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Circ_0022340 promotes colorectal cancer progression via HNRNPC/EBF1/SYT7 or miR-382-5p/ELK1 axis

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ABSTRACT

Circular RNAs (circRNAs) are characterized as a class of new noncoding RNAs and function in tumorigenesis of colorectal cancer (CRC). In our study, the molecule mechanism of circ_0022340 in CRC was investigated. For this aim, quantitative real-time polymerase chain reaction (RT-qPCR) was used to test gene expression in CRC cells. Cell function assays including 5-ethynyl-20-deoxyuridine (EdU), colony formation and transwell investigated the proliferation and migration capacity in CRC cells. Luciferase reporter and RNA immunoprecipitation (RIP)assays determined the interaction between circRNA, miRNA and mRNA. Western blot was used to test protein expression. An immunohistochemistry assay was used to assess the tumor growth in vivo. Results showed that Circ_0022340 was highly expressed in CRC cells. Circ_0022340 was formed from exon 5 to 6 of the synaptotagmin 7 (SYT7). Silencing of circ_0022340 suppressed CRC cell proliferation and migration. Functionally, circ_0022340 recruited heterogeneous nuclear ribonucleoprotein C (HNRNPC) to stabilize EBF1 mRNA and thereby activated SYT7. Moreover, circ_0022340 targeted miR-382-5p to up-regulate ETS transcription factor ELK1 (ELK1). It is concluded that Circ_0022340 promoted colorectal cancer progression via recruiting HNRNPC to stabilize EBF1 mRNA and thereby activated SYT7 or miR-382-5p/ELK1 axis, which might provide a novel target for CRC treatment.

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Introduction

Colorectal cancer (CRC) ranks as the third most frequent human cancer worldwide and causes serious deaths every year (1). With the development of CRC diagnosis and therapy, the 5-year survival rate has been improved. However, CRCpatients' prognosis is still not optimistic. A high incidence of distant metastasis and recurrence has been identified as the most crucial obstacle in treating this cancer (2-4). Thence, the inner mechanism underlying CRC should be further explored for searching of more effective diagnosis and treatment for CRC patients.

Circular RNAs (circRNAs) are covalently closed RNA with a closed loop and are active in modulating the expression of genes (5, 6). Accumulating evidence has proved the biological function of circRNAs in various human cancers, including CRC (7-9). They functioned as tumor-promoting roles or tumor-suppressing roles in the development of CRC (10, 11). Moreover, many circRNAs can act as prognostic biomarkers and therapeutic targets for CRC (12, 13). However, there are still some circRNAs remaining undiscovered in CRC.

Transcription factors are important regulators in recognizing specific DNA sequences to control chromatin and transcription, which forms a complex system that affects the expression of the genome(14). Increasingly studies have demonstrated that the abnormal expression of circRNAs is closely related to transcription factors (15, 16). Moreover, transcription factors are widely reported to exert functions in the development of cancers (17). However, the mechanism between transcription factors and circRNAs are needed to be further determined in CRC.

Furthermore, RNA binding proteins (RBPs) are closely involved in the progression of cancers (18). A previous study has found that circZKSCAN1 can bind FMRP to regulate gene expression in hepatocellular carcinoma(19). Besides, Barbagallo D et al have proposed thatcircSMARCA5 can recruit SRSF1 to affect the migration ability in glioblastoma(20). However, the relevant reports about circRNAs and RBP in CRC remain largely unclear.

MicroRNAs (miRNAs) are noncoding RNAs with a length of 20-22 nucleotides (21). It has been reported that circRNAs can function as competing endogenous RNAs (ceRNA) to target miRNA and thereby regulate downstream genes (22-28). For instance, circRNA_0084043 activates the cell growth, invasion and migration of melanoma via sponging to miR-153-3p and further up-regulating the expression of Snail(29). CircRNA_103809 enhances the progression of lung cancer by modulating miR-4302/ZNF121/MYC signaling (30). CircRNA-100338 regulates

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the cell invasion of hepatocellular carcinoma by modulating the expression of miR-141-3p(31). Despite these progresses, the mechanistic functions of circRNAs in CRC have not been deeply investigated.

In summary, our study focused on investigating the role and potential mechanism of circ_0022340 in CRC. First, circ_0022340, which was formed from exon 5 to 6 of the synaptotagmin 7 (SYT7) and was highly expressed in CRC cells. Knockdown of circ_0022340 impeded CRC cell proliferation and migration in vitro and vivo. Mechanistically, circ_0022340 recruited heterogeneous nuclear ribonucleoprotein C (HNRNPC) to stabilize EBF1 mRNA and thereby activated SYT7 and acted as a ceRNA to target miR-382-5p/ETS transcription factor ELK1 (ELK1) axis. All these results suggested that circ_0022340 presented oncogenic properties, which might be a candidate for CRC diagnosis and treatment.

Materials and Methods

Cell lines and reagents

Human normal colorectal mucosal cell line (FHC) and human CRC cell lines (LOVO, HT-29, HCT116, SW480) in this study, were available from American Type Culture Collection (ATCC, Manassas, VA). FHC and LOVO cells were cultured in F-12K medium (Invitrogen, Carlsbad, CA, USA). HT-29 and HCT116 cells were cultured in ATCC-formulated McCoy's 5a medium (Manassas, VA). SW480 cell was cultured in ATCC-formulated Leibovitz's L-15 medium (Manassas, VA). All mediums with 10%FBS (Gibco) and 1% antibiotics were utilized for cell culture under the condition of 37°C and 5% CO_2. Besides, 3 U/mg of RNase R from Epicentre Technologies (Madison, WI) and 2 mg/ml of Actinomycin D from Sigma-Aldrich (St. Louis, MO) were used to treat cell samples of HCT116 and SW480.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from SW480 and HCT116 cells with TRIzol reagent (Invitrogen, Carlsbad, CA), then converted into cDNA as per the user manual (Takara, Shiga, Japan). SYBR Green PCR Master Mix (Invitrogen) was employed on Step-One Plus System (Applied Biosystems, Foster City, CA) to perform quantitative analysis. RNA level was calculated by the $2^{-\Delta\Delta CT}$ method, with GAPDH or U6 as an internal control. All experiments were in triplicate.

Plasmid transfection

The specific short hairpin RNAs (shRNAs) were designed by Genepharma Company (Shanghai, China) to stably silence circ_0022340, ELK1, EBF1 and HNRNPC in HCT116 and SW480 cells. Besides, the full-length cDNA sequences of ELK1 or SYT7 were subcloned into the pcDNA3.1 vector (Invitrogen) for gene overexpression. The miR-382-5p mimics/inhibitor and NC mimics/inhibitor were also procured from Genepharma. 48 h of plasmid transfection was implemented in presence of Lipofectamine 3000 (Invitrogen). All experiments were in triplicate.

5-ethynyl-20-deoxyuridine (EdU) staining assay

Cells were put on sterile coverslips in 96-well plates with 5×10^4 cells in each well. EdU kit from RiboBio (Guangzhou, China) was used following guidebook. Cell nucleus was double-stained with EdU and DAPI dye (Beyotime, Shanghai, China). Images were captured by fluorescence microscope (Olympus, Tokyo, Japan).All experiments were in triplicate.

Colony formation

Clonogenic cells of HCT116 and SW480 in 6-well plates (500/well) were cultured for 14 days and then stained for 15 min with 0.5% crystal violet in 4% paraformaldehyde. Colonies containing more than 50 cells were counted. All experiments were in triplicate.

Cell migration assay

Cell samples suspended in serum-free medium were planted in the upper chamber of 24-well plates transwell insert (Corning Incorporated, Corning, NY) for migration assay. The lower chamber was supplemented with the conditioned culture medium. Migrated cells in the bottom were fixed in 4% paraformaldehyde and dyed with crystal violet, counted under a microscope. All experiments were in triplicate.

Subcellular fractionation

CRC cell samples (1×10^6) were subjected to PARIS™ Kit as instructed by the supplier (Invitrogen) for collecting cell nuclear and cytoplasmic fractions. RT-qPCR was performed for quantification of circ_0022340, U6 (nuclear control) and GAPDH (cytoplasmic control). All experiments were in triplicate.

Fluorescent in situ hybridization (FISH)

The synthesized circ_0022340-FISH probe was procured from Ribobio for culturing with hybridization buffer in presence of the air-dried cell samples. After double-staining cell nuclei with DAPI, samples were visualized using a fluorescence microscope. All experiments were in triplicate.

RNA immunoprecipitation (RIP)

RIP assay in CRC cells was performed by using EZ-Magna RIP RNA Binding Protein Immunoprecipitation Kit based on the protocol (Millipore, Billerica, MA). Cell lysates from RIP lysis buffer were conjugated with human Ago2, EBF1 or HNRNPC antibodies in magnetic beads. A normal mouse IgG antibody was applied to the control group. All experiments were in triplicate.

RNA pull-down

RNA pull-down assay in CRC cells was achieved with Pierce Magnetic RNA-Protein Pull-Down Kit as instructed (Thermo Fisher Scientific, Waltham, MA). Cell protein samples from RIPA lysis buffer were mixed with the biotin-labeled RNA probes for circ_0022340, miR-382-5p or EBF1. After adding magnetic beads for 1 h, the pull-downs were assayed by RT-qPCR or western blot. All experiments were in triplicate.

Luciferase reporter assay

The circ_0022340 or ELK1 fragments covering miR-382-5p binding sites (wild-type and mutated) were applied for generating circ_0022340 WT/MUT and ELK1 WT/MUT by use of pmirGLO luciferase vector (Promega, Massachusetts, MA). After co-transfection with pmirGLO

vectors and miR-382-5p mimics or NC mimics, CRC cell samples were examined by Luciferase Reporter System (Promega). Besides, cells were co-transfected with pGL3-Basic luciferase vector (Promega) containing SYT7 promoter and the shRNAs for circ_0022340 or EBF1 for promoter analysis. All experiments were in triplicate.

Western blot

Total protein samples were obtained and loaded onto12% SDS-PAGE and transferred to PVDF membrane (Millipore), blocked with 5% fat-free milk, followed by incubation with the primary antibodies against internal control GAPDH (CST, 1:1000) and ELK1 (Abcam, 1/500), SYT7 (Abcam, 1/1000), EBF1 (Abcam, 1/1000), PCNA (Abcam, 1/1000), Ki67 (Abcam, 1/5000) overnight. Afterward, proteins were hatched with the secondary antibodies for probing membranes after specific dilution. The final membranes were all visualized using an ECL detection system (Bio-Rad, Hercules, CA). All experiments were in triplicate.

Chromatin immunoprecipitation (ChIP)

ChIP assay in CRC cells was implemented utilizing Magna ChIP Kit as requested (Millipore). DNA-protein cross-links were sonicated to 200-500-bp fragments and immunoprecipitated withEBF1 or control IgGantibody. Precipitated chromatin was retrieved and assayed by RT-qPCR and western blot. All experiments were in triplicate.

In vivo tumor growth assay

TransfectedHCT116 cells with sh-circ_0022340 or sh-NC were inoculated subcutaneously intoBALB/c male nude mice (6-week old; the First Hospital of China Medical University). The animal-related assay was performed strictly in line with the protocol approved by the Animal Research Ethics Committee of the First Hospital of China Medical University. Tumor volume was calculated every 4 days. 4 weeks later, mice were sacrificed after which tumors were excised and weighed. All experiments were in triplicate.

Immunohistochemistry (IHC)

Paraffin-embedded tissues from in vivo tumor growth assay were sectioned at 4 μm thick and cultured with primary antibodies, including anti-Ki67, anti-ELK1, anti-SYT7 and anti-PCNA at 4°C overnight, then with secondary antibodies for 30 min at room temperature. All experiments were in triplicate.

Statistical analyses

Data were reported as mean ± standard deviation (SD) for three individual experiments. Prism 6 (GraphPad, San Diego, CA) was used for statistical analyses, with $p < 0.05$ as the threshold for significant level. Group differences were analyzed using Student's t-test or ANOVA.

Results

Circ_0022340 knockdown inhibits CRC cell proliferation and migration

It has been reported that SYT7 is overexpressed in CRC cells and can promote cell proliferation in CRC(32). Furthermore, the expression of certaincircRNAs was more than ten times higher than those of their canonical linear transcripts generated from the same genes(33), therefore we speculated that circRNAs that were produced by extensive pre-mRNA back-splicing could also promote CRC progression. To identify which circRNA expression was abnormal in CRC cells, we applied RT-qPCR to detect different expressions of 8 circRNAs which were generated from precursor SYT7 back-splicing in four of CRC cells (LOVO, HT-29, HCT116 and SW480) and human normal cell (FHC). As shown in Figure 1A, the circ_0022340 level was obviously up-regulated compared with other circRNAs in CRC cells. Then we determined that circ_0022340 was formed from exons 5 to 6 of the SYT7 gene. The back-spliced junction of circ_0022340 was confirmed by Sanger sequencing (Figure 1B). Fur-

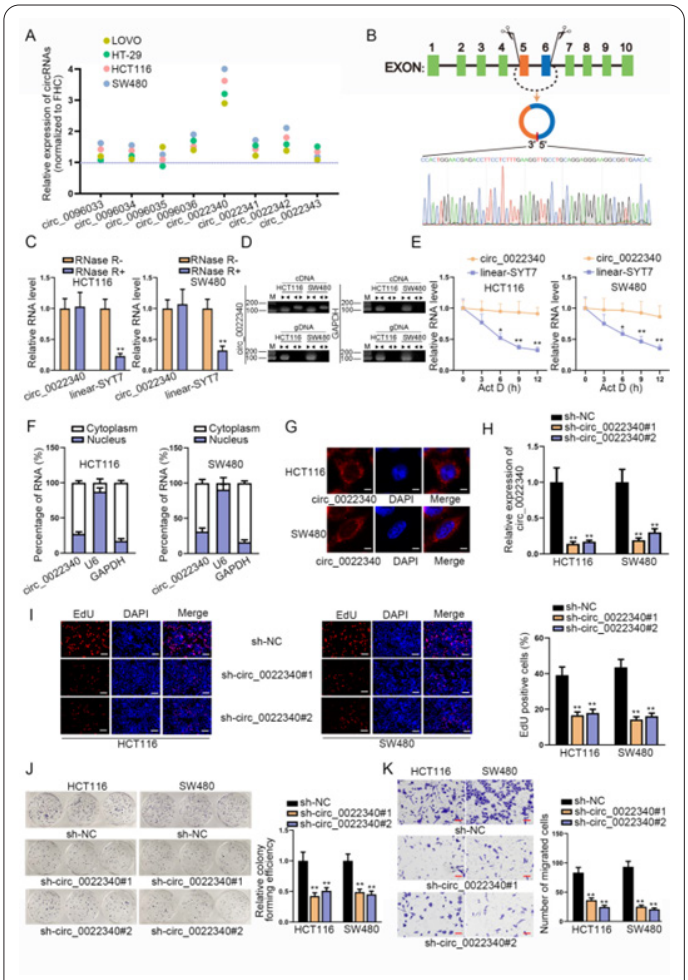

Figure 1. Circ_0022340 knockdown inhibits CRC cell proliferation and migration. A. RT-qPCR detected the expression levels of circRNAs expression in CRC cells (LOVO, HT-29, HCT116 and SW480) and human normal cell (FHC). B. Sequence diagram of cyclization site analysis of circ_0022340. C. RT-qPCR examined the level of circ_0022340 and linear-SYT7 in HCT116 and SW480 cells treated with RNase R. D.The existence of circ_0022340 was measured in HCT116 and SW480 cells by RT-qPCR with convergent or divergent primers and confirmed by Gel electrophoresis. E. Actinomycin D detected the stability of circ_0022340 in CRC cells. F-G. Nuclear separation and FISH assays were performed to confirm the subcellular location of circ_0022340. H. RT-qPCR detected the expression of circ_0022340 in HCT116 and SW480 cells transfected with shRNAs targeting circ_0022340. I-J. EdU and colony formation assays detected the proliferation ability in HCT116 and SW480 cells transfected with sh-circ_0022340#1 and sh-circ_0022340#2. K. Transwell assays detected migration ability in HCT116 and SW480 cells transfected with sh-circ_0022340#1 and sh-circ_0022340#2. $^{**}P<0.01$.

thermore, RT-qPCR confirmed that linear-SYT7 level was digested by adding RNase R to both HCT116 and SW480 cells, while circ_0022340 was counteractive to RNase R degradation (Figure 1C). In addition, circ_0022340 was further identified using either divergent primers that amplified only circ_0022340 or convergent primers that assessed linear-SYT7. Complementary DNA (cDNA) and genomic DNA (gDNA) from HCT116 and SW480 cells were as templates. The data showed that circ_0022340 was detected with divergent primers in cDNA but not gDNA (Figure 1D). Meanwhile, we also examined the stability of circ_0022340 in HCT116 and SW480 cells treated with Actinomycin D (Act D), an inhibitor of transcription. The result displayed a half-life of more than 12 h, implying that circ_0022340 was more stable than linear-SYT7 (Figure 1E). All these results suggested the presence of circ_0022340 in CRC cells. To investigate the potential mechanism of circ_0022340 in CRC cells, we carried out subcellular fractionation and FISH analysis and discovered that the subcellular location of circ_0022340 was major in the cytoplasm (Figure 1F and 1G), suggesting that circ_0022340 exerted functions in CRC cells via post-transcriptional regulation. Subsequently, we conducted loss-of-function assays to assess the role of circ_0022340 through stably silencing the level of circ_0022340 by transfecting two shRNAs into HCT116 and SW480 cells (Figure 1H). In EdU assays, we found that the EdU-positive cells were obviously decreased after the knockdown of circ_0022340 (Figure 1I). Likewise, the results from colony formation assays revealed that the colony forming efficiency was also inhibited by circ_0022340 deletion (Figure 1J). Besides, silencing of circ_0022340 led to a decrease in migration ability in CRC cells through transwell assays (Figure 1K). Totally, circ_0022340 is highly expressed in CRC cells and circ_0022340 knockdown inhibits cell proliferation and migration in CRC.

EBF1 binds to SYT7 promoter in CRC cells

TCGA database showed that SYT7 was obviously up-regulated in CRC cells (Figure 2A). Through luciferase reporter assay, we also discovered that the luciferase activity of SYT7 promoter was decreased when circ_0022340 was silenced in HCT116 and SW480 cells (Figure 2B), therefore we speculated that SYT7 might be regulated at the transcription level. Then we utilized the UCSC website (http://genome.ucsc.edu) to find 5 transcription factors combined with the SYT7 promoter. For further screening, we detected SYT7 expression in HCT110 and SW480 cells transfected with shRNAs targeting these five transcription factors, respectively. The results from Figure 2C showed that SYT7 expression was down-regulated by EBF1 deletion, while it had no change after the transfection of other transcription factors. Thus, we further guessed whether circ_0022340 combined with EBF1 to regulate the transcriptional level of SYT7. However, we found that the enrichment of circ_0022340 has no statistical significance in the EBF1 complex (Figure 2D). Furthermore, we stably down-regulated EBF1 expression and found that the mRNA level and protein level of SYT7 were markedly reduced by EBF1 deletion (Figures 2E and 2F). Through JASPAR software (http://jaspar.genereg.net/), we predicted the binding sites between the SYT7 promoter and EBF1 (Figures 2G and 2H). To verify the effectiveness of the binding sites, luciferase reporter assays were carried out. The data disclosed that the luciferase activity was obviously reduced in HCT116 and SW480 cells co-transfected with shRNAs targeting EBF1 and SYT7 promoter-WT, while there was no change in HCT116 and SW480 cells co-transfected with shRNAs targeting EBF1 and SYT7 promoter-MUT (Figure 2I). Additionally, ChIP assays further reflected that SYT7 was prominently abundant in Anti-EBF1 groups (Figure 2J). All these results indicate that EBF1 can activate SYT7 expression in CRC cells.

Circ_0022340 recruits HNRNPC to stabilize EBF1 mRNA in CRC cells

Due to the fact that circ_0022340 could not directly combine with EBF1 to regulate the SYT7 promoter, we doubted whether circ_0022340 recruited some certain protein to combine with EBF1 and thereby affected the SYT7 promoter in CRC cells. We next performed RNA pull-down, followed by SDS-PAGE and mass spectrometry assays to identify proteins associated with circ_0022340(Supplementary figure 1A). At the same time, starbase (http://starbase.sysu.edu.cn/) predicted 15

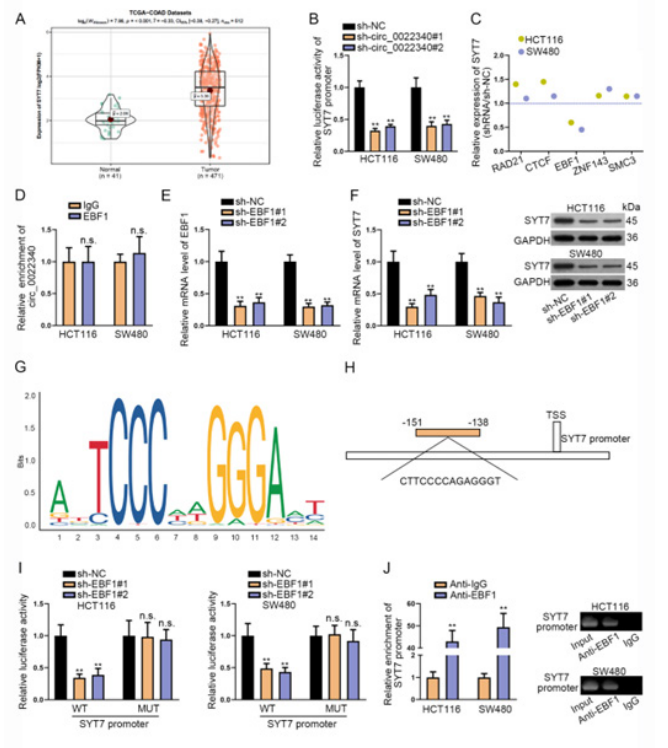

Figure 2. EBF1 can combine with SYT7 promoter. A. TCGA database of SYT7 expression in CRC and normal samples . B. Luciferase reporter assays detected the luciferase activity of SYT7 promoter in HCT116 and SW480 cells transfected with shRNAs targeting circ_0022340. C. RT-qPCR examined SYT7 expression in HCT116 and SW480 cells transfected with shRNAs targeting 5 transcription factors. D. RIP assays assessed the enrichment of circ_0022340 combined with EBF1 in HCT116 and SW480 cells. E. RT-qPCR detected the mRNA level of EBF1 in HCT116 and SW480 cells transfected with sh-EBF1#1 and sh-EBF1#2. F. RT-qPCR and western blot were used to test SYT7 mRNA level and protein level in HCT116 and SW480 cells transfected with sh-EBF1#1 and sh-EBF1#2. G-H. JASPAR software predicted the binding sites between SYT7 promoter and EBF1. I. Luciferase reporter assays further proved the effectiveness of the binding sites between SYT7 promoter and EBF1. J. ChIP assays were performed to reflect the enrichment of SYT7 promoter in Anti-EBF1 group. $^{**}P<0.01$. n.s.: no significance.

RBPs that could both bind to circ_0022340 and EBF1, among which only RBP was chosen for it was also specifically identified in mass spectrometry analysis (Supplementary figure 1B). Hence, we chose HNRNPC for further analysis. To further validate our assumption, we first inhibited the expression and protein level of HNRNPC (Figure 3A and 3B) and used RT-qPCR and western blot to analyze the mRNA level and protein level of EBF1 in CRC cells transfected with sh-HNRNPC#1 and sh-HNRNPC#2, respectively. The data showed that HNRNPC silencing dramatically decreased the mRNA level and protein level of EBF1 (Figure 3C and 3D). Besides, we found that circ_0022340 was highly enriched in Ago2 groups, while EBF1 was not, which excluded the existence of a ceRNA mechanism between circ_0022340 and EBF1 (Figure 3E). Meanwhile, RNA pull-down assays disclosed that HNRNPC was largely enriched in Bio-circ_0022340-WT groups and EBF1 groups, suggesting that HNRNPC could combine with both circ_0022340 and EBF1 in CRC cells (Figure 3F). Similarly, the RIP assay also demonstrated that circ_0022340 and EBF1 were both enriched in Anti-HNRNPC groups (Figure 3G). In addition, we found that the expression of EBF1 mRNA declined after circ_0022340 knockdown or HNRNPC deletion in CRC cells treated with Act D, suggesting that the stability of EBF1 was evidently reduced by the depletion of circ_0022340 or HNRNPC (Figure 3H). Conclusively, circ_0022340 recruits HNRNPC to stabilize EBF1 mRNA in CRC cells.

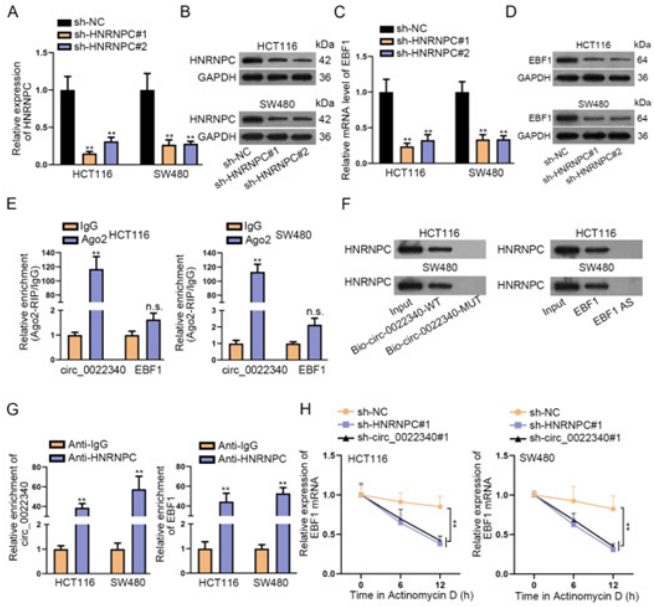

Figure 3. Circ_0022340 can recruit HNRNPC to stabilize EBF1 mRNA in CRC cells. A-D. RT-qPCR and western blot severally examined the expression and protein levels of HNRNPC and EBF1 in both HCT116 and SW480 cells transfected with shRNAs targeting HNRNPC. E. RIP assays displayed the enrichment of circ_0022340 and EBF1 in Ago2 groups. F. RNA pull down assays disclosed that the enrichment of HNRNPC in Bio-circ_0022340-WT group and EBF1 group. G. RIP assays demonstrated the enrichment of circ_0022340 and EBF1 in Anti-HNRNPC groups. H. RT-qPCR separately detected the EBF1 mRNA expressionin HCT116 and SW480 cells transfected with sh-NC, sh-circ_0022340#1 or sh-HNRNPC#1 upon adding Actinomycin D. $^{**}P<0.01$. n.s.: no significance.

MiR-382-5p is the downstream gene of circ_0022340 in CRC cells

Since circ_0022340 had been proven to play a promoting role in CRC cell proliferation and migration, we further determined its potential mechanism in CRC cells. Based on the previous mass spectrometry assay, we found that Ago2 protein was also pulled down by circ_0022340 (Supplementary Figure 1A). Therefore, we suspected circ_0022340 might function as a ceRNA to regulate CRC progression at the post-transcriptional level. To validate our assumption, RIP assay and RNA pull-down assays were carried on to further verify the association between circ_0022340 and Ago2, for Ago2 is a part of RNA-induced silencing complex (RISC) which contains miRNA ribonucleoprotein complexes(34). The data showed that-circ_0022430 was obviously enriched in Ago2 groups (Figure 4A and 4B), which proved our conjecture. Subsequently, we applied bioinformatics prediction to observe 16 miRNAs combined with circ_0022430 and performed RNA pull-down assay to filter the certain one that was targeted by circ_0022430 in CRC cells. The results dis-

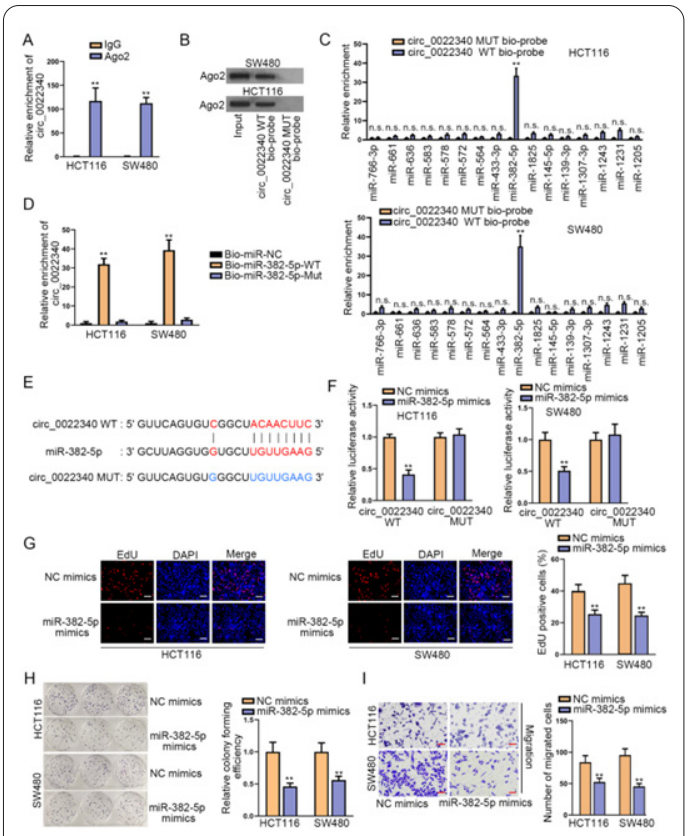

Figure 4. Circ_0022340targetsmiR-382-5pto promote proliferation and migration of CRC cells. A. RIP assays analyzed the enrichment of circ_0022340 in Ago2 groups. B. RNA pull down assays analyzed the enrichment of Ago2 in circ_0022340 WT bio-probe groups and circ_0022340 MUT bio-probe groups. C. RNA pull down assays displayed the richness of miRNAs combined with circ_0022340. D. RNA pull down assays demonstrated the enrichment of circ_0022340 in Bio-miR-382-5p-WT and Bio-miR-382-5p-MUT groups. E. Starbase predicted the binding sites between circ_0022340 and miR-382-5p. F. Luciferase reporter assays analyzed the combination of circ_0022340 and miR-382-5p. G-H. EdU and colony formation assays detected the proliferation ability in HCT116 and SW480 cells transfected with miR-382-5p mimics. I. Transwell assays detected the migration ability in HCT116 and SW480 cells transfected with miR-382-5p mimics. $^{**}P<0.01$. n.s.: no significance.

closed that the enrichment of miR-382-5p was apparently increased in circ_0022430 WT bio-probe groups whereas other miRNAs had no change (Figure 4C). We also used bio-probe to mark miR-382-5p and observed that circ_0022340 was concentrated in the Bio-miR-382-5p-WT group rather than in the Bio-miR-382-5p-MUT group (Figure 4D). Next, the binding sites between circ_0022340 and miR-382-5p were obtained through the starbase website (http://starbase.sysu.edu.cn), and the luciferase reporter assay further confirmed that the luciferase activity was evidently declined in HCT116 and SW480 cells co-transfected with miR-382-5p mimics and circ_0022340 WT, while no significant difference was observed in HCT116 and SW480 cells co-transfected with miR-382-5p mimics and circ_0022340 MUT(Figure 4E and 4F). To investigate the role of miR-382-5p in CRC, gain-of-function assays were conducted. In EdU and colony formation assays, miR-382-5p overexpression significantly reduced the cell proliferation in CRC (Figure 4G and 4H). In transwell assays, the number of migrated cells was also suppressed by miR-382-5p mimics (Figure 4I). Collectively, miR-382-5p is the downstream gene of circ_0022340, and miR-382-5p suppresses CRC cell proliferation and migration.

ELK1 serves as a direct target gene of miR-382-5p and ELK1 knockdown inhibits CRC cell proliferation and migration

MiRNAs primarily function via negatively regulating the expression of protein-coding genes through binding to the 3′untranslated regions (3′UTR) of mRNAs with imperfect base pairing, resulting in the regulation of gene expression in cells(35). To elucidate the target gene miR-382-5p in CRC cells, we used several bioinformatics programs including PITA, RNA22 and miRanda, the result of which is that nine mRNAs could combine with miR-382-5p (Figure 5A). Then we applied RT-qPCR to separately analyze the expression levels of miRNAs in HCT116 and SW480 cells transfected with sh-circ_0022340 or miR-382-5p mimics. As revealed in Figure 5B, only ELK1 and PDE8A expression were statistically significant, and RNA pull-down assay further confirmed that only ELK1 was evidently enriched in the bio-miR-382-5p WT group (Figure 5C). Moreover, RT-qPCR and western blot testified that the mRNA level and protein level of ELK1 were signally down-regulated in HCT116 and SW480 cells transfected with miR-382-5p mimics (Figure 5D). Intriguingly, the mRNA level and protein level of ELK1were decreased by the knockdown of circ_0022340, while enhanced by co-transfection of miR-382-5p inhibitor (Figure 5E). RIP assay further reflected that circ_0022340, miR-382-5p and ELK1 were all positively enriched in Ago2 pallets, indicating that these three RNAs belonged to the RISC (Figure 5F). Additionally, we identified the binding sites of miR-382-5p on ELK1 3'UTR through the starBase database (Figure 5G), and we found that the luciferase activity of ELK1 WT was significantly reduced by miR-382-5p mimics, which was different from that in ELK1 MUT groups (Figure 5H). Finally, we stably knocked down ELK1 expression (Figure 5I) and assessed the effect of ELK1 on CRC cell proliferation and migration. It was revealed in EdU and colony formation assays that silencing of ELK1 suppressed CRC cell proliferation (Figure 5J and 5K). Synchronously, the capacity of migration in CRC cells was also impeded by ELK1 deletion via transwell assays (Figure 5L). Taken together, ELK1 is the target gene of miR-382-5p and ELK1 promotes CRC cell proliferation and migration.

Circ_0022340 facilitates CRC progression by targeting miR-382-5p to promote ELK1

To explore the interaction among circ_0022340, miR-382-5p and ELK1 in CRC progression, rescue experiments were conducted. As shown in Figure 6A, the EdU-positive cells were reduced by circ_0022340 deletion, while co-transfection of miR-382-5p inhibitor or pcDNA3.1-ELK1 partly restored this effect. Similarly, the reduced number of colonies mediated by circ_0022340 silencing could be partially enhanced by co-transfection of miR-382-5p inhibitor or pcDNA3.1-ELK1 (Figure 6B). Furthermore, miR-382-5p inhibitor or ELK1 overexpression partly reversed the inhibitory effect of circ_0022340 silencing on

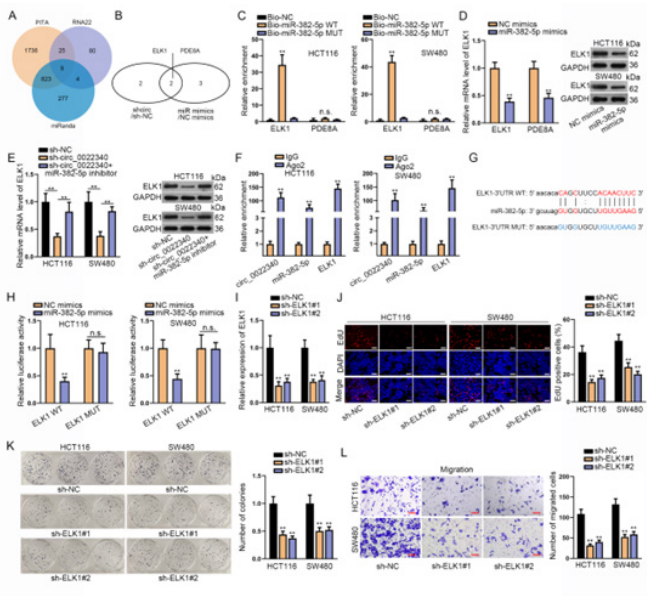

Figure 5. ELK1 serves as a direct gene of miR-382-5p and ELK1 knockdown inhibits CRC cell proliferation and migration. A. Using several bioinformatics programs including PITA, RNA22 and miRanda to predict that nine mRNAs could combine with miR-382-5p. B. RT-qPCR separately analyzed the expression of mRNAs in HCT116 and SW480 cells transfected with sh-circ_0022340#1/#2 or miR-382-5p mimics, respectively. C. RNA pull down assays detected the enrichment of ELK1 and PDE8A in Bio-miR-382-5p WT/MUT groups. D. RT-qPCR and western blot testified the mRNA level and protein level of ELK1 in HCT116 and SW480 cells transfected with miR-382-5p mimics. E. RT-qPCR and western blot detected the mRNA level and protein level of ELK1 in three groups (sh-NC, sh-circ_0022340 and sh-circ_0022340+miR-382-5p inhibitor) of HCT116 and SW480 cells. F. RIP assays reflected the enrichment of circ_0022340, miR-382-5p and ELK1 in Ago2 pallets. G. StarBase website analyzed a binding site of miR-382-5p on ELK1 3'UTR. H.Luciferase reporter assays confirmed the luciferase activity in HCT116 and SW480 cells co-transfected with ELK1 WT and miR-382-5p mimics or ELK1 MUT and miR-382-5p mimics. I. RT-qPCR examined ELK1 expression in HCT116 and SW480 cells transfected with shRNAs targeting ELK1. J-K. EdU and colony formation assays detected the proliferation ability in HCT116 and SW480 cells transfected with sh-ELK1#1 and sh-ELK1#2. L. Transwell assays detected the migration ability in HCT116 and SW480 cells transfected with sh-ELK1#1 and sh-ELK1#2. **P<0.01. n.s.: no significance.

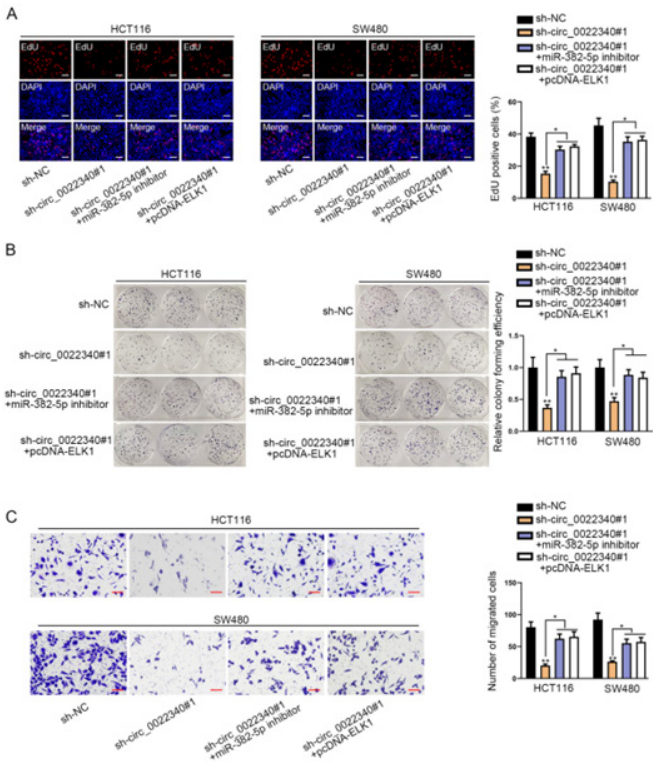

Figure 6. Circ_0022340 promotes CRC progression by targeting miR-382-5p to promote ELK1. Rescue experiments were performed in HCT116 and SW480 cells co-transfected with sh-NC, sh-circ_0022340#1, sh-circ_0022340#1+miR-382-5p inhibitor and sh-circ_0022340#1+pcDNA-ELK1. A-B. EdU and colony formation assays detected cell proliferation in HCT116 and SW480 cells. C. Tranwell assays assessed the cell migration in HCT116 and SW480 cells. *P<0.05, **P<0.01.

CRC cell migration (Figure 6C). All these data reveal that circ_0022340 facilitates CRC cell proliferation and migration by targeting the miR-382-5p/ELK1 axis.

Circ_0022340 promotes the progression of CRC through regulating ELK1 and SYT7

Finally, to elucidate the relationship among circ_0022340, ELK1 and SYT7 on CRC cell proliferation and migration, a series of rescue experiments were implemented. As shown in Figures 7A and 7B, the results from EdU and colony formation assays displayed that silencing of cir_0022340 inhibited the proliferation and migration in HCT116 cell, and this effect was partially rescued by co-transfection of pcDNA3.1-ELK1, while completely enhanced by co-transfection of pcDNA3.1-ELK1 and pcDNA3.1-SYT7. Besides, overexpression of ELK1 partly restored the inhibited number of migrated cells mediated by cir_0022340 deletion, while up-regulation of ELK1 and SYT7 could totally reverse this effect caused by cir_0022340 deletion (Figure 7C). Moreover, we also explored the impact of circ_0022340 on the tumor growth of CRC in vivo. As displayed in Figures 7D and 7E, the tumor volume and tumor weight were largely reduced by circ_0022340 knockdown in the HCT116 cell. Meanwhile, RT-qPCR and western blot corroborated that the expression levels of circ_0022340, SYT7 and ELK1 as well as the protein levels of SYT7 and ELK1 were depleted by circ_0022340 reduction in HCT116 cell (Figure 7F and 7G). In addition, IHC results further showed that

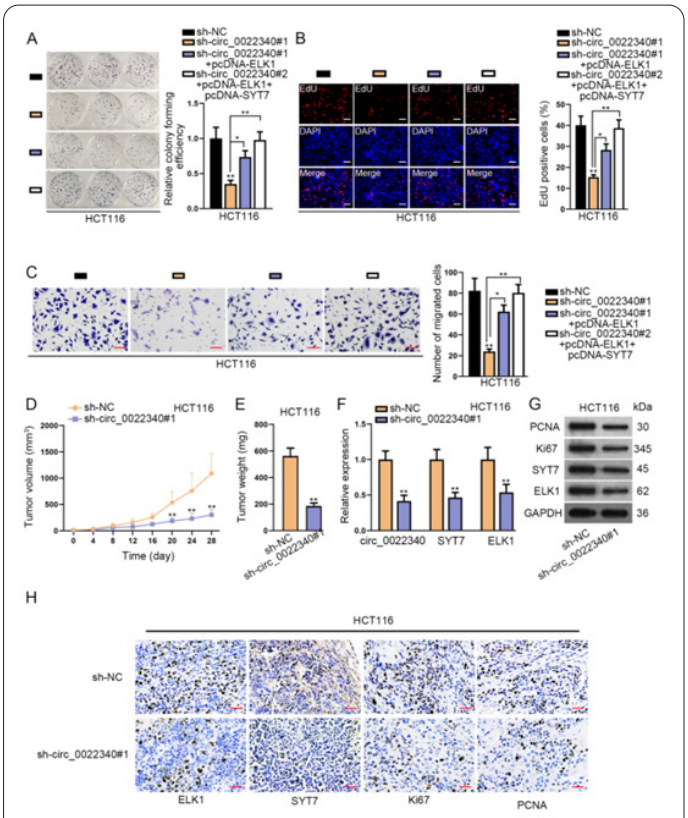

Figure 7. Circ_0022340 promotes the progression and tumor growth of CRC through regulating ELK1 and SYT7. A. Four groups including sh-NC, sh-circ_0022340#1, sh-circ_0022340#1+pcDNA-ELK1 and sh-circ_0022340#1+pcDNA-ELK1+pcDNA-SYT7 were transfected into HCT116 cells. Then rescue experiments were performed. A-B. EdU and colony formation assays assessed the proliferation capacity in HCT116 cells. C. Transwell assays assessed the migration capacity in HCT116 cells. D-E. Tumor volume and tumor weight were analyzed in HCT116 cell. F. RT-qPCR analyzed the expression of circ_0022340, SYT7 and ELK1 in HCT116 cell transfected with sh-circ_0022340#1. G. Western blot examined the protein levels of PCNA, Ki67, SYT7 and ELK1 in HCT116 cell transfected with sh-circ_0022340#1. H. IHC results of the positivity of PCNA, Ki67, SYT7 and ELK1 after knockdown of circ_0022340. *P<0.05, **P<0.01. n.s.: no significance.

ELK1, SYT7, the proliferation index Ki67 and PCNA all presented lower positivity upon circ_0022340 knockdown (Figure 7H). All these results suggest that circ_0022340 promotes CRC cell proliferation and migration of CRC cells through regulating ELK1 and SYT7.

The scientific concept map describes the functional mechanism of circ_0022340 in CRC cells

We concluded that circ_0022340 promoted CRC cell proliferation and migration through recruiting HNRNPC to stabilize EBF1 mRNA and thereby up-regulate its host gene SYT7 as well as sponging miR-382-5p to promote ELK1 expression (Figure 8).

Discussion

Recently, circRNAs have been a hot topic in human CRC research. They exhibit important roles in regulating signaling molecules associated with the progression of CRC, thereby providing novel strategies to improve CRC therapy(36). Accumulating evidence has revealed that circRNAs take part in the occurrence and develop-

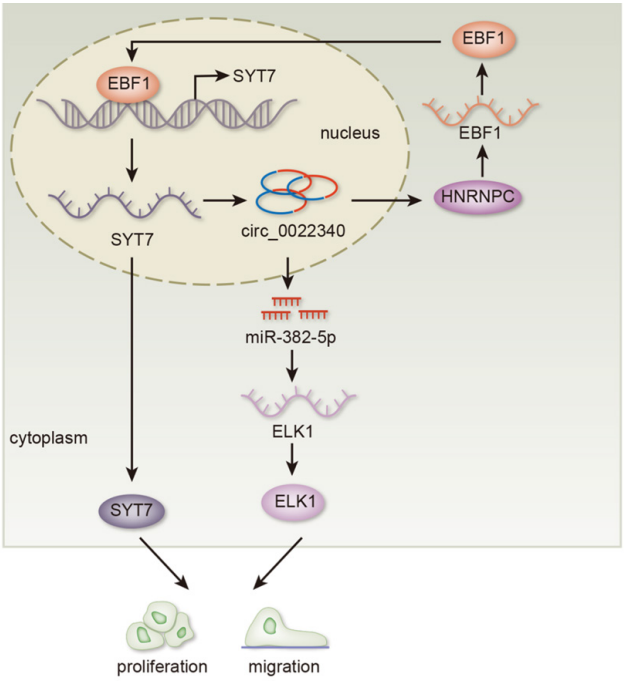

Figure 8. The scientific concept map describes the functional mechanism of circ_0022340 in CRC cells.

ment of CRC. Ruan H et al have found that circ_0002138 is down-regulated and impedes cell proliferation in CRC (37). Besides, hsa_circ_0009361 has been registered to act as a tumor suppressor in CRC via targeting miR-582 to regulate APC2 expression(38). Unlike these reports, our study identified that circ_0022340 was highly expressed in CRC cells. Moreover, we conducted loss-of-function assays to confirm thatcirc_0022340 deletion obviously suppressed the capacities of proliferation and migration in CRC, which was consistent with the previous study discovered by Zhang R et al(39). All these results could draw a conclusion that circ_0022340 played a tumor-promoting role in CRC development.

SYT7 is the host gene of circ_0022340 and functions as an oncogene in various cancers, such as glioblastoma, gastric cancer and colorectal cancer(32, 40, 41). In our study, it was found that SYT7 expression was up-regulated by EBF1. As reported previously, EBF1 functioned as a transcription factor reported to participate in the progression of various cancers, including CRC (42). Here, we found that EBF1 could bind to the SYT7 promoter and activate the expression of SYT7. Moreover, our study found thatcirc_0022340 recruited HNRNPC to stabilize EBF1 mRNA and thereby activated the transcription of SYT7. It has been reported that HNRNPC acts as an RNA-binding protein to promote the development of esophageal squamous cell carcinoma(43). Similarly, HNRNPC has been reported to contribute to tumor growth in non-small cell lung cancer via interaction with KHSRP(44). All these reports are in line with our findings. More importantly, we carried on a series of rescue experiments to validate that overexpression of ELK1 and STT7 could entirely restore the inhibitory effect mediated by circ_0022340 deletion on CRC cell proliferation and migration. Meanwhile, we performed in vivo experiments to find thatcirc_0022340 silencing could suppress the tumor growth in CRC.

It is well known that circRNA can serve as a ceRNA in CRC via post-transcriptional regulation (45). For example, circFMN2 can function as a miR-1182 sponge to regulate the expression of HTERT in CRC and activate cell growth and migration (46). In addition, hsa_circ_0000523 affects CRC cell proliferation and apoptosis via targeting miR-31 (47). Consistent with these studies, our present research identified the potential binding sites between circ_0022340 and miR-382-5p. Moreover, previous studies have reported the tumor suppressor role of miR-382-5p in glioma and osteosarcoma(48-50). In line with these references, our study also validated that overexpression of miR-382-5p obviously reduced CRC cell proliferation and migration. Notably, miR-382-5p has been documented to exert effects on breast cancer via targeting RERG (51). Accordingly, our research proved that ELK1 was the target gene of miR-382-5p. In addition, loss-of-function assays further verified that ELK1 played a tumor-promoting role in CRC. Similarly, previous studies have identified that ELK1 can function as an oncogenic role in breast cancer and prostate cancer(52, 53). Finally, rescue assays further revealed that circ_0022340 facilitated CRC cell proliferation and migration via modulating miR-382-5p/ELK1 axis. All these results supported the ceRNA hypothesis of circ_0022340 in CRC cells and suggested that circ_0022340 might be a potential target for CRC treatment. In conclusion, our study elucidated the functional role and potential mechanism of circ_0022340 in CRC. Our study confirmed that circ_0022340 promoted CRC cell proliferation and migration through modulating HNRNPC/EBF1/SYT7 or miR-382-5p/ELK1axis which might contribute to the exploration of CRC treatment.

Conflict of interest

The authors declare that they have no competing interest in this study.

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