Article

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CKAP2L Plays a Pivotal Role in Colorectal Cancer Progression via the Dual Regulation of Cell Cycle and Epithelial-Mesenchymal Transition

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Background: Cytoskeleton-associated protein 2 like (*CKAP2L*) has been demonstrated to mediate the cell cycle in cancer cells. However, it is unknown whether CKAP2L impacts colorectal cancer (CRC). The purpose of this study was to investigate the role of *CKAP2L* in CRC.
Methods: *CKAP2L* and regulatory factor X 5 (*RFX5*) expression profiles in colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) were analyzed in UALCAN. Human colorectal adenocarcinoma epithelial cells, DLD1, were transfected with small interfering RNA targeting *RFX5* and *CKAP2L*-overexpressing vectors (OE-CKAP2L). The interaction between *CKAP2L* and *RFX5* was identified by dual-luciferase assay and chromatin immunoprecipitation. Epithelial-mesenchymal transition (EMT)- and protein kinase B/mammalian target of the rapamycin (AKT/mTOR) pathway-associated proteins were evaluated by western blotting.
Results: *RFX5* and *CKAP2L* expression was increased in CRC based on the UALCAN database. *RFX5* downregulation inhibited proliferation, migration, invasion, and EMT while promoting G1/S phase arrest ($p < 0.01$). *RFX5* knockdown downregulated *CKAP2L* expression and mediated the inactivation of the AKT/mTOR pathway ($p < 0.001$). *RFX5* acted as an upstream transcription factor of *CKAP2L*. *CKAP2L* overexpression attenuated the restriction of *RFX5* downregulation on CRC cell malignant phenotypes ($p < 0.01$).
Conclusion: *CKAP2L* transcriptionally activated by *RFX5* accelerates CRC proliferation and metastasis by promoting the cell cycle and EMT. This study provides potential molecular targets for treating CRC.

Keywords: cytoskeleton-associated protein 2 like; regulatory factor X 5; colorectal cancer; protein kinase B/mammalian target of the rapamycin

Introduction

Colorectal cancer (CRC) accounts for approximately 9.7% of all cases and 9.2% of all deaths associated with all malignant tumors worldwide and ranks second in mortality among malignant tumors [1]. Approximately 50% of CRC patients develop metastases during the disease, which incurs a significant challenge in the control of CRC [2,3]. Thus, finding biomarkers for diagnosis and prognosis can promote the prevention and treatment of the disease.

Cytoskeleton-associated protein 2 like (*CKAP2L*) encodes a polypeptide located in centrosomes and mitotic spindles [4,5], where it impacts cell division and cell cycle progression in neural progenitor cells [6]. *CKAP2L* overexpression has been discovered in multiple cancers, resulting in cell cycle activation to accelerate tumor progression [7]. For instance, *CKAP2L* upregulation in progression and drug resistance of esophageal squamous cell carcinoma, and the blockage of *CKAP2L* depletion on G2/M transition are documented [8]. Additionally, *CKAP2L* upregulation facilitates the invasion of lung adenocarcinoma and is associated with poor prognosis [9]. However, the role of *CKAP2L* in CRC has not been clarified.

Regulatory factor X 5 (*RFX5*) from the RFX family encodes several transcription factors [10]. *RFX5* transcriptionally regulates histocompatibility class II (MHCII) in a complex with regulatory factor X-associated ankyrin-containing protein (*RFXANK*) and regulatory factor X-associated protein (*RFXAP*) [11]. *RFX5* mutations disrupt the normal function of the immune system, leading to immunological diseases [12]. Of note, *RFX5* participates in cancer progression via the transcriptional activation of cancer-associated target genes [13,14]. As reported, *RFX5* may be a common mutation target of DNA mismatch re-

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pair deficiency in colorectal tumorigenesis [15]. There is also evidence showing that *RFX5* is associated with the cell cycle by accelerating the transition of G0/G1 to the S phase, thus driving the progression of hepatocellular carcinoma [13].

In this study, we predict that *RFX5* is a transcriptional regulator of *CKAP2L* and report the positive correlation between *RFX5* and *CKAP2L* in CRC. Therefore, the transcriptional activation of *CKAP2L* by *RFX5* is considered to promote CRC progression.

Materials and Methods

Bioinformatics Analysis

RFX5 and *CKAP2L* expression patterns in colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) were analyzed in UALCAN (https://ualcan.path.uab.edu/). On the UALCAN website, The Cancer Genome Atlas (TCGA) was selected, and the gene name (*RFX5* or *CKAP2L*) was entered, followed by "colon adenocarcinoma" in the TCGA dataset section, and "Explore". Next, "Expression" was selected to obtain the *RFX5* and *CKAP2L* expression profiles in COAD. Similar procedures were performed to obtain *CKAP2L* expression in READ, except "rectal adenocarcinoma" was entered in TCGA.

The *RFX5* and *CKAP2L* correlations were conducted in GEPIA (http://gepia.cancer-pku.cn/). On the GEPIA website, Multiple Gene Analyses and "Correlation Analysis" were selected consecutively. *RFX5* and *CKAP2L* were entered in the box below Gene A and Gene B, and COAD Tumor was selected from "TCGA Tumor (Cancer name)" to add to the "Used Expression Datasets" on the right. Finally, "Plot" was selected to obtain the correlations.

The *CKAP2L* promoter sequence was acquired from National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). The upstream transcription factors of *CKAP2L* were predicted in GRNdb (http://www.grndb.com/), and the specific binding sites were obtained from JASPAR (http://jaspar.genereg.net/).

Cell Culture

Incubation of human colorectal adenocarcinoma epithelial cells DLD1 (AW-CELLS-H0078, AnWei-Sci, Shanghai, China) was conducted in 1640 RPMI medium (SNM-001B, Sunncell, Wuhan, China) containing 10% fetal bovine serum (FBS, AW-FBS-001, AnWei-Sci) and 1% penicillin–streptomycin (V900929, Sigma-Aldrich, St. Louis, MO, USA) in 96-well plates in an incubator (51032874, ThermoFisher, Waltham, MA, USA) (37 °C, 5% CO_2). DLD1 cells underwent authentication by short tandem repeat (STR) profiling and mycoplasma testing and were free from mycoplasma contamination.

Cell Transfection and Dual-Luciferase Assay

CKAP2L-overexpressing vectors (OE-CKAP2L) and negative control (OE-NC, pcDNA3.1(+) vector, VT1001) were purchased from YouBio (Changsha, China). The small interfering RNA against *RFX5* (si-RFX5, siG000005993A-1-5, 5′-CCTCGGGAACGGTCATCTAAA-3′) and si-NC (siN0000002-1-5, 5′-TTCTCCGAACGTGTCACGT-3′) were procured from RiboBio (Guangzhou, China). Liposome complexes of the above-mentioned vectors were prepared using Lipofectamine 3000 Reagent (L3000001, ThermoFisher, Waltham, MA, USA) and transfected (48 h, 37 °C) into DLD1 cells (1×10^4 cells/well) at 70–90% confluence.

Based on the predicted binding site of *RFX5*, wild-type (WT) and mutant (Mut) *CKAP2L* promoters were generated and cloned into the fluorescence vector psiCHECK-2 (C8021, Promega, Madison, WI, USA). After culturing DLD1 cells in 96-well plates overnight, WT-luc/Mut-luc and empty vector/pcDNA3.1-RFX5 (pcDNA-RFX5) were transfected into cells with Lipofectamine 3000 reagent, followed by luciferase activity detection (Luc-Screen Extended Glow luciferase reporter gene detection system, T1033, ThermoFisher, Waltham, MA, USA) and analysis (a chemiluminescence analyzer, 1410130, ThermoFisher, Waltham, MA, USA).

Chromatin Immunoprecipitation (ChIP) and Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

With the SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (#9005, Cell Signaling Technology, Beverly, MA, USA), cells in 15-cm culture dishes (4×10^7 cells/dish) were fixed (1% formaldehyde, #47746, Cell Signaling Technology, USA) (room temperature, 10 min) and digested. The digested cross-linked chromatin was incubated with the immunoprecipitation antibody against RFX5 (PA1-32133, ThermoFisher, Waltham, MA, USA) or Immunoglobulin G (IgG) (4 °C, overnight). The immunocomplexes were pulled down, and the DNA was purified using DNA Purification Columns and Collection Tubes for subsequent qRT-PCR.

As for qRT-PCR, DNA and primers were incubated with Fast SYBR Green Master Mix (4385612, ThermoFisher, Waltham, MA, USA) in a QuantStudio 7 Pro real-time PCR instrument (A43165, ThermoFisher, Waltham, MA, USA) at conditions of 95 °C (20 sec), followed by 40 cycles of 95 °C (3 sec) and 60 °C (30 sec). All data were processed using the $2^{-\Delta\Delta CT}$ method, with β-actin as the internal control. The primers were: *CKAP2L*, sense: 5′-GAGCCAAAACACCAAGCCTTA-3′; antisense: 5′-GGAGTTTAATGCTGATGGACCTT-3′; β-actin, sense: 5′-CTGGGACGACATGGAGAAAA-3′, antisense: 5′-AAGGAAGGCTGGAAGAGTGC-3′.

Cell Counting Kit-8 (CCK-8) Assay

DLD1 cells in 96-well plates (5×10^3 cells/well) were assayed for viability determination every day for 4 days by incubating (4 h, 37 °C, darkness) with 10 µL/well WST-8 solution in CCK-8 kit (ab228554, Abcam, Cambridge, UK). Finally, a multiwell plate cell reader (CytoSMART Omni, Axion BioSystems, Shanghai, China) was employed to measure the absorbance at 460 nm.

Colony Formation Assay

DLD1 cells (1×10^3/well) were cultured in 6-well plates for two weeks. Next, 4% paraformaldehyde (PN4204, G-CLONE, Beijing, China) and Crystal Violet Ammonium Oxalate Solution (G1063, Solarbio, Beijing, China) were used to fix and stain colonies (room temperature). After washing with phosphate-buffered saline (PBS, abs962, Absin, Shanghai, China), the colony number was calculated using a BX53F microscope (OLYMPUS, Tokyo, Japan).

Transwell Assay

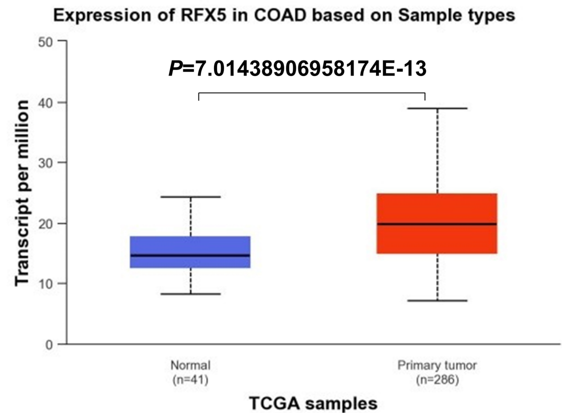
To assess DLD1 cell migration and invasion, DLD1 cells (1×10^4/well) were seeded in the upper chamber (8-µm pore filters, 140629, ThermoFisher, USA) with (for cell invasion)/without (for cell migration) Matrigel (E1270, Sigma Aldrich) for 48 h. After fixing with 4% paraformaldehyde (30 min, room temperature) and dyeing with Crystal Violet Ammonium Oxalate Solution, the migration and invasion rates were calculated as follows: number of stained cells/total cell number × 100% by observing under BX53F microscope.

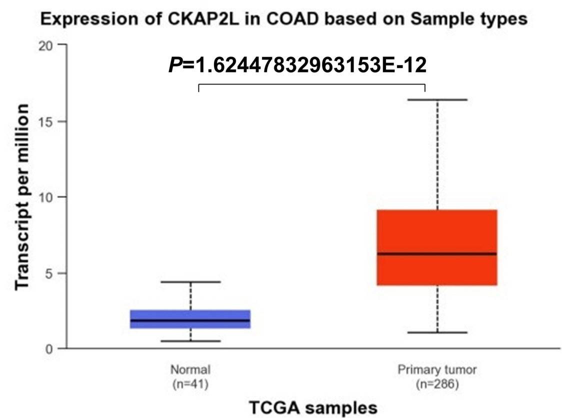
Flow Cytometry

The APC BrDU Kit (552598, BD Pharmingen, San Jose, CA, USA) was used to analyze the cell cycle. In brief, DLD1 cells were successively probed with 10 µM BrdU (1 h, 37 °C), Cytofix/Cytoperm buffer, DNase (1 h, 37 °C), and APC-conjugated anti-BrdU (20 min, room temperature). Before 7-AAD staining, cells were rinsed with washing buffer, followed by observation (Attune NxT flow cytometer, A24864, ThermoFisher, USA) and data analysis (Cell Quest software, BD Biosciences, San Diego, CA, USA).

Western Blot

Total proteins from DLD1 cells were extracted using RIPA Buffer (R0010, Solarbio, China) supplemented with protease and phosphatase inhibitors (P1261, Solarbio), followed by boiling for 5 min and protein concentration determination (BCA Protein Assay Kit, PC0020, Solarbio). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in NuPAGE 4–12%, Bis-Tris (NP0321BOX, ThermoFisher), followed by transfer onto polyvinylidene fluoride membranes (YA1701,





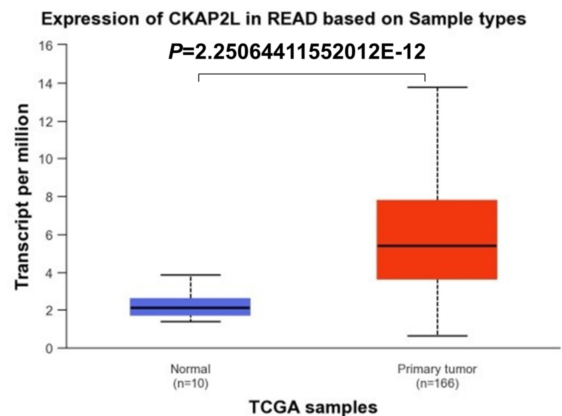


Fig. 1. *RFX5* and *CKAP2L* differential expression levels in colorectal cancer (CRC). (A) *RFX5* expression in normal tissues (n = 41) and primary COAD tissues (n = 286) from TCGA. (B) *CKAP2L* expression in normal tissues (n = 41) and primary COAD tissues (n = 286) from TCGA. (C) *CKAP2L* expression in normal tissues (n = 10) and primary READ tissues (n = 166) from TCGA. *RFX5*, regulatory factor X 5; *CKAP2L*, cytoskeleton-associated protein 2 like; COAD, colon adenocarcinoma; READ, rectal adenocarcinoma; TCGA, The Cancer Genome Atlas.

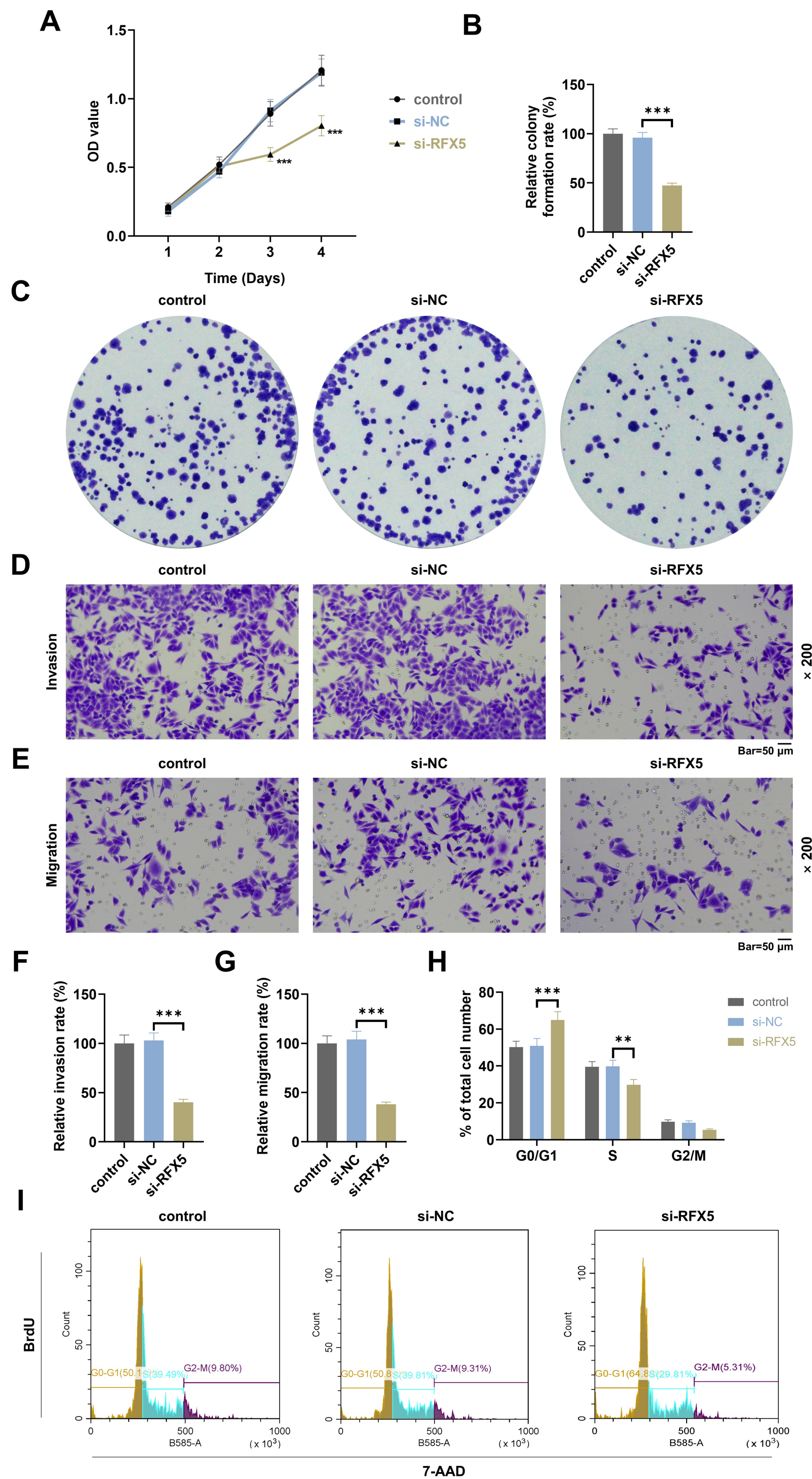


Fig. 2. Effects of *RFX5* downregulation on cell proliferation, invasion, migration, and cell cycle. (A) Cell viability from day 1 to 4 after transfection (Cell Counting Kit-8 (CCK-8) assay). (B,C) Cell proliferation (colony formation assay). (D–G) Cell invasion (D,F) and migration (E,G) (Transwell assay). Magnification: 200×; scale bar: 50 µm. (H,I) Cell cycle (flow cytometry). Each experiment was repeated at least thrice. Control: DLD1 cells without transfection; si-NC: DLD1 cells transfected with negative control small interfering RNA (siRNA); si-RFX5: DLD1 cells transfected with siRNA targeting RFX5. **$p < 0.01$; ***$p < 0.001$.

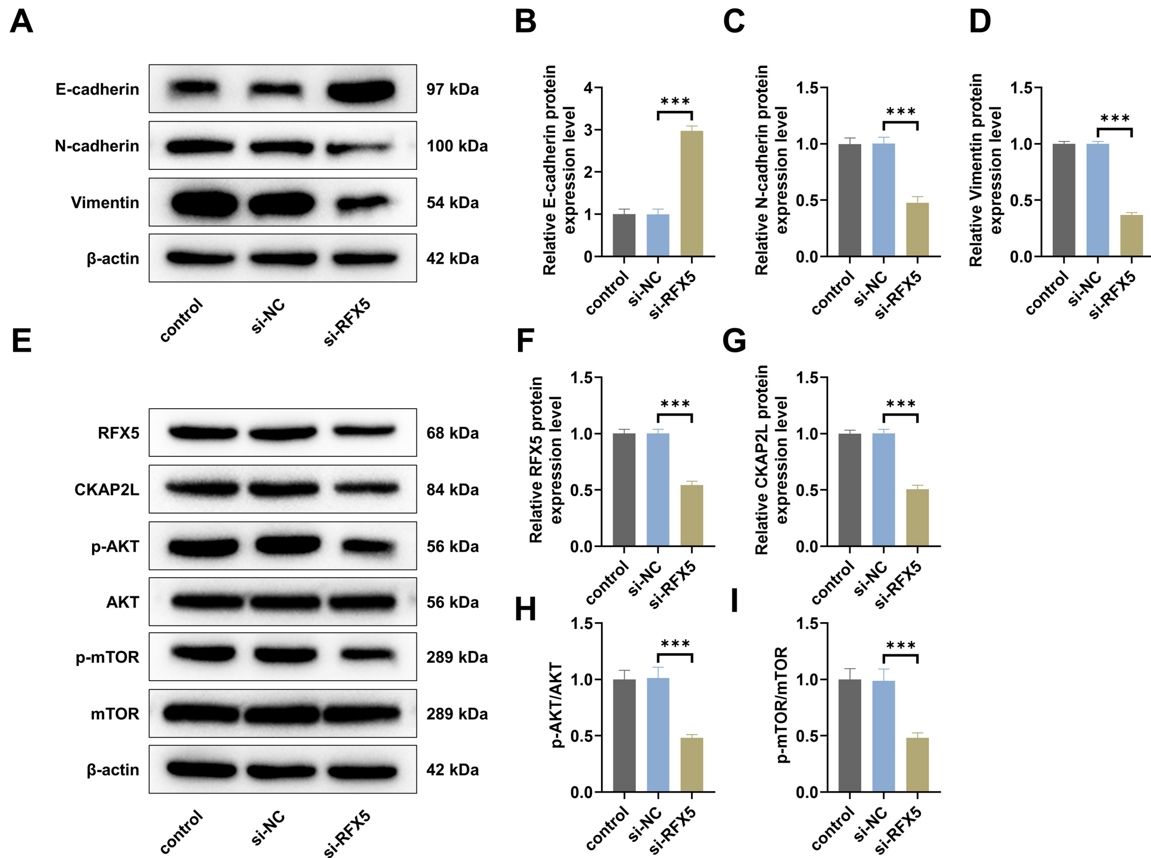


Fig. 3. Effects of RFX5 downregulation on EMT and AKT/mTOR pathway. (A–I) EMT-related protein expression (A–D) and AKT/mTOR pathway-related protein expression (E–I) (western blot, β-actin as the loading control). Each experiment was repeated at least thrice. Control: DLD1 cells without transfection; si-NC: DLD1 cells transfected with si-NC; si-RFX5: DLD1 cells transfected with si-RFX5. EMT, epithelial-mesenchymal transition; AKT, protein kinase B; mTOR, mammalian target of the rapamycin. ***$p < 0.001$.

Solarbio) and blocking (1 h, room temperature) with Blocking Buffer (37581, ThermoFisher, USA). Next, membranes were incubated with primary (4 °C, overnight) and secondary (1 h, room temperature) antibodies (Abcam, Cambridge, UK), including E-cadherin (ab231303, 97 kDa, 1:2000), N-cadherin (ab76011, 100 kDa, 1:5000), vimentin (ab20346, 54 kDa, 1:1000), CKAP2L (ab221897, 84 kDa, 1:1000), RFX5 (ab9255, 68 kDa, 1:1000), p-protein kinase B (AKT) (ab38449, 56 kDa, 1:1000), AKT (ab8805, 56 kDa, 1:500), p-mammalian target of the rapamycin (mTOR) (ab109268, 289 kDa, 1:1000), mTOR (ab134903, 289 kDa, 1:10000), β-actin (ab8226, 42 kDa, 1:1000), anti-rabbit IgG (ab175781, 1:10000), and anti-mouse IgG (ab205719, 1:2000).

Post-treatment with BeyoECL Plus (P0018S, Beyotime, Shanghai, China), analyses on band signals and data were carried out with iBright CL750 (ThermoFisher, Waltham, MA, USA) and Image J software (1.52s version, National Institutes of Health, Bethesda, MD, USA), respectively, with β-actin as the internal reference.

Statistical Analyses

All data from assays repeated thrice were depicted by mean ± standard deviation, after processing with GraphPad Prism 8 (GraphPad, Inc., La Jolla, CA, USA). Data dissection between groups and among groups was performed by independent samples t-test, and one-way analysis of variance (ANOVA) or two-way ANOVA. The statistical significance was defined at $p < 0.05$.

Results

RFX5 and CKAP2L were Increased in CRC

Transcriptome data from 41 normal and 286 COAD tumor samples in the UALCAN database were analyzed, revealing that *RFX5* and *CKAP2L* expression levels were higher in tumor samples than in normal tissues (Fig. 1A,B, $p < 0.001$). Additionally, transcriptome data of READ, including 10 normal tissues and 166 tumor samples, were anatomized in the UALCAN database, and increased *CKAP2L* expression was observed in READ tumor tissues relative to normal tissues (Fig. 1C, $p < 0.001$).

RFX5 Downregulation Inhibited CRC Cell Proliferation, Migration, and Invasion

RFX5 downregulation was detected in DLD1 cells via RNA interference. Through functional assays, we found that *RFX5* downregulation inhibited cell proliferation (Fig. 2A–C, $p < 0.001$) and reduced migration and invasion rates in DLD1 cells (Fig. 2D–G, $p < 0.001$).

RFX5 Downregulation Promoted Cell Cycle Arrest and Inhibited Epithelial-Mesenchymal Transition (EMT)

To distinguish different cells in the cell cycle, flow cytometry was used to display BrDU- and 7-AAD-positive cells. *RFX5* deficiency increased the proportion of DLD1 cells at the G0/G1 phase (Fig. 2H,I, $p < 0.001$) and decreased those at the S phase (Fig. 2H,I, $p < 0.01$), suggesting an inhibitory role for *RFX5* deficiency in the transition from G1 to S.

EMT allows tumor cells to become metastatic [16]. The level of E-cadherin, as an epithelial cell marker, was increased in *RFX5*-silenced DLD1 cells (Fig. 3A,B, $p < 0.001$), while the levels of interstitial cell markers vimentin and N-cadherin were decreased (Fig. 3A,C,D, $p < 0.001$). These findings suggest that the downregulation of *RFX5* inhibits the EMT.

RFX5 Downregulation Inhibited *CKAP2L* and Mediated the Inactivation of Protein Kinase B/Mammalian Target of the Rapamycin (AKT/mTOR) Pathway

Western blot showed that depletion of *RFX5* inhibited *RFX5* and *CKAP2L* expression (Fig. 3E–G, $p < 0.001$). The p-AKT/AKT and p-mTOR/mTOR levels were significantly declined after *RFX5* downregulation (Fig. 3E,H,I, $p < 0.001$), suggesting AKT/mTOR pathway inactivation.

RFX5 Regulated AKT/mTOR Pathway by Transcriptional Activation of *CKAP2L*

A strong correlation between *RFX5* and *CKAP2L* in CRC was identified in GEPIA (Fig. 4A, $p = 1.2 \times 10^{-9}$, $R = 0.36$). Furthermore, the prediction that *RFX5* is a transcription factor of *CKAP2L* was confirmed in GRNdb, and specific binding sites were obtained in JASPAR (Fig. 4B). To clarify the accuracy of the bioinformatics analysis, ChIP-qRT-PCR and dual-luciferase assay were performed. It was observed that the *CKAP2L* promoter was markedly enriched in immunocomplexes of the *RFX5* antibody (Fig. 5A,B, $p < 0.05$). Additionally, relative luciferase activity was higher in the WT-luc+pcDNA-RFX5 group than in the WT-luc+vector and Mut-luc+pcDNA-RFX5 groups (Fig. 5C, $p < 0.001$). As shown in Fig. 5D,E, the protein expression of *CKAP2L* in OE-CKAP2L group was higher than that in OE-NC group, it indicated that *CKAP2L* overexpression was successfully transfected. Relative to the si-RFX5+OE-NC group, *CKAP2L*, p-AKT/AKT, and p-mTOR/mTOR levels were upregulated in the si-RFX5+OE-CKAP2L group (Fig. 5F–I, $p < 0.001$). These findings demonstrate *RFX5* as the transcription activation factor of the *CKAP2L*-activated AKT/mTOR pathway.

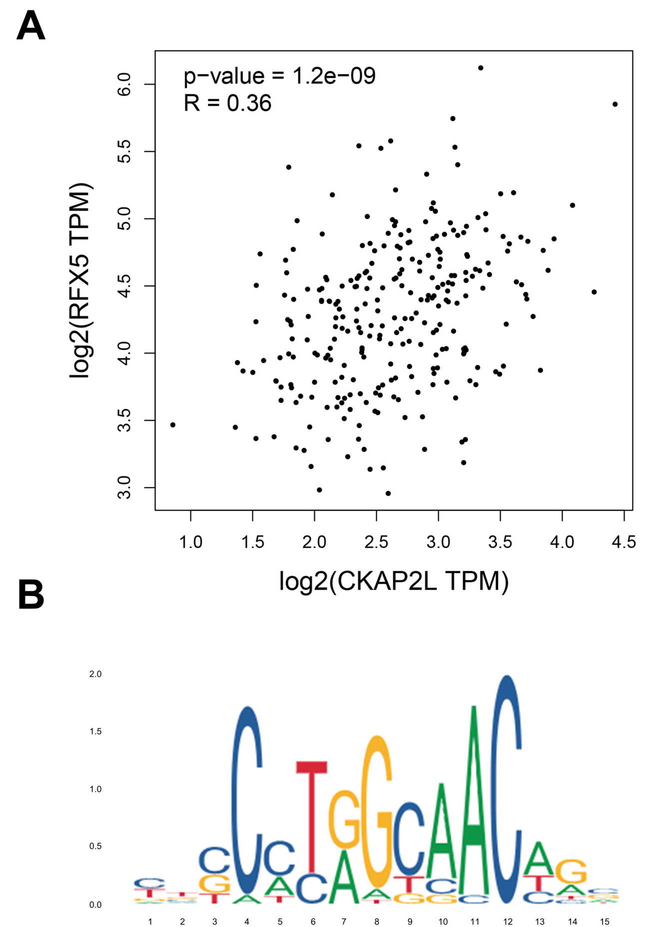


Fig. 4. Correlation and binding site analysis between *RFX5* and *CKAP2L*. (A) Correlation analysis between *RFX5* and *CKAP2L* in CRC, as determined by GEPIA. (B) The binding site of *RFX5* on the *CKAP2L* promoter, as determined by JASPAR.

CKAP2L Overexpression Attenuated the Restrictions of *RFX5* Downregulation on CRC Cell Malignant Phenotypes

We further explored the role of the *RFX5*/*CKAP2L* axis in CRC cell malignant phenotypes. *CKAP2L* overexpression promoted proliferation (Fig. 6A–C, $p < 0.01$), invasion (Fig. 6D,F, $p < 0.001$), and migration (Fig. 6E,G, $p < 0.001$) of *RFX5*-silenced DLD1 cells. In the si-RFX5+OE-CKAP2L group, the decrease in the proportion of DLD1 cells at the G0/G1 phase and the increase in the proportion of cells at the S phase (Fig. 6H,I, $p < 0.01$) indicates that *CKAP2L* overexpression promoted G1/S tran-

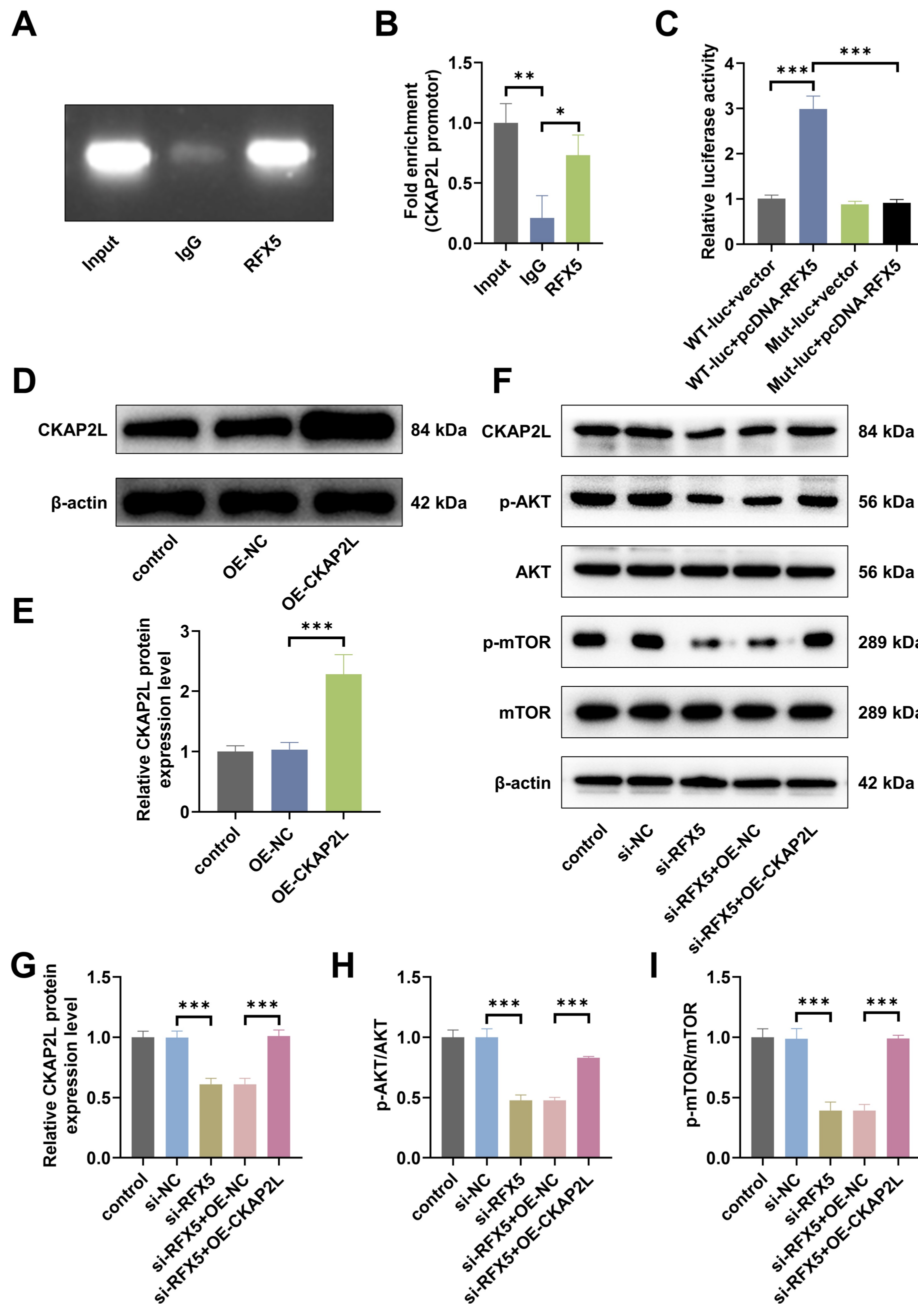


Fig. 5. Binding of *RFX5* with CKAP2L and effect of the *RFX5/CKAP2L* axis on the AKT/mTOR pathway. (A–C) ChIP-qRT-PCR (A,B) and dual-luciferase assay (C) were performed to identify the binding of *RFX5* on the *CKAP2L* promoter (β-actin as the loading control). (D,E) The protein expression of CKAP2L was detected after DLD1 cells transfected with CKAP2L-overexpressing vector. (F–I) *CKAP2L* and AKT/mTOR pathway-related protein expression (western blot, β-actin as the loading control). Each experiment was repeated at least thrice. Control: DLD1 cells without transfection; si-NC: DLD1 cells transfected with si-NC; si-RFX5: DLD1 cells transfected with si-RFX5. si-RFX5+OE-NC: DLD1 cells transfected with si-RFX5 and negative control overexpressed vector; si-RFX5+OE-CKAP2L: DLD1 cells transfected with si-RFX5 and CKAP2L-overexpressing vector. OE-NC, negative control pcDNA3.1(+) vector; OE-CKAP2L, *CKAP2L*-overexpressing vectors; ChIP-qRT-PCR, chromatin immunoprecipitation-quantitative reverse transcriptase polymerase chain reaction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

sition. In addition, *CKAP2L* overexpression enhanced the expression of vimentin and N-cadherin and reduced E-cadherin expression (Fig. 7A–D, $p < 0.001$).

Discussion

Improving the survival rates of CRC patients remains a great challenge, and elucidating the mechanism of CRC

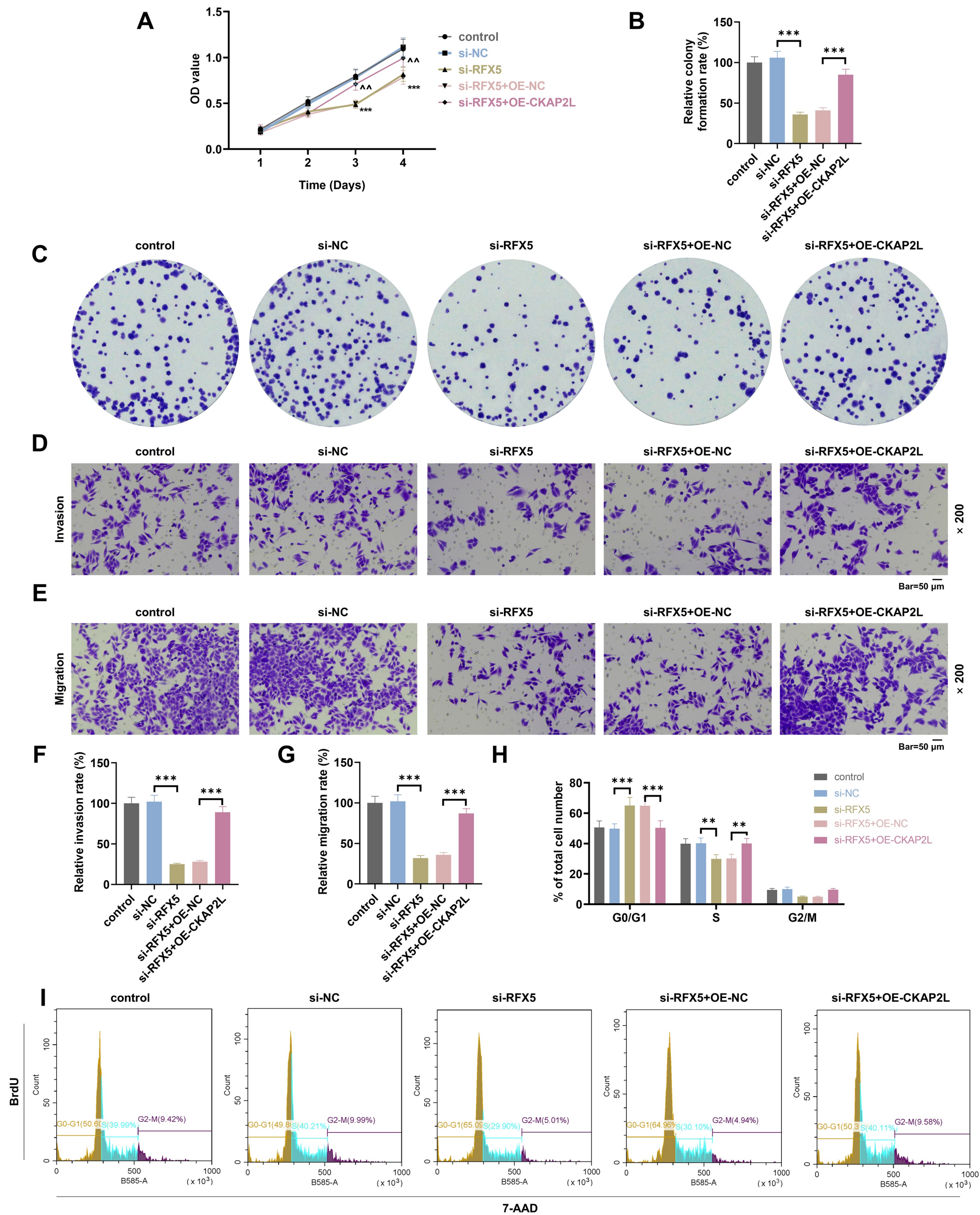


Fig. 6. Effects of *RFX5*/*CKAP2L* axis on cell proliferation, invasion, migration, and cell cycle. (A) Cell viability at day 1 to 4 after transfection (CCK-8 assay). (B,C) Cell proliferation (colony formation assay). (D–G) Cell invasion (D,F) and migration (E,G) (Transwell assay). Magnification: 200×; scale bar: 50 µm. (H,I) Cell cycle (flow cytometry). Each experiment was repeated thrice or above. Control: DLD1 cells without transfection; si-NC: DLD1 cells transfected with si-NC; si-RFX5: DLD1 cells transfected with si-RFX5. si-RFX5+OE-NC: DLD1 cells transfected with si-RFX5 and negative control overexpressed vector; si-RFX5+OE-CKAP2L: DLD1 cells transfected with si-RFX5 and CKAP2L-overexpressing vector. ** $p < 0.01$; *** $p < 0.001$. ^^ $p < 0.01$.

progression can offer new insights. Herein, we found that *RFX5* and *CKAP2L* were overexpressed in CRC, and the *RFX5*/*CKAP2L* axis promoted cell proliferation, migration, invasion, cell cycle, and EMT while activating the AKT/mTOR pathway in CRC cells.

Although transcription factor *RFX5* is required for the transcriptional activation of MHCII in immunoreaction [17], in hepatocellular carcinoma, *RFX5* binds to the promoter of tripeptidyl peptidase 1 (*TPP1*), whose upregulation is related to poor prognosis rather than MHCII promoter activity [18]. Chen *et al.* [13] reported that *RFX5* transcriptionally activates *KDM4A* to drive the progression of hepatocellular carcinoma by regulating the cell cycle. In addition, *RFX5* mutations are responsible for MHCII deficiency in CRC, resulting in the high infiltration of CD4-positive T cells and immune escape [15,19,20]. Herein, we

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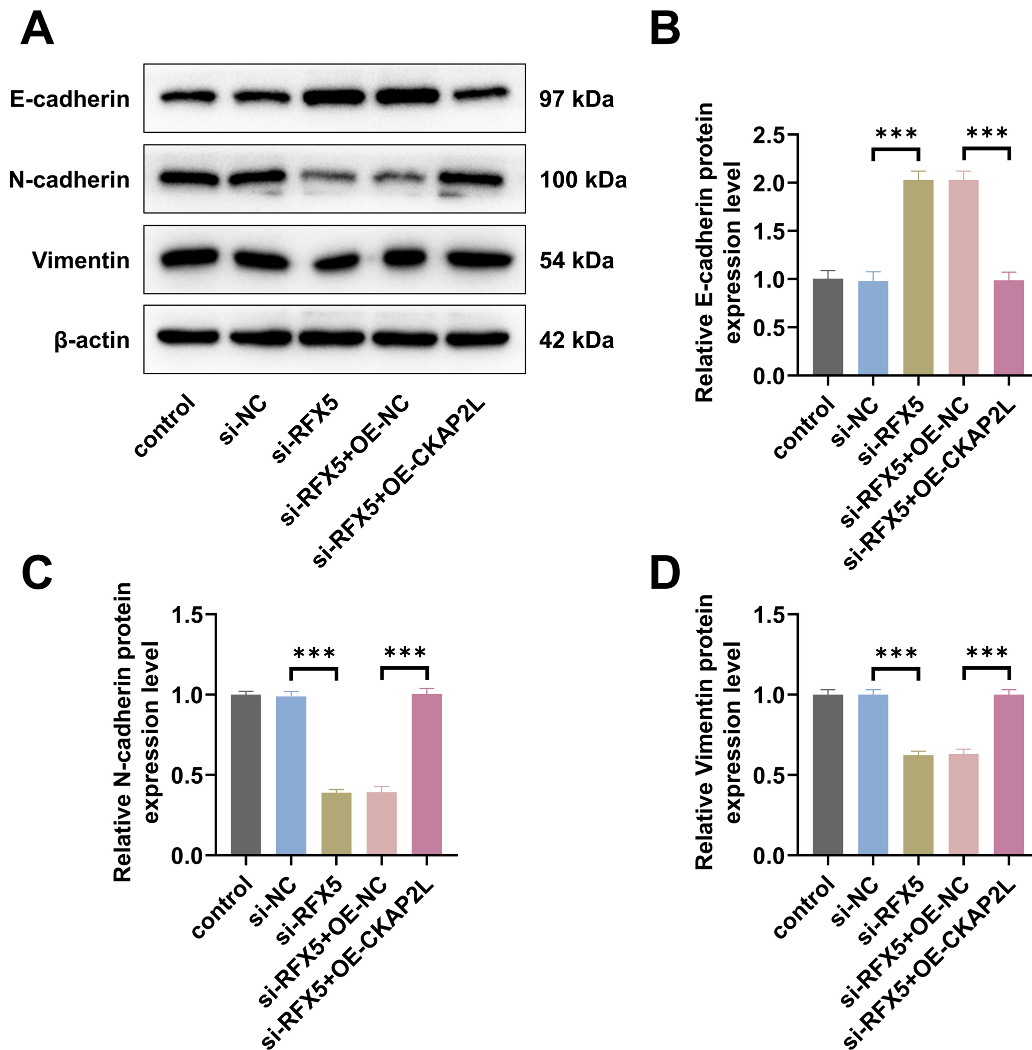

Fig. 7. Effects of the RFX5/CKAP2L axis on EMT. (A–D) EMT-related protein expression (western blot). Each experiment was repeated at least thrice. Control: DLD1 cells without transfection; si-NC: DLD1 cells containing si-NC; si-RFX5: DLD1 cells transfected with si-RFX5. si-RFX5+OE-NC: DLD1 cells transfected with si-RFX5 and negative control overexpressed vector; si-RFX5+OE-CKAP2L: DLD1 cells transfected with si-RFX5 and CKAP2L-overexpressing vector. ***$p < 0.001$.

showed that *RFX5* expression was high in COAD from data in UALCAN and found that *RFX5* silencing inhibited CRC cell proliferation, migration, invasion, and G1/S phase transition *in vitro*, consistent with findings in non-small cell lung cancer and hepatocellular carcinoma [14,21]. EMT is the process by which epithelial cells acquire a mesenchymal phenotype [22]. EMT is instrumental in tumor progression, metastases, and drug resistance, and its targeting is regarded as a potential therapeutic strategy in CRC [22,23]. Here, we have observed that depletion of *RFX5* led to EMT blockage. Therefore, the regulation of cell cycle and EMT may be the possible patterns by which RFX5 promotes CRC progression. Nevertheless, the specific mechanism remains unclear.

CKAP2L, a cell cycle-related protein, is acknowledged as a cancer-promoting factor. A pan-cancer analysis confirmed that *CKAP2L* contributes to cancer cell proliferation and metastases by driving the cell cycle from the G2 phase to the M phase [24]. The promoting influence of *CKAP2L* overexpression on tumor progression has been reported in lung cancer [25], glioma [26], ovarian cancer [27], clear cell renal cell carcinoma [28], and prostate cancer [7]. The *CKAP2L* homologous protein CKAP4 promotes migration, angiogenesis, and tumorigenesis in CRC [29]. A weighted gene co-expression network analysis revealed *CKAP2L* as one of the hub genes in CRC onset [30]. Herein, we confirmed the binding of *RFX5* to the *CKAP2L* promoter, and *CKAP2L* overexpression reversed the effects of *RFX5* downregulation on CRC cell proliferation, migration, invasion, cell cycle, and EMT. These outcomes suggest that *CKAP2L* transcriptionally activated by *RFX5* accelerates CRC progression.

As one of the most commonly dysregulated pathways in cancer, the AKT/mTOR signaling pathway is essen-

tial to the survival and proliferation of cancer cells [31–33]. There is evidence to indicate that AKT/mTOR pathway activation in CRC mediates proliferation, migration, invasion, and chemotherapy resistance [34–36]. Herein, we showed that *RFX5* downregulation decreased values of p-AKT/AKT and p-mTOR/mTOR, which was reversed by *CKAP2L* overexpression, suggesting the activation of AKT/mTOR signaling pathway by *RFX5*/*CKAP2L* axis. Moreover, Zhu *et al*. [37,38] demonstrated that *Arnebia euchroma* and acetylshikonin induce cell cycle at the G0/G1 phase and cell apoptosis by inhibiting AKT/mTOR pathway activation to exert an antitumor role in CRC. The phosphorylation of the AKT/mTOR pathway also mediates EMT in CRC cells, which creates favorable conditions for tumor metastasis [39,40]. Thus, targeting the AKT/mTOR pathway is a potential treatment strategy for CRC [41].

There are several limitations in this study. First, based on the findings that *RFX5* can activate the AKT/mTOR pathway in CRC cells, it is necessary to use agonists or inhibitors of the AKT/mTOR pathway to further explore the cancer-promoting effect of *RFX5* through the activation of the AKT/mTOR pathway. Second, we only conducted cell experiments and animal experiments are needed to confirm our findings.

Conclusion

In conclusion, *CKAP2L* transcriptionally activated by *RFX5* is capable of activating the AKT/mTOR pathway and promoting CRC proliferation and metastasis. This study provides potential molecular targets for treating CRC.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Substantial contributions to conception and design: QBL, BHZ. Data acquisition, data analysis and interpretation: CLW, YRW. Drafting the article and critically revising it for important intellectual content: All authors. Final approval of the version to be published: All authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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