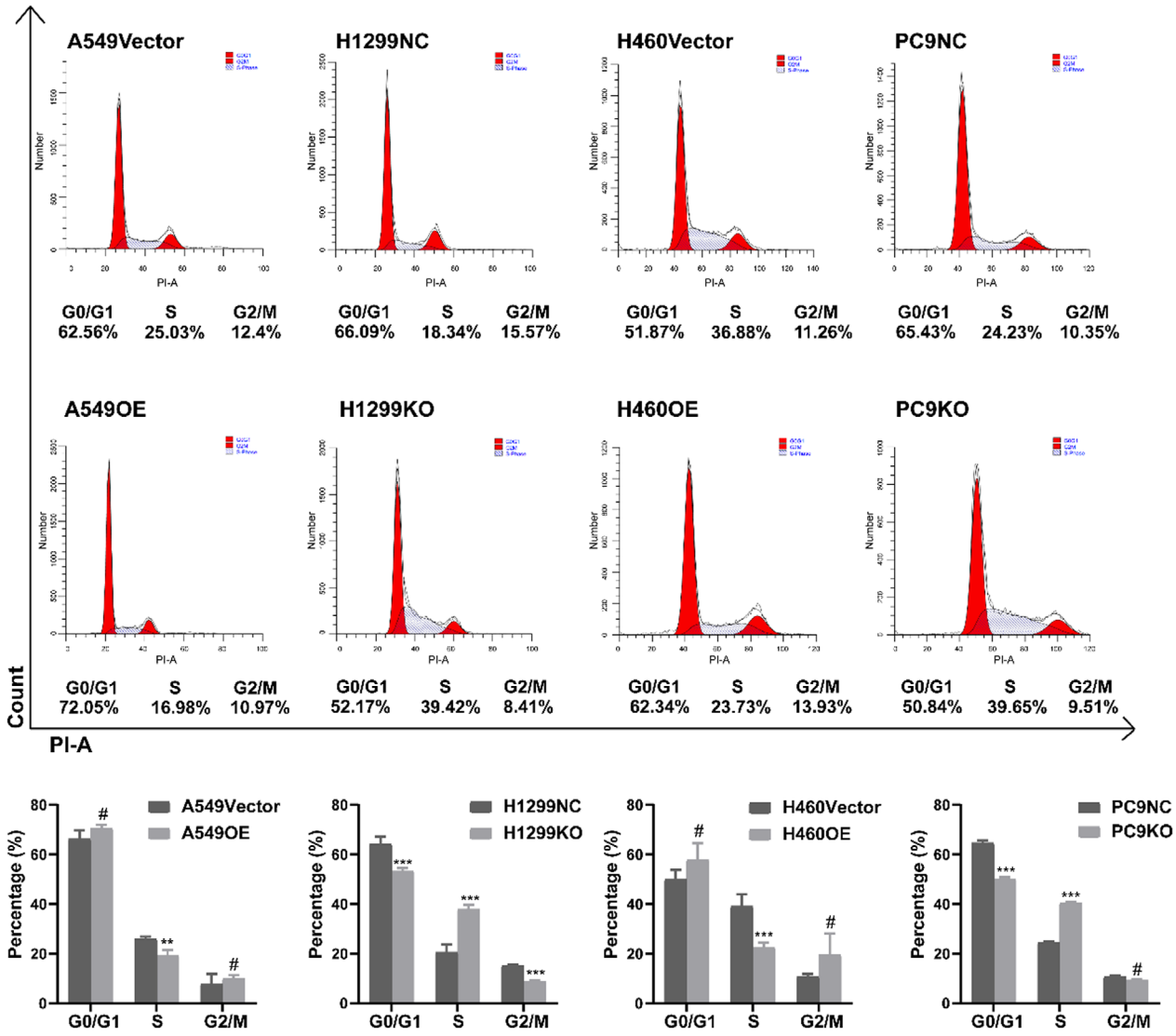


FIGURE 3 Cell division cycle-associated 4 (CDCA4) knockdown causes S-phase arrest. The downregulation of CDCA4 in H1299 caused S-phase arrest, whereas the proportion of S-phase was decreased in A549 overexpressing CDCA4 when compared with the control cells (mean ± standard deviation [SD], Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, #not significant)

CDCA4 affects cell proliferation mediated by the PI3K–AKT signaling pathway

The PI3K/AKT signaling pathway regulates cell life processes, such as promoting cell proliferation and metabolism, participating in cancer occurrence, chemotherapy resistance, and promoting blood vessel formation. In H1299 and A549 (Figure 4), we examined the effect of CDCA4 on the protein expression of the PI3K/AKT signaling pathway and discovered that the overexpression of CDCA4 in A549 caused a decrease in PTEN and an increase in p-PI3K and p-AKT. The expression of $P21^{Waf1/Cip1}$, a negative cell-cycle regulator, was inhibited. CDCA4 knockdown in H1299 was accompanied by an increase in PTEN and $P21^{Waf1/Cip1}$, and a reduction in p-PI3K and p-AKT. These results suggested that CDCA4 inhibits the PTEN protein expression and that CDCA4 could further activate the PI3K/AKT signaling pathway. Based on these results, we suggested that CDCA4 may participate in the regulation of LUAD cell proliferation and cell cycle through the PTEN/PI3K/AKT signaling pathway.

CDCA4 interacts with IGF2BP1 and promotes the post-transcriptional translation level of IGF2BP1

The binding proteins of the A549 overexpressed cell line were analyzed by mass spectrometry to further understand how CDCA4 controls the growth of LUAD cells at the molecular level. The results revealed that CDCA4 and IGF2BP1 possibly interacts (Figure 5(a)). Figure 5(b),(c) demonstrate that

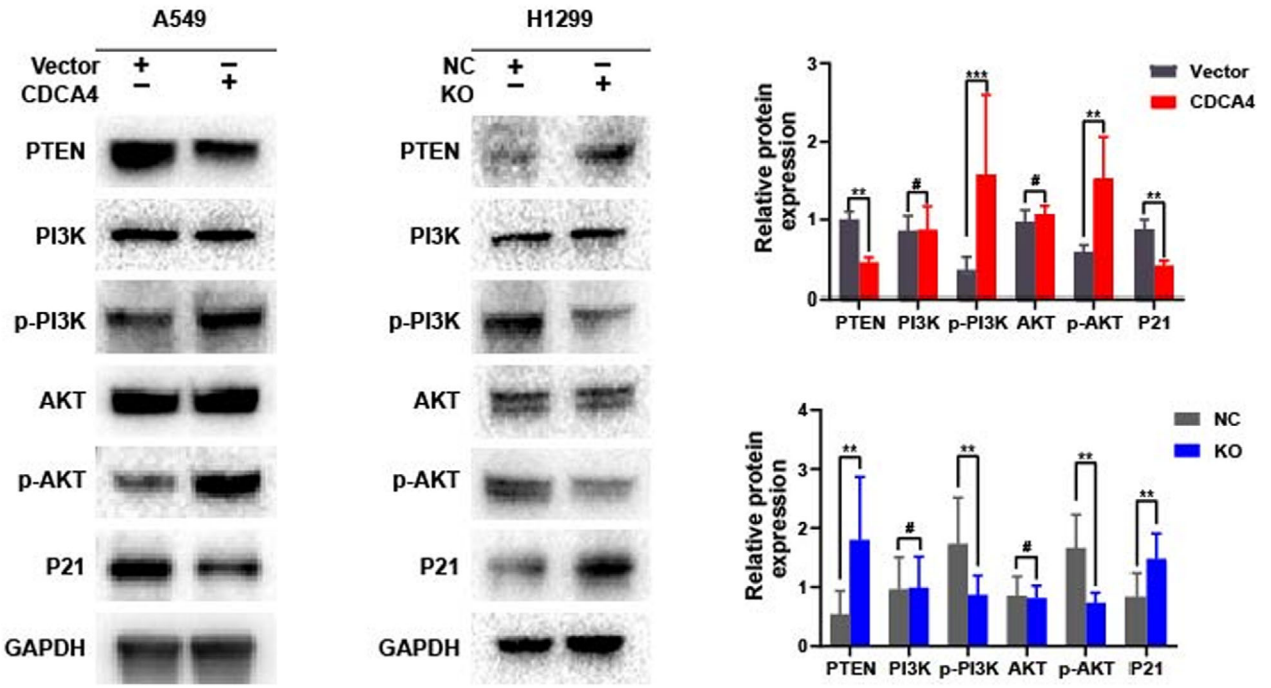


FIGURE 4 Cell division cycle-associated 4 (CDCA4) affects cell proliferation mediated by the phosphatidylinositol 3 kinase (PI3K)– protein kinase B (AKT) signaling pathway. Western blotting analysis indicates the expression of the PI3K–AKT signaling pathway-related proteins in H1299 and A549 cells (mean ± standard deviation [SD], Student's *t*-test, $^{**}p < 0.01$, #not significant)

inhibiting IGF2BP1 in A549 reduced the number of clones and the proliferation rate when compared to that in the control group, suggesting that IGF2BP1 plays a carcinogenic role in LUAD. Meanwhile, the downregulation of IGF2BP1 similarly led to an increase in PTEN protein and a decrease in P-PI3K and p-AKT (Figure 5(d)), suggesting that IGF2BP1 is involved in the regulation of the PTEN/PI3K/AKT pathway. The co-IP assay in A549 confirmed their binding interaction (Figure 5(e)). In H1299 and A549 cells, CDCA4 favorably regulated the IGF2BP1 protein expression (Figure 5(f),) but did not affect the IGF2BP1 messenger RNA (mRNA) levels (Figure 5(g)). IGF2BP1 was successfully downregulated at both the mRNA and protein levels in A549 cells following transfection with si-IGF2BP1, whereas the CDCA4 expression was unaffected (Figure 5(h),(i)). These data suggest that CDCA4 targeted IGF2BP1 and positively regulated IGF2BP1 post-transcriptional translation level in LUAD.

Reducing the expression of IGF2BP1 markedly reduced the proliferation-promoting effect induced by CDCA4 overexpression

As shown in Figure 6, si-IGF2BP1 was transfected into CDCA4-overexpressing cell A549 and CDCA4-knockout cell H1299 to evaluate whether IGF2BP1 is involved in the regulation of CDCA4 on the proliferation phenotype (Figure 6(a)). The outcomes demonstrated that, in comparison to the KO group, the downregulation of IGF2BP1 in CDCA4 knockout cells further hindered their ability to proliferate, whereas the downregulation of IGF2BP1 in CDCA4-overexpressing cells significantly reversed the proliferation-promoting effect, which was manifested as a decrease in the proliferation rate and the clone number (Figure 6(b)–(d)). The EdU-positive cell rate in the CDCA4-overexpressing group was further reduced after IGF2BP1 knockdown, whereas in H1299, the cumulative effect of CDCA4 knockout and IGF2BP1 knockdown resulted in the lowest EdU-positive cell rate (Figure 6(e)). These findings imply that CDCA4 promotes the development of LUAD cells by targeting IGF2BP1. Reducing the expression of IGF2BP1 could markedly reduce the proliferation-promoting effect caused by CDCA4 overexpression.

shCDCA4 inhibits the development of A549 cell xenografts

To investigate the effect of CDCA4 on the proliferation of lung cancer cells in vivo, we constructed a nude mice model using shNC and shCDCA4 transfected A549 cells (Figure 7(a)). After A549 cell injection, xenograft growth was observed on day 9, and the body weight and xenograft diameter were recorded every 3 days (Figure 7(b),(c)). On day 30 following injection, the transplanted tumors were dissected subcutaneously from the nude mice and their weight was measured (Figures 7(d),(e)). When compared to the control group, the shCDCA4 group had considerably lower tumor volume and weight.

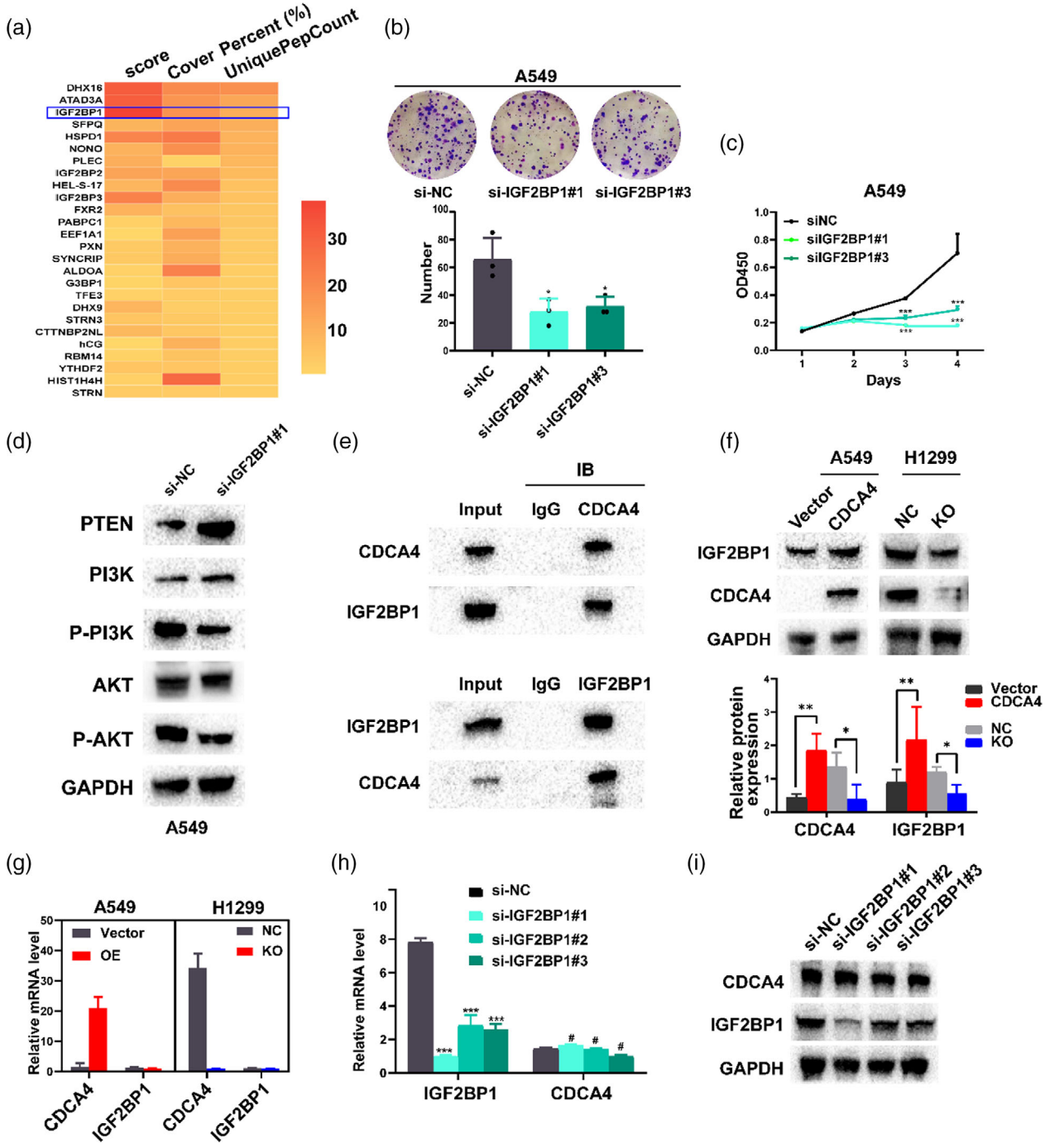
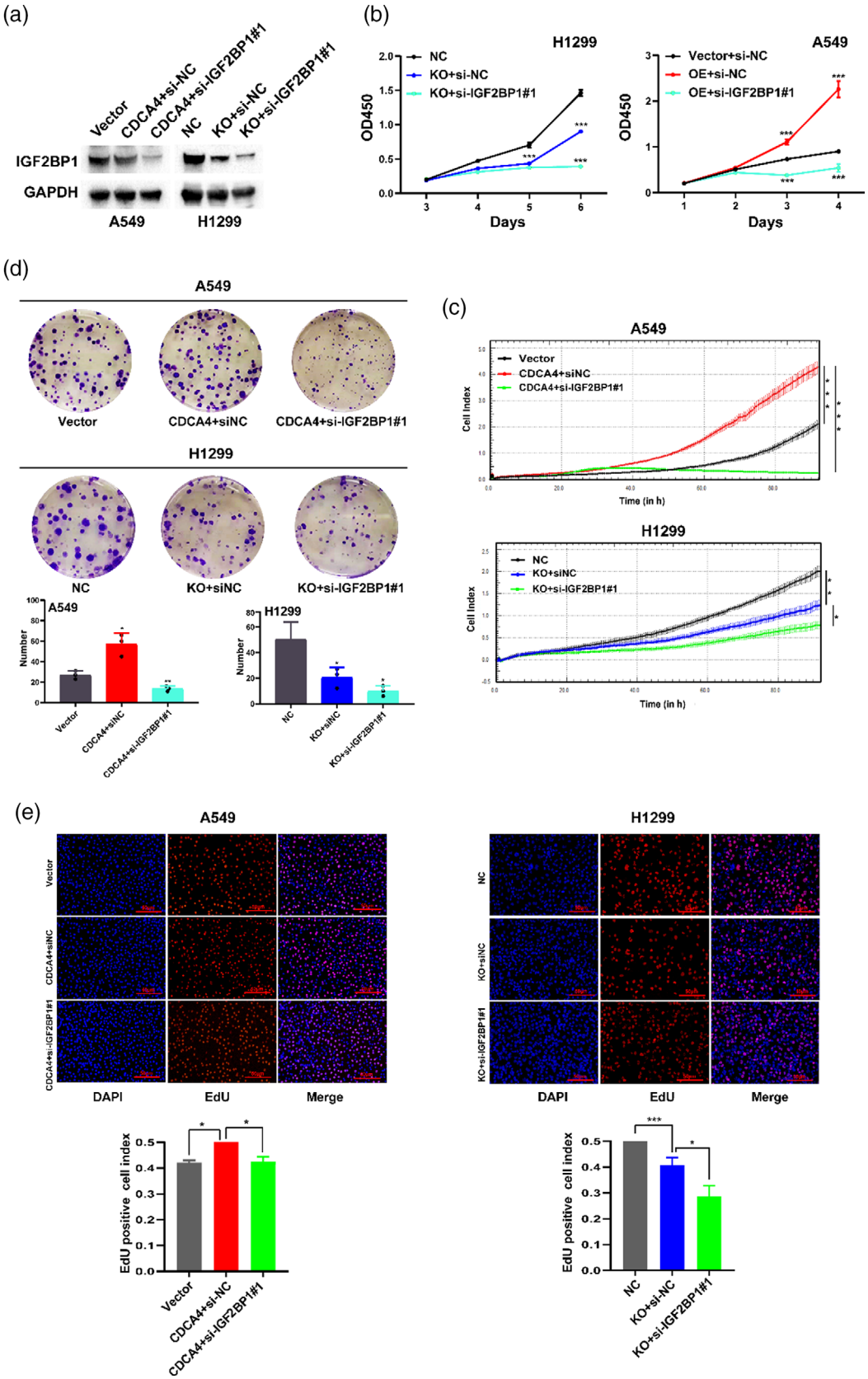


FIGURE 5 Cell division cycle-associated 4 (CDCA4) interacts with IGF2BP1 and promotes the post-transcriptional translation level of IGF2BP1. (a) The results of mass spectrometry analysis of differential fragments in A549 cells overexpressing CDCA4. (b) Reducing IGF2BP1 inhibits the clonogenic ability of A549 (mean ± SD, Student's *t*-test, $^{*}p < 0.05$). (c) The downregulation of CDCA4 in A549 inhibits cell proliferation (mean ± SD, Student's *t*-test, $^{***}p < 0.005$). (d) The effect of decreasing IGF2BP1 expression on the phosphatase and tensin homolog (PTEN)/phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway. (e) CO-immunoprecipitation (IP) assay demonstrating the binding of CDCA4 and IGF2BP1 in A549 cells. (f) Western blotting analysis indicating changes in the IGF2BP1 protein expression in stable transgenic strains A549 and H1299 (mean ± standard deviation [SD], Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (g) The effect of upregulation versus inhibition of CDCA4 on the messenger RNA (mRNA) level of IGF2BP1. (h) Quantitative polymerase chain reaction (qPCR) analysis indicating the changes in the CDCA4 expression in A549 transfection with si-IGF2BP1. (i) The effect of downregulation of IGF2BP1 in A549 on the expression of CDCA4 protein

Ki67 staining was weaker in the shCDCA4 group than in the shNC group, as per the immunohistochemical examination. Figure 7(f) shows that CDCA4 may promote tumor growth in vivo.

DISCUSSION

The bioinformatics analysis of *CDCA4* has significantly improved in recent years, and a growing number of analyses



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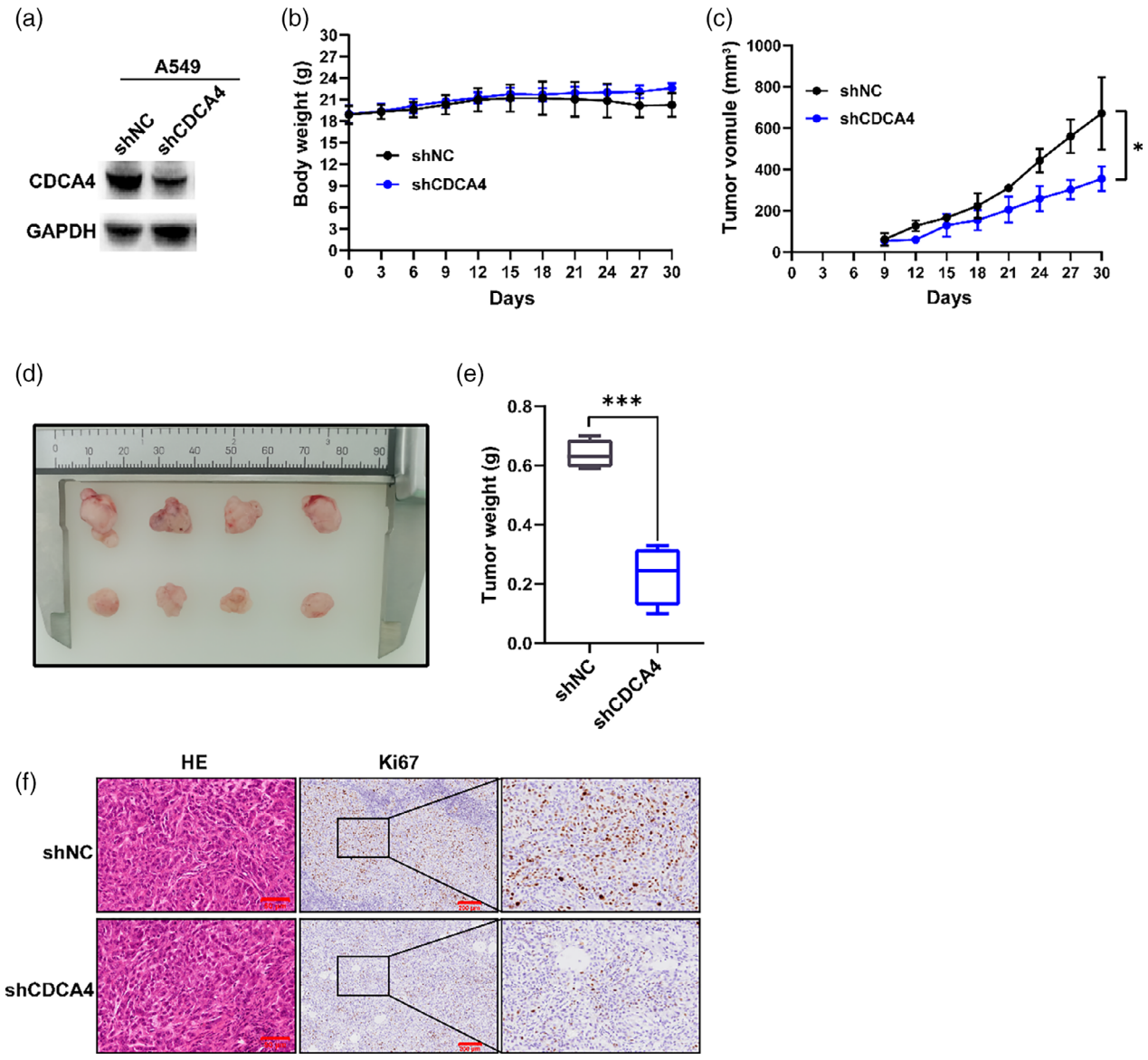

FIGURE 7 The knockdown of cell division cycle-associated 4 (CDCA4) inhibits the ability of lung adenocarcinomas (LUAD) cells to proliferate in vivo. (a) Western blotting analysis indicating the expression of CDCA4 protein in A549 transfection with shCDCA4. (b,c) Bodyweight changes and tumor volume changes in nude mice after subcutaneous injection of tumor cells (mean ± SD, Student's *t*-test, $^{*}p < 0.05$). (d,e) The downregulation of the CDCA4 expression significantly inhibits tumor growth in vivo (mean ± standard deviation [SD], Student's *t*-test, $^{***}p < 0.005$). (f) The results of KI67 staining of tumor sections

suggest that high CDCA4 levels play a catalytic role in tumor carcinogenesis to different extents.[18] Moreover, CDCA4 can function as a target gene in the control of the cell cycle and tumor cell resistance, whereas the CDCA4 expression is directly correlated to the level of immune infiltration. cDCA4 has the potential to be both diagnostic and prognostic.[19–21]

CDCA4, also known as SEI-3/HEPP. SEI-1, SEI-2, and CDCA4 have structural and functional similarities. Sei-1 can regulate the activity of CDK inhibitors. It influences CDK expression, which then regulates the cell proliferation.[22] According to past studies, the regulatory activation actions of SEI-1 and SEI-2 are based on the C-terminal transactivation region. Meanwhile, the C-terminal is one of the highly conserved

FIGURE 6 IGF2BP1 knockdown can alter the function of cell division cycle-associated 4 (CDCA4). (a) Western blotting analysis showing the expression of IGF2BP1 protein in H1299, A549 stable transfer strain transfection with si-IGF2BP1. (b) Effect of downregulation of IGF2BP1 on the proliferation efficiency of CDCA4 knockout cells H1299 and CDCA4 overexpressing cells A549 (mean ± standard deviation [SD], Student's *t*-test, $^{***}p < 0.005$). (c) Differences in the cell proliferation capacity as reflected by the real-time cell analysis (RTCA) results (mean ± SD, Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). (d) The effect of the downregulation of IGF2BP1 on the phenotype of CDCA4 clone formation (mean ± SD, Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$). (e) EdU assay indicated the effect of IGF2BP1 knockdown on the proliferation ability of lung adenocarcinomas (LUAD) cells with high and low expression of CDCA4 (mean ± SD, Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$)

characteristic sequences of CDCA4; therefore, CDCA4 can act as a nuclear factor to regulate E2F and p53-dependent transcriptional activation and participate in the cell-cycle progression.[23] Here, our results suggested that the high expression of CDCA4 is associated with poor prognosis in LUAD. Lowered CDCA4 expression inhibited the proliferation of LUAD cells and induced S-phase arrest in vitro. Meanwhile, the CDCA4 overexpression had the opposite result. Reduced CDCA4 expression exhibited low tumorigenicity in vivo. Previous research found that CDCA4 was primarily involved in the malignant phenotypes of tumors as a downstream target. For instance, Ren et al.[24] hypothesized that consuming LINC01116 prevented LUAD cell proliferation, migration, and invasion. However, increasing CDCA4 levels may reverse the biological consequences of LINC01116 knockdown on LUAD cells. Yuan et al.[15] pointed out that exosomal Mir-424-5p inhibited granulosa cell proliferation and induced polycystic ovary syndrome cell senescence by blocking the CDCA4-mediated Rb/E2F1 signaling. Meanwhile, CDCA4 may be a potential target of miR-15a-5p and the upregulation of CDCA4 promotes the proliferation and invasion of lung cancer cells.[14] The underlying regulating mechanism of CDCA4 in malignant tumors, however, remains unknown. We accordingly hypothesized that CDCA4 downregulation in LUAD cells is associated with decreased cell proliferation and cell-cycle inhibition. Therefore, it is crucial to investigate the potential mechanism of CDCA4 in affecting the activity of LUAD cells.

The PI3K/AKT pathway is the most commonly altered in a range of malignancies, and the aberrant activation of this system is connected to tumor cell proliferation, invasion, migration, and medication resistance.[25] The upregulation of the PI3K/AKT pathway mostly predicts the malignant progression of tumors. PTEN and PI3K may both phosphorylate the metabolite phosphatidylinositol 4,5-bisphosphate (PIP2) to PI(3,4,5) P3 (PIP3).[26] Therefore, the PI3K signaling pathway is terminated,[27] and PTEN loss is the key mechanism for the loss of the PI3K/AKT pathway.[28] We examined the effect of CDCA4 on the PI3K/AKT signaling pathway protein expression in H1299 and A549 cells and discovered that, when CDCA4 was overexpressed in A549 cells, the PTEN protein level decreased, whereas the phosphorylated PI3K and AKT expression increased. CDCA4 knockdown in H1299 had the opposite effects on the PTEN, P-PI3K, and P-AKT pathway proteins. Therefore, these results indicated that CDCA4 affected the LUAD cell activity through the PI3K/AKT pathway. Next, we verified that CDCA4 could interact with IGF2BP1 by silver staining, mass spectrometry, and co-IP screening in the A549 overexpression cell line. IGF2BP1 plays a cancer-promoting role in the occurrence and development of NSCLC,[29,30] and a low level of IGF2BP1 predicts a good prognosis in LUAD patients.[31] Past studies have demonstrated that IGF2BP1 participated in the defect process of mesenchymal stem cells by regulating the PI3K/AKT pathway.[32] In renal cell carcinoma, IGF2BP1 could promote renal tumorigenicity through multiple pathways, including enhancement of the stability of sphingosine-1-phosphate receptor 3 (S1PR3) mRNA, regulation of the expression of S1PR3, and upregulation of the PI3K/AKT pathway.[33] We found that IGF2BP1 promoted the malignant progression of LUAD cells as the proliferative and colony formation abilities of A549 were significantly inhibited after the downregulation of IGF2BP1. Meanwhile, the downregulation of IGF2BP1 could antagonize the expression of PTEN/PI3K/AKT pathway proteins. We, therefore, performed rescue studies to evaluate the regulatory relationship between CDCA4 and IGF2BP1 and found that IGF2BP1 reversed the proliferation-promoting effect of CDCA4. The downregulation of IGF2BP1 did not affect the CDCA4 expression. Meanwhile, CDCA4 positively regulated the IGF2BP1 protein level, but had no impact on the IGF2BP1 mRNA expression. These findings imply that CDCA4 could improve the proliferation activity of LUAD cells by upregulating IGF2BP1 post-transcriptional translation. Interference and regulation of any process, such as translation initiation, extension, and termination, can significantly affect the abundance of protein expression during the cell translation process.[34,35] In addition, ubiquitination modification after protein translation may also lead to protein degradation.[36,37] Therefore, based on these findings, we plan to continue studying the detailed mechanism of CDCA4 in regulating the IGF2BP1 post-transcriptional translation level.

In conclusion, this study elucidated the role of CDCA4 in the progression of LUAD and preliminarily explored the underlying mechanism therein. The CDCA4 expression was elevated in LUAD cells. CDCA4 operated as an oncogenic factor to control the growth of LUAD cells. The activation of the PI3K/AKT pathway may be the underlying mechanism through which CDCA4 influences LUAD cell proliferation and cell-cycle progression. Cancer treatment involves inhibition of the tumor cell growth, and targeting cell cycle components is an effective new anticancer strategy. Our cumulative data suggest that CDCA4 could be a new target for LUAD therapy.

AUTHOR CONTRIBUTIONS

Sitong Feng and Haixia Cao designed and conceived the study, and all authors participated in the data analysis and writing of the initial draft. All authors read and approved the final manuscript. Sitong Feng and Haixia Cao contributed equally in this study.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data supporting this research are presented in the article.

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