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Cdc37 facilitates cell survival of colorectal carcinoma via activating the CDK4 signaling pathway

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Cell division cycle 37 (Cdc37) is an important partner for heat shock protein 90 (HSP90), assisting in molecular chaperone activities, particularly with regard to the regulation of protein kinases. Given its influence on cell growth pathways, Cdc37 has been discussed as a potential intermediate in carcinogenesis. However, to date, the potential functional roles and molecular mechanisms by which Cdc37 regulates cell survival in colorectal carcinoma (CRC) remain unclear. Here, we investigated the expression of Cdc37 and its clinical significance in CRC, and systematically explored the role and the underlying mechanism of Cdc37 in CRC cell survival both in vitro and in vivo. Our results showed that Cdc37 was remarkably up-regulated in CRC, which facilitated cell survival mainly by promoting cell proliferation, G1-S transition, and inhibiting cell apoptosis. Our data further indicated that Cdc37 increased the stability of cyclin-dependent kinase 4 (CDK4) to activate the retinoblastoma 1 (RB1) signaling pathway, followed by increased expression of Bcl-2 and Bcl-xL, which ultimately promoted cell survival in CRC. Moreover, knockdown of CDK4 reversed the Cdc37-mediated effect in promoting the progression of CRC. Our findings showed that Cdc37 played a critical role in promoting CRC cell survival by increasing CDK4 stability to activate the RB1 signaling pathway. Thereby, Cdc37 might serve as a potential therapeutic target in CRC patients.

KEYWORDS
Cdc37, CDK4, cell survival, colorectal carcinoma, p-RB1

1 | INTRODUCTION

Colorectal adenocarcinoma is one of the most common digestive system malignancies, of which the morbidity is increasing nowadays. With the second highest incidence among all the malignancies, it is also the second most common cause of cancer-related mortality.[1,2] The carcinogenesis of colorectal adenocarcinoma is a multistep process in which multi-genes are involved.[2] Exploring the molecular pathogenesis of colorectal carcinoma is conducive to cancer prevention, early diagnosis and effective treatment. HSP90 is an important molecular chaperone, responsible for the conformational maturation of client proteins.[3] So far, several co-chaperones of HSP90 have been identified and proven to participate in the progression of malignancies, including stress-inducible protein 1 (Sti1/p60), Cdc37, p23, the activator of HSP90 ATPase-1 (AHA1), protein phosphatase (PP5) etc.[4]

Abbreviations: AR, androgen receptor; Cdc37, cell division cycle 37; CDK, cyclin-dependent kinase; Chx, cycloheximide; CRC, colorectal carcinoma; 5-FU, fluorouracil; HCC, hepatocellular carcinoma; HSP90, heat shock protein 90; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt; PI, propidium iodide; p-RB, phosphorylated-retinoblastoma; RB, retinoblastoma.

Cdc37, as a protein kinase-specific adaptor co-chaperone of HSP90, functions as a scaffold protein to recruit protein kinase client to HSP90, followed by protecting the protein kinase client from degradation, resulting in increased protein stability and kinase activity.[3] Through genetic screens in *Drosophila*, Cdc37, also named as p50, was identified to be an essential component of the MAPK signaling pathway.[5] So far, most previous studies on Cdc37 focused on its role in the occurrence and progression of HCC. Wei et al[6] reported that Cdc37 was overexpressed in HCC cells, where it functioned with HSP90 to regulate the activity of protein kinases in multiple oncogenic signaling pathways. Disrupting the HSP90/Cdc37 complex facilitated degradation and inhibited phosphorylation of protein kinase clients in the Raf/MEK/ERK and phosphatidylinositol-3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathways. Furthermore, Wang et al[7] reported that both the transcript and protein expression of Cdc37 were significantly increased in hepatitis B virus (HBV)-associated HCC patients. Cell proliferation was inhibited, cell cycle was arrested at the G1 phase, and apoptosis was enhanced after Cdc37 knockdown in HCC cells. Meanwhile, Cyclin D1, CDK4, and p-RB were down-regulated upon Cdc37 knockdown, resulting in the arrest of cell cycle progression at the G1 phase[7]. Guo et al[8] also reported that the expression levels of Cdc37 were up-regulated in HCC cell lines. Overexpression of Cdc37 in HCC cells increased cell proliferation, invasion activity, and protein levels of Akt, which suggested that Cdc37 promoted cell proliferation and invasion through stabilizing and activating Akt.

Although the functional roles of Cdc37 have been elaborately explored in HCC, little useful information is known in other malignancies. Stepanova reported that Cdc37 displayed increased expression in human prostatic tumors, neoplasias and certain pre-malignant lesions, suggesting an important function for Cdc37 in prostatic transformation.[9] Cdc37 was identified as a crucial HSP90-cofactor for KIT oncogenic expression in gastrointestinal stromal tumors through genome-wide functional screening.[10] Smith et al[11] showed that knockdown of Cdc37 in human colon cancer cells attenuated association of kinase clients with HSP90 and decreased levels of the clients ERBB2, CRAF, CDK4 and CDK6, as well as phosphorylated Akt. Moreover, combining Cdc37 knockdown with the HSP90 inhibitor 17-AAG induced more extensive depletion of kinase clients and potentiated cell cycle arrest and apoptosis. However, to date, the potential functional roles of Cdc37 in CRC are not clear. In the present study, we systematically investigated the expression of Cdc37 and its functional roles in CRC cell survival both in vitro and in vivo. More importantly, the underlying molecular mechanisms were thoroughly explored.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Primary antibodies used in this study are provided in Table S1. Detailed information of the reagents is described in Doc S1.

2.2 | Cell culture and tissue collection

Human colon cell lines were cultured in F12K medium supplemented with 10% FBS. Colorectal adenocarcinoma tissues and adjacent normal colorectal tissues were obtained from 120 patients at Affiliated Hospital of North Sichuan Medical College. Pathological examination further confirmed the absence of cancer cells in the normal collected tissues. The study was approved by the Ethics Committee of North Sichuan Medical College and written informed consent was obtained from all participants.

2.3 | qRT-PCR, western blot and IHC

RNA extraction, cDNA synthesis, and qRT-PCR reactions were carried out as described in Doc S1. Primer sequences were synthesized by Sangon Biotech (Shanghai, China) and are provided in Table S2. Western blot and immunohistochemistry (IHC) analysis was carried out as described in Doc S1.

2.4 | Knockdown and forced expression of target genes

siRNAs were transfected into cells with Lipofectamine 2000 according to the manufacturer's instructions. siRNAs were purchased form GenePharma Company (Shanghai, China). Cdc37 knockdown and overexpression plasmid were purchased and constructed from Gene Chem Company (Shanghai, China). The method for transfecting plasmid was similar to siRNA transfecting. Sequences of siRNAs and primers are listed in Table S2.

2.5 | Establishing the Lovo-shCdc37 and Lovo-Cdc37 cell lines

According to the Lipofectamine 2000 manufacturer's instructions, 2 μg Cdc37 knockdown or overexpression plasmid mixed with 5 μL Lipofectamine 2000 was transfected into Lovo cells, respectively. Two days later, the transfected Lovo cells were cultured in medium containing 1000 μg/mL G418. After 14 days, monoclonal cells were selected and cultured in the medium containing 500 μg/mL G418. The new constructed cell lines generated from the corresponding monoclonal cells were named Lovo-shCdc37 and Lovo-Cdc37, respectively.

2.6 | [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) assay

Cancer cells were plated into 96-well plates and incubated normally. MTS reagent (20 μL) was added to the well and incubated at 37°C for 2 hours and protected from light. The plate was read at the absorbance wavelength of 490 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.7 | Ethynyl deoxyuridine (EdU) incorporation assay

Proliferation activity of cancer cells was analyzed using an EdU incorporation assay kit (Ribobio, Guangdong, China) according to the manufacturer's instructions. Briefly, cells were incubated with 5 μM EdU in medium at 37°C for 2 hours, followed by fixing with 4% formaldehyde for 30 minutes at room temperature and permeating with 0.5% Triton X-100 for 20 minutes. Cells were incubated with 1× Apollo® reaction cocktail (Ribobio) for 30 minutes at room temperature. Then, the nuclear DNA was stained with Hoechst 33342 for 30 minutes and visualized under a fluorescent microscope.

2.8 | Cell cycle analysis

Cell cycle was measured by propidium iodide (PI) staining (BestBio, Shanghai, China) following the manufacturer's instructions. Briefly, cancer cells seeded in 6-well plates were collected and fixed in 70% ethanol at 4°C for 24 hours. Then cells were stained with PI (1 mg/mL) for 30 minutes and protected from light after digesting with RNase. Cell cycle analysis was carried out by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Proportion of cells in the G1, S, and G2/M phases was determined by their DNA content.

2.9 | Cell apoptosis assay

Cell apoptosis was measured by Annexin V-FITC detection Kit (BestBio) according to the manufacturer's protocol. Briefly, collected cells were incubated with 400 μL binding buffer and 5 μL Annexin V at 4°C for 15 minutes and protected from light. The cells were incubated at 4°C for 10 minutes and protected from light after adding 10 μL PI. Cell samples were analyzed by flow cytometry (Beckman Coulter).

2.10 | Nude mice xenograft model

All animal procedures were approved by the Institutional Animal Care and Use Committee of North Sichuan Medical College. Twenty male BALB/c nude mice (4 weeks old) were randomly divided into groups. Xenografts were initiated by s.c. injection of colon cancer cells into the back of nude mice (n = 5 in each group). Two week later, the mice was injected with 5-FU (50 mg/kg) by tumor regional injection every 3 days. One month later, the transplanted mice were

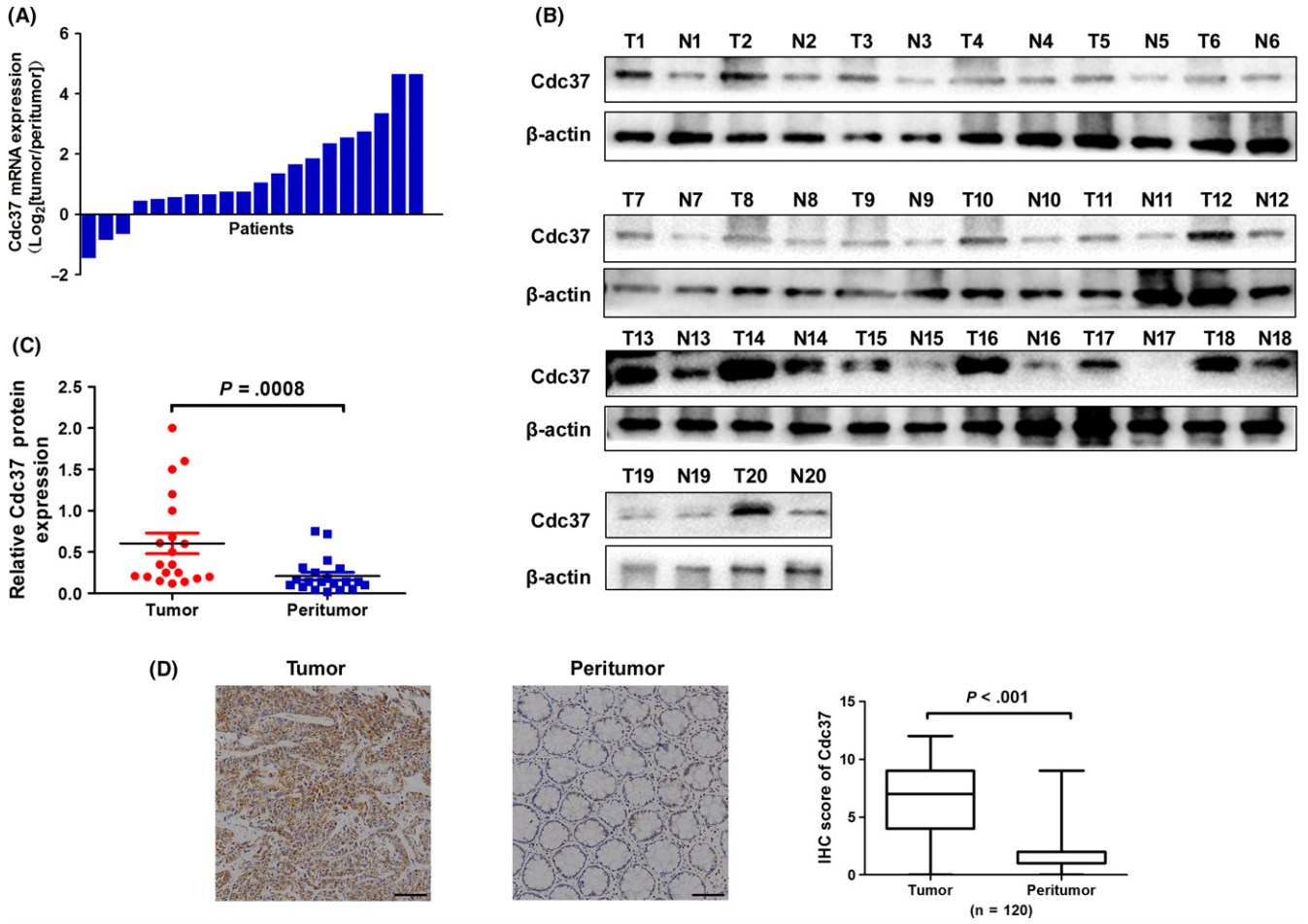


FIGURE 1 Cell division cycle 37 (Cdc37) is up-regulated in colorectal carcinoma (CRC) tissues. A, qRT-PCR and B, western blot analysis for mRNA and protein expression of Cdc37 in 20 paired tissues from colorectal carcinoma patients. β-Actin was used as internal control. C, Gray value analyses of Cdc37 protein expression. D, Representative immunohistochemical (IHC) staining images of Cdc37 (Left) and IHC analysis for Cdc37 protein expression in paired tissues from colorectal carcinoma patients (n = 120) (Right). Scale bar, 50 μm

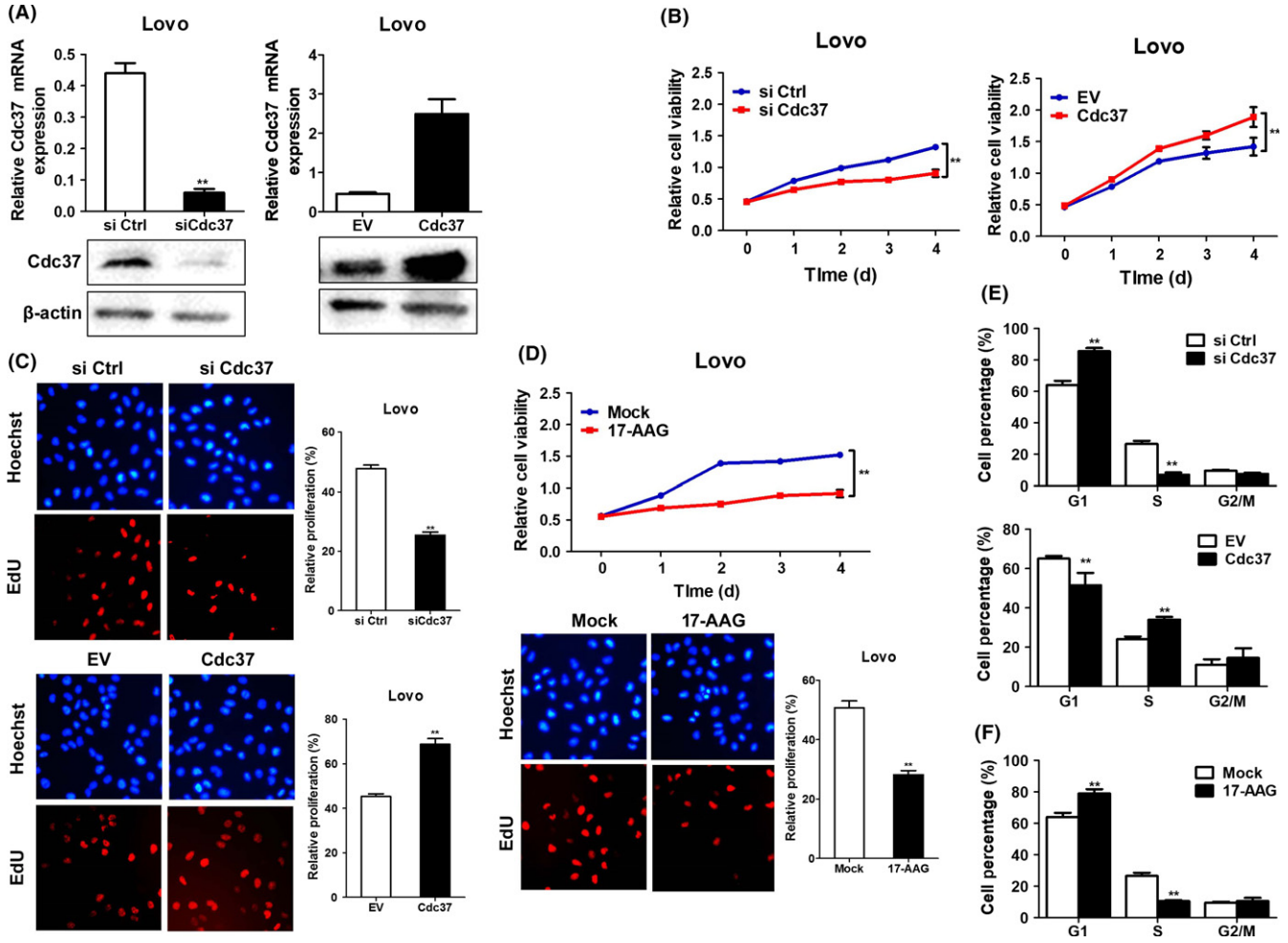


FIGURE 2 Cell division cycle 37 (Cdc37) promotes colorectal carcinoma (CRC) cell proliferation. A, qRT-PCR and western blot analysis of Cdc37 mRNA and protein expression in Lovo cells transfected with siRNA or plasmid as indicated. Cdc37 group, cells transfected with Cdc37 forced expression vector; EV group, cells transfected with the empty vector; siCdc37, siRNA against Cdc37; si Ctrl, negative control siRNA. B, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (MTS) cell viability assay in Lovo cells treated as indicated. C, Cell proliferation ability was evaluated using ethynyl deoxyuridine (EdU) incorporation assay 48 h after transfection with treatment as indicated. D, MTS and EdU assay in Lovo cells treated as indicated. Mock, DMSO; 17-AAG, 17-AAG (2.5 μmol/L). E,F Cell cycle analysis by flow cytometry in Lovo cells 72 h after treatment as indicated. Data are presented as mean ± SEM from three independent experiments. $^{**}P < .01$, unpaired *t* test

killed, at which point the tumor nodules were photographed and weights calculated. A time-volume curve was plotted to investigate xenograft growth. Tumor volume (mm^3) was calculated by the formula (length × $width^2$)/2.

2.11 | TUNEL assay

For analysis of apoptosis in xenograft tissues, TUNEL assay (Roche Applied Science, Rotkreuz, Switzerland) was carried out according to the manufacturer's protocol. Briefly, sections were incubated with Proteinase K (Sigma, Munich, Germany) at 37°C for 30 minutes, followed by incubating with TUNEL reaction buffer at 37°C for 1 hour protected from light. Then, cell nucleus was stained by DAPI (Beyotime). Images of TUNEL/DAPI-stained sections were taken by a fluorescence microscope (DM5000B; Leica, Heerbrugg, Switzerland).

2.12 | Protein stability assay

For protein stability experiments, cells were seeded in 6-well plates. Forty-eight hours later, cycloheximide (50 μg/mL) was added to the cells, and this time was marked as 0 hours. Cell samples were collected at 0 hours, 2 hours, 4 hours and 6 hours after cycloheximide treatment, respectively. Protein levels were analyzed by western blot.

2.13 | Co-immunoprecipitation

For immunoprecipitation experiments, cells lysate was incubated with 200 μL protein A beads (Beyotime) supplemented with primary antibody at 4°C overnight. After washing the protein A beads with 0.5 μL cold lysis buffer, normalized amounts of total cell lysates or immunoprecipitated samples were analyzed by western blot.

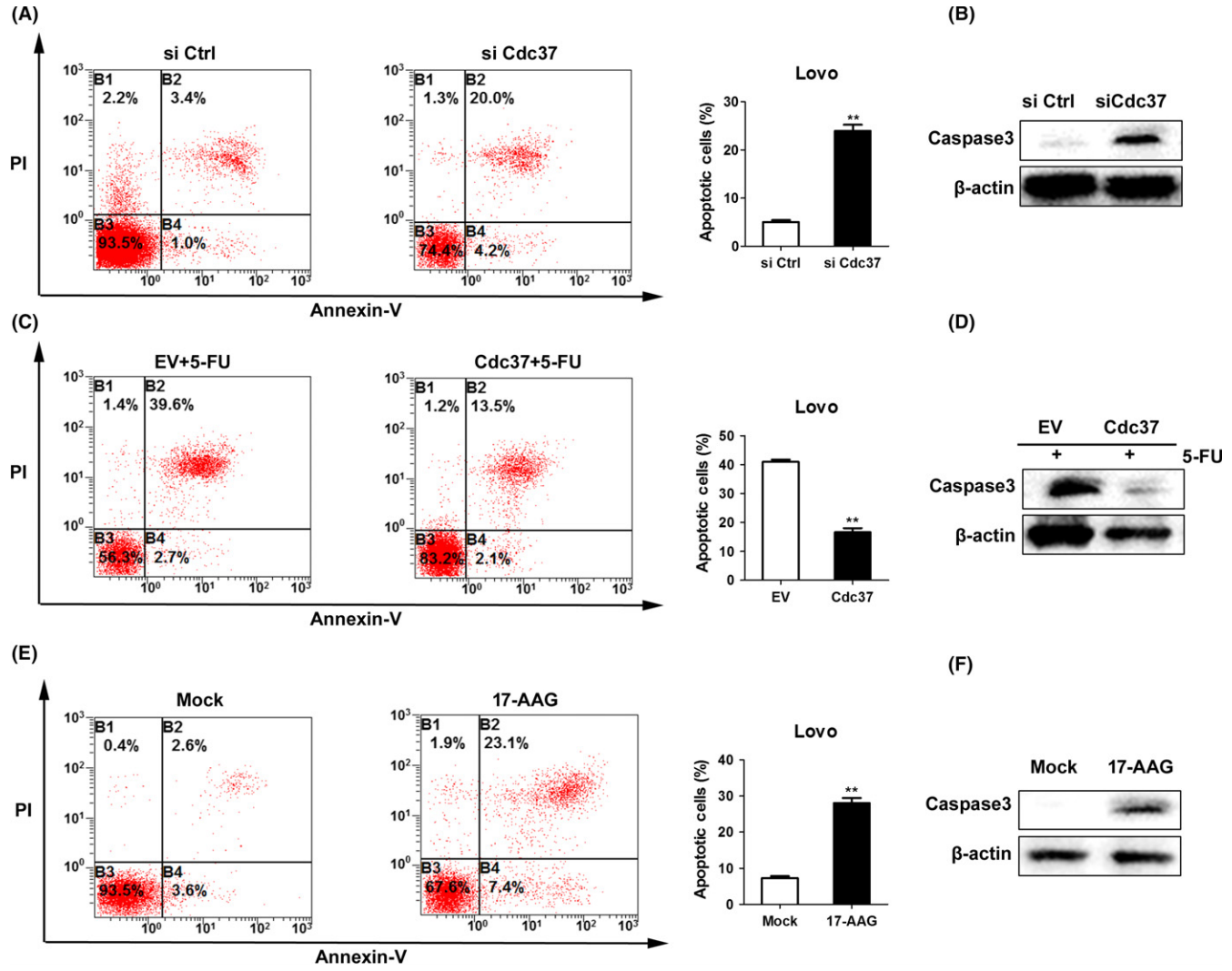


FIGURE 3 Cell division cycle 37 (Cdc37) inhibits colorectal cancer cell apoptosis in vitro. (A,C,E) Cell apoptosis analysis by flow cytometry 48 h after treatment as indicated. (B,D,F) Western blot analysis of Caspase 3 protein expression in Lovo cells 48 h after treatment as indicated. 5-FU, 5-fluorouracil (50 μm/L). Data are presented as mean ± SEM. $^{**}P < .01$, unpaired *t* test

2.14 | Statistical analysis

SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses and *P*-values less than .05 were considered to be statistically significant. Data were represented as mean ± SEM. Unpaired *t* tests were used for comparisons between two groups. One-way ANOVA was used for comparisons among 3 or more groups. Correlations between measured variables were tested by Pearson's *r* correlation analyses.

3 | RESULTS

3.1 | Cdc37 is up-regulated in CRC tissues

In order to explore the functional roles of Cdc37 in CRC, both mRNA and protein expression of Cdc37 was detected by qRT-PCR and western blot in 20 pairs of CRC tissues. Our results showed that both mRNA and protein expression of Cdc37 was up-regulated in CRC tissues when compared with paired non-tumor tissues ($P = .005$, $P = .0008$, respectively) (Figure 1A-C). Similarly, IHC staining results also showed that Cdc37 protein expression was significantly increased in CRC tissues compared with paired non-tumor tissues ($P < .001$) (Figure 1D). Furthermore, we analyzed the relationship between Cdc37 expression level and pathological characteristics of CRC patients (Table S3), and found that Cdc37 expression level in female patients was significantly higher than that in male patients ($P = .0450$). Moreover, Cdc37 expression level was gradually increased with the pathological development of CRC, but the difference among them was not statistically significant ($P = .1707$). Altogether, these results indicate that Cdc37 is up-regulated in CRC, which promotes the development of CRC.

3.2 | Cdc37 promotes CRC cell proliferation and G1-S phase transition

Next, we investigated the role of Cdc37 in cell growth. As shown in Figure S1, expression of Cdc37 was significantly increased in Lovo cells when compared with normal colon cells. MTS assays showed that growth rate of CRC cells was significantly decreased after Cdc37

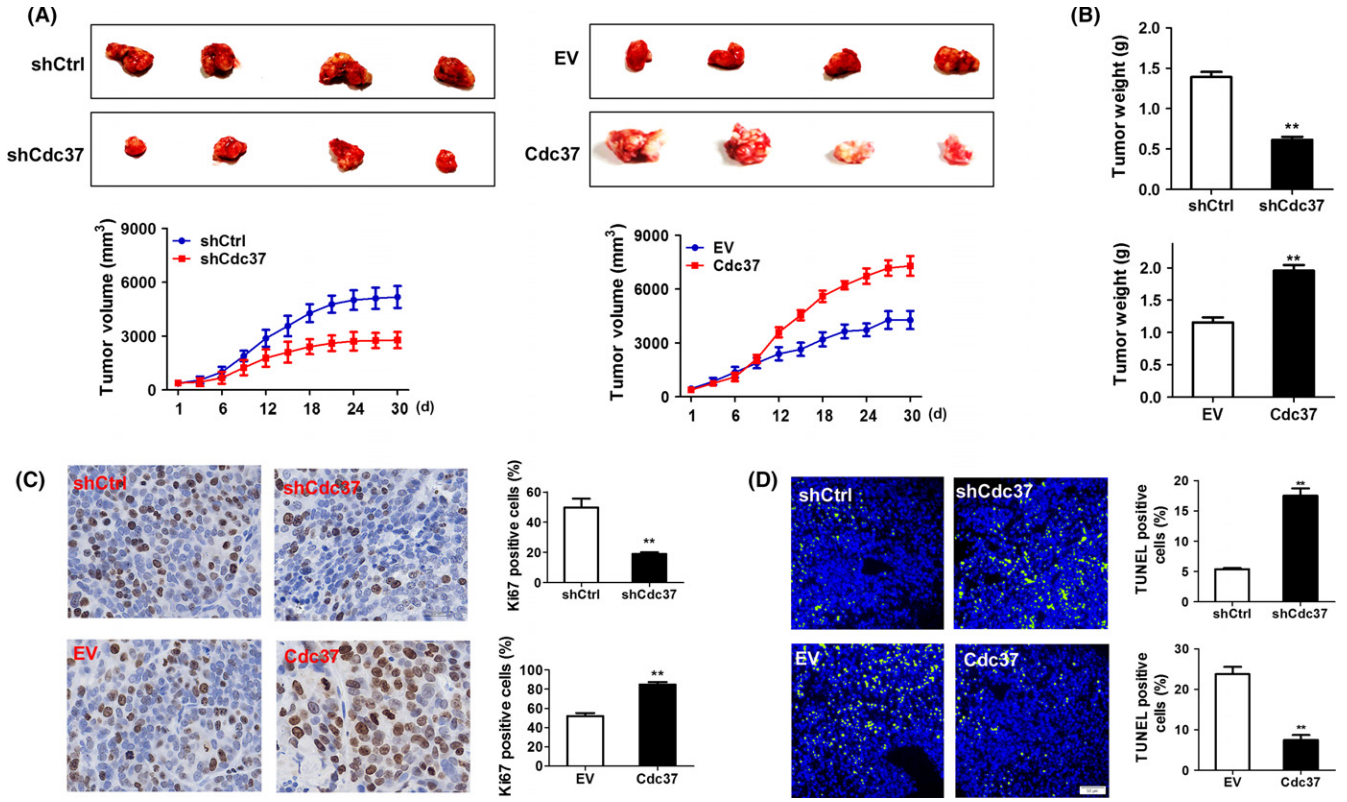


FIGURE 4 Cell division cycle 37 (Cdc37) promotes colorectal cancer growth in vivo. A, (Lower) Tumor growth curves of subcutaneous xenograft tumor model developed from stable cell lines and (Upper) dissected tumors from killed mice are shown. B, Tumor weights were calculated and are shown. C, Representative immunohistochemical (IHC) staining images of Ki67 in xenograft tumor treated as indicated. D, TUNEL staining in tumor tissues of nude mice xenograft model with treatment as indicated. Blue, Hoechst; Green, TUNEL-positive nucleus. Data shown are the mean ± SEM from three independent experiments. $^{**}P < .01$, unpaired *t* test

knockdown, whereas cell growth rate was significantly increased after overexpression of Cdc37 in Lovo cells (Figure 2A,B). Results of EdU incorporation assay further suggested that the expression level of Cdc37 was well correlated with the proliferation activity in CRC cells (Figure 2C). Previous studies reported that Cdc37 acting in concert with HSP90 is required for maturation of clients.[8,11] 17-AAG, an inhibitor of HSP90, restrained the binding of Hsp90 to its client proteins.[3] Thus, 17-AAG was used to disrupt the HSP90-Cdc37-client complex, and our results showed that cell growth rate and proliferation activity of CRC cells were significantly reduced after 17-AAG treatment (Figure 2D). Our results also showed that Cdc37 knockdown promoted cancer cells to accumulate in the G1 phase, whereas overexpression of Cdc37 facilitated cancer cells to enter into S phase (Figure 2E). Similarly, 17-AAG also arrested the cells in G1 phase (Figure 2F). Taken together, our results suggest that up-regulated Cdc37 facilitates CRC cell growth through promoting G1-S transition.

3.3 | Cdc37 inhibits CRC cell apoptosis in vitro

Next, we investigated the functional role of Cdc37 in CRC cell apoptosis. Flow cytometry showed that the apoptosis rate of Lovo cells was significantly increased after Cdc37 knockdown (Figure 3A), whereas cell apoptosis induced by 5-FU was significantly decreased after over-expression of Cdc37 in colorectal carcinoma cells (Figure 3C). In addition, our results showed that expression of the cleaved form of caspase 3 protein was significantly increased after Cdc37 knockdown (Figure 3B), whereas overexpression of Cdc37 protected CRC cells from 5-FU-induced cell apoptosis (Figure 3D). Similarly, the cell apoptotic rate and expression of the cleaved form of caspase 3 were significantly increased after 17-AAG treatment (Figure 3E,F). Together, these results indicate that Cdc37 promotes the progression of CRC through inhibiting cell apoptosis.

3.4 | Cdc37 facilitates CRC growth in vivo

Our previous data showed that up-regulated Cdc37 promoted cell survival in vitro, but the functional role of Cdc37 in vivo remains unclear. Stable cell models with knockdown or overexpression of Cdc37 were established, respectively (Figure S2). Here, our data showed that the growth capacity of xenograft tumors developed from Lovo-shCdc37 cells was significantly decreased than that of xenograft tumors developed from control cells, whereas the growth capacity of xenograft tumors developed from Lovo-Cdc37 was significantly increased compared with the control group (Figure 4A,B). We further investigated the effect of Cdc37 on cell apoptosis and proliferation activity in vivo using TUNEL staining assay and immunohistochemical staining of Ki67. Our data showed that overexpression of Cdc37 promoted the proliferation activity, and protected CRC from 5-FU-induced apoptosis

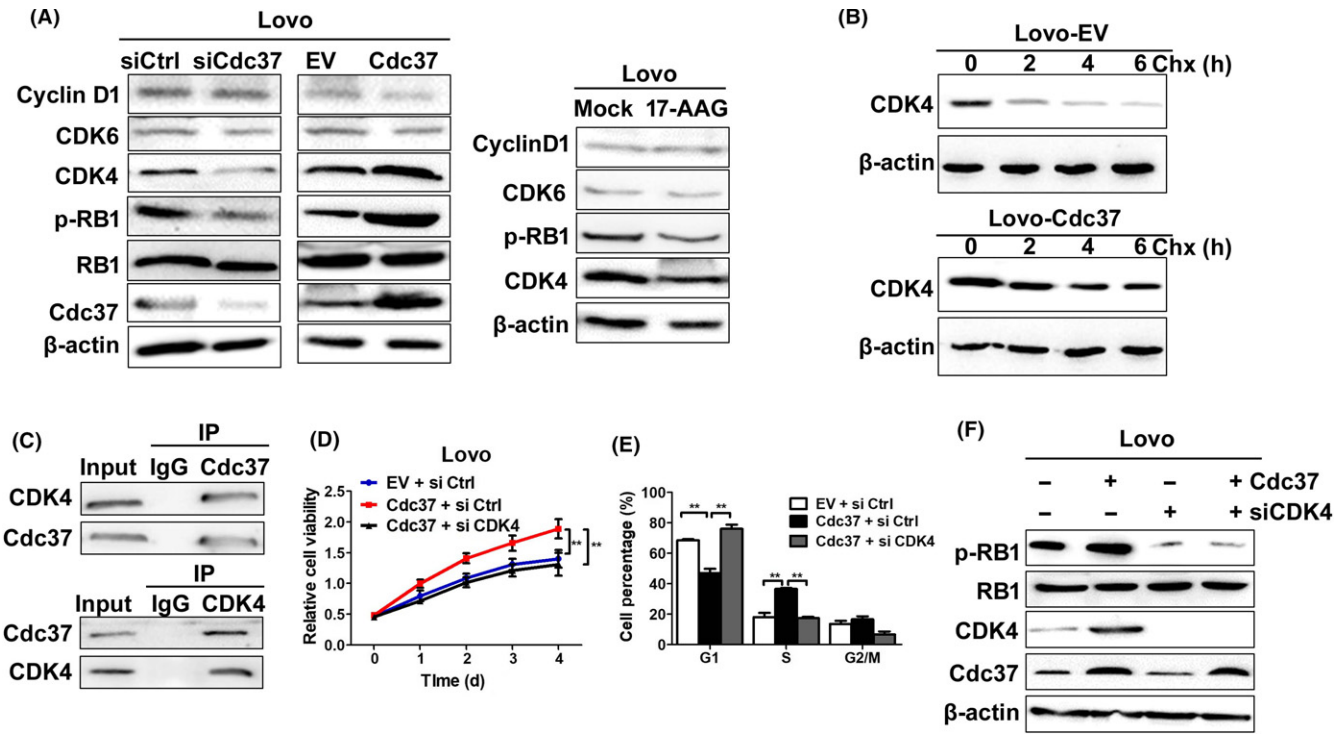


FIGURE 5 Cell division cycle 37 (Cdc37) promotes colorectal cancer cell growth by increasing CDK4 stability. A, Western blot analysis of Cyclin D1, CDK6, CDK4, p-RB1 and RB1 level in Lovo cells 48 h after treatment as indicated. B, Western blot analysis of the lifespan of CDK4 in Lovo cells after treatment as indicated. ChX, cycloheximide (50 μm/mL). C, Co-immunoprecipitation (co-IP) assay was carried out with anti-Cdc37 or anti-CDK4 antibody. Presence of Cdc37 or CDK4 was evaluated by western blot. D, Cell viability and E, cell cycle analysis in Lovo cells treated as indicated. F, Western blot analysis of CDK4 and p-RB1 levels after treatment as indicated. siCDK4, siRNA against CDK4. Data shown are the mean ± SEM from three independent experiments. $^{**}P < .01$, one-way ANOVA. CDK, cyclin-dependent kinase; p-RB1, phosphorylated retinoblastoma 1; RB1, retinoblastoma 1

in vivo, whereas Cdc37 knockdown inhibited the proliferation activity, and sensitized CRC cells to 5-FU-induced apoptosis in vivo (Figure 4C,D). Taken together, these in vivo data indicate the functional role of Cdc37 in promoting CRC growth.

3.5 | Cdc37 promotes CRC cell growth by increasing CDK4 stability

Cdc37 knockdown significantly reduced the protein level of CDK4 and the phosphorylation of RB1, whereas overexpression of Cdc37 significantly increased the protein expression of CDK4 and the phosphorylation of RB1 in Lovo cells (Figure 5A). Furthermore, our results showed that Cdc37 had no effect on the protein expression of Cyclin D1, CDK6, and RB1 in CRC. Similarly, the protein level of CDK4 and the phosphorylation of RB1were significantly decreased after 17-AAG treatment (Figure 5A). Our data further showed that overexpression of Cdc37 increased the lifespan of CDK4 (Figure 5B). In addition, co-immunoprecipitation (co-IP) results showed that Cdc37 protein specifically interacted with CDK4 in colorectal carcinoma cells (Figure 5C).

In order to further confirm whether Cdc37-induced phosphorylation of RB1 was conveyed by CDK4, expression of CDK4 was silenced by siRNA in the present study. Our data showed that CDK4 knockdown restrained the promoting growth effect of Cdc37 in CRC cells (Figure S3, Figure 5D). Moreover, our data showed that CDK4 knockdown inhibited the role of Cdc37 in promoting G1-S transition in CRC (Figure 5E). CDK4 knockdown significantly reduced the effect of Cdc37-induced phosphorylation of RB1 (Figure 5F). Altogether, our results indicate that Cdc37 increases the stability of CDK4 to activate RB1, which ultimately promotes G1-S transition.

3.6 | Cdc37 inhibits cell apoptosis by inducing the expression of Bcl-2 and Bcl-xL

Our data showed that overexpression of Cdc37 significantly increased the expression of Bcl-2 and Bcl-xL, but had no effect on the expression of Mcl-1, XIAP and survivin (Figure 6A,B). Furthermore, CDK4 knockdown dramatically reversed the anti-apoptotic effect of Cdc37 in CRC cells (Figures 6C, Figure S4). Moreover, CDK4 knockdown remarkably inhibited the effect of Cdc37-induced expression of Bcl-2 and Bcl-xL (Figure 6D,E). Altogether, our data strongly indicated that Cdc37 inhibits cell apoptosis by increasing the expression of Bcl-2 and Bcl-xL in CRC.

3.7 | Correlational analysis of Cdc37 and CDK4 in CRC tissues

We then investigated the protein expression of Cdc37 and CDK4 in 120 pairs of tumor and peri-tumor colorectal tissues through IHC

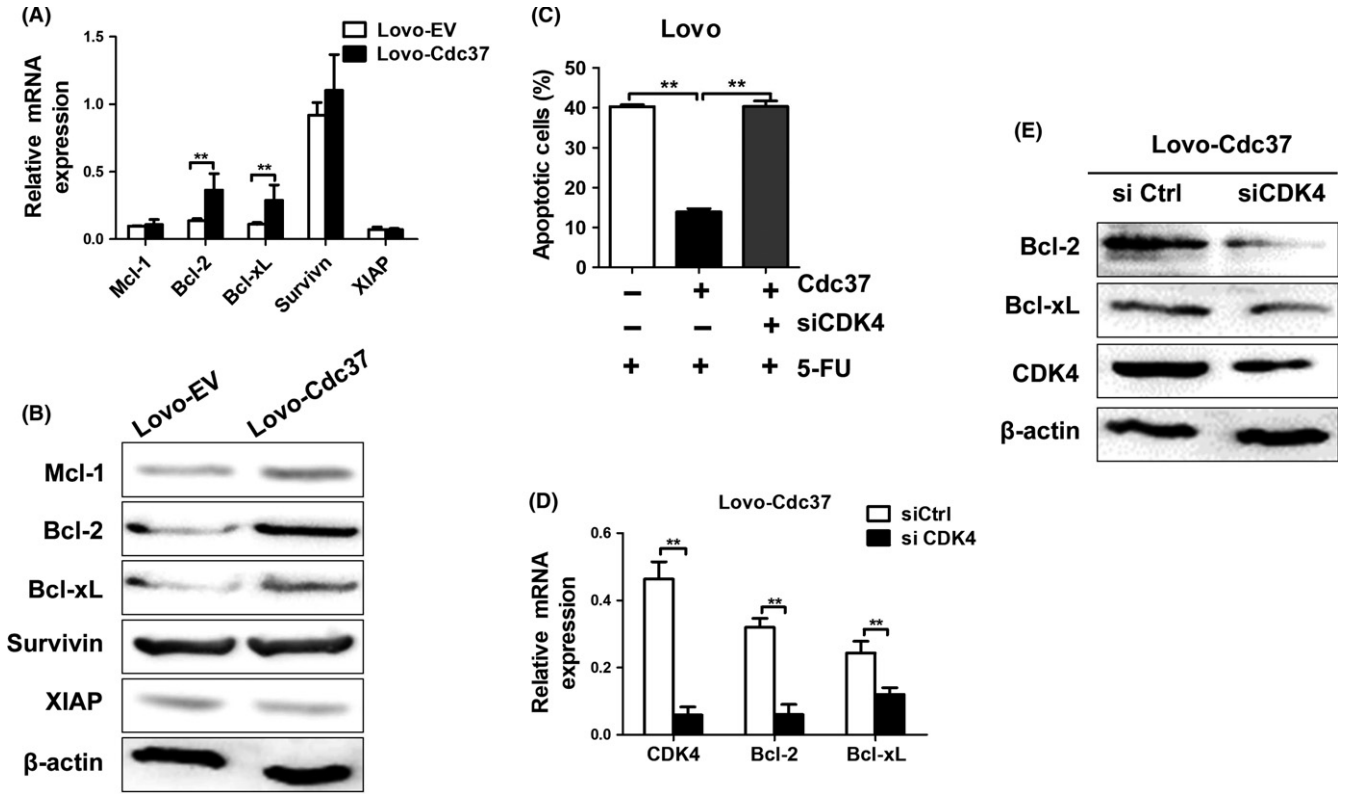


FIGURE 6 Cell division cycle 37 (Cdc37) inhibits cell apoptosis by inducing the expression of Bcl-2 and Bcl-xL. A, qRT-PCR and B, western blot analysis of Mcl-1, Bcl-2, Bcl-xL, XIAP and survivin mRNA and protein expression in Lovo cells as indicated. Lovo-Cdc37, Lovo cells stably expressing Cdc37; Lovo-EV, Lovo cells transfected with empty vector. C, Cell apoptosis analysis by flow cytometry 48 h after treatment as indicated. 5-FU, 5- fluorouracil (50 μm/L). D, qRT-PCR and E, western blot analysis of Bcl-2 and Bcl-xL mRNA and protein expression in Lovo cells as indicated. Data shown were the mean ± SEM from three independent experiments. $^{**}P < .01$, unpaired *t* test

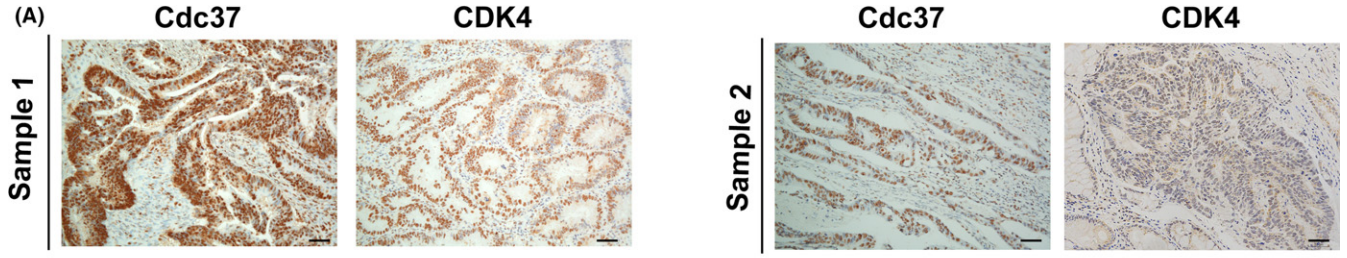
staining. Pearson *r* correlation analysis indicated a strongly positive correlation between Cdc37 expression and CDK4 expression in CRC tissues ($r = .514$, $P < .0001$), which provided further supporting evidence (Figure 7A,B). Collectively, our data showed that Cdc37 promotes proliferation and inhibits apoptosis in CRC cells through activating CDK4.

4 | DISCUSSION

Colorectal carcinoma is a common malignancy, and its morbidity rate ranks second for digestive system tumors.[1] As CRC is not easy to diagnose early, it has mostly reached through the serosa and lymph node metastasis has occurred, making it difficult to treat.[2] So far, many oncogenes (survivin, C-myc, Bcl-2 etc.) and anti-oncogenes (p53, DCC, nm23, APC, PTEN) have been identified to be related to the development and progression of CRC.[2] However, many oncogenes still have not been identified, and the functional role of these genes has also not been clarified. Therefore, it is meaningful to continue to look for new oncogenes associated with the development and progression of CRC, and investigate their functions in the process of tumor development.

Several previous studies have shown that Cdc37 expression is abnormal in various types of malignancy.[7,12] Schwarze et al[12] reported that Cdc37 was almost not expressed in normal human prostate tissues, but the proportion of Cdc37-positive cells was the highest in moderately differentiated prostate cancer, and the expression level of Cdc37 was also higher in most of the prostate intraepithelial neoplasia tissues. Wang et al[7] confirmed significant overexpression of CDC37 transcript and protein in HBV-associated HCC patients. Consistent with previous studies, our present study for the first time showed that both mRNA and protein expression of Cdc37 was significantly up-regulated in CRC. In addition, our additional data showed that the Cdc37 expression level was increased with the development of CRC, which strongly suggested that Cdc37 participates in the development of CRC. Rao et al[13] reported that Cdc37 bound to AR in rabbit reticulocyte lysates, and this binding occurred through the ligand-binding domain of the AR in a way that was partially dependent on Hsp90 and hormone. Expression of a dominant-negative form of Cdc37 in animal cells down-regulates full-length AR. The current study showed that the expression of Cdc37 in female patients was remarkably increased over that in male patients, which suggested that the overexpression of Cdc37 might be related with the AR or other hormones in CRC patients. Together, these results strongly suggest that overexpression of Cdc37 facilitates the development of CRC.

Previous studies have investigated the functional roles of Cdc37 in cancer cell proliferation and cell apoptosis. Wang et al[7] reported



(B)

Correlation analysis between Cdc37 and CDK4 expression in CRC tissues

Cdc37 expression	CDK expression (–)	(+)	(++)	(+++)	Total, N
(-)	15	1	5	1	22
(+)	5	1	2	5	13
(++)	10	9	18	7	44
(+++)	11	4	8	18	41
Total, N	41	15	33	31	120

Pearson r analysis, $r = 0.514$, $P < .0001$

(C)

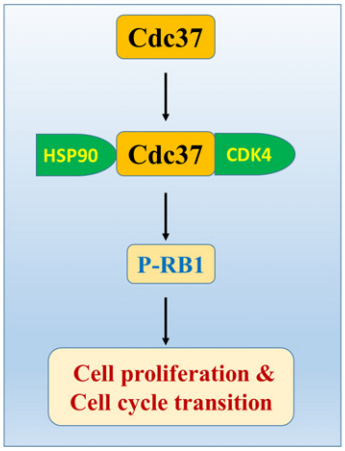


FIGURE 7 Correlational analysis of cell division cycle 37 (Cdc37) and cyclin-dependent kinase (CDK)4 in colorectal cancer tissues. A, Representative immunohistochemical (IHC) staining images of Cdc37 and CDK4 in colorectal carcinoma (CRC) tissues. Scale bar, 50 μm. B, Correlation between the protein expression levels of Cdc37 and CDK4 was evaluated in 120 colorectal carcinoma tissues based on IHC staining. C, Schematic depicting the regulation of cell survival by Cdc37 in colorectal carcinoma. HSP90, heat shock protein 90; p-RB1, phosphorylated retinoblastoma 1

that cell proliferation was inhibited, cell cycle was arrested at the G1 stage, and that cell apoptosis was enhanced after knockdown of Cdc37 in HCC cells. Wu et al[14] showed that Cdc37 increased prostate cancer cell proliferation selectively in Vav3-expressing cells, and that diminished Vav3-Cdc37 interaction would cause decreased prostate cancer cell proliferation. Results from Huang et al[15] showed that FW-04-806 inhibited proliferation and induced apoptosis in human breast cancer cells by disrupting Hsp90-Cdc37 complex formation. Smith et al[11] showed that Cdc37 silencing inhibited cell proliferation, and combining Cdc37 knockdown with the HSP90 inhibitor 17-AAG potentiated cell cycle arrest and apoptosis. Our findings were very consistent with previous reports, showing that the proliferation activity of CRC cells was reduced after knockdown of Cdc37 or 17-AAG treatment, whereas proliferation activity of CRC cells was enhanced after overexpression of Cdc37. In addition, Cdc37 knockdown or 17-AAG treatment promoted apoptosis in CRC cells, whereas overexpression of Cdc37 protected CRC cells from 5-FU-induced apoptosis. However, Smith's data showed that Cdc37 silencing resulted in promoting the kinase clients to enter into the proteasome-mediated degradation pathway independent from HSP90 binding.[16] This inconsistency could be as a result of differences between cell types. Altogether, our results indicate that Cdc37 facilitates the development of CRC by promoting cell proliferation and inhibiting cell apoptosis.

In budding yeast, Cdc37 is necessary for cells to complete division.[17] Protein kinase Cdc28 and Cak1 became unstable after Cdc37 mutation, and they could not be combined with cell cycle protein to form the cell cycle complex, which ultimately inhibited cell growth and division.[18] In addition, Cdc37 has been shown to be required for the activity and stability of protein kinases that regulate different stages of cell cycle progression.[19] Lange et al[20] showed that the loss of function of Cdc37 in *Drosophila* triggered defects in mitosis, and provided evidence that Aurora B interacted with and required the Cdc37/Hsp90 complex for its stability, which substantiated the functional role of Cdc37 as an upstream regulatory element of cell cycle kinases. In the present study, our results were consistent with previous findings that Cdc37 promoted G1-S transition in other cell types,[7] and our results also showed that Cdc37 increased the stability of CDK4. CRC cells were arrested in G1 phase and protein expression of CDK4 was decreased after knockdown of Cdc37 or 17-AAG treatment, whereas overexpression of Cdc37 facilitated CRC cells to enter into S stage and increased the protein expression level of CDK4. However, Cdc37 almost had no effect on the expression of Cyclin D1. Taken together, our results suggest that Cdc37 increases the stability of CDK4, resulting in promoting the CRC G1-S transition in CRC.

The activated kinase complex formed by Cyclin D and CDK4 or CDK6 could phosphorylate RB to p-RB protein, release transcription factor E2F, and then activate the expression of related genes, and ultimately promote the cell G1-S phase transition.[21,22] Herrera-Abreu et al[23] reported that phosphorylation of RB was abolished and that activation of S-phase transcriptional programs was inhibited after

reducing the levels of cyclin D1 and other G1-S cyclins. In the present study, our results showed that Cdc37 knockdown inhibited the phosphorylation of RB1, and vice versa, which strongly suggested that RB1 was the downstream regulated molecule of Cdc37 in CRC. Moreover, phosphorylation of RB1 was abolished after CDK4 knockdown in CRC. Taken together, our results indicate that Cdc37 facilitates the phosphorylating of RB1 through increasing CDK4 stability, which is consistent with the previous reports.[24,25]

In conclusion, our study showed that the Cdc37 gene was up-regulated in human colorectal adenocarcinoma. Furthermore, knockdown of Cdc37 effectively reduced cell proliferation activity, enhanced apoptosis, and inhibited G1-S transition in CRC cells, and vice versa. For the mechanism, Cdc37 increased CDK4 stability to promote the phosphorylation of RB1, which finally promoted the progression of CRC. In conclusion, up-regulated cdc37 promoted the development of colorectal cancer, and regulation of the Cdc37/CDK4/pRB1 axis might be a potential novel target in CRC treatment.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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